Reduced Chemical Weed Control Options in Virginia for Corn and Turfgrass and
Characterization of *Sorghum halepense* Expressing Multiple Resistance to Nicosulfuron and
Glyphosate

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ABSTRACT

Sustainable weed control in managed agricultural systems requires the judicious use of multiple weed control tactics and prevents over-reliance on any one tactic. In this context, sustainable weed management plays a critical role in the mitigation of one of agriculture's most pressing problems- herbicide resistance. Research conducted in Virginia sought to explore the effects of integrating multiple weed management tactics in corn and cool-season turfgrass. Additionally, research was conducted to confirm nicosulfuron and glyphosate herbicide resistance in Virginia johnsongrass and elucidate the molecular mechanisms conferring those resistances. Rye and hairy vetch cover crop residues, combined with reduced rates of preemergence herbicide and postemergence glyphosate applications, were shown to provide sufficient weed control and corn yield. Cover crop type or residue level did not augment weed control in corn production systems, but the use of glyphosate was essential for late-season weed control. Rye and vetch biculture as a cover crop increased corn yield compared to rye cover crop alone. In cool-season turfgrass, the addition of reduced preemergence herbicide rates to corn gluten meal, an organic herbicide product, reduced crabgrass 25%. Moreover, control was dependent on herbicide choice. Herbicides applied at half of recommended labeled rates or less did not control crabgrass at a commercially-acceptable level, regardless of corn gluten meal addition. In field experiments, Virginia johnsongrass expressed resistance to nicosulfuron and glyphosate. Glyphosate at 0.88 kg ae ha^{-1} controlled johnsongrass 65%. Nicosulfuron at 0.14 kg ai ha^{-1} controlled the same population 10%. Greenhouse experiments confirmed differential sensitivity of putative herbicide-resistant johnsongrass seedlings to nicosulfuron and glyphosate when compared to a susceptible population. Herbicide resistance was not conferred via target-site mutation. Five ALS-gene site mutations were confirmed absent in Virginia johnsongrass, while three others were located in coding regions that could not be elucidated in johnsongrass. Further investigations showed glyphosate resistance was not conferred via reduction in herbicide absorption or translocation. The susceptible johnsongrass caused an increase in a polar metabolite at $R_f = 0.17$ with concomitant reduction in glyphosate over time. Although the mechanism is not clear, these data suggests that glyphosate resistance in johnsongrass may be associated with differential metabolism.

To my wife

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Chapter 1: Literature Review

1a. Reduced Chemical Weed Control Options in Virginia for Corn and Turfgrass

Public perception of pesticide use in agriculture tends to be negative and exerts considerable influence in government policy, scientific research, and the marketplace (Dantzker et al. 2010; Dunlop 1992). While herbicides are the primary, safest, and most effective tools for weed control on a global scale (Duke and Powles 2008; Mazur and Falco 1989; Shaner 2004), interest in alternatives to their use has expanded rapidly and is an important influence in consumers' food, fiber, and home choices (Dantzker et al. 2010; Hammit 1990). The organic market is one of the fastest growing sectors in the country, with an approximate 8-20% growth each year for the last ten years (OTA 2011). Approximately 1.4 million hectares of US cropland are certificated organic (USDA 2012b). Crop producers and turfgrass managers interested in reducing chemical use and/ or organic production do so primarily to increase profits by reducing inputs and to meet market demand. Grain prices for conventional corn and soybean are $0.18 and $0.55 per kg, respectively, whereas organic alternatives are 245% and 208% the conventional price (Barchart 2012; USDA 2012a). In the turfgrass industry, public recreation centers, public sport arenas, and homeowners are interested in reduced chemical inputs (Dantzker er al. 2010). Moreover, homeowners, by their consumer choices, have driven the market for "green solutions" for pest control and fertilizer in the home and garden sector, as seen by the increase in organic and natural products for lawn care (Dantzker et al. 2010; Hammit 1990). Concerns regarding environmental and human health have been the dominant forces influencing such changes (Cooper 1987; Dantzker et al. 2010).

The public's negative perception of pesticides is primarily derived from the misunderstanding of scientific evidence and miscommunication with the scientific community (Perterson and Higley 1993). Research has shown that pesticide use causes changes in the environment, which may be deemed adverse, and can increase risk of exposure, which may have human health issues. The use of herbicides has led to documented groundwater detection of triazine and chloroacetamide herbicides (Thurman et al. 1991; Ritter 1990), herbicide resistant weeds (Holt 1992; Nandula et al. 2005; Powles and Yu 2010), and weed population shifts (Culpepper 2006; Warwick 1991). Other researchers have expressed concern over the consequences of pesticide exposure to humans (Carbonell et al. 1995; Antle and Pingali 1994), though it should be noted that herbicides differ in mammalian toxicity. The herbicide paraquat is highly toxic to mammals, whereas glyphosate is essentially non-toxic (Bus et al 1976; Dewey and Appleby 1983). While environmental changes are certain with pesticide use, it does not automatically follow that those changes are negative. However, generally speaking, individuals who possess a negative perception of pesticides will view most changes and circumstances adversely, regardless of whether the changes are or not. Furthermore, the concept of risk assessment is equally relevant in pesticide perception (Delaney 1993). Individuals are less likely to accept risk if the risk is imposed upon them. In the context of herbicide use, herbicide applications beyond an individual's control are less accepted than those within an individual's control (Delaney 1993). For the majority of applied herbicide, proper application provides excellent weed control with minimal environmental and human health risk (Duke and Powles 2008; Mazur and Falco 1989; Shaner 2004). For applicators, reducing chemical use and utilizing multiple weed control tactics may help change public perception of herbicide risk.

Employing multiple weed control tactics in an integrated weed management (IWM) approach can be extremely beneficial. Whether it be cultural, mechanical, or chemical weed control tactics, successful combinations can, depending on the circumstances, decrease reliance on a specific tactic (Swanton and Murphy 1996), decrease the risk of herbicide resistance (Zoschke 1994), contribute as a fertilizer (Christians 1993), or build soil organic matter (Reicosky et al. 1995). Other potential advantages include encouraging the growth of beneficial microorganisms (Harris et al 1994), soil erosion control (Dabney et al. 2001), nitrogen sequestration (Isse et al. 1999), and reducing herbicide persistence in the soil (Harris and Warren 1964). In summary, IWM is critical for the long-term sustainability of managed systems. An appropriate combination of weed control tactics can provide consistent, long-term control and can also maintain the efficacy and longevity of any specific tactic. This is an absolute necessity for herbicides, as herbicide resistance is agriculture's most pressing weed control problem (Swanton and Murphy 1996; WSSA 2012). In Virginia (VA), multiple weed control combinations were examined for efficacy in cool-season turfgrass and corn.

Herbicides and Corn Gluten Meal in VA Turfgrass

As stated, the public's negative perception of pesticides is important in the acceptance or rejection of herbicide use in the turfgrass and landscape business. Weed control tactics that reduce herbicide rates and incorporate natural products may be more accepted by the public. There are several natural products available to homeowners, and corn gluten meal (CGM), a by-product of the wet-milling process of corn, exhibits some herbicidal activity and is one of the most popular alternatives on the market.

Corn gluten meal inhibits root formation in several weed species without causing injury to established turf, and requires a period of moisture stress following application to be effective (Bingaman and Christians 1995; Christians 1993; Gardner et al. 1997). This makes CGM a selective preemergence herbicide safe for established turfgrass. This selectivity is possible because established turfgrass has developed root systems that are not affected, while root formation of emerging seedlings may be suppressed. Root suppression is due to Leu-Ser-Pro-Ala-Gln, a pentapeptide isolated from corn gluten hydrolysate as the bioactive root inhibitor in corn gluten meal (Liu and Christians 1996). CGM is categorized by the FDA as GRAS (Generally Recognized As Safe for food use), meaning that no known toxic effects have been observed in organisms or the environment (EPA 2013). Crabgrass (*Digitaria* spp.) is one species in which CGM exhibits some herbicidal activity, and is considered one the most problematic weeds in turfgrass (Kim et al. 2002). Research has reported 97% and 82% survival reduction at a 973 g m^{-2} CGM rate for smooth crabgrass (*D. ischaemum*) and large crabgrass (*D. sanguinalis*), respectively (Bingaman and Christians 1995).   However, other research has concluded that such application rates are excessive and suggests a spring application rate of 99 g m^{-2}, which provides 58% control (Christians 1993).

At 99 g m^{-2} CGM, crabgrass control of 58% does not meet industry standard of 80%. Moreover, a 99 g m^{-2} CGM rate is equivalent to 88 kg N ha^{-1} and acts as a slow-release N fertilizer (Christians, 1993). The recommended spring N rate for most cool-season turf is 49 kg N ha^{-1} (Goatley et al. 2009), and subsequent summer applications are not recommended. Under certain conditions, excessive N can lead to nitrogen loss and groundwater contamination (Morton et al. 1988; Guillard and Kopp 2004; Petrovic 1990). Further, excessive N fertilization can lead to summer stress and increased disease severity in cool-season turf (Couch 1966).

One disease partly influenced by excessive nitrogen applications is brown patch, caused by the fungal pathogen *Rhizoctonia solani* [*Thanatephouus cucumeris*] Kuhn (Couch 1966). Brown patch is considered one of the most important turfgrass diseases in the country (Smiley et al. 2005). It appears as an irregular shaped patch that eventually turns light brown due to leaf lesions (Smiley et al. 2005). It can be found in most cool- and warm-season grasses. The fungus's mycelium is tan to brown in culture and 4-15 µm in diameter. *R. solani* can survive as sclerotia found in plant debris or on the soil surface. The resumption of growth occurs in temperatures higher than 68°F (20°C).

Brown patch severity is often intensified by cultural practices, such as excessive irrigation, non-optimal mowing heights, and excessive fertilization (Fidanza and Dernoeden 1996). Brown patch severity can be increased by excessive N fertilization (Burpee 1995; Fidanza and Dernoeden 1996; Watkins and Witt 1993). The disease may be suppressed with proper turfgrass management, and a fungicide program is only needed when cultural practices are properly adjusted and the disease is still present. Managing soil fertility can suppress brown patch. The use of CGM at 99 g m^{-2} for weed control may increase brown patch severity. A lower rate of CGM at 49.5 g m^{-2} would meet spring N requirements, but likely provide minimal weed control. The use of preemergence herbicides at reduced rates, coupled with the use of reduced rates of CGM, could provide acceptable weed control while following recommended fertilizer rates.

Pendimethalin, oxadiazon, dithiopyr, and prodiamine are effective preemergence herbicides for crabgrass control in turf. All four herbicides are general use and sold to the general public. Pendimethalin (N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine) and prodiamine (N3-Ni-n-propyl-2,4-dinitro-6-(trifluoromethyl)-m-phenylenediamine) are

microtubule assembly inhibitors and belong to the dinitroaniline chemical family. Pendimethalin

has a pH of 7-8 and will solidify at approximately 0°C and has a boiling range of 85-100°C.

Vapor pressure is measured at 23.4 mbar (WSSA 2007). Pendimethalin has a molecular mass of

281.31 g/mol and is microencapsulated in an aqueous carrier (WSSA 2007). Prodiamine has a

solubility of 0.013ppm at 25°C in water. Prodiamine has a pH of 8-10 in a 1% suspension of

water and has a vapor pressure of <5.6x10(-6) mmHg at 68°C (WSSA 2007). For both

herbicides, toxicity is fairly low: oral LD_{50} is >5,000 mg/kg; inhalation LC_{50} is >5.23 mg/L for

pendimethalin and LC_{50} is >2.55 mg/L for prodiamine (4 hours of exposure resulted in no

mortality); and dermal LD_{50} is >5,000 mg/kg (WSSA 2007). For pendimethalin, no chronic

effects have been observed in humans, but pendimethalin has been observed to be acutely toxic

to fish and caution must be taken to prevent contamination of surface water. For prodiamine,

chronic toxicity has been observed. In rats, prodiamine produced benign thyroid tumors, caused

developmental and reproductive effects at 1 g/kg/day, created hormone imbalances in the

thyroid, decreased body weight, and enlarged and altered the liver (WSSA 2007). Additionally, it

is toxic to some aquatic animals and birds, but does not bioaccumulate, and is persistent and

immobile in soil.

Dithiopyr (S,S'-dimethyl 2-(difluoromethyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3,5-

pyridinedicarbothioate) belongs to the pyridine chemical family and acts as a microtubule

assembly inhibitor. Oxadiazon (2-tert-butyl-4-(2,4-dichloro-5-isopropoxyphenyl)-Δ-1,3,4-

oxadiazolin-5-one) belongs to the oxadiazole chemical family and acts as a protoporphyrinogen

oxidase (PPO) inhibitor. Both are emulsifible concentrates and do not readily dissolve in water

(WSSA 2007). Dithiopyr has a pH of 4.5. Oxadiazon has a pH of 6.8-7.8 in a 10% aqueous

solution. Both herbicides have slightly higher toxicity than the first two herbicides: oral LD_{50} is

>2,000 mg/kg and dermal LD$_{50}$ is >2,000 kg/kg (WSSA 2007). For dithiopyr, chronic toxicity effects on the liver, kidney, adrenal gland, thyroid, gall bladder, and blood have been discovered. Moreover, while the active ingredient was not shown to cause cancer, one of the solvents, naphthalene, was determined to be carcinogenic (WSSA 2007). Dithiopyr is relatively immobile in soil, whereas the solvents are more prone to leach. Both herbicides should not be applied to water and should not contact surface water (WSSA 2007). There is a restricted-entry interval of 24 hours for pendimethalin and a 12-hour restriction for the others. (Anonymous 2007; 2008; 2009a; 2009b).

Some research utilizing these herbicides has shown that reducing the application rate can be accomplished without a significant reduction in weed control. Reducing prodiamine rates to ¼ and ½ the recommended labeled rates resulted in 79 and 84% and 93 and 94% large crabgrass control in tall fescue in 1994 and 1993, respectively. Large crabgrass cover for non-treated plots in August was 59 and 89% for 1993 and 1994 (Johnson 1996). In the same research, reducing dithiopyr to ¼ and ½ the recommended labeled rates resulted in 7 and 11% and 16 and 24% large crabgrass control in tall fescue in 1994 and 1993, respectively, compared to 91 and 38% with a full rate (Johnson 1996). In other research, reduced rates of pendimethalin provided 92, 98, and 97% large crabgrass control for 1st, 2nd, and 3rd year applications in bermudagrass, and was insignificant from a full rate control of 96-100% (Johnson 1997a). Moreover, Johnson (1995) found 14 and 16% and 11 and 20% large crabgrass cover for a ½ rate of pendimethalin and oxadiazon in tall fescue in 1994 and 1993, respectively. For non-treated plots, large crabgrass cover was 33 and 38%, and cover greater than 10% was not deemed acceptable (Johnson 1995). At ⅓ the recommended labeled rate, oxadiazon and prodiamine provided approximately 80% and 93% control in one season, compared to full rate oxadiazon and prodiamine control of 86 and

99% in tall fescue (Johnson 1997a; Johnson 1997b). Coupling reduced herbicide rates with reduced CGM rates is an integrated approach that may provide adequate control with reduced herbicide and recommended N inputs. Gardner et al. (1997) showed that the combination of CGM and pendimethalin could provide sufficient control of large crabgrass. Using a ½ half of pendimethalin in conjunction with 49 g m^{-2} of CGM, the authors achieved 87% large crabgrass control in the greenhouse and 77% control in the field. Control of crabgrass using CGM alone at 49 g m^{-2} was 19-25%, depending on the location. CGM at 99 g m^{-2} controlled crabgrass 35 and 42%.

In some situations, it may not be economical to apply CGM as a granule and herbicides as liquids in separate applications. Impregnated herbicides on fertilizer are common ready-to-use products in turf and ornamental weed management. These fertilizer-herbicide products may allow for one or two granular applications in a season, reduced herbicide inputs, and prevent N over-fertilization. The objective of this research were to evaluate crabgrass control in cool-season turf using reduced rates of pendimethalin, prodiamine, oxadiazon, and dithiopyr impregnated on reduced rates of CGM. Additional research was conducted to assess the response of brown patch to increasing CGM applications in tall fescue turf.

Herbicides and Cover Crops in VA Corn

In 2011, corn was produced on 490,000 acres, with a 2011 production total of 40,120,000 bushels, bringing $274,822,000 to the state's economy (Virginia Ag Statistics 2012). The majority of corn producers plant transgenic crop varieties, rotated with soybean and wheat, and follow a recommended weed control program utilizing a residual preemergence herbicide with a

postemergence application of glyphosate (Hagood et al. 2012). Additionally, in lieu of winter wheat, some producers plant winter cover crops such as rye or other small grain and hairy vetch. Virginia producers who plant cover crops are offered financial incentives by local soil and water conservation districts for the purposes of offsetting some of the costs of cover crop planting. This incentive program promotes cover crop use and the specific cover crop benefits of nitrogen fixation (Isse et al. 1999) and a reduction in soil erosion (Dabney et al. 2001). The program is funded primarily because approximately 56% of Virginia falls within the Chesapeake Bay watershed (NRCS 2012), and nutrient sequestration and soil erosion are important factors in maintaining and fostering watershed preservation.

There are long and short-term benefits that can be derived from cover crops. Cover crops play an important role in reducing soil erosion (Dabney et al. 2001), and can improve soil structure and increase soil organic matter (Smith et al. 1987). Legume cover crops such as hairy vetch are recommended for their ability to fix nitrogen and their potential to reduce nitrogen inputs (Utomo et al. 1990). Rye is particularly successful as a weed control tactic due to its high biomass and allelopathic potential (Barnes and Putnam 1987; Smith et al. 2011; Weston 1990). The effectiveness of a rye cover crop for weed control is two-fold. It can actively compete against weeds during winter and early spring (Lemerle et al. 1995), but more importantly, rye can inhibit weed growth as a residue by providing a physical barrier to weed growth (Teasdale and Mohler 2000), by the release of allelochemicals that inhibit weed germination and growth (Barnes and Putnam 1987), and by light interception, thereby reducing weed seed germination (Teasdale and Mohler 1993).

These weed control benefits, however, come with a critical stipulation. Weed control resulting from cover crops is determined by the amount of cover crop residue on the soil surface.

There is an exponential inverse relationship between residue biomass and weed emergence (Teasdale and Mohler 2000). For Virginia producers planting corn in April, cover crop biomass levels are generally not high enough to provide effective weed control. For this reason, coupled with the possibility that cover crop residue might impede corn planting, most producers do not choose to use cover crops as a weed control tactic. Consequently, producers desiccate cover crops in sufficient time prior to corn planting to allow for almost complete residue decay. Allowing cover crops to grow until corn planting may offer producers the opportunity to reduce residual preemergence herbicide inputs while maintaining acceptable weed control with an integrated approach combining cover crop residue and reduced herbicide inputs.

Employing reduced herbicide rates is a common practice among producers (Hartzler 1993; Blackshaw et al. 2006). Research has shown that reducing herbicide rates can still provide adequate weed control or suppression. Using a ½ rate of bentazon reduced common cocklebur biomass and density from 710 g m^{-2} and 43 plants^{-2} in the weedy check to 222 g m^{-2} and 27 plants m^{-2} in plots receiving 0.6 kg ha^{-1} of bentazon (Buhler et al. 1993). Reduced herbicide rates can result in reductions in plant fitness, thereby increasing crop competiveness. Common cocklebur, velevetleaf, and jimsonweed were reported to have shorter statures and fewer leaves when treated with reduced rates of metribuzin (Weaver 1991). Moreover, reductions in plant fitness can also affect seed production. Zhang and Cavers (1994) found reduced fruit production and seed filling in common cocklebur treated with a ½ rate of bentazon. Reported control and suppression with reduced rates were often dependent on other factors, such as weed density (Pannell 1990), weed growth stage (Khan and Donald 1992), and environmental conditions such as humidity (Kudsk 1989). The use of reduced herbicide rate as a sole weed control tactic has associated problems such as herbicide resistance (Gressel 1995; Manalil et al. 2011) and

increasing weed seed banks (Blackshaw et al. 2006), and additional tactics, such as the use of

cover crops, could be integrated into the weed management programs to mitigate these problems.

The combination of cover crop mulch and preemergence herbicides may provide

acceptable weed control. Acceptable weed control was found with a combination of cover crop

residue with alachlor plus cyanazine in pumpkins and sweet corn (Galloway and Weston 1996).

The addition of alachlor plus cyanazine to cover crop residue significantly decreased weed dry

weight compared to cover crops alone. While looking at various cover crops and herbicide

program combination effects on soybean yield, Reddy (2001) found that cover crop residues may

provide enough early season weed suppression to reduce the need for preemergence herbicides.

Similar research was conducted with reduced preemergence herbicide rates in corn, finding that

the inclusion of preemergence herbicides did not significantly increase early season weed

suppression, but that a postemergence herbicide application was likely necessary for adequate

weed control (Teasdale 1993). The lack of necessity for preemergence herbicides in these

studies incorporating cover crop residue suggests that the inclusion of preemergence herbicides

at reduced doses may be used to increase weed control. It is important to note that both studies

had high cover crop biomass levels, an important factor in determining weed control (Teasdale

and Mohler 2000). Other research has shown that the combination of herbicides and cover crop

mulch can potentially have synergistic weed control effects (Teasdale et al. 2005). Using sub-

optimal rates of both hairy vetch residue and preemergence herbicide metolachlor, the authors

found that metolachlor activity was enhanced for smooth pigweed and common lambsquarters

control. They concluded that the synergism between the two tactics "could be explained by

carbohydrate deprivation of etiolated seedlings that prevented sufficient detoxification of

metolachlor at low rates." Seedlings growing under cover crop residue must partition much of

its available resources to growth in order to reach the residue surface that little is left to allow the

seedlings to detoxify low rates of metolachlor. This synergism was found in greenhouse

experiments where metolachlor was applied before residues were placed on the soil surface. The

authors warned that this synergism might not be found in field applications where cover crop

residue is already present on the soil surface.

Teasdale's concern reflects the pertinent issue of whether cover crop residue impedes a

preemergence herbicide application by acting as a physical barrier between the herbicide and the

soil surface.   There is evidence that cover crop residue may hinder preemergence herbicides

from reaching the soil surface or expose those herbicides to increased photodegradation or

volitization, thereby diminishing herbicide efficacy (Buhler 1992; Erbach and Lovely 1975;

Kells et al. 1980; Locke and Bryson 1997). Reduced green foxtail control was seen when

comparing tillage systems and herbicide combinations and was attributed to corn residue on the

soil surface intercepting the herbicide (Buhler 1992). Other research concluded that plant

residue on the soil surface did not impact atrazine treatment at a full rate, but when rates were

reduced, increasing residue levels resulted in poorer weed control (Erbach and Lovely 1975). In

contrast, Gaston et al. (2001) found that hairy vetch cover crops slowed the release of herbicide

into the soil and expanded the length of acceptable weed control.

Most investigations in cover crops for early season weed suppression conclude that,

while cover crops are effective at reducing weed pressure, they do not eliminate the need for

preemergence herbicides (Yenish et al. 1996; Reddy 2001; Teasdale 1996). This inconsistency

in control is due to the inherent variability that comes with cover crop growth. Cover crop

biomass levels are highly dependent on soil conditions, climatic conditions, and soil moisture.

Relying on cover crops as a singular weed control tactic may not provide sufficient control, and integrating other tactics into a weed management program can remove some of that risk.

For Virginia producers, integrated weed management utilizing cover crops and herbicides is of pertinent interest. With the risk of herbicide resistance and the rising costs of production agriculture, coupled with the fluctuations and instability in the commodities market, producers find that utilizing multiple weed control tactics gives them cost-effective and reliable weed control. Therefore, the objective of this research were to determine if rye or a rye/ hairy vetch biculture, desiccated at three different dates prior to corn planting can provide enough supplemental weed suppression to allow the use of reduced preemergence herbicide rates.

