

**EVALUATING METHIOZOLIN PROGRAMS FOR GOLF PUTTING GREENS AND
INVESTIGATING POTENTIAL MODES OF ACTION**

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Evaluating methiozolin programs for golf putting greens and investigating potential modes of action

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ABSTRACT

Annual bluegrass is a winter annual grass that is problematic on golf putting greens due to its light green color, prolific seedhead production and intolerance to stress. On creeping bentgrass putting greens, herbicides for annual bluegrass control are limited. A new herbicide, methiozolin, developed by Moghu Research Center, LLC, in Daejeon, South Korea, safely and selectively controls annual bluegrass in creeping bentgrass and several other turfgrass species. Methiozolin typically controls annual bluegrass over several weeks, allowing desirable turfgrass time to grow into areas previously infested by annual bluegrass with little surface disruption. The mode of action of methiozolin is unknown, but has been proposed to act as either a cell wall biosynthesis inhibitor (CBI) or an inhibitor of tyrosine aminotransferase (TAT). Field studies were conducted at Virginia Tech to investigate strategies promoting surface recovery on putting greens following atypically rapid annual bluegrass loss resulting from methiozolin application, intensive core-cultivation as well as potential interactions with plant growth regulators (PGR's), like ethephon. In the rapid annual bluegrass removal study, all treatments receiving additional fertility via synthetic fertilizer with or without trinexapac-ethyl or biostimulant recovered 1 to 3 weeks more quickly than treatments that did not include additional fertility. Addition of the PGR trinexapac-ethyl inconsistently regulated speed of canopy recovery, both increasing and decreasing recovery speed. Under normal maintenance conditions, methiozolin does not negatively influence putting green recovery, however, if the putting green is exposed to droughty

conditions, methiozolin can reduce recovery time by several weeks. Core-cultivation should be avoided in conjunction with methiozolin and ethephon applications because when this procedure was conducted on the same day as herbicide application it significantly damaged creeping bentgrass, reducing cover to 19% at 2000 g ai ha⁻¹, compared to the non-treated at 62%.

Regarding the question of methiozolin mode of action, laboratory studies supported the claim that addition of exogenous 4-hydroxyphenylpyruvate (4-HPP) alleviates symptoms of methiozolin exposure in lesser duckweed, a model monocot species, but feeding various turfgrass species and annual bluegrass exogenous 4-HPP did not alleviate symptoms. Creeping bentgrass secondary root length and density was not affected by methiozolin, although annual bluegrass, Kentucky bluegrass and perennial ryegrass secondary root lengths were reduced. Based on these data, it does not appear that TAT inhibition is a primary mode of action of methiozolin in turfgrass.

Studies were conducted to determine if methiozolin inhibited cell wall biosynthesis in desirable turfgrass species and annual bluegrass. All species exhibited decreased enrichment of ¹³C in cell-wall sugars from ¹³C-glucose in response to methiozolin and a known cell wall biosynthesis inhibitor, indaziflam. Indaziflam and methiozolin at 0.01 μM inhibited ¹³C enrichment of all sugars less than methiozolin at 1.0 μM, for xylose, arabinose and glucose, but not galactose.

Addition of 4-HPP increased incorporation of ¹³C into xylose, but had no other influence on ¹³C incorporation into other cell wall sugars. Lack of species specific response indicates that cell wall biosynthesis inhibition is probably not the source of interspecific species responses observed in the field.

“The question is not ‘how far.’ The question is, ‘do you possess the constitution, the depth of faith, to go as far as needed?’”

For my family and friends, who were always there.

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ATTRIBUTIONS

Chapter 2 – Improving Creeping Bentgrass Recovery on Golf Greens Treated with Methiozolin. Shawn D. Askew, Ph.D., is an associate professor of Turfgrass Weed Science in the Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. He assisted with the experimental and treatment design of the field studies, as well as statistical data analyses. Erik H. Ervin, Ph.D., is a professor in the Department of Crop and Environmental Science at Virginia Tech. He assisted with design of the biostimulant and fertility programs. Suk-Jin Koo, Ph.D. is the owner of Moghu Research Center, LLC, in Daejeon, South Korea and provided product and funding for these studies.

Chapter 3. Influence of Plant Growth Regulators on Creeping Bentgrass Response to Methiozolin

Shawn D. Askew, Ph.D., is an associate professor of Turfgrass Weed Science in the Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. He assisted with formulating the experimental and treatment design of the field studies, as well as statistical data analyses. Angela R. Post, Ph.D. is an assistant professor of Weed Science in Department of Plant and Soil Sciences at Oklahoma State University. She assisted with the PGR study design and evaluation.

Chapter 4. Investigating Tyrosine Aminotransferase as a Potential Target Site for Annual Bluegrass Control by Methiozolin

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Chapter 5. Effect of Methiozolin on ^{13}C -Glucose Accumulation in Annual Bluegrass and Three Turfgrasses.

Shawn D. Askew, Ph.D., is an associate professor of Turfgrass Weed Science in the Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. He assisted with formulating the experimental and treatment design of this greenhouse experiment, as well as statistical data analyses. Eva Collakova, Ph.D., is an associate professor in the Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech. Her expertise is in transcriptomics, metabolomics, and fluxomics. She formulated the protocol and helped with data collection and interpretation. Suk-Jin Koo, Ph.D. is the owner of Moghu Research Center, LLC, in Daejeon, South Korea and product and funding for these studies.

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Chapter 1. Literature Review

INTRODUCTION

Across the United States, golf courses are a popular destination for tourists and local residents. Economically, golf courses are important sources of revenue, not only as an individual business, but for the surrounding areas (Markwick 2000). Tourists who travel to play golf tend to spend more money than those who choose to opt out of golf (Haydu et al. 2008; Hutchinson et al. 2009). Golfers will tend to revisit areas which have either served them well, or where they have had a satisfying experience (Hutchinson et al. 2009). Clearly, golf course visitors intend on spending a significant amount of time and money at the facility. In 2003, the US Amateur Public Links Championship was held at the Blue Heron Pines Golf Club in Punta Gorda, Florida. The United States Golf Association outlined a “Pace of Play” in order to keep golfers moving through the course. Based on these times, a golfer in this tournament would spend between 4 and 4.5 hours on the course (Caruso and Morrisett 2003). As a result of the time required to play 18 holes, it can be assumed a significant portion of the game is played on putting greens, although there are no peer-reviewed publications to reinforce this statement. It is quite clear that aesthetics and playability of this surface are important for golfers (McCullough et al. 2007).