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1b. Herbicide Resistance Development in Agronomic Crops and Johnsongrass (*Sorghum halepense*)

Herbicide Resistance

Herbicides are the world's most effective means for managing weeds (Zimdahl 1999). In 2007, there was a $40 billion global pesticide market with herbicides accounting for 39% of expenditures. This equates to approximately 951 million kilograms of herbicide active ingredient applied (EPA 2007). There are over 200 registered herbicide active ingredients in the world (WSSA 2013), and the majority can be categorized in one of twenty-five known mechanisms of action (MOA) (WSSA 2007; Heap 2013). An herbicide's MOA is the plant process inhibited by the herbicide that results in plant death (Cobb and Reade 2010). A summary of most herbicide MOAs as referenced by the Weed Science Society of America (WSSA) is provided below.

Table 1: Listing and descriptions of herbicide modes of action (WSSA 2007).

Group	MOA	Description of Inhibition
1	ACCase Inhibitor	ACCase is an enzyme essential for lipid biosynthesis, and the inhibition of the enzyme leads to plant death due to its inability to make new cell membranes. ACCase herbicides are only used for grass weed species, as most dicots have insensitive ACCcase (Burton 1989).
2	ALS Inhibitor	Inhibition of ALS prevents the production of branch chained amino acids leucine, valine, and isoleucine (LaRossa and Schloss 1984).
5, 6, 7	Photosystem II Inhibitor	Inhibits various processes related to photosynthesis. By binding to the D1 protein in the photosystem II complex, the disruption in photosynthesis leads to the creation of singlet oxygen and cell death via lipid

		peroxidation (Devine et al. 1993).
22	Photosystem I Inhibitor	Herbicides that accept electrons in photosystem I, leading to the creation of hydroxyl radicals and cell death via lipid peroxidation (Dodge 1982).
14	PPO Inhibitor	Inhibits protoporphyrinogen oxidase, leading to an accumulation of protoporphyrin IX (PPIX). Plants reacting to an over-accumulation of PPIX create singlet oxygen and causes plant death via lipid peroxidation (Duke 1991).
12, 28, 11	Carotenoid Biosynthesis Inhibitor	Carotenoids are essential in protecting plants from singlet oxygen generated by photosystem II. In plants treated by carotenoid biosynthesis inhibitors, singlet oxygen is not readily dissipated and leads to lipid peroxidation and cell death (Bartels and Watson 1978; Sandman and Böger 1989).
9	EPSP Synthase Inhibitor	Glyphosate inhibits 5-enolpyruvyl-shikimate-3-phosphate synthase, an enzyme responsible for the production of aromatic amino acids (Amrhein 1980).
10	Glutamine Synthetase Inhibitor	Inhibition of glutamine synthetase prevents the conversion of glutamate and ammonia to glutamine. The accumulation of ammonia causes plant death (Lea 1984; Tachibana 1986).
18	Dihydropteroate Synthase Inhibitor	Asulam inhibits 7,8-dihydropteroate synthase, an enzyme for folic acid production. It also plays a role in inhibiting cell division in plant meristems (Fedtke 1982; Kidd et al. 1982).
3, 23,15	Mitosis Inhibitor	Inhibits mitosis by either binding to tubulin, inhibiting cell division, or inhibiting very long chain fatty acid synthesis (Vaughn and Lehnen 1991).
20, 21, 27	Cellulose Inhibitor	Inhibits cell wall biosynthesis (Heim et al. 1990).
24	Oxidative Phosphorylation Uncoupler	Dinoterb uncouples the process of oxidative phosphorylation leading to subsequent membrane destruction (WSSA 2007)
8, 16, 26	Fatty Acid and Lipid Biosynthesis Inhibitor	The inhibition of fatty acid and lipid biosynthesis affects cuticle production, gibberellin production, and the production of other proteins and flavonoids (WSSA 2007).
4	Synthetic Auxins	Mimics endogenous auxin (IAA), causing uncontrolled and random growth that eventually leads to plant death. (WSSA 2007)
19	Auxin Transport Inhibitor	Inhibits IAA transport and concentrates IAA in meristematic shoots and roots, leading to uncontrolled growth and plant death. (WSSA 2007)

An understanding of available herbicide MOAs is essential in light of one of agriculture's most pressing issues: herbicide resistance (WSSA 2012). Herbicide resistance is defined by the WSSA as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type… In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis" (WSSA 2013). The first reported case of herbicide resistance in a weed was wild carrot (*Daucus carota* L.) to 2,4-D in 1957 (Whitehead and Switzer 1967). At the time of this writing, there are over 400 herbicide resistant biotypes, representing 217 plant species and twenty-one herbicide MOAs (Heap 2013). Herbicide resistant weeds can now be found in sixty-one countries and the United States leads with 143 different herbicide resistant weed biotypes (Heap 2013). ALS inhibiting herbicides are responsible for more resistant biotypes than any other MOA, primarily due to the fact that there are eight possible target-site mutations that confer ALS resistance (Tranel et al. 2013). In the state of Virginia, there are eleven reported herbicide resistant biotypes from four herbicide MOAs (Heap 2013).

There are numerous factors that influence herbicide resistance that can be grouped into four categories: genetic, plant biology, herbicidal, and operational (Norsworthy et al. 2012; Powles and Yu 2010). Genetic influences generally refer to the presence and dominance of resistance genes in a given plant population, whether the expression of those genes is revealed under herbicide pressure, and whether expressing those genes is worth the fitness costs in subsequent generations (Powles and Yu 2010). In the context of plant biology, phenotypic plasticity and a weed's reproductive abilities and seed capabilities are also important in influencing herbicide resistance, specifically seed viability and dispersal and means of pollination (self vs. cross-pollinated) (Powles and Yu 2010). Moreover, the use and rotation of

herbicide MOAs and herbicide application tactics play a considerable role in herbicide resistance (Vencill et al. 2012; Norsworthy et al. 2012). Using a single herbicide MOA over an extended period of time will select plants that express herbicide resistance by removing susceptible plants and allowing plants resistant to the herbicide MOA to propagate. Herbicide resistance can also be exasperated by inadequate spray applications that result in reduced herbicide rates, improper coverage, or inappropriate herbicide selection for the targeted weed (Vencill et al. 2012).

The development of efficacious herbicides and transgenic cropping technology allowing specific herbicides to be applied in tolerant crops has drastically changed herbicide use and application tactics (Young 2006; Brooks and Barfoot 2011). With the introduction of herbicide-resistant crops, the use of multiple MOAs decreased because complete weed control could now be achieved with one MOA without risk of crop injury (Young 2006). The reliance on one herbicide MOA created a selection pressure that has, and will continue to, perpetuate the risk of herbicide resistance (Beckie 2006; Powles et al 1997). Where one herbicide MOA is consistently applied, weeds have evolved resistance to those herbicides (Vencill et al. 2012). The use of multiple MOAs in any given cropping system is a critical component in maintaining herbicide efficacy and mitigating future cases of herbicide resistance (Norsworthy et al. 2012).

There are several known molecular mechanisms of herbicide resistance. Target-site mutations within the plant's DNA can confer herbicide resistance. These mutations, in the form of amino acid substitutions, are found at the site of herbicide action, and often, but not always, result in high-level herbicide resistance (Avila-Gracia et al. 2012; Tranel et al. 2013). Another mechanism of resistance is metabolic deactivation, where a plant's inherent metabolism has the capacity to break down an herbicide into non-toxic metabolites (Anderson and Gronwald 1991; Carey et al. 1997). Reduced absorption and translocation are also known to confer herbicide

resistance. This mechanism prevents the absorption of the herbicide into the plant and/ or reduces the movement of systemic herbicides to susceptible plant parts (Riar et al. 2013; Dilpreet et al. 2011; Nandula et al. 2008; Powles and Preston 2006). Other herbicide resistance biotypes have evolved the ability to sequester herbicides into the vacuole or cell walls and prevent the movement of herbicide (Ge et al. 2010). Most recently, gene amplification in resistant biotypes was found to diminish the herbicide's relative toxicity via overproduction of targeted protein(s) (Gaines et al. 2010; Salas et al. 2012).

Herbicide resistance has numerous consequences. Of primary concern are yield loss (Legleiter et al. 2009) and weed seed contamination (Llewellyn and Allen 2006), increased production costs (Beckie et al. 1999; Norsworthy et al. 2007; Mueller et al. 2005), and the production changes that are required to combat herbicide resistance, such as the inclusion of tillage, crop rotations, or alterative weed management strategies (Norsworthy et al. 2012; Smith et al. 2011). Legleiter et al. (2009) saw a 23-24% soybean yield decrease due to glyphosate-resistant waterhemp (*Amarenthus rudis*). Weed interference and yield loss is well documented (Hall et al. 199; Van Acker et al. 1993; Weaver and Tan 1983; Zimdahl 1988). Managing herbicide resistant wild oat (*Avena fatua*) in small grains cost producers $4 million annually, increasing the cost of production from $0.75/ha - $22.78/ha, depending on the given crop and alternative herbicide options (Beckie et al. 1999). Norsworthy et al. (2007) suggested that it took an additional $65/ha to control propanil and quinclorac-resistant barnyardgrass in rice. Glyphosate-resistant horseweed (*Conyza canadensis*) increased herbicide costs $30.46 ha^{-1} in soybean, glyphosate-resistant spiderwort increased herbicide costs $35.07 ha^{-1} in cotton, and glyphosate-resistant common waterhemp could potentially increase costs $44.25 ha^{-1} in corn and soybean (Mueller et al. 2005). Utilizing multiple weed control tactics, such as tillage, cover

crops, crop and MOA rotations, while critical for the long-term sustainability of herbicides,

require time and equipment and can present additional challenges in addition to concerns

regarding resistance (Norsworthy et al. 2012).

Herbicide Resistance in Glyphosate and Nicosulfuron

Two herbicides commonly used in field corn are nicosulfuron and glyphosate.

Glyphosate is considered the world's most important herbicide, a "once-in-a-century" herbicide

(Duke and Powles 2008). It is the primary herbicide used in 170.3 million hectares of biotech

crops planted worldwide (James 2012). It is also an important herbicide in orchards and forestry.

Glyphosate is a broad-spectrum, post-emergence herbicide that was first synthesized in 1950 by

the Swiss pharmaceutical company Cilag (Franz et al. 1997). Finding no pharmaceutical use for

the compound, glyphosate was never reported in pharmaceutical literature. In the early 1970s,

Monsanto was synthesizing compounds for use as potential water-softening agents. Two

synthesized compounds expressed low herbicidal activity. Monsanto chemists proposed that the

compounds were metabolized differently in the plants and decided to synthesize possible

metabolites; glyphosate was the third compound synthesized (Halter 2007). Glyphosate was first

commercialized as Roundup® in 1973, and given its nonselective nature, its use was restricted to

pre-plant "burndown" applications, weed control in fallow fields, and spot treatments along

roadways and irrigation ditches. The advent of transgenic crop technology, which allowed for

certain crop species including corn, soybean, cotton, and canola to tolerate glyphosate, permitted

this herbicide to be sprayed over the crops, revolutionizing modern agriculture. Glyphosate is

registered in 130 countries and has over 160 annual and perennial weeds labeled for control

(Research and Markets 2008). Of the sixty-nine million hectares planted in corn, soybean, and cotton in the USA, approximately 77% is planted with glyphosate-resistance crops (Brooks and Barfoot 2011).

Glyphosate's MOA is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSPS) (Amrhein et al. 1980). EPSPS is only found in plants, bacteria, and fungi (Kishore and Shah 1988) and is part of the shikimate pathway, which is responsible for the production of aromatic amino acids, specifically tryptophan, tyrosine, and phenylalanine. Aromatic amino acids are partly responsible for the biosynthesis of lignin and auxin secondary products. Visual symptomology includes foliar chlorosis in immature leaf tissue within 10-20 days for most weed species, followed by eventual necrosis and plant death. Glyphosate is moderately absorbed by plant tissue and can interfere with its own absorption if spray droplets are too large and cell damage at the site of contact occurs before absorption is complete (Prasad and Cadogan 1992). Given glyphosate's MOA, translocation primarily occurs via symplast towards meristematic tissues (Sprankle et al. 1973). Apoplast translocation has been reported in tall morningglory species (Dewey and Appleby 1983). Glyphosate is rapidly bound to soil and adsorption is dependent on vacant phosphate sorption sites (Giesy et al. 2000). Microbial degradation during the growing season can vary depending on climatic and soil conditions, with 10-70% of the chemical being transformed into CO_2 (Franz et al. 1997). Glyphosate's half-life is approximately 47 days (Wauchope et al. 1992).

Glyphosate is essentially non-toxic to animals, given its MOA involving an enzyme found only in plants, fungi, and bacteria (Kishore and Shah 1988). Some glyphosate formulations have different surfactants that may cause various degrees of toxicity (Folmar et al. 1979). Recent toxicity studies have primarily focused on aquatic organisms, which are generally

more sensitive to herbicidal chemicals than other organisms (Dill et al. 2010). Research has reported that the lowest LC_{50} for a fish species is 1.7mg a.e. L^{-1} (Edginton et al. 2004). In amphibians, Edginton et al. (2004) found the lowest LC_{50} for *Xenopus laevis* was 0.88 mg a.e. L^{-1}. Research has reported that glyphosate concentration was highest in 51 water bodies in the U.S. Corn Belt region at 0.0000087mg a.e. L^{-1}, with 95% of all monitored concentrations falling between 0.00000045 and 0.000001.5mg a.e. L^{-1} (Scribner et al. 2003). Monitoring results from Ontario, Canada reported that 99% of all samples fell under 0.000021mg a.e. L^{-1} (Struger et al. 2008). Glyphosate concentrations, in some cases, are over 1000 fold less than the lowest LC_{50} for some species. In all cases, glyphosate concentrations in water bodies did not exceed EPA standards nor pose a risk to sensitive aquatic organisms (Edginton et al. 2004).

Glyphosate resistance has been documented in eighteen different weed species in sixteen countries (Heap 2013). The first documented case of glyphosate resistance was rigid ryegrass (*Lolium rigidum*) near Orange, New South Wales, Australia in 1997. The orchard with resistant rigid ryegrass had received two or three annual applications of glyphosate for fifteen years (Pratley et al. 1996). There are three known mechanisms of glyphosate resistance in weeds. Some species have evolved decreased glyphosate absorption and translocation and/ or vacuolar sequestration (Dinelli et al. 2008; Koger and Reddy 2005; Feng et al. 2004; Ge et al. 2010; Perez-Jones et al. 2007; Nandula et al. 2008; Lorraine-Colwill et al. 2003; Wakelin et al. 2004). In others, an amino acid substitution in the genomic coding region for EPSPS has been observed (Baerson et al. 2002; Ng et al. 2003,2004; Perez-Jones et al. 2007; Jasieniuk et al. 2008; Wakelin and Preston 2006; Yu et al. 2007; Simarmata and Penner 2008). Research has shown that resistant biotypes expressing this mechanism of resistance have substituted Pro_{106} for Ser, Thr, or Ala (Baerson et al. 2002; Ng et al. 2003,2004; Kaundan et al. 2008; Perez-Jones et al. 2007;

Jasieniuk et al. 2008; Wakelin and Preston 2006; Yu et al. 2007; Simarmata and Penner 2008). It

is theorized that the amino acid substitution changes the structure of the α-helix (Stallings et al.

1991; Zhou et al. 2006). This would reorient the target site of glyphosate and reduce glyphosate

binding (Zhou et al. 2006). Most recently, Jalaludin (2013) discovered a double amino acid

substitution (Thr_{102} and Pro_{106}) in *Eleusine indica*, conferring significantly increased resistance to

glyphosate. And, in two weed species, increased gene amplification and expression of the

EPSPS was discovered (Gaines et al. 2010; Salas et al. 2012). Mechanisms of resistance have

not been determined for all resistant weed species, leaving the possibility that new mechanisms

of resistance have yet to be observed.

Nicosulfuron, 2-[[[[4,6-dimethoxy-2-pyrimidyl)amino]carbonyl]amino]sulfonyl]-N,N-

dimethyl-3-pyridinecarbozamide], is an ALS inhibiting herbicide, a member of the sulfonylurea

family, and is a selective post emergence herbicide used in corn (Camacho et al. 1991). It

controls a variety of weeds, most importantly annual and perennial grass weeds. Nicosulfuron

inhibits acetolactate synthase, the first enzyme in the biosynthesis of valine, leucine, and

isoleucine (LaRossa and Schloss 1984). Like EPSP synthase, ALS is localized in the

chloroplast, and thus only found in photosynthetic organisms and carries low mammalian

toxicity (LaRossa and Schloss 1984). Addtionally, LaRossa and Schloss (1984) discovered that

the reaction sequence of ALS is highly conserved across several plant species. The authors

concluded that a pyruvate molecule binds to thiamine pyrophosphate, yielding an enzyme-

substrate complex and CO_2 and a second pyruvate molecule reacts with the complex releasing

acetolactate. Herbicides that inhibit ALS prevent the second pyruvate molecule from binding.

Symptomology of nicosulfuron includes the cessation of plant growth after a few hours

of application, followed by chlorotic and necrotic injury 1-2 weeks after application (WSSA

2007). Acute toxicity (oral LD_{50}) is greater than 5,000 mg kg^{-1} in rats. Daphnia has a 48-h LC_{50} greater than 1000 ml L^{-1} (WSSA 2007). In chronic exposure, nicosulfuron is neither oncogenic nor teratogenic, and had minimal effects on weight gain and litter size in rat reproduction studies (WSSA 2007). The half-life of nicosulfuron is 21 days (Augustijn-Beckers et al. 1994).

Nicosulfuron resistance has been documented in thirty-three biotypes across the world (Heap 2013). Where the molecular mechanism of nicosulfuron resistance has been documented, target site mutations are the mechanism of resistance. Target-site mutations in late watergrass (*Echinochloa orzicola*) confer cross-resistance to ALS inhibiting herbicides, including nicosulfuron (Kalaoumenos et al. 2013; Volenberg et al. 2001). Cross-resistance has been documented in other weed species as well (Osuna et al. 2003; Volenberg et al. 2001). Metabolic deactivation has also been suggested as a mechanism of resistance for ALS herbicides (Yu et al. 2009). In the state of Virginia, nicosulfuron resistance has been documented in shattercane (*Sorghum bicolor*) (King and Hagood 2006).

Johnsongrass and Herbicide Resistance in Johnsongrass

Johnsongrass (*Sorghum halepense* (L.) Pers.) is a troublesome weed in production agriculture (Webster and Nichols 2012). It is a member of the Poaceae family and originates from the Middle East. It was introduced as a forage crop and now can be found throughout most of the United States (Uva et al. 1997). It is a perennial plant with thick persistent rhizomes, can grow three to five meters tall, and can reproduce both vegetatively and via prolific seed dispersal (Uva et al. 1997). It is commonly found in cultivated fields, pastures, railroad and road sides, open woodlands, and waste sites, all of which are typically disturbed areas (Bryson and DeFelice

2009). In cultivated fields, johnsongrass is common in reduced tillage operations due to its

perennial nature and is known to spread with the use of farm equipment (Uva et al. 1997). It is

an aggressive competitor because of its reproduction capabilities, high biomass production, and

its ability as a C_4 plant to efficiently utilize available resources (Dilpreet et al. 2011; Sage and

Pearcy 1987). With the rise of herbicide technology, several efficacious control options were

made available for johnsongrass control in agronomic crops. However, johnsongrass has also

developed resistance to several herbicides and has become a serious concern in many parts of the

world.

Johnsongrass has been shown to reduce crop yield in major agronomic cropping systems.

Williams and Hayes (1984) found that full-season johnsongrass competition reduced soybean

yields up to 88%.   In cotton, 32 plants - 9.8m of cotton row resulted in 70% yield loss (Bridges

and Chandler 1987). In corn, after five years of increased johnsongrass populations in

monocultured corn plots, grain yield dropped to zero (Bendixen 1986). Similar yield reductions

have been seen when comparing soybean cultivars (McWhorter and Hartwig 1968). Such yield

loss translates into economic loss. In the states of Arkansas, Louisiana, and Mississippi,

johnsongrass reduced annual cotton profits by $5.9±1.9 and soybean profits by $23.7±0.6 million

dollars (McWhorter 1993). An additional concern is the indirect impact of johnsongrass as a

host and reservoir for various maize viruses (Bendixen 1986; Eberwine and Hagood 1995). In

grain sorghum, johnsongrass presents a unique concern with its ability to cross-pollinate and

hybridize (Arriola and Ellstrand 1996). Other non-agronomic impacts include infestations of

conservation lands. In Alabama and Mississippi, recently converted conservation lands often

become dominated by johnsongrass and the seed produced offered little to no value to wildlife.

Land managers managing for wildlife conservation and diversity found that johnsongrass

affected northern bobwhite quail populations. Attempts at mowing and replanting with legumes led to decreases in johnsongrass presence (Arner et al. 1993).

Herbicide options for controlling johnsongrass are specific to the cropping system. In soybean, johnsongrass can be controlled using clethodim, sethoxydim, and other graminicides (Hagood 2011). Control options in corn are nicosulfuron, primisulfuron, and rimsulfuron, as well as sethoxydim in tolerant varieties (Hagood 2011). In glyphosate and glufosinate-tolerant corn varieties, glyphosate and glufosinate are used to control the weed. All of these products are applied postemergence, as most preemergence products do poorly for johnsongrass control because of johnsongrass's perennial nature. For effective herbicide applications, an understanding of weed-free period is important for postemergence herbicides. The weed-free period is the period of time in which a crop must grow under weed-free conditions in order to meet its yield potential (Zimdahl 1988). In soybean, research has shown that for johnsongrass, the weed-free period was four weeks after planting and that soybean could not tolerate more than five weeks of heavy infestations (Williams and Hayes 1984). However, in cotton, that weed-free period was not sufficient and resulted in significant yield reductions (Bridges and Chandler 1987). In corn, the weed-free period was 3.5- 6 weeks after corn planting (Ghosheh et al. 1996). Utilizing this knowledge as a control tactic allows for the judicious application of herbicides, thus providing another tool for decreasing the risk of herbicide resistance.

The repeated use of a single herbicide for johnsongrass control brings the risk of resistance. As previously mentioned, herbicide resistance develops with the exclusive use of specific herbicides, creating a selection pressure for biotypes expressing resistant genes. To date, there are eighteen confirmed johnsongrass biotypes resistant to various herbicides (Heap 2013). Most of these biotypes can be found in North America, but some can also be found in Central

and South America and Europe (Heap 2013). Records confirm johnsongrass resistance to glyphosate, nicosulfuron, propaquizafop, quizalofop, fluazifop, haloxyfop, foramsulfuron, primisulfuron, rimsulfuron, fenoxaprop, pendimethalin, sethoxydim, clethodim, imazethapyr, and iodosulfuron (Heap 2013). Most of these cases involve three modes of action: ACCcase inhibitors, ALS inhibitors, and EPSPS inhibition (Heap 2013). ALS resistance is likely due to an amino acid substitution, though none have been confirmed in johnsongrass (Tranel et al. 2013). ACCase resistance is conferred via target-site resistance and has been shown to spread via pollen (Burke et al. 2006; Burke et al. 2007; Tardif and Powles 1993). For glyphosate resistance, reduced absorption and translocation were confirmed to confer herbicide resistance to johnsongrass (Riar et al. 2011; Vila-Aiub et al. 2012).

In Virginia, a suspect johnsongrass population believed to be resistant to both nicosulfuron and glyphosate was found in Rockingham County. The objectives of this research were to confirm herbicide resistance to nicosulfuron and glyphosate and to determine the molecular mechanisms conferring resistance to those herbicides.

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Chapter 2: Effects of Rye and Hairy Vetch Cover Crops on Preemergence Herbicide Inputs for Weed Control in Field Corn

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Abstract

Rye and hairy vetch are common cover crops that can be planted for erosion control, soil fertility, or weed control in no-tillage production systems. Corn producers in Virginia plant small grain winter cover crops for erosion control and some utilize the addition of hairy vetch to increase nitrogen levels in the soil. Most producers desiccate these cover crops early, allowing for minimal residue levels that do not impede corn planting. If allowed to grow, rye and hairy vetch cover crops may produce more residues and provide supplemental weed suppression that might allow for a reduction in herbicide inputs without a loss in weed control or crop yield. Experiments were conducted in Virginia to determine if rye and rye/ vetch cover crops provide enough additional weed suppression to allow reduced herbicide rates. Six different herbicide combinations were applied in three different cover crop residue levels. Cover crop residue levels were determined by desiccating cover crops at 20 days early pre-plant (EPP), 10 EPP, or at corn planting. A three-way mix of atrazine, s-metolachlor, and mesotrione was applied at a full rate

(0.7+1.87+0.187 kg a.i. ha^{-1}, respectively) or at a half rate (0.35+0.935+0.0935 kg a.i. ha^{-1}, respectively). Atrazine + simazine was applied at a half rate (1.12+1.12 kg a.i. ha^{-1}, respectively). Both half-rate preemergence (PRE) treatments were applied either alone or in sequence with a postemergence (POST) glyphosate treatment at a rate of 0.88 kg a.e. ha^{-1}. The PRE full rate treatment and the glyphosate POST treatment were also applied alone. At corn planting, rye residue levels ranged from 0 to 5,889 kg ha^{-1} and rye/ vetch residue levels ranged from 0 to 4,309 kg ha^{-1}. At most locations, residue levels were significantly higher when cover crop desiccation occurred at the latest date, corn planting. At 4 weeks after treatment (WAT), cover crop type and cover crop desiccation date played no role in early-season weed suppression. At most locations, a full rate of atrazine + metolachlor + mesotrione provided the best weed control, but control was not significantly different from control with the half rate of atrazine + metolachlor + mesotrione or with the half rate of atrazine + simazine. At 12 WAT, all treatments containing glyphosate POST provided significantly greater weed control. For NK-11 and M1-11, weed control ranged from 97-100% from treatments utilizing glyphosate. Corn yield increased with the use of rye/ vetch cover crops over rye cover crops. Corn yields in rye/ vetch averaged 7,646 kg ha^{-1} and in rye averaged 6,805 kg ha^{-1}. A nitrogen effect from rye and vetch is the likely factor in increasing corn yield when compared to rye alone. With respect to herbicide treatments, few yield differences were observed with herbicide treatment and cover crop desiccation date. In a few instances, the addition of PRE herbicides followed by POST glyphosate had higher yields than POST glyphosate applied alone. These data suggest that the addition of PRE herbicides, integrated with rye/ vetch cover crops, and followed by a POST application could provide sufficient weed control, competitive corn yield, and could decrease expenditures by decreasing inputs.

Nomenclature: glyphosate; atrazine; mesotrione; s-metolachlor; simazine; corn, *Zea mays* L.; rye, *Secale cereale* L.; hairy vetch, *Vicia villosa* Roth.

Key Words: preemergence herbicides, tank-mixed, winter cover crops.

Introduction

Corn (*Zea mays* L.) production in Virginia currently is 198,300 ha, with a 2011 production total of 40,120,000 bushels, bringing $274,822,000 to the state's economy (Virginia Ag Statistics 2012). The majority of corn producers plant transgenic crop varieties, rotated with soybean and wheat, and follow a recommended weed control program utilizing a residual preemergence herbicide with a postemergence application of glyphosate (Hagood et al. 2012). Some producers, in lieu of wheat, may plant winter cover crops such as rye (*Secale cereal* L.) or other small grain and hairy vetch (*Vicia villosa* Roth). Virginia producers who plant cover crops are offered financial incentives by local soil and water conservation districts for the purposes of offsetting some of the costs of cover crop planting. This promotes cover crop use and the specific cover crop benefits of nitrogen fixation (Isse et al. 1999) and a reduction in soil erosion (Dabney et al. 2001). This is funded primarily because approximately 56% of Virginia falls within the Chesapeake Bay watershed (NRCS 2012), and nutrient sequestration and soil erosion are important factors in maintaining and fostering watershed preservation.

There are other long and short-term benefits that can be derived from cover crops. Cover crops can improve soil structure and increase soil organic matter (Smith et al. 1987). Legume cover crops such as hairy vetch are recommended for their nitrogen fixation ability and the potential to reduce nitrogen inputs (Utomo et al. 1990). Rye is particularly useful as a weed control tactic due to its high biomass and allelopathic potential (Barnes and Putnam 1987; Smith et al. 2011; Weston 1990). These weed control benefits, however, come with a critical stipulation. Weed control resulting from cover crops is determined by the amount of cover crop residue on the soil surface. There is an exponential inverse relationship between residue biomass and weed emergence (Teasdale and Mohler 2000). For producers planting corn, cover crops

desiccated prior to planting may not have biomass levels high enough to provide effective weed control (Teasdale and Mohler 2000; Wagger 1989). For this reason, coupled with the possibility that cover crop residue might impede corn planting, most producers do not choose to use cover crops as a weed control tactic. Consequently, producers desiccate cover crops in sufficient time prior to corn planting to allow for almost complete residue decay. Allowing cover crops to grow until corn planting may offer producers the opportunity to reduce residual preemergence herbicide inputs while maintaining acceptable weed control with an integrated approach combining cover crop residues and reduced herbicide inputs.

Employing reduced herbicide rates is common practice among producers (Hartzler 1993; Blackshaw et al. 2006). Research has shown that reducing herbicide rates can still provide adequate weed control or suppression. Using a ½ rate of bentazon reduced common cocklebur biomass and density from 710 g m^{-2} and 43 plants^{-2} in the weedy check to 222 g m^{-2} and 27 plants m^{-2} in plots receiving 0.6 kg ha^{-1} of bentazon (Buhler et al. 1993). Common cocklebur, velvetleaf, and jimsonweed were reported to have shorter statures and fewer leaves when treated with reduced rates of metribuzin (Weaver 1991) and Zhang and Cavers (1994) found reduced fruit production and seed filling in common cocklebur treated with a ½ rate of bentazon. However, use of reduced herbicide rate as a sole weed control tactic has associated problems including herbicide resistance (Gressel 1995; Manalil et al. 2011) and increasing weed seed banks (Blackshaw et al. 2006). Additional tactics, such as the use of cover crops, could be integrated into the weed management programs to mitigate these problems.

The combination of cover crop residue and preemergence herbicides may provide acceptable weed control. Acceptable weed control was found with a combination of cover crop residue with alachlor + cyanazine in pumpkins and sweet corn (Galloway and Weston 1996).

While looking at various cover crops and herbicide program combination effects on soybean yield, Reddy (2001) found that cover crop residues may provide enough early season weed suppression to reduce the need for preemergence herbicides. Similar research was conducted with reduced preemergence herbicide rates in corn, and showed that the inclusion of preemergence herbicides did not significantly increase early season weed suppression, but that a postemergence herbicide application was likely necessary for adequate weed control (Teasdale 1993).

Most research involving cover crops for early season weed suppression has concluded that, while cover crops are effective at reducing weed pressure, they do not eliminate the need for preemergence herbicides (Yenish et al. 1996; Reddy 2001; Teasdale 1996). This is due to the inherent variability that comes with cover crop growth. Relying on cover crops as a singular weed control tactic may not provide sufficient control, and integrating other tactics into a weed management program can remove some of that risk. Most of the research reported involving reduced doses of herbicides and cover crops is done with single mode of action herbicides. Little research has been conducted with pre-mix or tank-mixed herbicide options. Having multiple modes of action at reduced rates may reduce the risk of escaped weeds and the potential onset of herbicide resistance.

Virginia producers have expressed interest in knowing if pre-mixed or tank-mixed preemergence herbicide options at reduced rates can be combined with cover crops to provide acceptable weed control (Davis, personal communication). Moreover, VA producers are interested in knowing when to desiccate cover crops and whether increased residue levels will affect corn planting. The objectives of this study were to determine if rye or a rye/ hairy vetch

biculture, desiccated at three different dates prior to corn planting could provide enough supplemental weed suppression to allow the use of reduced rates of preemergence herbicide.

Material and Methods

Field experiments were established in 2010 and 2011 in New Kent, Virginia and Blacksburg, Montgomery County, Virginia. The soil type in New Kent was a Pamunkey sandy loam (fine-loamy, mixed, thermic, Ultic Hapladalfs). The soil types in Blacksburg were a Unison loam (fine, mixed, semiactive, mesic Typic Hapludults) and a Groseclose loam (fine, mixed, semiactive, mesic Typic Hapludults). Experiments conducted in 2010 in New Kent are designated NK-10 and experiments conducted in 2011 in New Kent and Montgomery County are designated NK-11, M1-11, and M2-11, respectively. Rye ('Wren Abruzzi') and hairy vetch cover crops were planted at all locations in October of each year at a seeding rate of 67.2 kg ha^{-1} and 11.2 kg ha^{-1}, respectively. Cover crops were planted using a no-till drill with 15.2 cm spacing. No fertilizer was applied to cover crops.

Cover crops were terminated at 20 days early pre-plant (EPP), 10 EPP, or at corn planting using 2,4-D and glyphosate or paraquat at 0.53, 0.88, or 0.77 kg a.i. ha^{-1}, respectively. This corresponded to three different cover crop biomass levels, with cover crops terminated at 20 EPP having less biomass than cover crops terminated at corn planting (Figure 2). Glyphosate-tolerant corn varieties were planted at 64,220 seed ha^{-1} with starter fertilizer (44.8 kg N ha^{-1}). Individual plots consisted of four rows planted at 76.2 cm row spacing and 7.6 m in length, where the center 1.83 m received herbicide treatment.

Preemergence herbicides were applied at corn planting and postemergence glyphosate was applied 4 WAP. Herbicide treatments were applied using a CO_2-powered backpack sprayer delivering 210 L ha^{-1} of spray solution at 234 kPa. Six herbicide treatments were compared to a non-treated check. A three-way mix of atrazine, s-metolachlor, and mesotrione (AMM) was applied at a full rate (0.7+1.87+0.187 kg a.i. ha^{-1}, respectively) or at a half rate (0.35+0.935+0.0935 kg a.i. ha^{-1}, respectively). Atrazine + simazine (AS) tank-mixed was applied at a half rate (1.12+1.12 kg a.i. ha^{-1}, respectively). Both half-rate preemergence treatments were applied either alone or in sequence with a postemergence glyphosate treatment at a rate of 0.88 kg a.e. ha^{-1}. The preemergence full rate treatment and the glyphosate postemergence treatment were also applied alone.

At corn planting, cover crop biomass was estimated by cutting all vegetation from a 0.25-m^2 quadrat from each sub-plot, providing four samples per cover crop type and desiccation date at each location. Plant material was dried at 60° C for 72 hours to derive total cover crop biomass at each site. At 4 WAT, weed counts and visual estimates of weed control were taken. Weed counts and/ or visual weed ratings were taken monthly until corn harvest in September. Weed counts were measured using 0.25-m^2 quadrats randomly placed in each plot. All weeds within that quadrat were identified and counted. Visual weed control was taken based on % weed cover compared to the non-treated check (NTC). Corn was harvested either by hand or with a plot combine from the center two rows of each plot.

The experimental design was a randomized complete block design with four replicates per site. Treatments were arranged in a split-split plot design with the main plot being cover crop type, sub-plots being cover crop biomass level/ desiccation date, and sub-sub plots being herbicide treatment. Data were analyzed as a split-split plot design and subjected to ANOVA

using PROC MIXED in SAS [SAS (Version 9.3)]. To meet the assumptions of ANOVA, early season weed counts were transformed using $\log_{10}$. Cover crop type, cover crop desiccation date, and herbicide treatments were considered fixed effects. Mean separations were generated using Fisher's Protected LSD at $P < 0.05$. Simple linear regression analysis was utilized where appropriate.

Results and Discussion

Cover Crop Biomass. Cover crop biomass varied by location. Biomass levels in response to cover crop type and cover crop desiccation date were reported by location; no significant interactions between main effects were observed (Figure 1, 2). For NK-10, rye and rye/ vetch yielded 3,721 and 2,982 kg ha^{-1}, respectively (Figure 1). Over cover crop type, biomass levels in plots desiccated at corn planting were 4,762 kg ha^{-1} (Figure 2).   For NK-11, rye had a biomass level of 1,594 kg ha^{-1}, and rye/ vetch yielded 2,046 kg ha^{-1} (Figure 1). Biomass levels in plots desiccated at 20 EPP were 497 kg ha^{-1}, while 10 EPP and at-planting biomass levels were 1,927 kg ha^{-1} and 3,035 kg ha^{-1}, respectively (Figure 2). For M2-11, rye biomass levels were 4,876 kg ha^{-1} and rye/ vetch biomass levels were 4,365 kg ha^{-1} (Figure 1). Cover crops desiccated at 20 EPP were 2,713 kg ha^{-1}, whereas cover crops desiccated at-planting were 5,722 kg ha^{-1} (Figure 2). For M1-11, rye and rye/ vetch cover crops yielded significantly different biomass levels (Figure 1). Rye was 1,679 kg ha^{-1} and rye/ vetch was 2,874 kg ha^{-1} (Figure 1). In plots desiccated at 10 EPP, cover crops were 2,263 kg ha^{-1}, and at 20 EPP, cover crops yielded 1,477 kg ha^{-1} (Figure 2). Cover crop biomass levels in plots desiccated at-planting were 3,089 kg ha^{-1} (Figure 2). In general, rye and rye/ vetch cover crops produced similar biomass levels (Figure

1). Delaying cover crop desiccation to corn planting increased biomass production, and in some cases, produced more biomass than 10 EPP desiccation dates. Delaying cover crop desiccation until corn planting increased biomass production over cover crops desiccated at 20 EPP in all cases. Overall, cover crop biomass levels were low and would not be considered sufficient for adequate weed suppression alone (Smith et al. 2011; Teasdale and Mohler 2000; Wagger 1989). Because cover crop termination must precede corn planting, and corn planting generally takes place in late April, these cover crops were not allowed enough time to produce adequate biomass. Delaying corn planting would allot more time to cover crop growth, potentially producing biomass levels high enough to provide additional weed control (Teasdale and Mohler 2000; Wagger 1989). However, the utilization of this system coupled with a late April corn planting would likely require additional weed control.

Weed Control. At 4 WAT, herbicide treatment was the only factor that contributed to early-season weed suppression (Figure 3). Neither cover crop type nor desiccation date played a role in early-season weed suppression. Additionally, no significant interactions between main effects were observed. Preemergence herbicide treatment effects on early-season weed counts varied by location. For all locations, all preemergence herbicide treatments provided significantly greater control than the non-treated check (NTC) (Figure 3). For NK-10, the NTC averaged 247 weeds m^{-2}, whereas a full rate of atrazine + metolachlor + mesotrione averaged 8.6 weeds m^{-2}, a significant reduction in weed counts when compared to the NTC (Figure 3). The full rate of atrazine + metolachlor + mesotrione was also significantly different from weed counts observed in the half rate of atrazine + metolachlor + mesotrione or the half rate of atrazine + simazine (Figure 3). For NK-11, atrazine + metolachlor + mesotrione at the full rate averaged 0.6 weeds

m^{-2} (Figure 3), a significant reduction in weed counts when compared to the NTC average of 20.3 weeds m^{-2} (Figure 3). The full rate of atrazine + metolachlor + mesotrione was not significantly different from counts resulting from the half rate treatment of atrazine + simazine, which averaged 1.3 weeds m^{-2} (Figure 3). Effects of preemergence herbicide treatments on weed counts in the Montgomery County experiments followed a similar pattern to NK-11. For M1-11, all three herbicide treatments performed similarly (Figure 3). The full rate of atrazine + metolachlor + mesotrione and the half rates of atrazine + metolachlor + mesotrione and atrazine + simazine averaged 9.1, 8.1, and 7.2 weeds m^{-2}, respectively (Figure 3). For M2-11 the full rate atrazine + metolachlor + mesotrione averaged 0.22 weeds^{-2}, and the half rates of atrazine + metolachlor + mesotrione and atrazine + simazine averaged 1.3 and 6.5 weeds m^{-2} (Figure 3). The full and half rates of atrazine + metolachlor + mesotrione were not significantly different (Figure 3). Utilizing reduced rates of herbicides is a common practice in crop production (Hartzler 1993; Blackshaw et al. 2006), and research has observed weed suppression when utilizing reduced rates (Buhler et al. 1993; weaver 1991; Zhang and Cavers 1994). These results suggest that reduced preemergence herbicide rates could be utilized in this system, but would be highly dependent upon individual circumstances and assessed risks (Gressel 1995; Manalil et al. 2011).

At 12 WAT, all main effects, cover crop type, cover crop desiccation date, and herbicide treatment, played some significant role in late-season weed control (Figure 4, 5, 6). No significant interactions between main effects were observed. For NK-10, over herbicide treatment and cover crop desiccation date, rye controlled weeds better than rye/ vetch (Figure 4). That difference was not seen at the other locations (Figure 4). Pooled over herbicide treatment and cover crop type, NK-11 was the only site to see a significant increase in weed control due to

delaying desiccation date (Figure 5). Other sites saw a reduction or no change in weed control across desiccation dates (Figure 5). This decrease or lack of control may be due to insufficient biomass levels (Mohler and Teasdale 1993; Smith et al. 2011), where cover crop biomass levels are high enough to optimize soil conditions for plant growth, but too low to provide physical weed interference.

For herbicide treatments, all preemergence herbicides followed by glyphosate resulted in the best control (Figure 6). At all locations, preemergence herbicide only treatments were not able to maintain sufficient control throughout the season (Figure 6). For NK-10, atrazine + metolachlor + mesotrione at the full rate maintained 54% control, and atrazine + metolachlor + mesotrione and atrazine + simazine at the half rates controlled weeds 59% and 39%, respectively (Figure 6). For NK-11, atrazine + metolachlor + mesotrione at the full rate maintained 74% control, and the half rates of atrazine + metolachlor + mesotrione and atrazine + simazine controlled weeds 51 and 65%, respectively (Figure 6). For M1-11 and M2-11, control was insufficient for most preemergence herbicide only treatments, ranging from 47-55% and 22-64% control, respectively (Figure 6). All treatments containing glyphosate POST provided significantly greater weed control, except at the NK-10 location. For NK-11 and M1-11, weed control ranged from 97-100% for treatments utilizing glyphosate (Figure 6). For NK-10, control was insufficient. Despite the POST application of glyphosate, weed control ranged from 51-66% (Figure 6). For M2-11, all treatments receiving glyphosate POST controlled weeds 84-87% (Figure 6). The inclusion of glyphosate is a critical component for weed control in this system (Teasdale 1993). The removal of emerged weeds is not only important for yield and harvest ability, but controlling weeds POST prevents weed seed spread and contamination (Llewellyn and Allen 2006).

Yield. Across all locations, corn yield increased with the use of rye/ vetch cover crops (Figure 7). Corn yields in rye/ vetch averaged 7,645 kg ha^{-1} and yields in rye averaged 6,805 kg ha^{-1} (Figure 7). Given that rye/ vetch did not provide a major additional weed control advantage over rye cover crops (Figure 4), it is likely that a nitrogen effect from vetch is the primary factor in increasing corn yield. This has been documented in other work involving legume cover crops (Beale et al. 1995; Hoyt and Hargrove 1986; Utomo et al. 1990).

There was a significant interaction between desiccation date and herbicide treatment (Table 1). For NK-10, corn planted in cover crops desiccated at 20 EPP and treated with atrazine + metolachlor + mesotrione at the half rate yielded 6,846 kg ha^{-1} (Table 1). In cover crops desiccated at planting, corn yield for the same herbicide program was 8,964 kg ha^{-1} (Table 1). However, across locations, most significant differences seen between desiccation dates held no biologically relevant trend, suggesting that cover crop desiccation date generally did not have an effect on corn yield (Table 1). Additionally, few differences were seen between herbicide treatments (Table 1). For NK-10, all herbicide treatments had significantly better yield than the NTC, which yielded 2,986-4,875 kg ha^{-1} (Table 1). For NK-11, yield from most herbicide treatments was significantly different from yield observed from the NTC (Table 1). This difference was also seen in M1-11 and M2-11 (Table 1). In few instances, PRE followed by POST herbicide treatments had higher corn yields than POST alone, suggesting that the addition of PRE herbicides reduced weed competition during corn's critical weed free period (Table 1). For M1-11, the highest yield was seen with atrazine + metolachlor + mesotrione at the half rate followed by glyphosate in at-planting desiccated cover crops at 7,195 kg ha^{-1}, and was

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significantly different from POST alone at the same cover crop desiccation date at 2,542 kg ha^{-1} (Table 1).

These data show a lack of differences between PRE herbicide treatments, significant weed control increases seen with the use of glyphosate, and yield increases seen with rye/ vetch cover crops. These data suggest that the addition of PRE herbicides, integrated with rye/ vetch cover crops, and followed by a POST herbicide application can provide sufficient weed control, competitive corn yield, and could decrease expenditures by decreasing inputs. Moreover, a producer could potentially benefit from other cover crop advantages, such as a reduction in soil and water erosion, increased soil health, and increased organic matter (Dabney et al. 2001; Isse et al. 1999; Smith et al. 1987; Utomo et al. 1990). Teasdale (1996) suggested that a POST application was necessary for corn grown in cover crops and treated with reduced PRE rates and Reddy (2001), in a system utilizing cover crop residue, suggested that while PRE herbicides may or may not be necessary for early season weed control, a POST application of glyphosate was essential. Reducing herbicide rates is a concern as it has been shown to promote herbicide resistance (Manalil et al. 2011). For this system, an appropriate crop and herbicide rotation is critical for maintaining herbicide sustainability.

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Figure 1: Comparison of cover crop biomass levels by cover crop type, separated by location. Within locations, means followed by the same letter are not significant different based on Fisher's Protected LSD test at P< 0.05. Abbreviation: NK, New Kent; M1, Montgomery County site 1; M2, Montgomery County site 2; 10; Year 2010; 11, Year 2011.
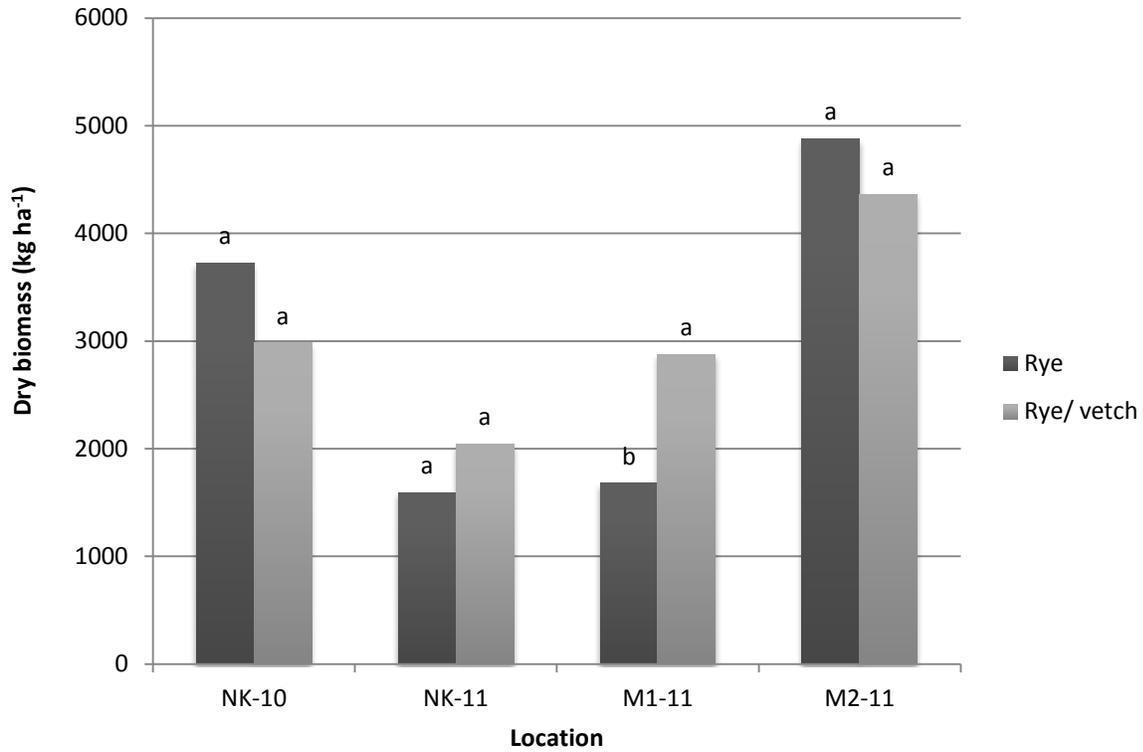
Figure 2: Cover crop biomass level response to desiccation date, separated by location.