Golf course putting greens in the transition zone of the United States are most commonly constructed using creeping bentgrass (*Agrostis stolonifera* L.) (McCullough et al. 2007; O’Brien 1981). Creeping bentgrass is a fine textured turfgrass that can tolerate the low heights of cut preferred by golfers. Although creeping bentgrass can withstand low heights of cut, turfgrass quality, rooting and tolerance to stresses can be decreased as a result (Beard 1973; Fagerness and Yelverton 2001; Salaiz et al. 1995). For example, core-cultivation is utilized to reduce thatch,

improve water infiltration and improve soil air movement on creeping bentgrass putting greens. Although the benefits are well characterized, putting green quality is reduced following core-cultivation (McCarty et al. 2007). The act of cultivating a putting green damages turfgrass shoots and decreases rooting following cultivation. Murphy et al. (1993) observed a 16% decrease in root weight density following cultivation, most likely due to major trauma to the roots and disruption of the soil. In addition. Further, when soil moisture is reduced, roots are more susceptible to injury than when soil is moist (Murphy et al. 1993). In order to mitigate some of the damage to greens following core-cultivation, trinexapac-ethyl and fertility products are often applied to pre-condition and recover areas. Although commonly reported in warm-season turfgrass species like bermudagrass, increases in root growth as a direct result of trinexapac-ethyl application are inconsistent (Beasley et al. 2005; McCann and Huang 2007; Wherley and Sinclair 2009). In cool-season turfgrasses, Wherley and Sinclair (2009) and Beasley et al. (2005) did not observe changes in creeping bentgrass or Kentucky bluegrass root growth following applications of trinexapac-ethyl, but suggested that rooting increases could be a direct result of nitrogen applications. Trinexapac-ethyl has been found to increase tiller density in Kentucky bluegrass under traffic stress, improving quality, but rooting was unaffected (Ervin and Koski 2001). Putting greens can be a stressful environment for turfgrass and creeping bentgrass is not the only turfgrass species that can withstand such conditions.

Annual bluegrass (*Poa annua* L.) is a problematic, cosmopolitan, grassy weed on highly maintained turfgrass like putting greens, fairways, lawns and various other managed turfgrass areas. Annual bluegrass has tremendous genetic variability and can be found on every continent, including Antarctica. This inherent variability allows annual bluegrass to colonize many different environments and successfully survive under a wide range of environmental conditions

(Dionne et al. 2010; Molina-Montenegro et al. 2012). The light green color and clumpy growth habit of annual bluegrass disrupt the overall, uniform aesthetic of infested areas by creating a blotched appearance (Beard 1973; Sprague and Burton 1937; Tutin 1957).

Annual bluegrass is an allotetraploid ($2n=4x=28$) with progenitor species presumed to be *Poa infirma* Shrad. and *Poa supina* Kunth. ($2n=2x=14$), the suspected source of the genetic variability within the species (La Mantia and Huff 2011; McElroy et al. 2012; Tutin 1957). Annual bluegrass mainly reproduces sexually, not via apomixis, as observed in other *Poa* species (Kelly et al. 1999; Koshy 1969). The seedhead is a greenish-white, open, pyramidal panicle, roughly 4 to 6 cm long with several spikelets. Leaves are light green and have a boat-shaped leaf tip, much like Kentucky bluegrass (*Poa pratensis* L.). The ligule is a membrane that is roughly 1 to 2 mm in length. The leaves can also appear wrinkled (Tutin 1957; Uva et al. 2007). Both perennial (*Poa annua* L. var. *reptans* Hausskn.) and annual (*Poa annua* L.) types exist on putting greens in the United States, but the perennial type is almost only found in highly maintained areas where the height of cut is less than 0.32 cm (Gaussoin and Branham 1989; La Mantia and Huff 2011; Lush 1989; Till-Bottraud et al. 1990). Differentiation between annual and perennial types can be difficult, but research has been conducted to elucidate the differences for weed control and potential breeding programs. Carson et al. (2007) found that annual and near-annual types grouped on the same bootstrap in cladograms, separate from other perennial types, indicating some level of genetic variability between the types, although they do not separate into species or sub-species. The perennial type appears to grow in a more prostrate fashion, and produces fewer seedheads during peak reproduction compared to the annual biotype (Johnson and White 1998; Lush 1989; Tutin 1957). Annual biotypes expand by aggressive tillering and perennial biotypes will root at tiller nodes (Tutin 1957). Annual bluegrass

germinates throughout the year, but large flushes of germination typically occur in fall and early spring (Johnson and White 1998; Uva et al. 2007). Johnson and White (1997) found that annual and strong perennial types flower after vernalization and/or variable daylength, and typically, vernalization was the major factor for spring flowering of perennial types. Annual, or less perennial types, were more sensitive to a combination of daylength, vernalization or neither when germinating (Johnson and White 1997). Annual types are capable of producing between 150,000 and 675,000 seed per square meter per year (Lush 1988).