Abbreviation: NK, New Kent; M1, Montgomery County site 1; M2, Montgomery County site 2;

10; Year 2010; 11, Year 2011.
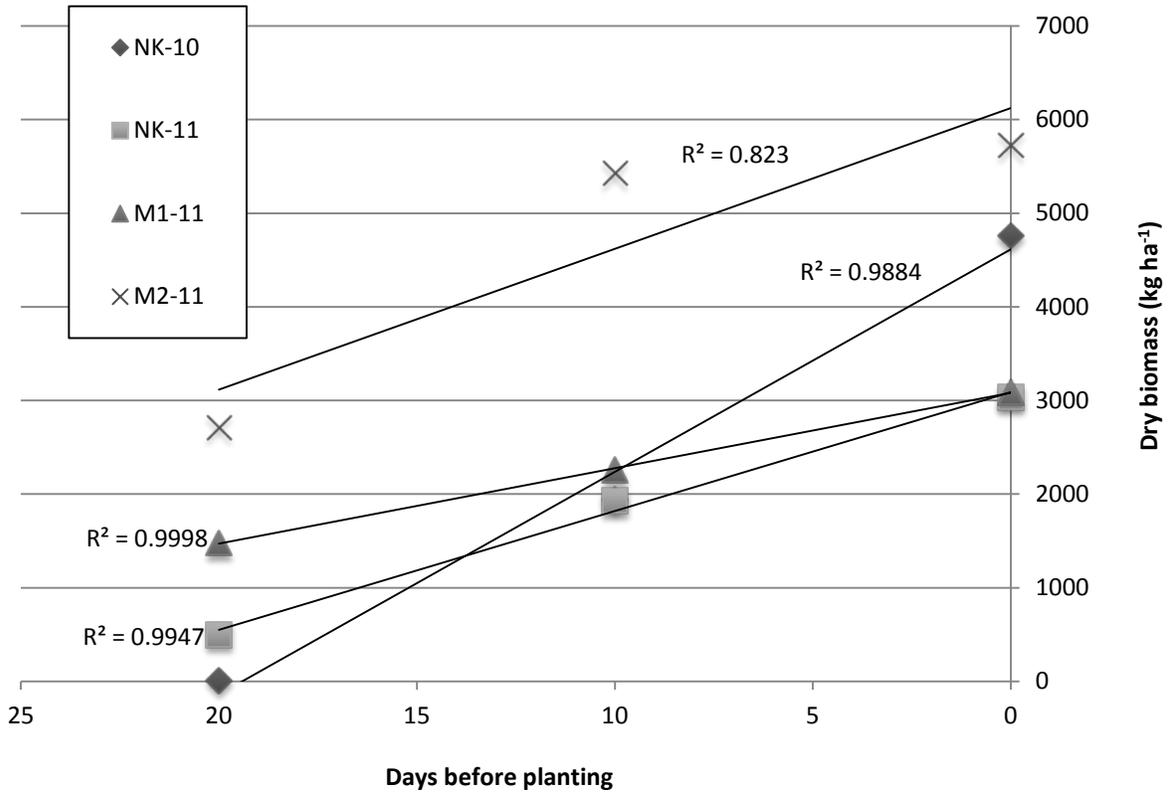
Figure 3: Herbicide treatments response on early-season weed counts, separated by location. Within locations, means followed by the same letter are not significant different based on Fisher's Protected LSD test at P< 0.05. Abbreviation: NK, New Kent; M1, Montgomery County site 1; M2, Montgomery County site 2; 10; Year 2010; 11, Year 2011; AMM, atrazine + metolachlor + mesotrione; AS, atrazine + simazine; NTC, non-treated check; 1x, labeled herbicide rate; 0.5x, half of labeled herbicide rate; NTC, non-treated check.
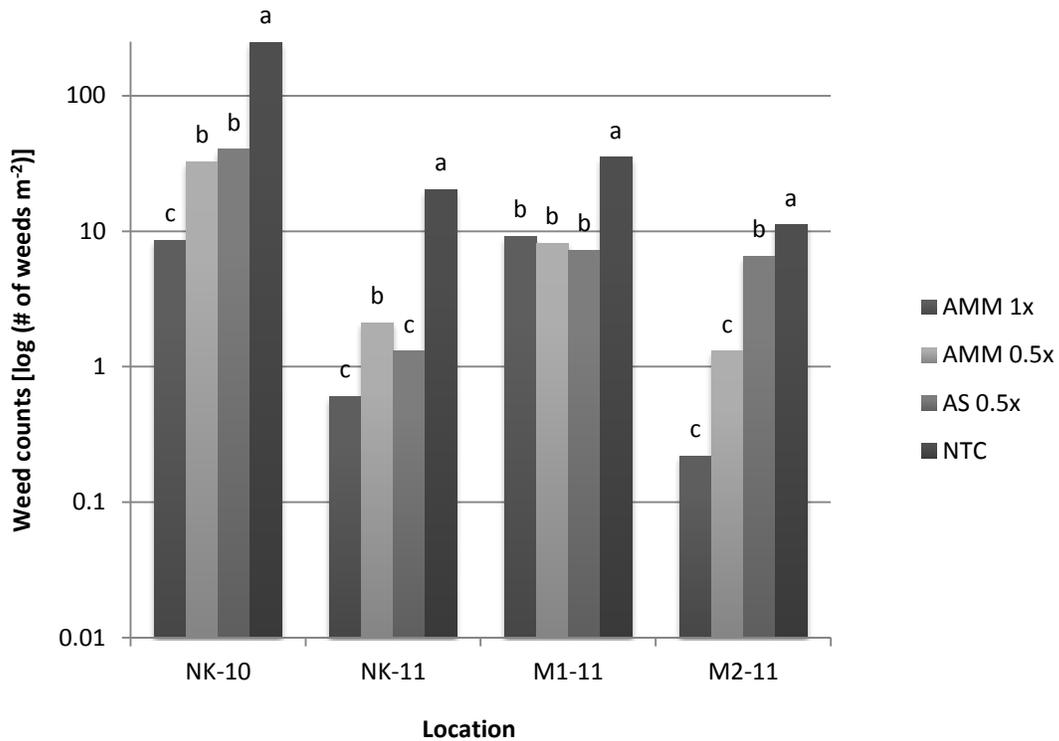
Figure 4: Cover crop type response on late-season weed control, separated by location. Within locations, means followed by the same letter are not significant different based on Fisher's Protected LSD test at P< 0.05. Abbreviation: NK, New Kent; M1, Montgomery County site 1; M2, Montgomery County site 2; 10; Year 2010; 11, Year 2011.
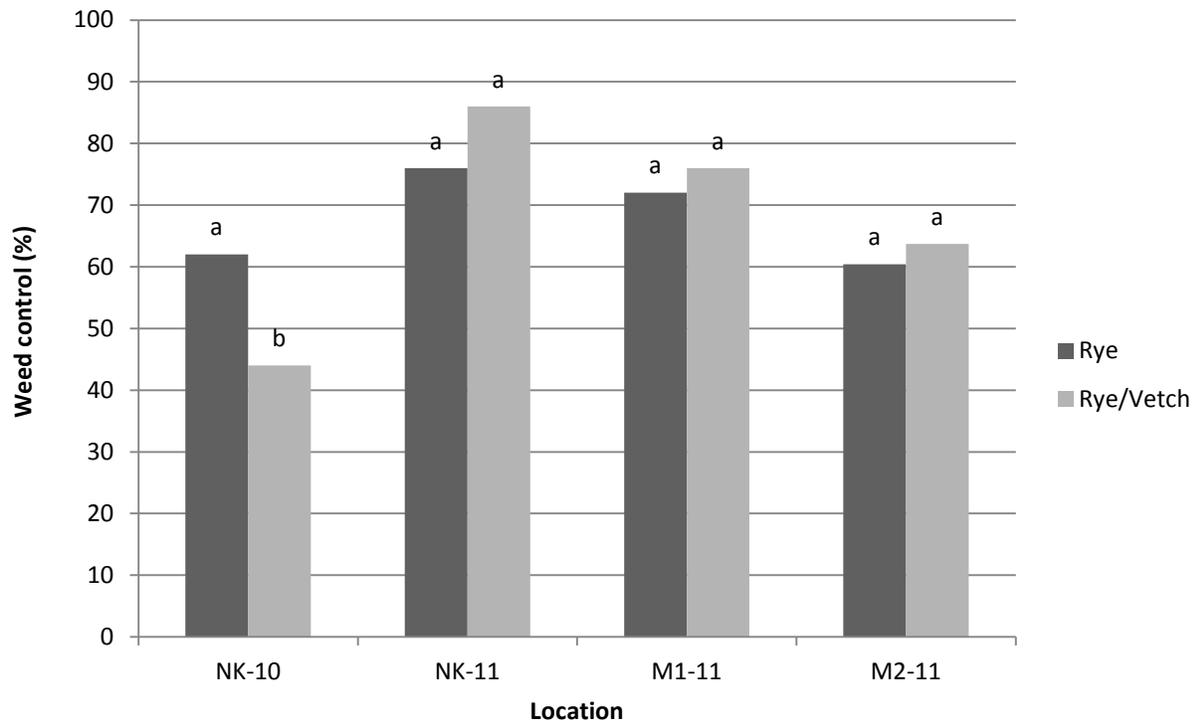
Figure 5: Weed control response to cover crop desiccation date, separated by location.

Abbreviation: NK, New Kent; M1, Montgomery County site 1; M2, Montgomery County site 2;

10; Year 2010; 11, Year 2011.
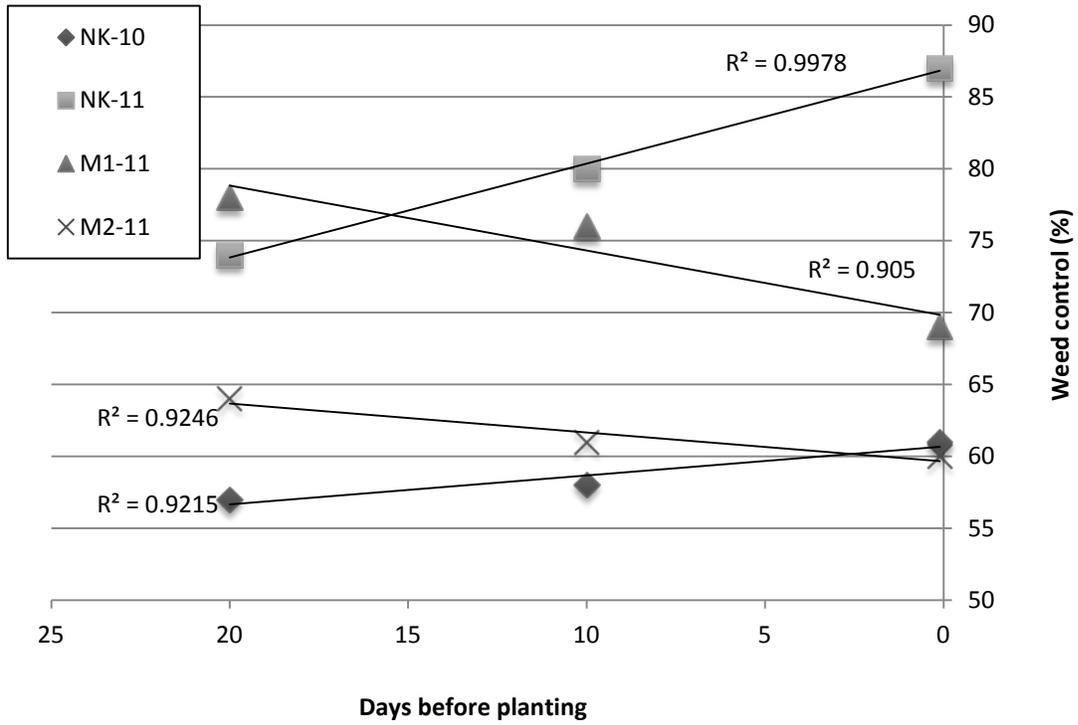
Figure 6: Herbicide treatments response on late-season weed control, separated by location. Within locations, means followed by the same letter are not significant different based on Fisher's Protected LSD test at P< 0.05. Abbreviation: NK, New Kent; M1, Montgomery County site 1; M2, Montgomery County site 2; 10; Year 2010; 11, Year 2011; AMM, atrazine + metolachlor + mesotrione; AS, atrazine + simazine; NTC, non-treated check; 1x, labeled herbicide rate; 0.5x, half of labeled herbicide rate; POST, postemergence glyphosate.
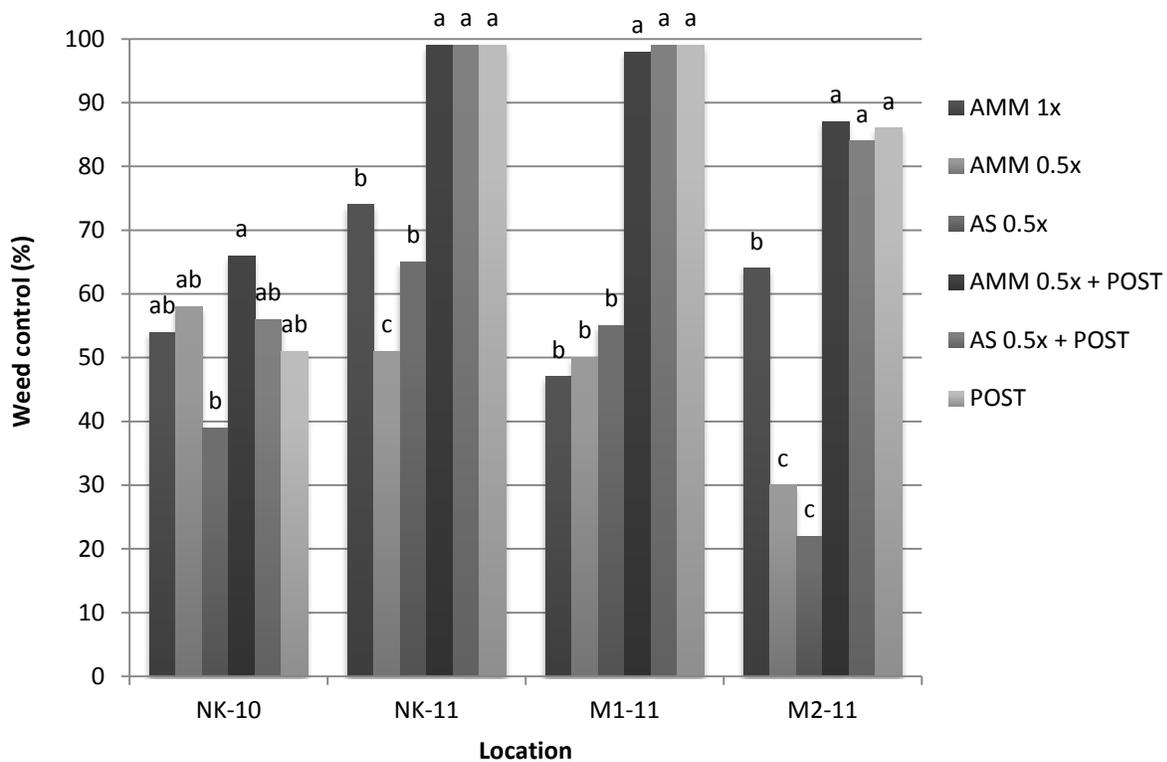
Figure 7: Effects of cover crop type on corn yield. Means followed by the same letter are not significant different based on Fisher's Protected LSD test at P< 0.05.
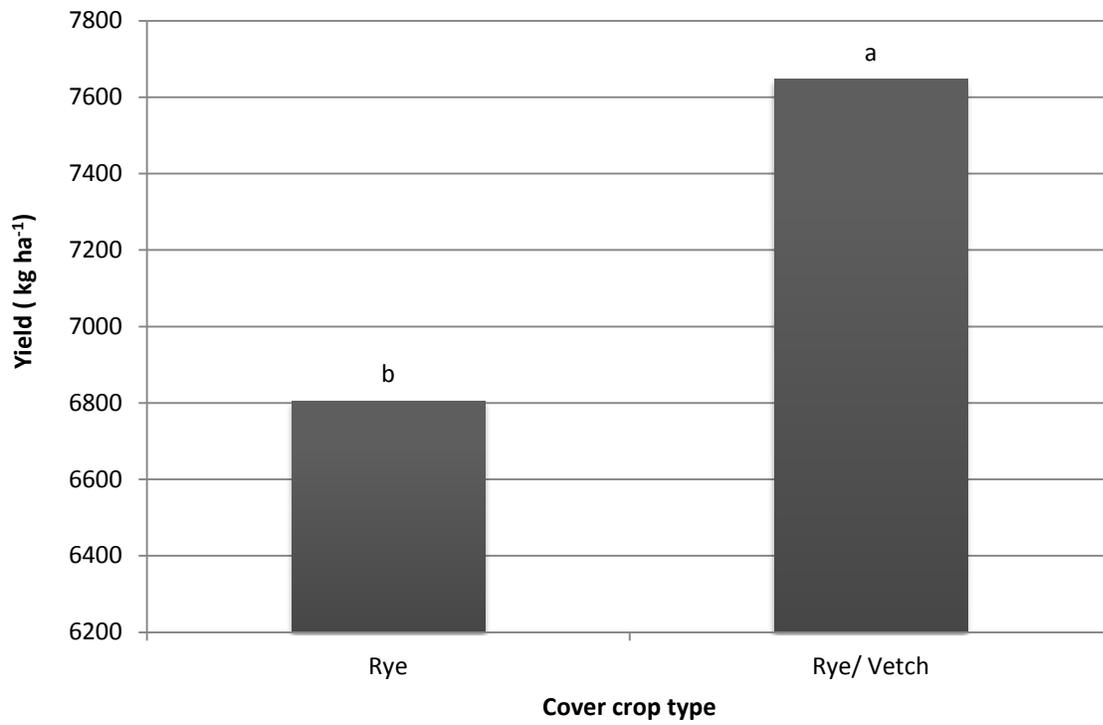
Table 1: Herbicide treatment and cover crop desiccation date effects on corn yield, separated by location.[a]

Herbicide Treatment[b]	NK-10				NK-11			
	20 EPP[b]	10 EPP	Planting		20 EPP	10 EPP	Planting	
AMM 1x	7705 ab	7837 a	8124 a	NS	8106 a	8090 ab	8413 a	NS
AMM 0.5x	6846 ab	7808 a	8964 a	*	7622 a	8001 ab	8130 a	NS
AS 0.5x	6373 b	8463 a	8871 a	NS	8139 a	8292 ab	8357 a	NS
AMM 0.5x fb POST	7130 ab	7644 a	9395 a	NS	8171 a	8744 a	8478 a	NS
AS 0.5x fb POST	8322 a	7709 a	9068 a	NS	8865 a	8599 ab	8817 a	NS
POST only	7582 ab	7298 a	8031 a	NS	7404 a	7565 b	8421 a	NS
NTC	4365 c	2986 b	4875 b	NS	4344 b	6120 c	7743 a	*
Herbicide Treatment[b]	M1-11				M2-11			
	20 EPP	10 EPP	Planting		20 EPP	10 EPP	Planting	
AMM 1x	5166 a	3766 cd	3260 cd	*	7379 a	8490 ab	10377 a	*
AMM 0.5x	5605 a	4286 bcd	5173 b	NS	6877 a	4229 d	6649 bc	NS
AS 0.5x	3011 b	4572 abcd	4770 bc	NS	6755 a	5249 cd	5211 c	NS
AMM 0.5x fb POST	6221 a	5598 ab	7195 a	NS	7493 a	7934 abc	7721 abc	NS
AS 0.5x fb POST	5569 a	5891 a	6470 ab	NS	7729 a	10240 a	6603 bc	*
POST only	5056 a	5239 abc	5393 b	NS	8041 a	7562 bcd	8452 ab	NS
NTC	2835 b	3509 d	2542 d	NS	4595 b	5835 bcd	6283 bc	NS

[a] Within columns and locations, means followed by the same letter are not significantly different based on Fisher's Protected LSD test at P < 0.05. Within rows and locations, cover crop desiccation effects were identified as non-significant (NS) or significant (*) at P < 0.05.

[b] Abbreviations: AMM, atrazine + metolachlor + mesotrione; AS, atrazine + simazine; NTC, non-treated check; 1x, labeled herbicide rate; 0.5x, half of labeled herbicide rate; EPP, early pre-plant; WAIT, weeks after initial treatment; POST, postemergence glyphosate treatment; NS, non-significant

Chapter 3: Reduced Chemical Programs for Crabgrass Control in Cool-season Turfgrass Using Corn Gluten Meal Combined with Synthetic Herbicides

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Abstract

Corn gluten meal (CGM) is an organic weed control product that can be used for preemergence control of crabgrass. Research has shown that CGM controls crabgrass 58% when applied at 99 g m^{-2}. At 99 g m^{-2}, CGM is equivalent to 88 kg nitrogen (N) ha^{-1} and the recommended spring N rate for cool-season turf is 49 kg N ha^{-1}. Excessive nitrogen is often a precursor for disease problems and can encourage weed growth where the turf stand is not competitive. To minimize risk of nitrogen over-fertilization while improving weed control, reduced rates of CGM were impregnated with ¼ and ½ the recommended labeled rates of pendimethalin, prodiamine, oxadiazon, and dithiopyr and applied to tall fescue and Kentucky bluegrass lawn-height turf. Turf injury, turf quality, and crabgrass control were evaluated. Turf injury or increased turf quality from CGM applications was not observed. Corn gluten meal did not augment crabgrass control. Additionally, herbicide rate did not play a significant role in crabgrass control. Herbicide treatment was the only factor that significantly reduced crabgrass coverage. The use

of prodiamine reduced crabgrass coverage 25% when compared to the non-treated check.

Pendimethalin reduced crabgrass coverage 22% and oxadiazon reduced crabgrass coverage 18%.

Dithiopyr reduced crabgrass coverage 13%. Though significant, control seen with prodiamine

and pendimethalin was unacceptable; an 80% reduction in crabgrass coverage is the minimum

accepted control. Other research reported 77% crabgrass control with reduced CGM and

pendimethalin rates applied in separate applications. Impregnating herbicides on CGM may

have diminished crabgrass control.

Nomenclature: corn gluten meal; dithiopyr; oxadiazon; pendimethalin; prodiamine; tall fescue

'Crossfire II', *Festuca arundinacea* Schreb.; Kentucky bluegrass 'Midnight', *Poa pratensis* L.;

smooth crabgrass, *Digitaria ischaemum* Schreb. ex Schweig.

Key Words: crabgrass control, integrated weed management

Introduction

Public perception of pesticides tends to be negative and exerts considerable influence in the public and private sectors (Child Safe Playing Fields Act 2010; Dantzker et al. 2010; Dunlop 1992). Interest in alternatives to pesticide use has rapidly expanded and is now an important factor in research, public policy, and consumer choices (Child Safe Playing Fields Act 2010; Dantzker et al. 2010; Balogh and Walker 1992). Public recreation centers, public sport arenas, and homeowners are interested in reduced herbicide inputs for turfgrass and ornamentals (Child Safe Playing Fields Act 2010). Moreover, homeowners, by their consumer choices, drive the market for "green solutions" for weed and pest control and fertilizers in the home and garden sector (Dantzker et al. 2010; Hammit 1990). Concerns regarding environmental and human health are the dominant forces influencing such changes (Child Safe Playing Fields Act 2010; Dantzker et al. 2010; Cooper 1987).

Under certain circumstances, residential use of outdoor pesticides and fertilizers has led to pesticide and fertilizer detection in storm water, pest resistance, and accidental herbicide exposure (Iskander 1994; Leonas and Yu 1992; Nishioka et al. 1996; Raupp et al. 1992). Environmental changes that arise from pesticide use might be perceived as detrimental by individuals and can influence an individual's purchasing and voting decisions. Additionally, public perception is influenced by risk assessment (Delaney 1993). Individuals are less likely to accept risk if the risk is imposed upon them. Pesticide applications beyond an individual's control are less accepted than those within an individual's control (Delaney 1993). The use of alternative weed control tactics in turf can potentially downplay that risk in the public eye. Reduced herbicide rates and the incorporation of natural products for turf weed control may be

more publicly accepted. Corn gluten meal (CGM), a by-product of the wet-milling process of corn, exhibits some herbicidal activity and may serve as a potential alternative.

Corn gluten meal inhibits root formation in several weed species without causing turf injury in established turf, and requires a period of moisture stress following application to be effective (Bingaman and Christians 1995; Christians 1993; Gardner et al. 1997). This root inhibition makes CGM a preemergence herbicide safe for established turfgrass. Crabgrass (*Digitaria* spp.) is one of the tested species in which CGM exhibits some herbicidal activity, and is considered one the most problematic weeds in turfgrass (Kim et al. 2002). Research has reported 97% and 82% survival reduction at a 973 g m^{-2} CGM rate for smooth crabgrass (*D. ischaemum* Schreb. ex Schweig.) and large crabgrass (*D. sanguinalis* (L.) Scop.), respectively (Bingaman and Christians 1995).   However, other research has concluded that such application rates are excessive and suggests an application rate of 99 g m^{-2}, which controls crabgrass 58% (Christians 1993).

At a 99 g m^{-2} CGM rate, crabgrass control of 58% is not sufficient in the turfgrass and landscape industry, where 80% control is the acceptable minimum standard (Chism and Bingham 1991). Moreover, a 99 g m^{-2} CGM rate is equivalent to 88 kg N ha^{-1} and acts as a slow-release N fertilizer (Christians, 1993). The recommended spring N rate for most cool-season turf is 49 kg N ha^{-1} (Goatley et al. 2009), and subsequent summer applications are not recommended. Excessive N fertilization can lead to summer stress and increased disease severity in tall fescue (Couch 1966). Even more, under certain conditions, excessive N can potentially lead to nitrogen losses and groundwater contamination (Guillard and Kopp 2004; Morton et al. 1988; Petrovic 1990). The use of preemergence herbicides at reduced rates,

coupled with the use of reduced rates of CGM, may improve weed control while reducing N fertilizer inputs.

Pendimethalin, oxadiazon, dithiopyr, and prodiamine are effective preemergence herbicides used for crabgrass control in turfgrass (Goatley et al. 2009). Some research utilizing these herbicides has shown that reducing the application rate can be accomplished without a significant reduction in weed control (Johnson 1995; Johnson 1996; Johnson 1997a; Johnson 1997b). Reducing prodiamine rates to ¼ and ½ the recommended labeled rates controlled large crabgrass 79 to 94% (Johnson 1996). In the same research, dithiopyr at ¼ and ½ the recommended labeled rates controlled large crabgrass 7 to 24% (Johnson 1996). In other research, reduced rates of pendimethalin controlled large crabgrass 92, 98, and 97% for 1st, 2nd, and 3rd year applications in bermudagrass, compared to a full rate control of 96-100% (Johnson 1997a). Moreover, Johnson (1995) reported that a ½ rate of pendimethalin and oxadiazon resulted in 4 to 16% and 11 to 20% large crabgrass cover in tall fescue, respectively. At ⅓ the recommended labeled rate, oxadiazon and prodiamine controlled crabgrass 80% and 93% in one season, compared to full rate oxadiazon and prodiamine control of 86 and 99% in tall fescue (Johnson 1997a; Johnson 1997b). Coupling reduced herbicide rates with reduced CGM rates is an integrated approach that may provide adequate control with reduced herbicide rates while meeting recommended N inputs. Gardner et al. (1997) showed that the combination of CGM and pendimethalin could control large crabgrass. Using a ½ rate of pendimethalin in conjunction with 49 g m^{-2} of CGM, crabgrass control was 87% in the greenhouse and 77% in the field (Gardner et al. 1997). Control of crabgrass using CGM alone at 49 g m^{-2} was 19-25%, depending on the location. CGM at 99 g m^{-2} controlled crabgrass 35 and 42% (Gardner et al. 1997).

In some situations, it may not be economical to apply CGM as a granule and liquid herbicides in separate applications. Impregnated herbicides on fertilizer are common ready-to-use products in turf and ornamental weed management. No research has been reported in the peer-reviewed literature on impregnated preemergence herbicides on CGM. These fertilizer-herbicide products may allow for one or two granular applications in a season, reduce herbicide inputs, and prevent N over-fertilization. The objective of this study was to evaluate smooth crabgrass control in cool-season turf using reduced rates of pendimethalin, prodiamine, oxadiazon, and dithiopyr impregnated on CGM being applied once at 49.5 g m^{-2} or in a split application at 24.8 g m^{-2} per application.

Materials and Methods

Research was conducted on tall fescue 'Crossfire II' (*Festuca arundinacea* Schreb.) and Kentucky bluegrass 'Midnight' (*Poa pratensis* L.) at the Turfgrass Research Center in 2010 and the Glade Road Research Facility in Blacksburg, VA in 2011. The soil type was a Groseclose (fine, mixed, semiactive, mesic Typic Hapludult) with 3% organic matter and a pH of 6.2 at both locations; turfgrass stands were established in 2002 and 2006, respectively. Tall fescue was mowed at 7.6 cm and Kentucky bluegrass was mowed at 6.35 cm. Plots were 1.8 m x 1.8 m and all granular treatments were applied using a 1.2 m x 1.8 m modified shaker box to ensure even distribution (Kelly and Coats 1999).

Herbicide treatments were created using an atomizer (Preval Atomizer, IL, USA) to impregnate herbicide solutions on 1000g of CGM or an inert carrier (500g sand). The final products were thoroughly blended, dried, and divided into individual plot amounts. Herbicide

solutions consisted of 10 ml of distilled water mixed with herbicide amounts that allowed final impregnated products to be applied at the following rates: dithiopyr at 0.14 and 0.28 kg ai ha^{-1} for ¼ and ½ the recommended labeled rates, respectively, oxadiazon and pendimethalin at 0.56 and 1.1 kg ai ha^{-1} for ¼ and ½ the recommended labeled rates, respectively, and prodiamine at 0.42 and 0.84 kg ai ha^{-1} for ¼ and ½ the recommended labeled rates, respectively. Impregnated CGM was applied once at 49.5 g product m^{-2} in April or in a split application at 24.75 g product m^{-2} in April and June. Impregnated sand was applied once in April at 109 g product m^{-2}. Plots receiving split applications of CGM received a ⅛ herbicide rate per application for plots receiving ¼ herbicide rates, and a ¼ herbicide rate per application for plots receiving ½ herbicide rates. In summary, all designated plots, regardless of carrier application type, received either a ¼ or ½ total herbicide rate during the season.

Turf injury and quality were evaluated 2 WAT, 4 WAT, and monthly for six months. Grid counts for crabgrass cover were taken in late summer. Crabgrass control was also visually estimated against the non-treated check (NTC). Turf cover and injury were visually estimated using a 0 to 100% scale, where 0% represented no turf cover or turf injury and 100% representing full turf cover or complete death of all turf due to injury. Turf quality was visually estimated using a 1 to 9 scale, where 9 represented optimal turf quality and 6 represented minimum accepted turf quality. Crabgrass control was visually estimated using a 0 to 100% scale, where 100% represented complete crabgrass control. Crabgrass counts were measured using a 98-point grid that covered an area of 2.2 m^{-2} for each plot. At each line intersection, either turfgrass or crabgrass was noted and recorded. Resulting counts were converted to percentage crabgrass reduction compared to the NTC.

Experimental design was a RCBD with four replications and included a NTC. Treatments were arranged as a three by two by four factorial design, with three herbicide carrier application types, two herbicide rates, and four herbicide types. Additionally, CGM was applied alone either at 49.5 g product m^{-2} in April or in a split application at 24.75 g product m^{-2} in April and June. Sites were combined and data was subjected to ANOVA and mean separations were generated from Fisher's Protected Least Significant Differences (LSD) at $P < 0.05$ using PROC MIXED [SAS 9.3]. Herbicide carrier, herbicide rate, and herbicide type were considered fixed effects and blocks were considered random effects.

Results and Discussion

Turf injury was not observed during the season. Additionally, no late-season turf quality differences were seen with in plots receiving CGM. Crabgrass control was poor and potential differences in turf quality were likely diminished by high crabgrass pressure and weed competition (Figure 1). Corn gluten meal did not augment weed control. There were no differences between impregnating herbicides on CGM and impregnating herbicides on sand. Even more, herbicide rate did not play a significant role in weed control. The ½ rate of herbicide did not preform better than the ¼ rate of herbicide. There were no significant interactions between herbicide carrier, herbicide rate, and herbicide type. The only factor that significantly reduced crabgrass coverage was herbicide type (Figure 1). The use of prodiamine reduced crabgrass coverage 25% when compared to the NTC (Figure 1). Pendimethalin reduced crabgrass coverage 22% and oxadiazon reduced crabgrass coverage 18% (Figure 1). Dithiopyr

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reduced crabgrass coverage 13% (Figure 1). Though significant, control seen with prodiamine and pendimethalin are unacceptable; an 80% reduction in crabgrass control is the minimum accepted control (Chism and Bingham 1991). These results are in contrast with Gardner et al. (2007), who achieved 87% large crabgrass control in the greenhouse and 77% control in the field using a half rate of pendimethalin in conjunction with 49 g m^{-2} of CGM. It should be noted that Gardner et al. (2007) applied CGM as a granular application and pendimethalin as a spray application. Although a ½ rate of pendimethalin was used, uniform coverage of the spray application likely increased control. Impregnating herbicides on CGM may have resulted in a decrease in herbicide application uniformity or may have prevented the herbicides from adequately reaching the soil surface, thereby decreasing herbicide control efficacy.

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Figure 1: Preemergence herbicide treatment effect on late-season crabgrass reduction compared to the non-treated check. Means followed by the same letter are not significantly different based on Fisher's Protected LSD test at $P < 0.05$.
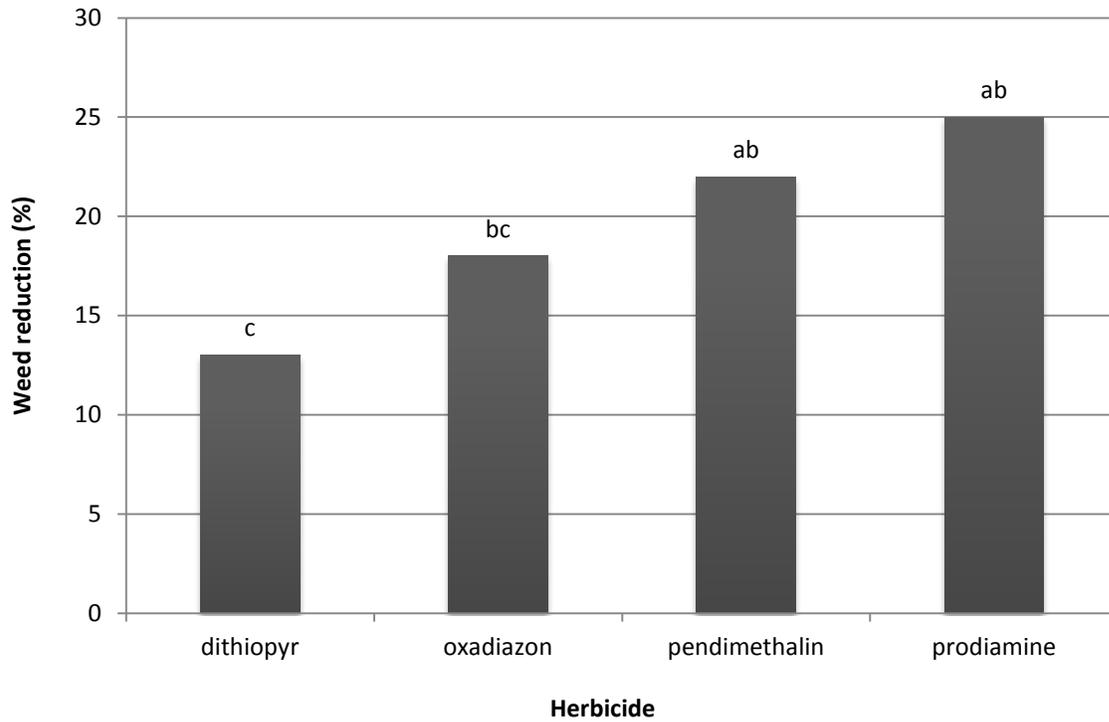
Chapter 4: Corn Gluten Meal Affects Brown Patch Severity in Tall Fescue Turfgrass

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Abstract

Corn gluten meal (CGM) is an organic preemergence herbicide for crabgrass control in cool-season turfgrass. CGM is 9% nitrogen (N) by weight, and the recommended spring CGM application rate of 20lbs/ 1000 ft^2 equates to approximately 1.8lbs N/ 1000 ft^2. In Virginia, the recommended spring N application is approximately 0.9lbs N/ 1000 ft^2. Excessive N inputs have been shown to encourage brown patch disease, a problematic disease in turf. The objective of this study was to assess the effects of CGM and synthetic N application rates on brown patch severity in tall fescue turf. CGM and synthetic N were applied at 0.45lbs, 0.9lbs, 1.8lbs, 1.8lbs applied twice, or 1.8lbs N/ 1000ft^2 applied three times in mid-April, mid-June, and mid-July. Single applications were applied in mid-April. The study was replicated three times over three years at two locations. An upward trend in turf quality at some locations suggested that 1.8lbs N/ 1000 ft^2 applied twice and three times increased turf quality over nitrogen rates at 0.45lbs N/ 1000 ft^2 in un-blighted turfgrass. However, at all six sites, brown patch cover increased with increased N rates. At several locations, there was an increased rate of blight incidence beginning

at 1.8lbs N/ 1000 ft^2 applied once. CGM at nitrogen rates ranging from 0.9 to 1.8lbs N/ 1000 ft^2 can increase turfgrass health and potentially decrease the presence and spread of brown patch. At rates higher than 1.8lbs N/ 1000 ft^2, nitrogen fertilizers could induce brown patch development with minimal benefit in terms of turfgrass quality.

Introduction

Corn gluten meal (CGM) is an organic preemergence (PRE) herbicide used in home lawns for weed control. It is the by-product of the wet milling process of corn and is sold as a granular product. Research has shown that CGM has herbicidal activity by inhibiting root formation in emerging weeds (Bingaman and Christians 1995). Emerged weeds without developed root systems die under moisture stress, a critical factor for successful weed control when utilizing CGM (Christians 1993a). CGM is a popular product for organic weed control; its popularity may be due to the fact that public perception of pesticide use tends to be negative, which has, in turn, spurred the use of natural products for pest management and soil fertility (Bahlai et al. 2010, Hammit 1990, Peterson 2000).

CGM contains 9% nitrogen (N) by weight (Christians 1993a), allowing CGM to be advertised as a "weed and feed" product that provides both weed control and acts as a slow-release N fertilizer. At the recommended rate of 20lbs/ 1000 ft^2, expected large crabgrass (*Digitaria sanguinalis*) control is 58% (Christians 1993a). Acceptable weed control in turfgrass is greater than 80% (Chism and Bingham 1991). At 58% control, one application of CGM may not meet customer expectations and subsequent applications of CGM may be made during the season. If these subsequent applications occur after weed emergence, CGM is not effective

(Christians 1993b). Multiple applications of CGM can result in N over-fertilization and promote

excessive foliar growth, making the turf susceptible to disease (Couch 1966).

The recommended spring N fertilization rate for cool-season turfgrass is 0.9lbs N/ 1000

ft^2 and ensures healthy turfgrass growth, reduces disease and weed pressure, and mitigates

potential N loss risks associated with excessive N inputs (Goatley et al. 2009). CGM is a slow-

release fertilizer and N release is dependent upon the product being broken down by soil

microbes (Patton 2007).   Applying CGM at the recommended rate of 20lbs/ 1000 ft^2 equates to

approximately 1.8lbs N/ 1000 ft^2. Subsequent applications of CGM could result in the addition

of up to 5.4lbs N/ 1000 ft^2. Under certain conditions, excessive N fertilization can potentially

lead to N losses and groundwater contamination (Guillard and Kopp 2004; Morton et al. 1988;

Petrovic 1990). Stress and foliar damage from disease and insects, due to increased foliar

growth, can decrease turfgrass competition with weeds, potentially increasing weed pressure

(Cutulle 2011). Moreover, increased N inputs have been shown to promote turfgrass disease,

such as brown patch (Couch 1966; Fidanza and Dernoeden 1996; Haygood et al. 1989).

Brown patch is considered one of the most troublesome turfgrass diseases in the US and

its severity is partly determined by N inputs (Fidanza and Dernoeden 1996). In certain tall

fescue cultivars, increasing monthly applications of N from 0.5lbs N/ 1000ft^2 to 0.9lbs N/

1000ft^2 doubled brown patch cover (Burpee 1995). Watkins and Wit (1993) found similar

results, showing that increasing N inputs led to increased brown patch coverage, with the most

severe brown patch corresponding with 3.6lbs N/ 1000 ft^2. Other research has found similar

results suggesting that increasing N rates can increase brown patch severity (Bloom and Couch

1960; Couch 1966; Haygood et al. 1989). The use of natural N fertilizers has been shown to

decrease brown patch when compared to a non-treated check (Soika and Sanders 1991). In

contrast, research conducted by Green et al. (1994) showed no interaction between brown patch severity and fertilizer source.

Research regarding CGM effects on brown patch disease has not been reported. Under current use patterns of CGM, excessive spring N may increase the severity of brown patch disease later in the season. The objective of this study was to assess the effects of CGM and synthetic N application rates on brown patch severity in tall fescue turf.

Materials and Methods

Research was conducted on tall fescue turf mowed at approximately 3.5 inches in 2010, 2011, and 2012 at Brookmeade Sod Farm in Richmond, VA and at the Glade Road Research Center in Blacksburg, VA. The soil type at Brookemeade Sod Farm was a Norfolk fine sandy loam (fine-loamy, kaolinitic, thermic Typic Kandiudults) with a pH of 5.5 and was a Groseclose soil (fine, mixed, semiactive, mesic Typic Hapludult) at the Glade Road Research Center with a pH of 6.5. Experiments conducted in Richmond, VA in 2010, 2011, and 2012 are designated as R-10, R-11, and R-12, respectively. Experiments conducted in Blacksburg, VA in 2010, 2011, and 2012 are designated as B-10, B-11, and B-12, respectively.

Plots were 6 by 6 feet. Granular CGM was applied at 0.45lbs, 0.9lbs, 1.8lbs, 1.8lbs applied twice, or 1.8lbs N/ 1000ft^2 applied three times in mid-April, mid-June, and mid-July. All singular treatments were applied in mid-April. CGM was compared to treatments of synthetic slow-release nitrogen fertilizer at the same N rates. A non-treated check (NTC) was included. Treatments were applied using a 4 by 6 ft modified shaker box to ensure even distribution of the products (Kelly and Coats 1999). Turf cover, turf quality, and brown patch severity were rated

bi-monthly. Turf cover was rated on a 0 to 100% cover scale. Turf quality of non-blighted turf was rated on a 1 to 9 quality scale, with 9 representing optimal quality and 6 representing minimum accepted turf quality. Disease severity was assessed using a visual estimation of percent blighted turf in each plot. Experimental design was a randomized complete block design with four replications. The trial was replicated six times over three years. Treatments were designed as a 2 x 5 factorial, with two fertilizer types and five fertilizer rates. Disease cover data and turf quality data were subjected to linear and non-linear regression analysis to show turf quality and disease response to increasing N rates.

Results and Discussion

At three sites, there were minimal turfgrass quality differences between application rates and fertilizer types (Figure 1). For these trials, increasing nitrogen rates did not seem to affect turfgrass quality in non-blighted turfgrass (Figure 1). For R-10, R-11, and B-12, an upward trend in turfgrass quality was observed with increasing nitrogen rates (Figure 1). For these trials, 1.8lbs N/ 1000 ft^2 applied twice and three times provided increased turf quality over nitrogen rates at 0.45lbs N/ 1000 ft^2 in un-blighted turfgrass (Figure 1). At higher rates, where blight was absent, turfgrass was denser and maintained better green color. However, these same nitrogen rates increased brown patch coverage (Figure 2).

At all six sites, there was increased brown patch coverage with increased nitrogen rates (Figure 2). There were minimal differences in fertilizer type, suggesting that CGM does not induce additional blight symptoms compared to similar N rates from synthetic fertilizers (Figure 2). At several locations, there was an increased blight incidence beginning at 1.8lbs N/ 1000 ft^2

applied once (Figure 2). Additionally, a curvilinear response was noted where brown patch

cover was least at a single application between 0.9lbs N and 1.8lbs N/ 1000 ft^2 (Figure 2). This

range of nitrogen application (0.9 to 1.8lbs N/ 1000 ft^2) is the recommended nitrogen rate for

cool-season turfgrass (Goatley et al. 2009), creating optimal growing conditions that would

reduce blight symptoms. Low nitrogen rates likely leave the turfgrass in stressed conditions that

may induce brown patch, and high nitrogen rates create optimal conditions for disease with

excessive top growth and an overabundance of nitrogen in the soil surface (Cutulle et al. 2014).

These data suggest that use of CGM at nitrogen rates from 0.9 to 1.8lbs N/ 1000 ft^2 can

increase turfgrass health and potentially decrease the severity and incidence of brown patch

(Cutulle et al. 2014). At rates higher than 1.8lbs N/ 1000 ft^2, nitrogen fertilizers induce brown

patch development with minimal benefit in turfgrass quality.   CGM is currently marketed as an

organic preemergence herbicide and, when applied at a rate that equates to 1.8lbs N/ 1000 ft^2,

controls crabgrass approximately 59% (Christians 1993a). Individuals that use CGM may or

may not understand the concept of a preemergence herbicide, and when 59% control is achieved

but deemed unacceptable, the misuse of additional CGM may lead to decreased turf health and

increased disease. CGM, used at recommended N rates, can be an effective nitrogen fertilizer for

maintaining healthy turf that will naturally compete with many grassy and broadleaf weeds.