Despite thriving in harsh environments, annual bluegrass is sensitive to various abiotic stressors. When compared to desired turfgrass species like Kentucky bluegrass (*Poa pratensis* L.) or perennial ryegrass (*Lolium perenne* L.), annual bluegrass is less tolerant to heat under low fertility and water conditions (Beard 1973; Whener and Watschke 1981). Annual bluegrass has a shallow root system, which could exacerbate symptoms of stress in the plant, especially under low heights of cut (Bogart and Beard 1973; Murphy et al. 1994). Murphy et al. (1994) observed that annual bluegrass had significantly less roots, and root recovery following summer stress than creeping bentgrass mown at fairway height. Physical counts of annual bluegrass roots decreased from 313 ± 47 to 88 ± 16 between June and September. Creeping bentgrass root counts ranged from 760 ± 47 to 761 ± 65 in the same time period (Murphy et al. 1994). Not only does the shallow rooting of annual bluegrass impact its tolerance to heat and drought, but nutrient uptake can be impacted. Pare et al. (2006) noted that, when compared to creeping bentgrass, annual bluegrass rooting depth was insufficient to prevent soluble nitrogen from leaching out of a putting green. Creeping bentgrass also utilized more nitrogen than annual bluegrass, due to a more extensive root system (Pare et al. 2006). In order to maintain optimal canopy coloration and health of plants, nitrogen fertility of 244 kg ha^{-1} should be applied to mixed stands per year

(Schlossberg and Schmidt 2007). Due to its need for intensive management, annual bluegrass can be a challenge.

Abiotic stresses like heat, drought and poor fertility are not the only problems turfgrass managers face when maintaining annual bluegrass. Annual bluegrass weevils (*Listronotus maculicollis* Dietz) feed and reproduce preferentially on annual bluegrass (Miltner et al. 2004). Evidence of feeding can be observed on greens, fairways and any areas where turfgrass is maintained below 3.8 cm (Vargas and Turgeon 2004). Female annual bluegrass weevils lay eggs inside the leaf sheath, and emerged grubs feed inside plant stems (Diaz and Peck 2007). Two complete generations occur in most areas where weevils are found, the first generation matures by mid-summer and the second, late summer (Vittum and Tashiro 1987). Mature adults overwinter in debris found up to 60 m from the maintained turfgrass area, and up to 10 m into forested areas (Diaz and Peck 2007). During active feeding and reproduction, weevils prefer to feed on annual bluegrass mown at fairway height or lower, and enter areas from the edges, often where heavy feeding can be observed (McGraw and Koppenhofer 2009b). Annual bluegrass weevils are mainly controlled by insecticide applications, although researchers have investigated the potential for use of entomopathogenic nematodes, important as reports of pyrethroid resistance have been confirmed (McGraw and Koppenhofer 2009a, 2009b, Ramoutar et al. 2009).

Anthracnose (*Colletotrichum cereal* Manns sensu lato Crouch, Clarke & Hillman) is a problematic fungal disease that infects both annual bluegrass and creeping bentgrass (Smiley et al. 2005). The fungus can cause two different symptoms on infected plants, a foliar blight or basal rot. Acervuli are visible on the leaf surface during foliar infection, but are not obvious when the plant is infected with basal rot, crowns simply appear black. Although this disease

infects both annual bluegrass and creeping bentgrass, infection of annual bluegrass can occur at any time during the growing season, whereas creeping bentgrass is more likely to be infected in summer/early fall (Smiley et al. 2005). Anthracnose is thought to enter the plant through wounding. Mowing height has a significant impact on anthracnose severity in annual bluegrass. When mowing height was decreased from 3.2 mm to 2.8 mm, disease severity increased from 3 to 17% in 2005 and 13 to 21% in 2006, respectively (Inguagiato et al. 2009a). Applications of trinexapac-ethyl, a plant growth regulator, reduced anthracnose infection up to 27% when applied every 7 days during high disease pressure (Inguagiato et al. 2009b). Increasing nitrogen levels can reduce the amount of disease activity up to 24% if applied once every seven days at 4.9 kg ha⁻¹ (Inguagiato et al. 2008).

Annual bluegrass can be an aesthetically pleasing putting surface if the green is managed properly. Unfortunately, annual bluegrass can die unexpectedly due to environmental stress and requires more inputs of pesticides and water compared to creeping bentgrass. Most superintendents consider annual bluegrass a weed but struggle to control it due to lack of chemicals controls that are a) safe for use on putting greens, b) labeled for use on putting greens and c) effective in removing annual bluegrass.

CHEMICAL CONTROLS

Currently, only three herbicides, bensulide, dithiopyr and oxadiazon, are labeled for use PRE on creeping bentgrass putting greens for annual bluegrass control. Both bensulide and dithiopyr are commonly used to control annual bluegrass in mature swards of creeping bentgrass and other cool-season turfgrass species, and provide a significant soil residual (Landschoot et al., 1993, Yelverton and McCarty 2001). Bensulide has been shown to control annual bluegrass

PRE since the 1960's (Bingham and Schmidt 1967). Callahan and McDonald (1992) found that applications of bensulide can control annual bluegrass up to 97% over 4 years of applications of 11 kg ha⁻¹ applied in January plus 6 kg ha⁻¹ in February and March. In the same study, other application regimes resulted in up to 5% stand loss after 4 years, indicating the potential for a small amount of stand loss after several years of applications (Callahan and McDonald 1992). Similarly, Hart et al. (2004) found that although both bensulide and dithiopyr are safe to use on creeping bentgrass and did not impact visual ratings of turfgrass cover, applications of either herbicide reduced root mass at 1 month after treatment in fall and the following spring, but that applications of bensulide in the fall did not impact root mass to the same degree as dithiopyr. Oxadiazon is a shoot-inhibiting herbicide that can be used to effectively control annual bluegrass. Based on the timing of application, annual bluegrass control can decrease from 90% or greater to 23% if applied 60 days versus 30 days prior to fall overseeding of roughstalk bluegrass on a bermudagrass putting green (Toler et al. 2003). Increasing the frequency of application by splitting the rate will not increase control of annual bluegrass with oxadiazon, unlike the increases in control observed with multiple, split applications of dithiopyr (Toler et al. 2003).