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Figure 1: Tall fescue turf quality response to increasing nitrogen rates at six locations. Nitrogen rates higher than 1.8lbs N/ 1000 ft^2 were applied in sequential applications of 1.8lbs N/ 1000 ft^2. Abbreviation: CGM, corn gluten meal.
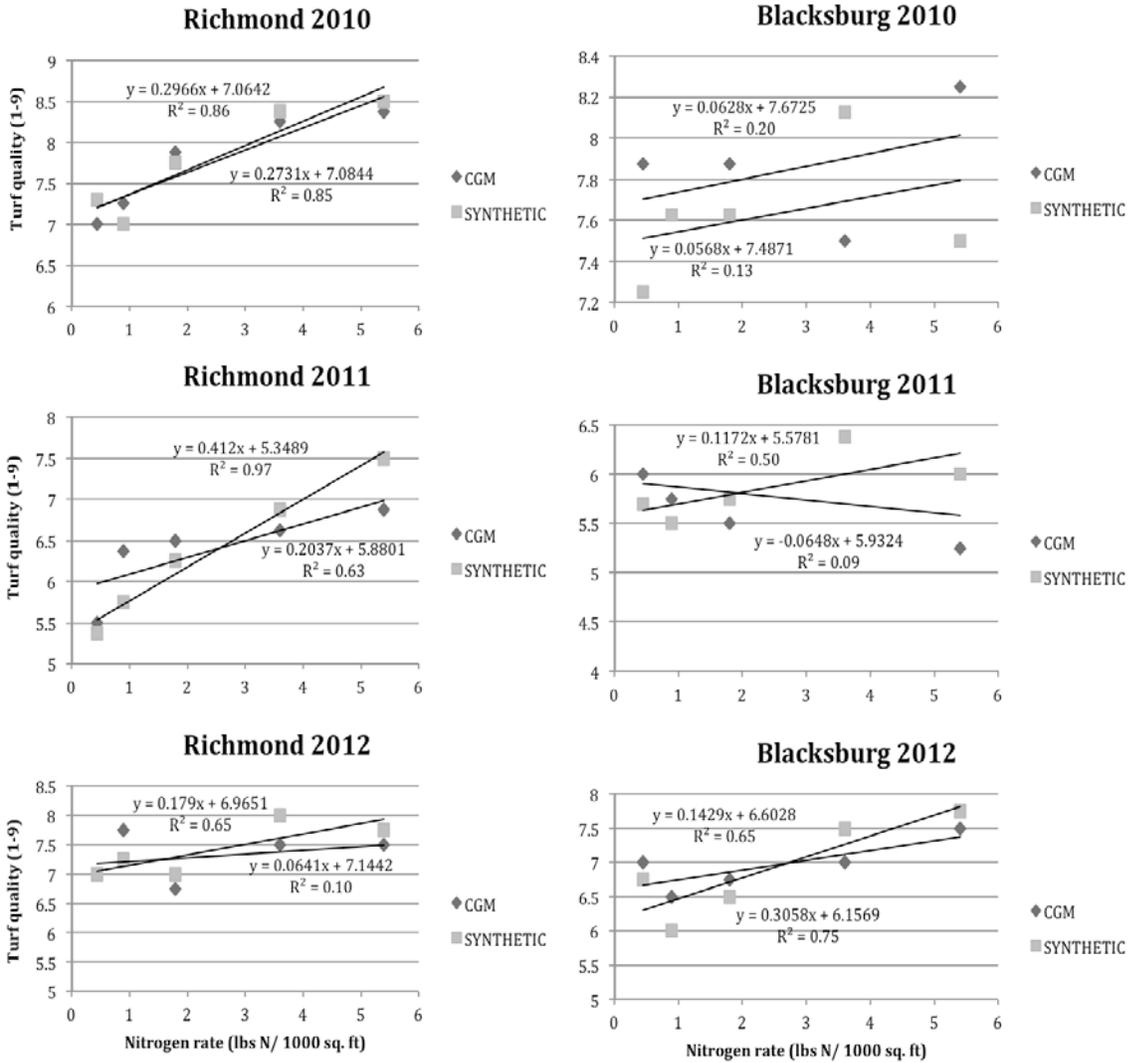
Figure 2: Brown patch cover response when compared to the non-treated check in tall fescue to increasing nitrogen rates at six locations. Nitrogen rates higher than 1.8lbs N/ 1000 ft^2 were applied in sequential applications of 1.8lbs N/ 1000 ft^2. Abbreviation: CGM, corn gluten meal.
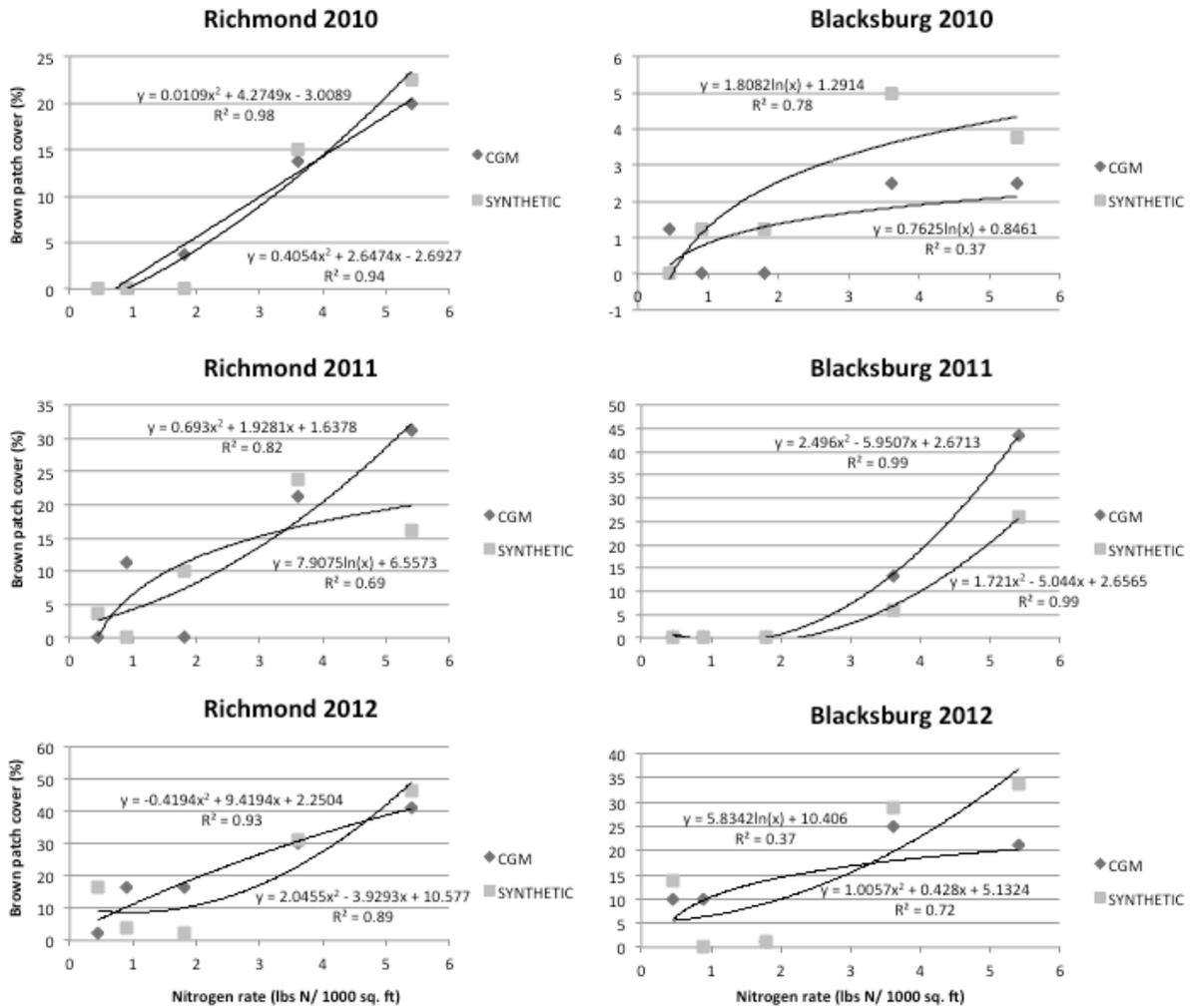
Chapter 5: Characterization of Johnsongrass (*Sorghum halepense*) Expressing Multiple Resistance to Nicosulfuron and Glyphosate

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Abstract

Herbicide resistance is a ubiquitous concern in agriculture that potentially jeopardizes the long-term sustainability and effectiveness of herbicide technology. There are over 400 unique weed biotypes expressing herbicide resistance worldwide. A putative nicosulfuron and glyphosate-resistant johnsongrass population was reported in continuous corn in Rockingham County, VA. Research was conducted to determine if the population was resistant to nicosulfuron and glyphosate and to further elucidate the mechanisms conferring those resistances. Field and greenhouse experiments subjected rhizomatous and seedling putative herbicide-resistant johnsongrass plants to four rates of nicosulfuron and five rates of glyphosate. In the field experiment, the 0.88 kg ae ha^{-1} rate of glyphosate controlled putative herbicide-resistant johnsongrass 65%, while control with at the 3.52 kg ae ha^{-1} rate was 90%. At 0.14 kg ai ha^{-1}, nicosulfuron controlled johnsongrass 9%. In greenhouse experiments, nicosulfuron at 0.14 kg ai ha^{-1} failed to control putative herbicide-resistant johnsongrass seedlings. Surviving seedlings

had increased plant vigor, taller statures, and increased plant weights when compared to the susceptible johnsongrass population. The putative herbicide-resistant johnsongrass population was additionally subjected to DNA sequencing to assess target-site mutations and glyphosate absorption, translocation, and metabolism to assess non-target site mechanisms of resistance. Neither nicosulfuron or glyphosate resistance were conferred via target-site mutation. Five of eight reported ALS-gene site mutations were confirmed absent in Virginia johnsongrass, while three others were located in coding regions that could not be elucidated in johnsongrass. Further investigations showed glyphosate resistance was not conferred via a reduction in herbicide absorption or translocation. The susceptible johnsongrass caused an increase in a polar metabolite at $Rf = 0.17$ with concomitant reduction in glyphosate over time, suggesting that glyphosate resistance in johnsongrass may be associated with differential metabolism, although the mechanism is not clear.

Nomenclature: Johnsongrass, *Sorghum halepense* (L.) Pers.; nicosulfuron; glyphosate

Key Words: glyphosate absorption and translocation, glyphosate metabolism, multiple herbicide resistance, target-site mutation

Introduction

Johnsongrass (*Sorghum halepense* (L.) Pers.) is a troublesome weed in production agriculture (Webster and Nichols 2012). It is a perennial plant with thick persistent rhizomes, can grow 3 to 5 meters tall, and can disperse both vegetatively and via prolific seed production (Uva et al. 1997). It is commonly found in cultivated fields and disturbed areas (Bryson and DeFelice 2009). In crops, johnsongrass is common in reduced tillage operations due to its perennial nature and is known to spread with the use of farm equipment (Uva et al. 1997). It is an aggressive competitor because of its high biomass production and its ability as a C_4 plant to efficiently use available resources (Riar et al. 2011; Sage and Pearcy 1987). With developing herbicide technology, several efficacious control options were made available for johnsongrass control in agronomic crops. However, johnsongrass has also developed resistance to several herbicides and has remained a serious concern in many parts of the world.

Johnsongrass has been shown to reduce crop yield in major agronomic cropping systems. Williams and Hayes (1984) found that full-season johnsongrass competition reduced soybean yields up to 88%. In cotton, 32 plants per 9.8m of cotton row resulted in 70% yield loss (Bridges and Chandler 1987). In corn, after five years of increased johnsongrass populations in monocultured corn plots, grain yield dropped to zero (Bendixen 1986). Similar yield reductions have been seen when comparing soybean cultivars (McWhorter and Hartwig 1968). Such yield losses translates into economic losses. In the states of Arkansas, Louisiana, and Mississippi, johnsongrass reduced annual cotton profits by $5.9±1.9 million and soybean profits by $23.7±0.6 million dollars (McWhorter 1993). An additional concern is the indirect impact of johnsongrass as a host and reservoir for various maize viruses (Bendixen 1986; Eberwine and Hagood 1995).

In grain sorghum, johnsongrass presents a unique concern with its ability to cross-pollinate and hybridize with grain sorghum (Arriola and Ellstrand 1996).

Herbicide options for controlling johnsongrass are specific to the cropping system. In soybean, johnsongrass can be controlled using graminicides. Control options in corn are nicosulfuron, primisulfuron, rimsulfuron, and sethoxydim (in tolerant varieties) (Hagood 2011). In glyphosate and glufosinate-tolerant corn varieties, glyphosate and glufosinate are used to control the weed. All of these products are applied postemergence, as most preemergence products do poorly for johnsongrass control. The repeated use of a single herbicide for johnsongrass control brings the risk of resistance, creating a selection pressure for biotypes expressing resistant genes. To date, there are eighteen confirmed johnsongrass biotypes resistant to various herbicides (Heap 2013). Most of these biotypes can be found in the North America, but some can also be found in Central and South America and Europe (Heap 2013). Records confirm johnsongrass resistance to glyphosate, nicosulfuron, propaquizafop, quizalofop, fluazifop, haloxyfop, foramsulfuron, primisulfuron, rimsulfuron, fenoxaprop, pendimethalin, sethoxydim, clethodim, imazethapyr, and iodosulfuron (Heap 2013). Most of these cases fall under three sites of action: ACCcase inhibitors, ALS inhibitors, and EPSPS inhibition (Heap 2013). ALS resistance is likely due to an amino acid substitution, though none have been confirmed in johnsongrass (Tranel et al. 2013). ACCase resistance is conferred via target-site resistance and has been shown to spread via pollen (Burke et al. 2006; Burke et al. 2007; Tardif and Powles 1993). For glyphosate resistance, reduced absorption and translocation were confirmed to confer herbicide resistance in johnsongrass (Riar et al. 2011; Vila-Aiub et al. 2012).

In Virginia, a putative herbicide-resistant johnsongrass population believed to be resistant to both nicosulfuron and glyphosate was found in Rockingham County, VA. The objectives of

this research were to confirm herbicide resistance to nicosulfuron and glyphosate, and to determine the molecular mechanisms conferring resistance to those herbicides.

Materials and Methods

Site Location. The putative herbicide-resistant population was located in Rockingham County, VA in a field used for continuous corn harvested for dairy silage. The field had a history of transgenic corn varieties with exclusive use of nicosulfuron and glyphosate for weed control. The producer observed nicosulfuron resistance several years ago, which led to the subsequent switch to glyphosate and glyphosate-tolerant corn varieties to address the issue. The use of continuous glyphosate gave way to suspected glyphosate resistance. The producer had experienced glyphosate failure for two seasons prior to contacting the university. The soil type was a Carbo silt loam (very-fine, missed, active, mesic Typic Hapludalfs).

Plant Material and Dose-Response Studies. The field experiment was initiated in spring 2010 in V2 corn with 15 to 30-cm johnsongrass. Glyphosate-tolerant corn was planted on 76.2 cm rows at a seeding rate of 64,200 seed ha^{-1}. The trial was a RCBD with four repetitions that subjected putative herbicide-resistant johnsongrass to four rates of nicosulfuron and five rates of glyphosate. Plots were 3.6 m x 15.24 m. The rates were as follows: nicosulfuron at 0.14, 0.07, 0.035, and 0.0175 kg ai ha^{-1} and glyphosate at 3.52, 1.78, 0.88, 0.44, and 0.22 kg ae ha^{-1}. Visually estimated weed control was measured at 2, 4, 6, and 8 weeks after treatment (WAT). Surviving plants treated with 1.78 and 3.52 kg ae ha^{-1} of glyphosate were marked 2 WAT with flags and marking tape. All surviving plants were allowed to mature. Mature seed was collected

from marked individual plants and labeled and separated as individual collections, while seed

from plots treated with all rates of nicosulfuron or glyphosate at a rate of 0.88 kg a.e. ha^{-1} or

lower were collected from multiple surviving plants and combined as one collection. In the

greenhouse, select putative herbicide-resistant collections were sown in 28 x 54 cm greenhouse

flats with commercial greenhouse soil mix. Susceptible johnsongrass was also sown for

comparison. Putative herbicide-resistant and susceptible johnsongrass seedlings were grown to 2

to 3-leaf stage and transferred to 10 cm pots and allowed to acclimate. For greenhouse

experiments, pots containing 3 to 4-leaf seedlings were arranged into a RCBD with four

repetitions replicated six times. Johnsongrass seedlings were subjected to nicosulfuron at 0.14,

0.07, and 0.035 kg ai ha^{-1} and glyphosate at 3.52, 1.78, 0.88, 0.44, and 0.22 kg ae ha^{-1}. Plant

height and vigor was measured 7, 14, 21, and 28 days after treatment (DAT). At 28 DAT, plants

were cut at the soil surface and fresh weights were measured. For the field and greenhouse

experiments, data was subjected to ANOVA using PROC MIXED [SAS 9.3] and non-linear

regression analysis. For both experiments, herbicide treatments were considered fixed effects

and blocks were considered random effects. For greenhouse experiments, there was no

replication by treatment interaction, allowing a pooled analysis.

Glyphosate Absorption and Translocation. Putative herbicide-resistant and susceptible

johnsongrass seedlings were germinated in separate 28 x 54 cm greenhouse flats containing sand

and were irrigated with quarter-strength modified Hoagland's solution. At the 2 to 3-leaf stage,

seedlings were cleaned and transferred to 50 ml centrifuge tubes containing quarter-strength

modified Hoagland's solution and were allowed to acclimate for three days using supplemental

light at 300 μmol m^2 sec^{-1} PAR for a 12-hour photoperiod. All plants remained in the centrifuge

tubes and Hoagland's solution was checked and replenished over the course of the experiment.
[14]C glyphosate (glyphosate [phosphonomethyl-14C]) was obtained from Helena Chemical Company (Collierville, TN) with a purity of 99%. [14]C glyphosate was dissolved in sterile water with 0.25% v/v NIS containing 38.4 kbq radioactivity. At the 4-leaf stage, seedlings were spotted with 4 1-µl droplets of ^{14}C glyphosate to the leaf immediately below the newest expanding leaf. Plants were harvested at 48, 72, 96, and 120 hours after treatment (HAT). Two treated plants (one putative herbicide-resistant and one susceptible) were harvested for each harvest timing from each repetition. Plants were partitioned into treated leaf, plant material above treated leaf, aboveground plant material below treated leaf, and roots. Treated leaves were cut at the point where the leaf enters the leaf sheath. Plant foliage and roots were separated at the crown. At harvest, the treated leaf was immediately submersed in 3 ml of deionized water and vortexed for 30 sec to remove any herbicide still residing on the leaf surface. The treated leaf was then submersed into 3 ml of methanol and vortexed for 30 sec to remove the leaf cuticle and any herbicide residing in the cuticle. Both water and methanol rinses were added to 17 ml of scintillation cocktail (SciniVerse) and radioactivity counts were determined using LS 600 liquid scintillation spectroscopy (LSS) (Beckman Coulter Inc., Indianapolis, IN). Additionally, a 1 ml aliquot of Hoagland's solution was added to 19 ml of scintillation cocktail to determine the radioactivity of the growing medium. The plant partitions were dried at 60°C for 24 hours, oxidized using a biological oxidizer OX500 (R.J. Harvey Instrument Corporation, Tappan, NY), and assayed for total radioactivity via LSS. For two replications, the treated leaf was processed for glyphosate metabolism. The treated leaf partitions were ground using 5 ml deionized water in a mortar and pestle into a homogenous aqueous solution. The pestle was rinsed with 1 ml water. Two ml of the total solution was centrifuged at 13,000 RPM and the pellet was re-

suspended and centrifuged three more times using 1 ml water. The supernatant fluid and washes were combined to create a final solution of 5 ml. The final solution was transferred to scintillation vials, 15 ml of scintillation cocktail was added, and radioactivity was assessed via LSS. The remaining 4 ml of solution was dried using a Nitrogen evaporator NVAP 112 (Organomation Associates, Inc., Berlin, MA) and stored in a freezer until samples were ready for metabolism processing.

The experimental design was a split-split plot design with four repetitions replicated three times. The four harvest timings were main plots, putative herbicide-resistant and susceptible populations were sub-plots, and the four plant partitions acted as the sub-sub plots. Absorption data was transformed from disintegrations per minute (DPM) to % of applied ^{14}C glyphosate. Translocation data was transformed from DPM to % of absorbed ^{14}C glyphosate for treated leaf, plant material above or below the treated leaf, and roots. As a split-split plot treatment design, main effects were subjected to ANOVA using PROC MIXED in SAS (9.3). Repetitions were considered random. Fisher's LSD test at $P < 0.05$ was used to perform mean separations where appropriate. There were no differences between replication, allowing a pooled analysis.

Glyphosate Metabolism. Glyphosate metabolism was performed utilizing the same treated putative herbicide-resistant and susceptible johnsongrass treated leaves from the absorption and translocation trials. Dried solutions were re-suspended in 300 μl of methanol, vortexed, and a 100-μl aliquot from each solution was spotted on 20 by 20 cm 500-μm cellulose thin-layer chromatography (TLC) plates. Plates were partitioned into 10 2-cm lanes. Five 1-μl droplets of stock ^{14}C glyphosate were spotted on each plate as a corresponding standard. Plates were developed to a 185 mm solvent front using a solvent system containing ethanol:water: 15 H

NH₄OH:TCA:17 N acetic acid (55:35:2.5:3.5 g:2, (v/v/v/w/v)) (Sprankle et al. 1978) and were

chromatographed using a BioScan System 200 (Bioscan, Inc., Washington, DC). Data was

analyzed with WinScan software (LabLogic, Washington, DC) with a smoothing set to 7-point

cubic and background excluded from the peak area calculation. Peaks below 1% of total

radioactivity were rejected. Parent herbicide and radioactive metabolites were identified by

comparing R_f values to the corresponding standard.

Isolation of EPSPS and ALS Protein Sequences. Putative herbicide-resistant johnsongrass and

susceptible seedlings were germinated in separate 28 x 54 cm greenhouse flats containing

commercial potting mix and were irrigated as needed. At the 3 to 4-leaf stage, seedlings were

transferred to 10 cm pots with commercial potting mix and allowed to acclimate. Putative

herbicide-resistant johnsongrass seedlings were subjected to a 0.44 kg ae ha^{-1} rate of glyphosate

or a 0.035 kg ai ha^{-1} of nicosulfuron and surviving seedlings were allowed to regrow. DNA was

extracted from nicosulfuron-treated putative herbicide-resistant and non-treated susceptible

seedlings using DNeasy Plant Mini kit for genomic DNA (Qiagen, Germany). RNA was

extracted from glyphosate-treated putative herbicide-resistant and non-treated susceptible

seedlings using RNeasy Plant Mini kit for RNA (Qiagen, Germany).

 The ALS gene was sequenced from extracted DNA. Primers were designed based on a

hypothetical sorghum ALS sequence from GenBank (Accession number XM_002452104). The

first primer set (ALS-1) was: Forward primer- 5' TGCAGTGGATAAGGCGGATC 3' and

reverse primer 5' GGAAGCTACCATCTCCGTCG 3'. The second primer set (ALS-2) was

forward primer 5' GGCGGCACAGTACTACACTT 3' and reverse primer 5'

AGAAAGGCAGGGAGATGTGC 3'. Using these primers, DNA from putative herbicide-

resistant and susceptible johnsongrass was amplified using a iProof™ High-Fidelity DNA polymerase (Bio-Rad, CA, USA) at annealing temperature 63°C. PCR reactions were loaded into 1% agarose gel for gel electrophoresis and bands were excised and isolated using QIAquick gel extraction kit (Qiagen, Germany). Gel-purified PCR products were ligated into *E. coli* cells using a pENTR™/D-TOPO® cloning kit (Invitrogen, NY, USA) and plasmid and ALS integration was confirmed via surviving *E. coli* colonies from LB-Kanamycin plates. Selected colonies were amplified using GoTaq® DNA polymerase (Promega, WI, USA) with the aforementioned primers and PCR products were loaded into 1% agarose gel for gel electrophoresis. Cultures that amplified the correct band were subjected to plasmid isolation using QIAprep Spin MiniPrep Kit (Qiagen, Germany). Plasmid DNA from putative herbicide-resistant and susceptible johnsongrass was sent to the Virginia Bioinformatics Institute (VBI) for sequencing.

EPSPS for putative herbicide-resistant and susceptible johnsongrass samples were sequenced using cDNA amplified from extracted RNA. cDNA was derived using RT-PCR and High Capacity cDNA Reverse Transcription Kit (Qiagen, Germany) and random primers. EPSPS was amplified from putative herbicide-resistant and susceptible johnsongrass cDNA using GoTaq® DNA polymerase (Promega, WI, USA) at annealing temperature 60°C. Primers were designed using johnsongrass EPSPS sequence (Accession number HQ436353) and were as follows: forward primer - 5' AGAGCTGTTGTTGTTGGCTG 3' and reverse primer 5' TCCCTCCAGTAATTGCAGCA 3'. PCR reactions were confirmed by running a 5- µl aliquot of each reaction on a 1% agarose gel to confirm band size. PCR reactions were cleaned using QIAquick PCR purification kit (Qiagen, Germany) and were sent to VBI for sequencing. For ALS and EPSPS sequences, resulting sequences were subjected to ClustalW2 multi-sequence

alignment using Geneious (Biomatters, New Zealand). For ALS coding sequences, johnsongrass

sequences were aligned to barnyardgrass (Accession numbers JX415268; JX415271) and

Arabidopsis thaliana (Accession number NM114714). For EPSPS coding sequences,

johnsongrass sequences were aligned to *Sorghum bicolor* (XP002436426), *Zea mays* (X63374),

and *Sorghum halepense* (HQ436351; HQ426352). The sequences from this research have been

submitted to GenBank. ALS sequences are reported as accession numbers __. EPSPS sequences

are reported as accession numbers __.

Results and Discussion

Dose-Response Experiments. In the field experiment, putative herbicide-resistant johnsongrass

was not controlled by nicosulfuron (Figure 1). At the 0.14 kg ai ha^{-1} rate of nicosulfuron,

johnsongrass control was 9% (Figure 1). Glyphosate control increased with increasing

glyphosate rate. The 0.88 kg ae ha^{-1} rate of glyphosate controlled johnsongrass 65% and the 3.52

kg ae ha^{-1} rate of glyphosate controlled johnsongrass 90% (Figure 1). No herbicide treatment

controlled johnsongrass 100% in the field. In the greenhouse experiments, 4-leaf stage

johnsongrass was not controlled by nicosulfuron. At 28 DAT, the putative herbicide-resistant

population had significantly better plant vigor when compared to the susceptible population at all

tested nicosulfuron rates (Figure 2). Plant vigor reduction was 25% for the putative herbicide-

resistant population treated with the 0.14 kg ai ha^{-1} nicosulfuron rate, whereas the susceptible

population had 100% plant vigor reduction at the same rate (Figure 2). Additionally, the putative

herbicide-resistant population had significantly lower reductions in plant heights and plant fresh

weights when compared to the susceptible populations at all tested nicosulfuron rates (Figure 3,

4). There was differential sensitivity in plant vigor and plant height between populations when

johnsongrass seedlings were treated with the 0.22 to 0.44 kg ae ha^{-1} rate of glyphosate (Figure 2,

3). There were no differences observed between populations treated with glyphosate for

harvested plant weight (Figure 4). Overall, glyphosate resistance was not observed in the

greenhouse trials (Figure 2, 3, 4). Putative herbicide-resistant seedlings did not express the same

level of glyphosate resistance that was observed in the field experiments (Figures 1, 2, 3, 4).

Greenhouse grown plants are known to be more susceptible to environmental stresses, which

may have affected plant response to glyphosate (Fletcher and Johnson 1990). Additionally, the

higher levels of glyphosate resistance expressed in the field trials could be attributed to the

mature rhizomatous development of the johnsongrass population in the field, whereas seedling

stage johnsongrass might be more susceptible to lower rates of glyphosate (Burke et al. 2006).

Another factor playing a role in differences seen between field and greenhouse experiments may

be the heritability of herbicide resistance in this population (Burke et al. 2007; Jasieniuk et al.

1996).

Glyphosate Absorption and Translocations Studies. There were no differences in ^{14}C

glyphosate absorption (Figure 5). For both populations, ^{14}C glyphosate absorption ranged from

3-5% of applied ^{14}C glyphosate (Figure 5). Though insignificant, the putative herbicide-resistant

population had higher absorption rates when compared to the susceptible population (Figure 5).

Reduced glyphosate absorption as a mechanism of herbicide resistance in johnsongrass has been

reported in individual accessions (Vila-Aiub et al. 2012), but all research regarding johnsongrass

and glyphosate resistance has observed that a reduction in glyphosate translocation is the primary

means of resistance (Riar et al. 2011; Vila-Aium et al 2012). For this putative herbicide-resistant population, reduced absorption does not appear to confer glyphosate resistance.

Additionally, there were no differences in ^{14}C glyphosate translocation for this putative herbicide-resistant population (Figure 6). The distribution of absorbed ^{14}C glyphosate was similar between johnsongrass populations across all harvest timings and plant partitions (Figure 6). In the treated leaf, both populations retained 43-55% of absorbed ^{14}C glyphosate (Figure 6). At 48 hours, both populations retained approximately 49% of absorbed ^{14}C, but by 120 hours, slightly more ^{14}C glyphosate had translocated to other plant parts (Figure 6). In the above treated leaf plant partition, the putative herbicide-resistant population retained slightly more ^{14}C glyphosate when compared to the wild-type population, though insignificant (Figure 6). The above treated leaf plant partition for the susceptible population retained a range of 9-13% of percent absorbed ^{14}C glyphosate, whereas the putative herbicide-resistant population retained a range of 9-19% of percent absorbed [14]C (Figure 6). The only significant difference seen in ^{14}C glyphosate translocation occurred at the 48 hour harvest for the below treated leaf plant partition (Figure 6). The putative herbicide-resistant population had significantly higher ^{14}C glyphosate (Figure 6). The susceptible population retained an average 15% of absorbed ^{14}C glyphosate and the putative herbicide-resistant population retained an average 24% of absorbed ^{14}C glyphosate (Figure 6). That difference was not seen in later harvest timings (Figure 6). For root partitions, no differences were seen in retained ^{14}C glyphosate (Figure 6).

The lack of ^{14}C glyphosate translocation differences between populations suggests that ^{14}C glyphosate translocation may not be the mechanism of herbicide resistance for this putative herbicide-resistant johnsongrass population. In other research showing reduced translocation of ^{14}C glyphosate in glyphosate-resistant johnsongrass, ^{14}C absorption levels were significantly

higher (Riar et al. 2011; Vila-Aium et al 2012) than the absorption levels presented in this research. Herbicide absorption can be affected by several factors, namely environmental factors affecting plant health such as light, humidity, and moisture (Hammerton, 1967). Additionally, glyphosate absorption can be influenced by glyphosate solution or herbicide application (Liu et al. 1996). The lack of differences in translocation may have been affected by low absorption rates.

Nicosulfuron and Glyphosate Target-site Mutations. There are eight known amino-acid substitutions that confer ALS resistance: Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654} (Tranel et al. 2014). The resulting ALS sequence from the putative herbicide-resistant population presented no single nucleotide polymorphisms (SNP) at these sites to confer ALS resistance (Figure 7). A silent mutation was observed at the Ser_{653} position (Figure 7). Other SNPs were observed in the sequence, but none suggested potential ALS herbicide resistance (Figure 7). Amino acid positions Ala_{122}, Pro_{197}, and Ala_{205} were not sequenced (Figure 7). The lack of the ability to sequence these positions may be due to inherent differences in the ALS gene amongst species. After ClustalW2 alignment with *Echinochloa crus-galli var. curs-galli*, *Zea mays*, and *Arabidopsis thaliana*, is was observed that both populations of johnsongrass did not have conserved sequences until the Asp_{376} position. Additionally, comparing the hypothetical ALS sequence *Sorghum bicolor* to the aforementioned references and both johnsongrass populations showed a similar lack of sequence conservation. The lack of a observed SNP conferring nicosulfuron resistance suggest that either the putative-herbicide resistant population has a non-target site mechanism of resistance or that the johnsongrass ALS gene possess genetic differences not conserved across related species. Enhanced metabolic

deactivation has been documented as a non-target site mechanism of resistance for ALS herbicide resistance (Yu et al. 2009).

Glyphosate resistance can be conferred via a target-site mutation at the Pro_{106} position. The resulting EPSPS sequence from the putative herbicide-resistant population did not show a SNP at the Pro_{106} position (Figure 8). No other SNPs were observed in the sequence (Figure 8). The lack of a target-site mutation at the Pro_{106} position suggests that glyphosate resistance is a non-target site mechanism of resistance. All other research regarding glyphosate resistance in johnsongrass concludes that glyphosate resistance is conferred via a non-target site mechanism of resistance (Riar et al. 2011; Vila-Aium et al 2012).

Glyphosate Metabolism. At 120 hours, metabolism results suggest that there may be differential metabolism between the putative herbicide-resistant and susceptible johnsongrass seedlings. Putative herbicide-resistant johnsongrass treated leaf samples resulted in a single peak with an average R_f value of 0.41 (Table 1, Figure 9). The peak accounted for an average of 92% of the counted radioactivity (Table 1). susceptible johnsongrass treated leaf samples resulted in two peaks. The R_f values for those peaks averaged 0.17 and 0.42 (Table 1, Figure 9), with these peaks accounting for an average of 45 and 46% of measured radioactivity, respectively (Table 1). The R_f peak for ^{14}C glyphosate ranged from 0.41-0.5 accounting for approximately 96% of measured radioactivity (Table 1). The polar metabolite observed in susceptible johnsongrass suggests that glyphosate resistance in johnsongrass may be associated with differential metabolism, although the mechanism is not clear.

If metabolic deactivation is the mechanism of resistance for this population, then metabolism-based resistance might be responsible for multiple resistance to nicosulfuron and

glyphosate. It was observed in the field and the greenhouse that putative herbicide-resistance johnsongrass plants treated with a tank mix of nicosulfuron and glyphosate was completely controlled (data not shown), while plants treated with nicosulfuron and glyphosate in separate applications survived (Figure 1, 2, 3, 4). It could theorized that this putative herbicide-resistant johnsongrass population developed metabolism-based high-level resistance to nicosulfuron, which potentially allowed the population to express low-level resistance to other phytotoxic compounds, such as glyphosate. This may explain why the putative herbicide-resistant population succumbed to a tank mix of the two herbicides; the plants were potentially so overwhelmed by phytotoxic chemical that plants cannot metabolize the chemicals in sufficient time to stay alive. The cytochrome P450 family of enzymes has correlative evidence that herbicide resistance can be conferred via P450-based metabolism (Yuan et al 2007; Yu et al 2009). Even more, there is evidence that multiple resistance can be conferred by P450 metabolism-based resistance (Letouze et al. 2003). For this putative herbicide-resistant population, it appears that multiple herbicide resistance may be conferred via differential metabolism. Additional research should evaluate this possibility by examining P450 activity in response to nicosulfuron and glyphosate treatments.

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Table 1: Rf values for glyphosate as the corresponding standard compared to the average Rf values of parent material glyphosate and metabolites found in putative herbicide-resistant and susceptible johnsongrass treated leaves at 120 hours. Means are the average of 4 repetitions from Trial 1. The peak generated by glyphosate is represented as ROI 1. Each peak accounted for a percentage of counted radioactivity. Within the Rf column and between populations, Rf values followed by the same letter are not significantly different based on Fisher's Protected LSD test at P < 0.05. Abbreviations: ROI, regions of interest; Rf, retardation factor; WT, susceptible johnsongrass population; RC, Rockingham County putative herbicide-resistant johnsongrass population.

Sample	ROI	Rf	% of counted ^{14}C
glyphosate	**1**	**0.41-0.5**	**96**
RC	1	.41 a	92
WT	2	0.17 b	45
	1	0.42 a	46

Figure 1: Putative herbicide-resistant johnsongrass response to nicosulfuron and glyphosate herbicide applications in the field experiment.
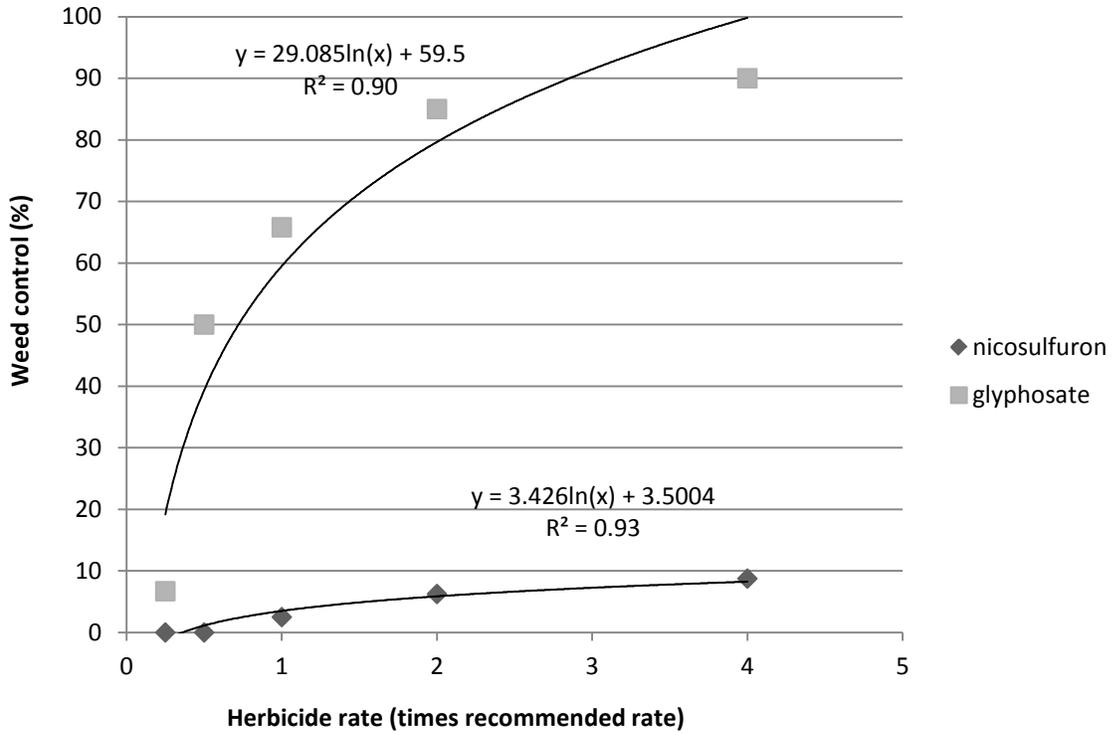
Figure 2: Plant vigor reduction response of putative herbicide-resistant and susceptible 4-leaf stage johnsongrass seedlings to nicosulfuron and glyphosate applications 28 DAT in the greenhouse. Abbreviation: WT, susceptible johnsongrass population; RC, Rockingham County putative herbicide-resistant johnsongrass population.
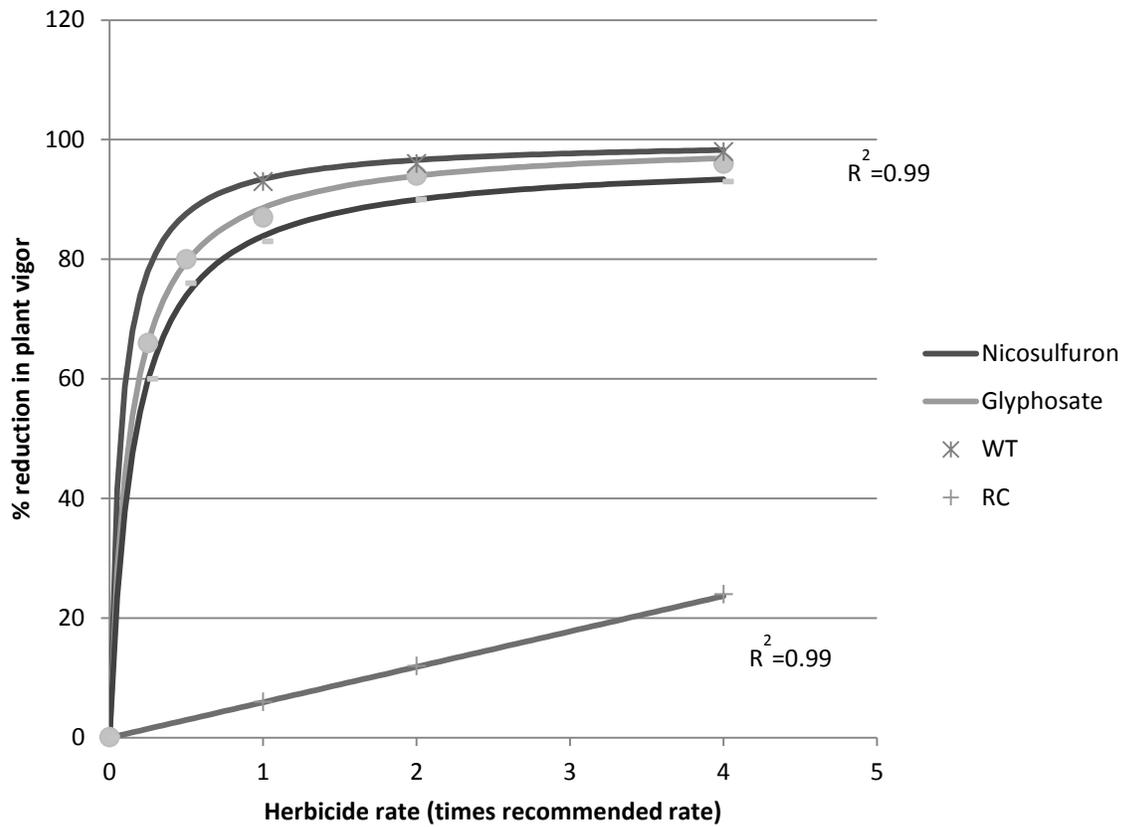
Figure 3: Plant height reduction response of putative herbicide-resistant and susceptible 4-leaf stage johnsongrass seedlings to nicosulfuron and glyphosate applications 28 DAT in the greenhouse. Abbreviation: WT, susceptible johnsongrass population; RC, Rockingham County putative herbicide-resistant johnsongrass population.
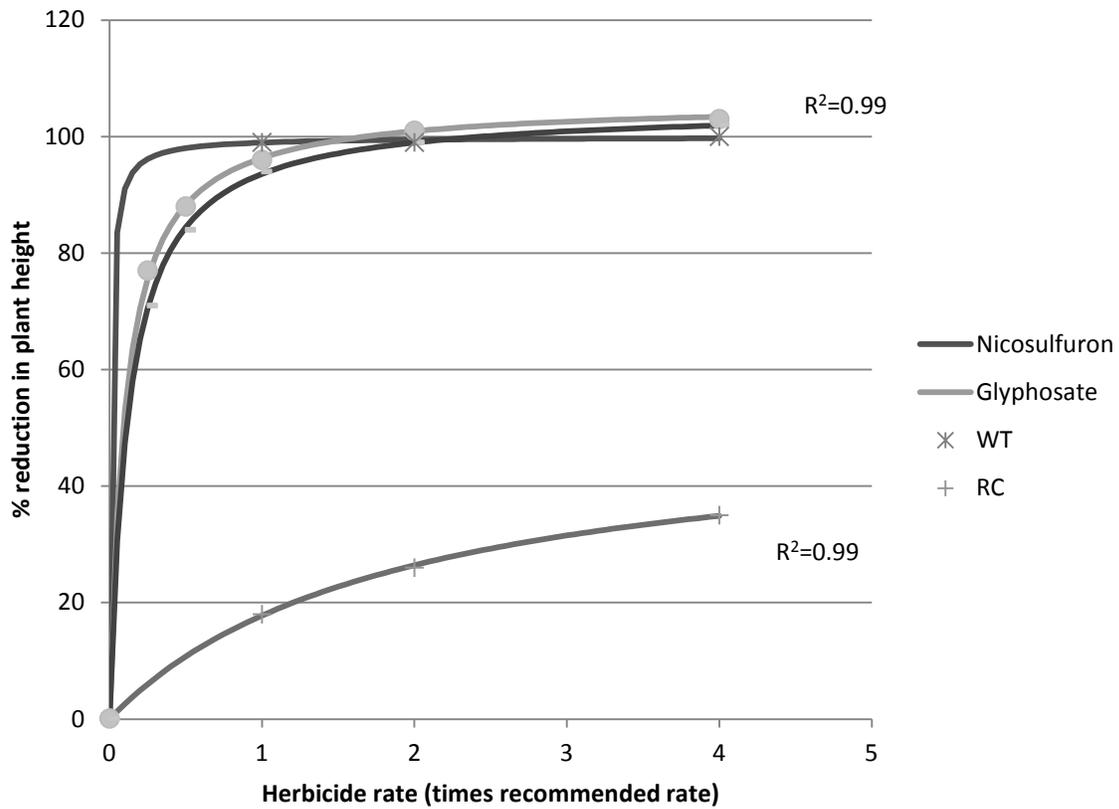
Figure 4: Harvested plant fresh weight reduction of putative herbicide-resistant and susceptible 4-leaf stage johnsongrass seedlings in response to nicosulfuron and glyphosate applications in the greenhouse 28 DAT. Abbreviation: WT, susceptible johnsongrass population; RC, Rockingham County putative herbicide-resistant johnsongrass population.
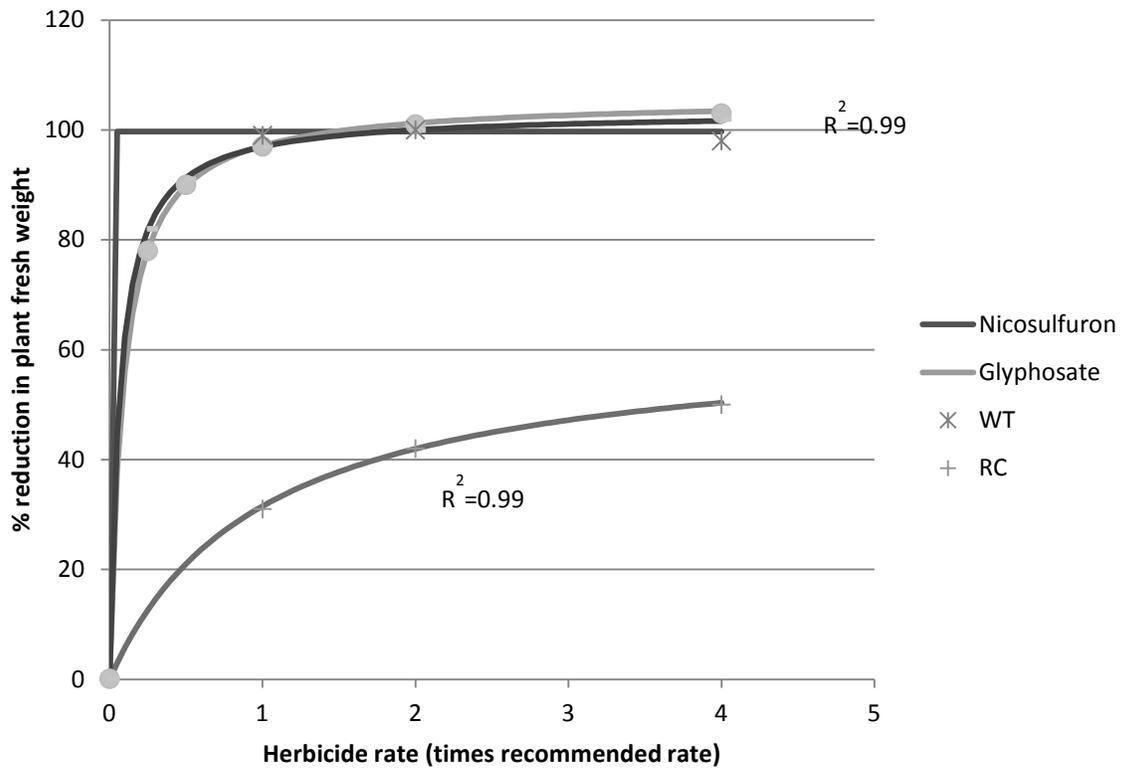
Figure 5: ^{14}C glyphosate absorption response between putative herbicide-resistant johnsongrass and susceptible wild-type populations over harvest timings. Abbreviations: RC, Rockingham County putative herbicide-resistant population; WT, susceptible population.
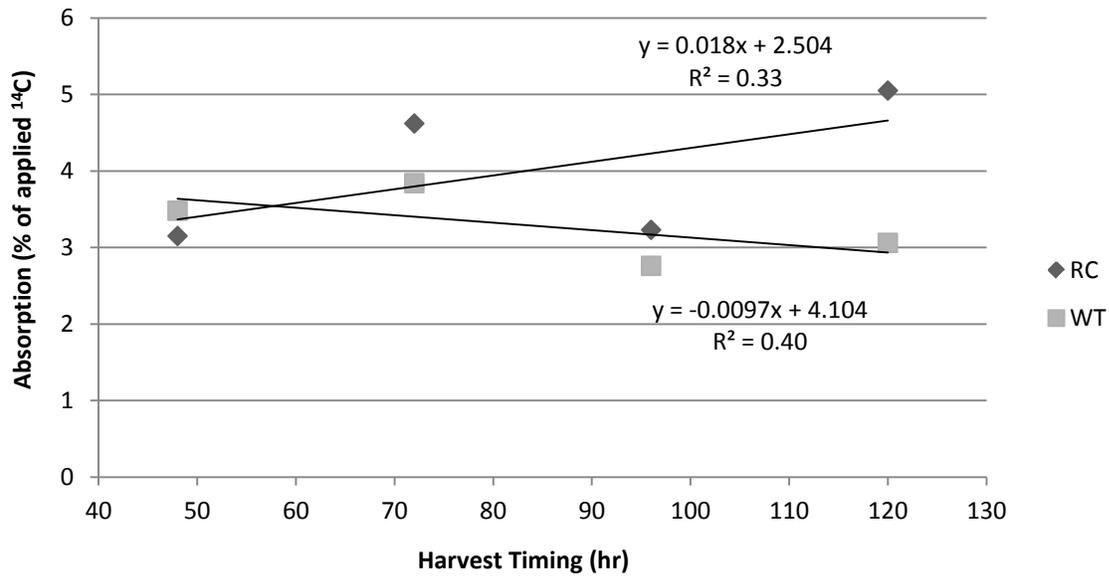
Figure 6: Comparison of the distribution of absorbed ^{14}C glyphosate to other plant parts between putative herbicide-resistant and susceptible johnsongrass populations over harvest timings. Error bars represent ± standard error. Abbreviations: RC, Rockingham County putative herbicide-resistant population; WT, susceptible johnsongrass population.
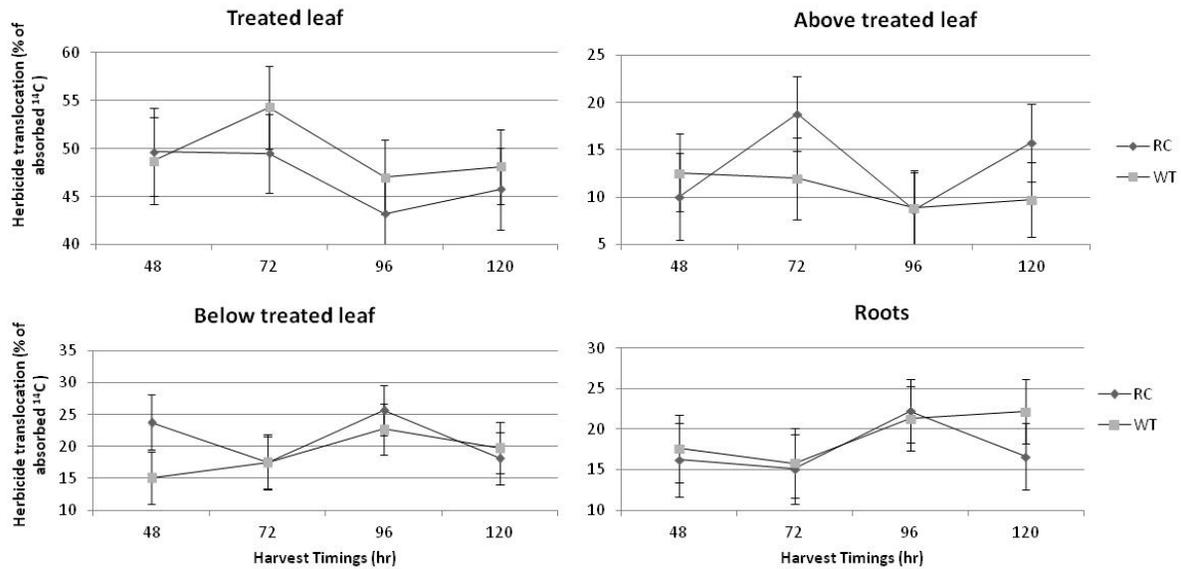
Figure 7: ALS nucleotide sequence alignment of putative herbicide-resistant johnsongrass and susceptible johnsongrass to published ALS resistant and susceptible barnyardgrass (Riar et al. 2013). Ala_{122}, Pro_{195}, and Ala_{205} substitutions were not sequenced in johnsongrass. The five other common substitutions are highlighted; no mutations conferring ALS resistance were observed. Abbreviations: RC, Rockingham County putative herbicide-resistant population; WT, susceptible population; R, resistant; S, susceptible.