Although dithiopyr, bensulide and oxadiazon can effectively control annual bluegrass PRE, there are concerns over decreasing turfgrass quality and stand vigor with some mixtures containing bensulide or oxadiazon (Johnson 1987; Neylan et al. 1997; Shim and Johnson 1992). In 1997, Neylan et al. found that, in combination with endothall, oxadiazon helped to improve annual bluegrass suppression, reducing cover 21% more than endothall alone. The combination of oxadiazon and ethofumesate decreased turfgrass quality from 6 to 4.5 on a 1 to 9 scale (Neylan et al. 1997). Similarly, combinations of oxadiazon and bensulide reduced creeping

bentgrass quality to 80% and 88% of the nontreated at 2 and 3 WAT, respectively, when applied at 13.4 kg ha⁻¹ bensulide and 3.4 kg ha⁻¹ oxadiazon. Opposing results were noted by Johnson (1987), as the same combination injured creeping bentgrass less than when oxadiazon was applied alone in Georgia. Shim and Johnson (1992) noted injury greater than 38% with either the 2G or 50WP formulations of oxadiazon when applied PRE. Despite the injury, plants recovered completely after 6 weeks (Shim and Johnson 1992). Although dithiopyr, bensulide and oxadiazon can effectively control annual bluegrass, concerns of maintenance of turfgrass quality are warranted.

There are several plant growth regulating compounds (PGR's) that are being used to suppress established annual bluegrass on putting green surfaces (Reicher et al. 2015). These PGR's are classified in three main groups: Type I, Type II and Other (Ervin and Zhang 2008, Watschke et al. 1992). Due to the number of plant growth regulating compounds, including herbicides and compounds derived from natural sources, further classifications have been developed within the types over time (Ervin and Zhang 2008).

Type I PGR's are categorized as synthetic compounds which regulate or inhibit cell division (Watschke and DiPaola 1995; Watschke et al. 1992). Typically, these are known as the "seedhead suppressors" as they inhibit cell division in the meristems, thus reducing the number of new vegetative and seedhead-bearing shoots produced by the plant following application. A popular Type I PGR used to manage annual bluegrass on putting greens is mefluidide (Beard and Beard 2005; Senseman 2007; Watschke and DiPaola 1995). Mefluidide is most commonly used to suppress annual bluegrass seedhead production, as it is less effective at suppressing vegetative shoot growth (Beard and Beard 2005). Cooper et al (1987) found that during periods of heavy seedhead production, mefluidide treated annual bluegrass increased rooting depth from between

50 to 57 mm to 57 to 68 mm. Rooting depth of nontreated plants declined from 50 to 57 mm to approximately 47 mm during the same period. Although mefluidide treatments appeared to have a positive impact on root growth, turfgrass quality decreased for 3 to 4 weeks (Cooper et al. 1987). Further, Cooper et al. (1988) found increased amounts of fructose and glucose in annual bluegrass roots when mefluidide was applied at 0.14 and 0.21 kg ai ha⁻¹ versus 0.0 and 0.7 kg ai ha⁻¹, possibly indicating improvements in rooting as a result of those applications.

Timing of application is important for achieving optimal seedhead suppression with mefluidide. Petrovic et al. (1985) controlled annual bluegrass seedheads 64 to 99% when mefluidide was applied over several years and across various rates. Applications were made based on when seedhead development was observed, not a specific calendar date (Petrovic et al. 1985). Danneberger et al. (1987) found that using calendar dates to apply mefluidide resulted in inconsistent control due to differing local climates in areas where mefluidide is used. Although acceptable levels of seedhead suppression was achieved using 15 to 30 growing degree days (GDD) with a base temperature of 13 C, further research needed to be conducted in order to develop a more exact GDD model (Danneberger et al. 1987). Inguagiato et al. (2010) found that mefluidide reduced annual bluegrass seedhead cover 12 to 15% relative to the nontreated, but that these numbers would have been higher if application timing was improved. More recently, unpublished work by Askew and Smith (2012) indicated that applying mefluidide prior to the commonly accepted 50 GDD₅₀ could help decrease the number of seedheads produced overall, but mefluidide applied prior to, or at 50 GDD₅₀ were not statistically different. In research performed by Haguewood et al. (2013), significant phytotoxicity approximately 3 weeks after initial treatment (WAIT) was visible on creeping bentgrass following applications of mefluidide for seedhead suppression.

Class A, Type II PGR's are represented by trinexapac-ethyl, and inhibit gibberellin (GA) biosynthesis late in the pathway (Ervin and Zhang 2008). More specifically, trinexapac-ethyl is a member of the acylcyclohexanedione family of chemicals, and functions as a mimic of 2-oxoglutaric acid. 2-oxoglutaric acid acts as a co-substrate for various dioxygenases and disruptions in function late in the pathway result in reduced plant growth (Rademacher 2000). Trinexapac-ethyl specifically impacts the conversion of GA₂₀, an inactive 3-deoxy form to the active, 3 β -hydroxylated GA, GA₁ (Rademacher 2000; Ridoutt et al. 1996). In experiments performed by Sarkar et al. (2004), barley seedlings demonstrated a reduction in active GA₁, and increased levels of inactive GA₂₀ following trinexapac-ethyl application. GA₁ was present as 3.63 ng g⁻¹ dry weight versus 44.06 ng g⁻¹ GA₂₀ (Sarkar et al. 2004). GA functions can be restored with GA that is already active, further supporting the mode of action as GA inhibition further down the pathway (Rademacher 2000). In fine turf, like putting greens, trinexapac-ethyl is used to reduce plant growth in order to improve color, stress tolerance and reduce clippings produced during mowing (Goss et al. 2002; Krueser and Soldat 2011; McCann and Huang 2007; Qian and Engleke 1999). Trinexapac-ethyl does not consistently increase tillering while reducing vertical growth in turfgrass species, but does consistently increase chlorophyll content and overall cell density (Ervin et al. 2002; Ervin and Zhang 2007). Inguagiato et al (2010) reported an 8 to 22% decrease in anthracnose disease on annual bluegrass following applications of trinexapac-ethyl applied every 7 days. Protections from abiotic and biotic stresses provided by trinexapac-ethyl could allow annual bluegrass plants to become healthier, as this product has been shown to improve the overall health of the green (Branham and Sharp 2007; Inguagiato et al. 2010).

Class B, Type II PGR's, including flurprimidol and paclobutrazol, inhibit gibberellin biosynthesis early in the pathway, by impacting precursor molecules. More specifically, these chemicals inhibit the monooxygenases which catalyze the oxidation reaction of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher 2000). Based on research performed by Rademacher et al. (1987), flurprimidol and other members of the triazole-type chemical family, inhibit the oxidative activities of cytochrome P-450 thus blocking catalysis of *ent*-kaurenoic acid. Early inhibition of GA synthesis could be the reason that Class B PGR's tend to injure turfgrass more than those that inhibit biosynthesis later in the pathway, as both flurprimidol and paclobutrazol can injure turfgrass following application (Baldwin and Brede 2011; Johnson and Murphy 1995). Han et al. (1998) reported a decrease in overall total non-structural carbohydrates (TNC) following applications of flurprimidol and paclobutrazol. These products are also used to decrease plant growth and reduce clippings. Flurprimidol and paclobutrazol are utilized to selectively control annual bluegrass. Paclobutrazol has been found to regulate annual bluegrass growth more severely and for a longer length of time than creeping bentgrass (Branham and Sharp 2007). Although these compounds regulate the growth of each species to different degrees, the plants will exhibit "rebound" growth when coming out of regulation, growing up to 4 times as quickly as the untreated (Branham and Sharp 2007). Post inhibition growth can also be observed via an increase in TNC content following a decline, possibly due to the plants partitioning energy for growth (Han et al. 1998).

Ethephon is neither a Type I nor II PGR, but it is used to suppress annual bluegrass seedheads in cool-season turf (Eggens et al. 1989). When applied alone, or in conjunction with another PGR, like trinexapac-ethyl, ethephon controls seedhead production up to 97%, depending on season and timing (Haguewood et al. 2013). Ethephon works by reacting with

intracellular water and releasing ethylene, phosphate and chloride, signaling plant stress or damage, thus reducing growth (McCullough et al. 2005a; Zhang and Wen 2010). In response to elevated levels of ethylene signaling, the plant will reduce shoot growth and reproduction. Eggens et al. (1989) found an increase in overall creeping bentgrass quality and a decrease in lateral growth of annual bluegrass following ethephon application, demonstrating selectivity on annual bluegrass. Despite these findings, it has been proposed that applications of ethephon could reduce creeping bentgrass vigor and quality as a result of senescence triggered by increased levels of ethylene (McCullough et al. 2005a). There are some conflicting data regarding the potential for ethephon to cause injury to creeping bentgrass (Haguewood et al. 2013; McCullough et al. 2005a). Dernoeden and Pigati (2009) found that treatments containing ethephon (alone or in combination with trinexapac-ethyl) caused severe scalping injury due to crown rising for 35 to 56 days after the second application. In addition to concerns surrounding delayed scalping injury from applications of ethephon, root loss can be a concern on creeping bentgrass putting greens (McCullough et al. 2005a). When applied at 3.8 kg ai ha⁻¹ on a three week interval, root mass decreased 35% by 9 WAIT. After 3 weeks, root length decreased 28% (McCullough et al. 2005a). In perennial ryegrass, ethephon has been found to reduce root length density by 25 and 28% at 0 to 10 cm and 10 to 20 cm depths, respectively after 2 applications at 3.3 kg ai ha⁻¹ (Jiang and Fry 1998). Ethephon reduced rooting of bermudagrass (*Cynodon dactylon x transvaalensis*) ‘TifEagle’ in a linear fashion as application rates increased, but this effect appeared to be mitigated by mixing with trinexapac-ethyl (McCullough et al. 2005b). This could be a result of varying weather, site or management practices, and is worth considering prior to making applications. In addition to reductions in rooting from ethephon applications, reductions in turfgrass quality due to ethylene can be exacerbated if mechanical stress or

artificial wear is applied to the turfgrass in conjunction with ethephon applications (Brosnan et al. 2010).

Ethofumesate is used to control annual bluegrass POST on fairway-height creeping bentgrass, Kentucky bluegrass, tall fescue, bermudagrass and perennial ryegrass (Anonymous 2015; Coats and Krans 1986; Johnson 1983). Ethofumesate impacts the synthesis of waxes and cutins, the end result of an inhibition of very long chain fatty acids in the plant (Abulnaja et al. 1992; Senseman 2007). Soil-applied ethofumesate reduced shoot dry weight 80% at 5.0 kg ha⁻¹, and foliar- and over-the-top applied ethofumesate reduced shoot dry weight 50 and 30%, respectively (Kohler and Branham 2002). Annual bluegrass absorbed 21.5% of ¹⁴C-ethofumesate at 1 DAT, versus 7.6% and 5.8% absorbed by perennial ryegrass and creeping bentgrass at the same date, respectively (Kohler and Branham 2002). Absorption at 14 DAT was similar, annual bluegrass absorbed significantly more (41.8%) versus perennial ryegrass (13.2%) and creeping bentgrass (18.7%) (Kohler and Branham 2002). In field trials, Coats and Krans (1986) found that annual bluegrass was controlled greater than 75% when ethofumesate was applied two or more times to dormant bermudagrass, but bermudagrass growth post-dormancy was delayed when treatments exceeded 1.1 kg ha⁻¹, regardless as to when (December, January or February and combinations therein) applications were made. Further, Coats and Krans (1986) and Meyer and Branham (2006) observed differences in herbicide tolerance in studies evaluating different turfgrass species and varieties following applications of ethofumesate PRE or POST. In studies conducted by Woosley et al. (2003), ethofumesate, applied at 0.85 kg ha⁻¹, beginning in November, did not control annual bluegrass as effectively as applications of paclobutrazol, applied at 0.14 or 0.28 kg ha⁻¹, beginning in March. Paclobutrazol applied in March also gave higher overall quality ratings than applications of ethofumesate (Woosley et al. 2003). Meyer

and Branham (2006) observed between 47 and 62% control with ethofumesate applied at 20.2 kg ha⁻¹ in 1999 and 2001, respectively, and less than 5% control with the same rate in 2000. This apparent lack of control could be due to a rain event occurring less than 24 hours after application (Meyer and Branham 2006). Fall experiments conducted by Hart and McCullough (2007) were unsuccessful in controlling annual bluegrass with ethofumesate. Based on these studies, it appears that ethofumesate can be an effective tool for annual bluegrass control, but efficacy and safety can vary both temporally and spatially (Coats and Krans 1986; Hart and McCullough 2007; Johnson 1983; Meyer and Branham 2006; Woosley et al. 2003)