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RC                 -----------------------------------------------------
WT                 -----------------------------------------------------
barnyardgrass R GTCGAGGCCCTCGAGCGCTGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGTCTCC
barnyardgrass S GTCGAGGCCCTCGAGCGCTGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGCCTCC
                                                                  Ala122

RC                 -----------------------------------------------------
WT                 -----------------------------------------------------
barnyardgrass R ATGGAGATCCACCAGGCGCTCACCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCAC
barnyardgrass S ATGGAGATCCACCAGGCGCTCACCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCAC

RC                 -----------------------------------------------------
WT                 -----------------------------------------------------
barnyardgrass R GAGCAGGGGGAGGCCTTCGCCGCCTCCGGGTTCGCGCGCTCGTCCGGCCGCGTCGGCGTC
barnyardgrass S GAGCAGGGGGAGGCCTTCGCCGCCTCCGGGTTCGCGCGCTCGTCCGGCCGCGTCGGCGTC

RC                 -----------------------------------------------------
WT                 -----------------------------------------------------
barnyardgrass R TGCGTCGCCACCTCGGGCCCCGGCGCCACCAACCTCGTCTCCGCGCTCGCCGACGCGCTG
barnyardgrass S TGCGTCGCCACCTCGGGCCCCGGCGCCACCAACCTCGTCTCCGCGCTCGCCGACGCGCTG

RC                 -----------------------------------------------------
WT                 -----------------------------------------------------
barnyardgrass R CTCGACTCCATCCCCATGGTCGCCATCACCGGCCAGGTGCCCCGCCGCATGATCGGCACC
barnyardgrass S CTCGACTCCATCCCCATGGTCGCCATCACCGGCCAGGTGCCCCGCCGCATGATCGGCACC
                                                          Pro197
RC                 -----------------------------------------------------
WT                 -----------------------------------------------------
barnyardgrass R GACGCCTTCCAGGAGACGCCAATCGTCGAGGTCACCCGCTCCATCACCAAGCACAACTAC
barnyardgrass S GACGCCTTCCAGGAGACGCCAATCGTCGAGGTCACCCGCTCCATCACCAAGCACAACTAC
                    Ala205
RC                 -----------------------------------------------------
WT                 -----------------------------------------------------
barnyardgrass R CTCGTCCTCGACATCGACGACATCCCCCGCGTCATACAGGAGGCGTTCTTCCTCGCCTCC
barnyardgrass S CTCGTCCTCGACATAGACGACATCCCCCGCGTCATACAGGAGGCGTTCTTCCTCGCCTCC

RC                 ------------------------------------------------------
WT                 ------------------------------------------------------
barnyardgrass R TCTGGCCGGCCGGGGCCGGTGCTCGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCC
barnyardgrass S TCTGGCCGGCCGGGGCCGGTGCTCGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCC
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RC              -----------------------------------------------------
WT              -----------------------------------------------------
barnyardgrass R GTGCCGGTCTGGAACACGCCCATGAGTCTGCCGGGGTACATTGCGCGCCTGCCCAAGCCT
barnyardgrass S GTGCCGGTCTGGAACACGCCCATGAGTCTGCCGGGGTACATTGCGCGCCTGCCCAAGCCT

RC              -----------------------------------------------------
WT              -----------------------------------------------------
barnyardgrass R CCGGCAACTGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAGTCGCGGCGCCCTGTT
barnyardgrass S CCGGCAACTGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAGTCGCGGCGCCCTGTT

RC              ------------------------------------------------------
WT              ------------------------------------------------------
barnyardgrass R CTTTATGTTGGTGGTGGTTGCGCTGCATCCGGTGAGGAGCTGCGCCGCTTTGTGGAGATG
barnyardgrass S CTTTATGTTGGTGGTGGTTGCGCTGCATCCGGTGAGGAGCTGCGCCGCTTTGTGGAGATG


RC              ------------------------------------------------------
WT              ------------------------------------------------------
barnyardgrass R ACCGGAATCCCAGTGACAACTACTCTGATGGGCCTTGGCAACTTCCCCAGTGATGACCCA
barnyardgrass S ACCGGAATCCCAGTGACAACTACTCTGATGGGCCTTGGCAACTTCCCCAGTGATGACCCA

RC              ------------------------------------------------------
WT              ------------------------------------------------------
barnyardgrass R CTGTCTCTGCGCATGCTCGGTATGCACGGTACTGTATATGCAAATTATGCAGTGGATAAG
barnyardgrass S CTGTCTCTGCGCATGCTCGGTATGCACGGTACTGTATATGCAAATTATGCAGTGGATAAG

RC              ---------------------GGCGTGCGGAT-GATGATCGTGTGACAGGAAAGATTGAG
WT              ---------------------GGAGTGCGGAT-GATGATCGTGTGACAGGAAAGATTGAG
barnyardgrass R GCCGACCTGTTGCTGGCATTTGGTGTGCGGTTCGATGATCGTGTGACAGGGAAAATTGAG
barnyardgrass S GCCGACCTGTTGCTGGCATTTGGTGTGCGGTTCGATGATCGTGTGACAGGGAAAATTGAG
                                                    Asp₃₇₆/Arg₃₇₇
RC              GCTTTTGCAAGCAGGGCTAAGATTGTGCACATTGATATTGATCCCGCTGAGATTGGCAAG
WT              GCTTTTGCAAGCAGGGCTAAGATTGTGCACATTGATATTGATCCCGCTGAGATTGGCAAG
barnyardgrass R GCTTTTGCAAGCAGGGCCAAGATTGTGCACATTGATATTGATCCAGCTGAGATTGGCAAG
barnyardgrass S GCTTTTGCAAGCAGGGCCAAGATTGTGCACATTGATATTGATCCAGCTGAGATTGGCAAG

RC              AACAAGCAGCCACATGTGTCCATCTGTGCAGACGTTAAGCTTGCTTTGCAGGGCATGAAT
WT              AACAAGCAGCCACATGTGTCCATCTGTGCAGACGTTAAGCTTGCTTTGCAGGGCATGAAT
barnyardgrass R AACAAGCAGCCACATGTGTCCATCTGTGCGGATGTCAAGCTTGCTTTGCAGGGCATGAAT
barnyardgrass S AACAAGCAGCCACATGTGTCCATCTGTGCGGATGTCAAGCTTGCTTTGCAGGGCATGAAT

RC              GCTCTTCTGGAAGGAAGCACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCAAGCTGAG
WT              GCTCTTCTGGAAGGAAGCACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCAAGCTGAG
barnyardgrass R GCTCTTCTGGAAGGAATCATATCAAAGAAGAGTTTTGACTTTGGCTCATGGCAGGATGAG
barnyardgrass S GCTCTTCTGGAAGGAATCATATCAAAGAAGAGTTTTGACTTTGGCTCATGGCAGGATGAG

RC              TTGGATCAGCAGAAGAGAGAGTTCCCCCTTGGGTATAAAACTTTTGATGACGAGATCCAG
WT              TTGGATCAGCAGAAGAGAGAGTTCCCCCTTGGGTATAAAACTTTTGATGACGAAATCCAG
barnyardgrass R TTGGATCAGCAGAAGAGGGAATTCCCCCTGGGGTACAAAACTTTCGATGAGGAGATTCAG
barnyardgrass S TTGGATCAGCAGAAGAGGGAATTCCCCCTGGGGTACAAAACTTTCGATGAGGAGATTCAG
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RC   CCACAATATGCTATTCAGGTTCTTGATGAGCTGACAAAAGGGGAGGCCATCATTGCCACA
WT   CCACAATATGCTATTCAGGTTCTTGATGAGCTGACAAAAGGGGAGGCCATCATTGCCACA
barnyardgrass R CCACAGTATGCTATCCAGGTTCTGGATGAGCTGACGAAAGGGGAGGCCATCATTGCCACT
barnyardgrass S CCACAGTATGCTATCCAGGTTCTGGATGAGCTGACGAAAGGGGAGGCCATCATTGCCACT

RC   GGTGTTGGGCAGCACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAG
WT   GGTGTTGGGCAGCACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAG
barnyardgrass R GGTGTTGGGCAACACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGACCAAGGCAG
barnyardgrass S GGTGTTGGGCAACACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGACCAAGGCAG

RC   TGGTTGTCTTCAGCTGGTCTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCT
WT   TGGTTGTCTTCAGCTGGTCTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCT
barnyardgrass R TGGTTGTCTTCAGCTGGTCTTGGAGCTATGGGATTTGGTTTGCCAGCTGCTGCTGGTGCT
barnyardgrass S TGGTTGTCTTCAGCTGGTCTTGGAGCTATGGGATTTGGTTTGCCAGCTGCTGCTGGTGCT

RC   GCTGTGGCCAACCCAGGTATCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATG
WT   GCTGTGGCCAACCCGGGTATCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATG
barnyardgrass R GCTGTGGCCAACCCAGGTGTTACAGTTGTTGACATCGATGGGGATGGCAGCTTCCTCATG
barnyardgrass S GCTGTGGCCAACCCAGGTGTTACAGTTGTTGACATCGATGGGGATGGCAGCTTCCTCATG

RC   AACATTCAGGAGCTAGCTATGATCCGAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTA
WT   AACATTCAGGAGCTAGCTATGATCCGAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTA
barnyardgrass R AACATTCAGGAGTTGGCTATGATCCGCATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTA
barnyardgrass S AACATTCAGGAGTTGGCTATGATCCGCATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTA

RC   AACAACCAGCACCTGGGGATGGTGGTGCAG<mark>TGG</mark>GAGGACAGGTTCTATAAGGCCAATAGA
WT   AACAACCAGCACCTGGGGATGGTGGTGCAG<mark>TGG</mark>GAGGACAGGTTCTATAAGGCCAATAGA
barnyardgrass R AACAACCAACACCTTGGGATGGTGGTGCAG<mark>TGG</mark>GAGGACAGATTCTACAAGGCCAACCGA
barnyardgrass S AACAACCAACACCTTGGGATGGTGGTGCAG<mark>TGG</mark>GAGGACAGATTCTACAAGGCCAACCGA
                    Trp574
RC   GCACACACATACTTGGGAAACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACA
WT   GCACACACATACTTGGGAAACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACA
barnyardgrass R GCACATACATACTTGGGGAACCCAGATAATGAGAGCGAGATATATCCGGATTTCGTGACC
barnyardgrass S GCACATACATACTTGGGGAACCCAGATAATGAGAGCGAGATATATCCGGATTTCGTGACC

RC   ATTGCCAAAGGGTTCAACATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTCCATGCA
WT   ATTGCCAAAGGGTTCAACATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTCCATGCA
barnyardgrass R ATTGCCAAAGGGTTTAACATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTACGTGCA
barnyardgrass S ATTGCCAAAGGGTTTAACATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTACGTGCA

RC   GCAATCAAGAAGATGCTTGAGACTCCAGGGCCATACCTCTTGGATATAATCGTCCCGCAC
WT   GCAATCAAGAAGATGCTTGAGACTCCAGGGCCATACCTCTTGGATATAATCGTCCCGCAC
barnyardgrass R GCAATCAAGAAGATGCTCGAGACTCCAGGGCCATACCTGTTGGATATCATTGTCCCGCAC
barnyardgrass S GCAATCAAGAAGATGCTCGAGACTCCAGGGCCATACCTGTTGGATATCATTGTCCCGCAC

RC   CAGGAGCATGTGTTGCCTATGATCCCT<mark><u>AGTGGT</u></mark>GGGGCTTTCAAGGATATGATCCTGGAT
WT   CAGGAGCATGTGTTGCCTATGATCCCT<u>AGT<mark>GGT</mark></u>GGGGCTTTCAAGGATATGATCCTGGAT
barnyardgrass R CAGGAACATGTGTTGCCTATGATCCCG<mark><u>AGCGGT</u></mark>GGCGCTTTCAAGGACATGATCCTGGAT
barnyardgrass S CAGGAACATGTGTTGCCTATGATCCCG<mark><u>AGCGGT</u></mark>GGCGCTTTCAAGGACATGATCCTGGAT
                  Ser653/Gly654
RC   GGTGATGGCAGGACTGTGTATTGATCTAAATTTCAGCATGCACATCTCCCTGCCTTTCTC
WT   GGTGATGGCAGGACTGTGTATTGATCTAAATTTCAGCATGCACATCTCCCTGCCTTTCTC
barnyardgrass R GGTGATGGCAGGACTGTGTAT--------------------------------------
barnyardgrass S GGTGATGGCAGGACTGTGTAT--------------------------------------

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Figure 8: EPSPS nucleotide sequence alignment of putative herbicide-resistant johnsongrass and wild-type johnsongrass to corn EPSPS sequence. Abbreviations: RC, Rockingham County putative herbicide-resistant population; WT, susceptible population.

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WT       GCGGGCGCCGAGGAGATCGTGCTGCAGCCCATCAAGGAGATCTCCGGCACCGTCAAGCTG
Z. mays  GCGGGTGCCGAGGAGATCGTGCTGCAGCCCATCAAGGAGATCTCCGGCACCGTCAAGCTG
RC       ------------------------------------------------------------

WT       CCCGGGTCCAAGTCGCTGTCCAACCGGATCCTCCTGCTCGCCGCCCTGTCCGAGGGGACA
Z. mays  CCGGGGTCCAAGTCGCTTTCCAACCGGATCCTCCTACTCGCCGCCCTGTCCGAGGGGACA
RC       ------------------------------------------------------------

WT       ACAGTGGTTGATAATCTGTTGAACAGTGAGGATGTTCACTACATGCTTGGGGCCTTGAAC
Z. mays  ACAGTGGTTGATAACCTGCTGAACAGTGAGGATGTCCACTACATGCTCGGGGCCTTGAGG
RC       ------------------------------------------------------------

WT       ACTCTTGGGCTCTCTGTCGAAGCAGACAAAGTTGCCAAAAGAGCTGTTGCTGTTGGCTGT
Z. mays  ACTCTTGGTCTCTCTGTCGAAGCGGACAAAGCTGCCAAAAGAGCTGTAGTTGTTGGCTGT
RC                                         ----------------------------------TCTCAGAGCTGTTGTTGTTGGCTGT

WT       GGTGGAAAGTTCCCAGTTGAGGACGCTAAAGAGGAAGTGCAGCTCTTCTTGGGGAATGCT
Z. mays  GGTGGAAAGTTCCCAGTTGAGGATGCTAAAGAGGAAGTGCAGCTCTTCTTGGGGAATGCT
RC       GGTGGAAAGTTCCCAGTTGAGGACGCTAAAGAGGAAGTGCAGCTCTTCTTGGGGAATGCT

WT       GGAACTGCAATGCGGCCATTGACAGCAGCTGTTACTGCTGCTGGTGGAAATGCAACTTAC
Z. mays  GGAACTGCAATGCGGCCATTGACAGCAGCTGTTACTGCTGCTGGTGGAAATGCAACTTAC
RC       GGAACTGCAATGCGGCCATTGACAGCAGCTGTTACTGCTGCTGGTGGAAATGCAACTTAC
                          Pro106
WT       GTGCTTGATGGAGTACCAAGAATGAGGGAGAGACCCATTGGTGACTTGGTTGTCGGATTG
Z. mays  GTGCTTGATGGAGTACCAAGAATGAGGGAGAGACCCATTGGCGACTTGGTTGTCGGATTG
RC       GTGCTTGATGGAGTACCAAGAATGAGGGAGAGACCCATTGGTGACTTGGTTGTCGGATTG

WT       AAGCAGCTTGGTGCGGACGTTGATTGTTTCCTTGGCACTGACTGCCCACCCGTTCGTATC
Z. mays  AAGCAGCTTGGTGCAGATGTTGATTGTTTCCTTGGCACTGACTGCCCACCTGTTCGTGTC
RC       AAGCAGCTTGGTGCGGACGTTGATTGTTTCCTTGGCACTGACTGCCCACCCGTTCGTATC

WT       AATGGAATTGGAGGGCTACCTGGCGGCAAGGTTAAGCTCTCTGGCTCCATCAGCAGTCAG
Z. mays  AATGGAATCGGAGGGCTACCTGGTGGCAAGGTCAAGCTGTCTGGCTCCATCAGCAGTCAG
RC       AATGGGATTGGAGGGCTACCTGGCGGCAAGGTTAAGCTCTCTGGCTCCATCAGCAGTCAG

WT       TACTTGAGTGCCTTGCTGATGGCTGCTCCTTTGGCTCTTGGGGATGTGGAGATTGAAATC
Z. mays  TACTTGAGTGCCTTGCTGATGGCTGCTCCTTTGGCTCTTGGGGATGTGGAGATTGAAATC
RC       TACTTGAGTGCCTTGCTGATGGCTGCTCCTTTGGCTCTTGGTGATGTGGAGATTGAAATC

WT       ATTGATAAATTAATCTCCATTCCCTATGTCGAAATGACATTGAGATTGATGGAGCGTTTT
Z. mays  ATTGATAAATTAATCTCCATTCCGTACGTCGAAATGACATTGAGATTGATGGAGCGTTTT
RC       ATTGATAAATTAATCTCCATTCCCTATGTTGAAATGACATTGAGATTGATGGAGCGTTTT

WT       GGTGTGAAAGCAGAGCATTCTGATAGCTGGGACAGATTCTACATTAAGGGAGATCAAAAA
Z. mays  GGTGTGAAAGCAGAGCATTCTGATAGCTGGGACAGATTCTACATTAAGGGAGGTCAAAAA
RC       GGCGTGAAAGCAGAGCATTCTGATAGCTGGGACAGATTCTACATTAAGGGAGGTCAAAAA
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Figure 9: Radioactivity counts from thin-layer chromatography of parent material glyphosate and its polar metabolites found in treated leaves of putative herbicide-resistant and susceptible (wild-type) johnsongrass seedlings at 120 hours. Mean Rf values are the average of 4 repetitions from Trial 1. Glyphosate as a corresponding standard had an R_f range of 0.41-0.5.