Bispyribac-sodium is an acetolactate synthase (ALS) inhibiting herbicide used to selectively control annual bluegrass POST on creeping bentgrass and perennial ryegrass fairways (Anonymous 2010; Senseman 2007). Inhibition of ALS results in plant death after several weeks via a lack of three branched-chain amino acids: valine, leucine and isoleucine (Anonymous 2010; McCarty and Estes 2005; Senseman 2007). Despite the label excluding use of bispyribac-sodium on putting greens, research has been performed evaluating turfgrass safety and application programs on both fairways and putting greens (Lycan and Hart 2006a, 2006b, 2006c; McCullough and Hart 2010a; Shortell et al. 2008, Teuton et al. 2007). In absorption and translocation studies, more than 90% of ¹⁴C-bispyribac-sodium remained in the treated leaf, and did not translocate elsewhere in the plant, and absorption increased with the addition of both surfactant and trinexapac-ethyl (37 and 30%, respectively) versus 21% of herbicide alone after 8 hours (Lycan and Hart 2006b; McCullough and Hart 2010b). Further, Lycan and Hart (2006b) found that, when averaged across creeping bentgrass, annual bluegrass and Kentucky bluegrass, soil and soil plus foliar applications of bispyribac-sodium injured turfgrass 66 and 64%, respectively, whereas foliar alone injured turfgrass 36%. McCullough and Hart (2010a) found

that as bispyribac-sodium rate increased from total rates of 148, 222 and 296 g ai ha⁻¹, which were divided into 2, 3 and 6 split-rate applications, injury to turfgrass increased. Applications that were made at 1 week intervals injured turfgrass more than those applied at 2 week intervals. Annual bluegrass was controlled between 81 to 83 and 91%, across 148, 222 and 296 g ai ha⁻¹, respectively and 78 to 83 and 94% across 2, 3 and 6 applications, respectively (McCullough and Hart 2010a). At 8 WAIT, Teuton et al. (2007) controlled annual bluegrass 75 and greater than 95% with bispyribac-sodium applied at 12 and 24 g ai ha⁻¹, respectively, weekly for 12 weeks. Although high levels of control were achieved with 12 weekly applications, at the conclusion of the study at 16 WAIT, annual bluegrass was controlled 96%, number of applications above 8 did not influence total control ratings (Teuton et al. 2007). Creeping bentgrass injury in 2003 and 2005 differed significantly. At 4 WAIT in 2003 the most severe injury ranged between 46 to 50% with weekly applications of bispyribac-sodium applied at 24 g ai ha⁻¹, but all injury observed was transient and turfgrass recovered. In 2005, however, turfgrass was injured between 80 and 85% with bispyribac-sodium applied weekly at 24 g ai ha⁻¹ at 8 WAIT. At the trial conclusion, plots were injured between 30 and 60%, and needed to be re-sodded (Teuton et al. 2007). Differences in these two studies could be due to trial initiation differing between March and May/June, as it has been reported that applying this product at different times of year can adversely impact both control and turfgrass injury (Lycan and Hart 2006c, McCullough and Hart 2006, McCullough and Hart 2010a, Teuton et al. 2007). On fairway height creeping bentgrass, bispyribac-sodium applications were found to reduce cover to 48 and 7% when applied twice at 74 and 146 g ai ha⁻¹, versus 78% in the nontreated. When applied in conjunction with nitrogen fertility, discoloration of both creeping bentgrass and annual bluegrass was reduced. Despite

mitigated discoloration, bispyribac-sodium applied at 146 g ai ha⁻¹ to annual bluegrass was not influenced by nitrogen application after 4 and 5 weeks (McCullough et al. 2011).

METHIOZOLIN

Methiozolin (5{2, 6-difluorobenzyl}oxymethyl-5-methyl-3, 3{3-methylthiophen-2-yl}-1, 2-isoxazoline) is a new herbicide developed and manufactured by Moghu Research Center in Daejeon, South Korea for the safe and selective control of annual bluegrass. Methiozolin is a member of the isoxazoline class of chemistry, a class that includes anti-parasitic agents and inhibitors of corrosion (Koo et al. 2013; Gassel et al. 2013; Heckerroth et al. 2011; Ryu et al. 2002; Yildirim and Cetin 2008). Methiozolin was discovered when examining derivatives of 5-benzyloxymethyl-1, 2-isoxazoline for herbicidal activity and safety in rice (*Oryza sativa* L.) production. These derivatives were found to have safety to rice plants and selectivity on grassy weeds commonly found in paddy fields, including barnyardgrass (Hwang et al. 2006; Ryu et al. 2002). Nam et al. (2012) found that, when synthesized, methiozolin exists as two isomers, (*S*)- and (*R*)-, as well as a racemic mixture of the two. In experiments evaluating isomeric efficacy, the (*S*)-isomer and racemic mixture demonstrated herbicidal activity, but the (*R*)-isomer did not (Nam et al. 2012). Similar findings regarding herbicide degradation have been observed with isomers of mecoprop (Tett et al. 1994). Despite finding activity on weedy species, the mode of action of this compound, coded EK-5229, or methiozolin, is unknown. Methiozolin has an LD₅₀ of >2000 mg kg⁻¹ body weight, meaning, it is non-toxic in rats. Further, it has been found to absorb via the gastrointestinal tract and is excreted via urine and feces with no tissue accumulation within 2 days (Hwang et al. 2013b). In soil, methiozolin has an approximate half life of 49 days under aerobic conditions, but the half-life under anaerobic conditions is not known, as it exceeded the time limits of the experiment (Hwang et al. 2013a). Norsworthy et al.

(2011) found that PRE activity on barnyardgrass in rice lasted until approximately 6 weeks after application, indicating some PRE activity is an attribute of this product. Researchers have proposed two different potential modes of action for this product (Grossmann et al. 2011b; Lee et al. 2007).

Lee et al. (2007) proposed that cell wall biosynthesis inhibition could be a direct or indirect effect of this herbicide in susceptible species. When compared to known cell wall biosynthesis inhibitors (CBI's), morphological characteristics are not similar. Researchers examined the effects of methiozolin on germinating barnyardgrass (*Echinochloa crus-galli* P. Beauv.) in comparison to dichlobenil, butachlor and pendimethalin. In this study, EK-5229 showed long, thin, purple coleoptiles, unlike symptoms observed with the known CBI treatments. Pendimethalin treated plants have been reported to be darker-green, stunted and have swollen stems/leaves (Smith 2004; Lee et al. 2007). In kale, dichlobenil caused stem-splitting and severe root damage (Leach and Biddington 1971). Similarly, Tresch and Grossmann (2003) found that dichlobenil will cause clubbing of root tips in barnyardgrass and reduced cell division following treatment. A newer, potent CBI, indaziflam, produces swelling of roots, and increased lignification, symptoms not observed when examining methiozolin treated seedlings (Brabham et al. 2014). Although seed germination occurred between 81 to 93%, seedling leaf and root length decreased at rates above 1 and 0.1 μ M methiozolin, respectively (Lee et al. 2007). No bleaching, burning or rapid necrosis of the tissue was observed either, indicating a lack of effect on carotenoids, protoporphyrinogen oxidase (PPO) or Photosystem I (Grossmann et al. 2011a; Lee et al. 2007).

Corn root tips were examined to determine the extent to which ^{14}C -glucose was incorporated into the cell wall components (Koo et al. 1996; Lee et al. 2007). Cellulose is

composed of β (1 \rightarrow 4) linked D-glucose subunits (Carpita and Gibeaut 1993). Hemicellulose is more intricate, composed of several different sugars, but with a high ratio of arabinose to xylose, although these ratios differ between species (Kozlova et al. 2014; Sun et al. 1995; Verbruggen et al. 1995). Grass cell walls are composed mainly of the hemicellulose, glucuronoarabinoxylan, where it makes up 20 to 40% of the weight of the primary cell wall, and 40 to 50% of the weight of the secondary cell wall (Scheller and Ulvskov 2010). When cellulose microfibril structure is altered as a result of hemicellulose deficiency, plants tend to become more brittle and prone to breakage or lodging (Scheller and Ulvskov 2010; Vogel 2008). Corn was found to be sensitive to methiozolin, and barnyardgrass root tips were more affected by herbicide application relative to other plant parts (Lee et al. 2007). Inhibition of corn cell wall growth occurred after 6 hours of treatment and incorporation was inhibited at concentrations beginning at 0.1 μ M and was completely inhibited after 12 hours of treatment at 1 μ M. Incorporation of 14 C-glucose into cellulose decreased from 149.2% to 59.1% between 6 and 12 hours (relative to the untreated) and hemicelluloses, GAX 1 and GAX2 ((2'-O-(4-O-methyl- α -D-glucopyranosyluronic acid)-D-xylose (methylglucuronoxylose and 2'-O-(4-O-methyl- α -D-glucopyranosyluronic acid)-D-xylobiose (methylglucuronoxylobiose, respectively), decreased from 158.9% to 80.8% and 132.2% to 62.4%, relative to the nontreated, respectively (Lee et al. 2007; Qian et al. 2003). Previous research on quinclorac, which is currently categorized as either a cellulose inhibitor or synthetic auxin, utilized similar methods to determine its mode of action (Koo et al. 1996; Koo et al. 1997). Other CBI herbicides reduced incorporation of labeled glucose into cell walls, including cellulose and hemicellulose (Heim et al. 1990; Monezinos and Delmer 1980). It is the inhibition of cellulose production and/or deposition that results in symptoms of characterized CBI's, and although Lee et al. (2007) concluded that although cell wall biosynthesis was affected

as a result of methiozolin application, CBI may not be a primary mode of action, but it could be acting secondarily on cell wall biosynthesis (Brabham et al. 2014; Sabba and Vaughn 1999).

The second proposed mode of action is inhibition of the enzyme tyrosine aminotransferase (TAT) (Grossmann et al. 2011b). Tyrosine aminotransferase catalyzes the reaction which converts L-tyrosine to 4-hydroxyphenylpyruvate (4-HPP) and is dependent on pyridoxalphosphate (Lee and Facchini 2011; Prabhu and Hudson 2010). Following conversion of L-tyrosine to 4-HPP, 4-HPP dioxygenase (4-HPPD) catalyzes the formation of homogentisate (Fielder et al. 1982, Moran 2005). This reaction is important in the initial steps of the prenylquinone pathway and inhibition results in a disruption in the eventual synthesis of plastoquinones, tocopherols, rosmarinic acid and other compounds (Diamondstone 1966; Lopukhina et al. 2001; Prabhu and Hudson 2010). When plants are aging or under stress, they require more protectant compounds, including tocopherols. To produce higher levels of protectant compounds, higher levels of precursor, homogentisic acid via transamination of 4-HPP by TAT is required (Lee and Facchini 2011; Leipman and Olsen 2004; Hollander-Cytko et al. 2005; Rippert et al. 2009). In *Arabidopsis thaliana* (L.) Heynh. there are 44 genes which encode for aminotransferases, and seven of those genes are TAT genes (Grossmann et al. 2011b; Prabhu and Hudson 2010; Riewe et al. 2012). Grossmann et al. (2011) observed an inhibition of the isoenzyme TAT7 in *A. thaliana* following applications of cinmethylin, methiozolin and two compounds structurally similar to methiozolin, ISO1 and ISO2 at, or above, 200 μM . Based on the high rates of methiozolin needed to inhibit TAT7, Grossmann et al. (2011b) proposed that TAT isoenzymes in more sensitive species could be inhibited at much lower rates.

Grossmann et al. (2011b) inhibited the growth of duckweed (*Lemna aequinoctialis* Welw.) with methiozolin at 0.2 μM . In contrast to the physical effects observed by Lee et al.

(2007) on barnyardgrass and corn seedlings, Grossmann et al. (2011b) observed bleaching of duckweed (*Lemna aequinoctialis* Welw.) fronds followed by necrosis of the meristematic regions after approximately 72 hours of exposure to methiozolin. The observed bleaching symptom was thought to indicate a disruption in carotenoid biosynthesis and function in the plant as similar symptoms are evident from other inhibitors of 4-HPPD and phytoene desaturase (PDS) (Grossmann et al. 2011b; Viviani et al. 1998). In order to determine which part of the pathway was disrupted, Grossmann et al. (2011b) added three different compounds back into the duckweed growth media following methiozolin application in an attempt to reverse the symptoms and learn the location of pathway disruption. Addition of exogenous L-tyrosine did not affect plants, nor did exogenous homogentisate. Re-addition of exogenous 4-HPP, however, returned plants to nearly normal growth indicating the disruption was occurring between L-tyrosine and 4-HPP and 4-HPP is needed for homogentisate production (Grossmann et al. 2011b). Similar results were observed for cinmethylin and ISO1. Based on the differing conclusions by both Lee et al. (2007) and Grossmann et al. (2011b), the primary mode of action of methiozolin in annual bluegrass is currently unknown, and needs to be evaluated.

Several researchers have investigated potential PRE activity of methiozolin against annual bluegrass, application placement as it related to efficacy and absorption and translocation of radio-labeled methiozolin. Hwang and Koo (unpublished) achieved complete annual bluegrass control with methiozolin applied PRE at 500 g ai ha⁻¹ and 125 g ai ha⁻¹ when seed were planted at a depth of 1 and 5 mm. Annual bluegrass was controlled 91% when seed were planted at 10 mm depth. Further, Nam et al (2012) found that the S-isomer of methiozolin imparted PRE control of annual bluegrass beginning at 31.3 g ai ha⁻¹, but the R-isomer did not. A racemic mixture of both inhibited annual bluegrass germination beginning at 15.6 g ai ha⁻¹ (Nam et al.

2012). Flessner et al. (2013) found that methiozolin does inhibit seed germination at 1.68 kg ai ha⁻¹, providing support to Hwang and Koo (unpublished) and Nam et al. 2012. McCullough et al. (2013) found that reseeding cool-season turfgrass species should be delayed approximately two weeks following methiozolin application, further supporting the findings of Hwang and Koo (unpublished) and Flessner et al. (2013). Creeping bentgrass cover was reduced to 18% at 8 weeks after seeding (WAS) at 0.56 kg ai ha⁻¹, and 6% at both 1.12 and 2.24 kg ai ha⁻¹ at 8 WAS. When seeding was delayed two weeks, cover was 65%, 58% and 44% at 0.56, 1.12 and 2.24 kg ai ha⁻¹, respectively. Perennial ryegrass and tall fescue seed germination was affected similarly (McCullough et al. 2013). Methiozolin controls 2-tiller annual bluegrass plants approximately 50%, but controls plants 6 tillers or larger approximately 28% (Flessner et al. 2013). Hwang and Koo (unpublished) also observed similar effects to annual bluegrass when treated at the 2- and 4-tiller growth stages. Askew and McNulty (2014) noted that methiozolin reduced annual bluegrass cover 25% in the first three months following fall treatment, but did not control existing bluegrass, perhaps due to PRE activity. After two years of single treatments in spring and fall, annual bluegrass cover was 34% in plots treated with 500 g ai ha⁻¹ methiozolin and 56% in the non-treated (Askew and McNulty 2014).

¹⁴C-Methiozolin is absorbed by both foliage and roots, but displays mainly acropetal movement (Yu et al. 2014). Flessner et al. (2013) found limited translocation from the point of herbicide application, either on the leaf or via hydroponic solution. In areas above the treated leaf, 10% of labeled, foliar-applied methiozolin was recovered and 1.4% was detected below the treated leaf. All other evaluated plant parts contained 1.3% of applied methiozolin (Flessner et al. 2013). When treated via roots, the majority of methiozolin is translocated from the roots to the crown, as evidenced by an increase in methiozolin in crowns versus leaves, approximately 30

μg and $8 \mu\text{g}$, respectively (Flessner et al. 2013). This is due to the low $\log K_{ow}$ value for methiozolin, which does not allow for ease of transport in xylem or phloem (Flessner et al. 2013). Non-radio-labeled studies also indicated that foliar-only and soil-only applications of methiozolin did not control annual bluegrass as effectively as soil-plus-foliar-treated plants with a single application (Brosnan et al. 2013b). Flessner et al. (2013) observed similar results, but found that soil-plus-foliar applications of methiozolin were nearly equivalent to foliar-only treatments at 1.68 kg ha^{-1} . Both soil-plus-foliar and foliar-only treatments were more effective than soil-only treatments at 3.36 kg ha^{-1} . These data indicate that absorption via soil- and foliar-applied methiozolin is important for annual bluegrass control. To date, only one study has been published investigating an apparent differential selectivity to methiozolin between annual bluegrass and creeping bentgrass. McCullough et al. (2013) found that, when absorbed through the roots at warmer temperatures, $30/25 \text{ }^\circ\text{C}$ day/night, and annual bluegrass translocates more ^{14}C -labeled methiozolin to the shoots than creeping bentgrass. This difference, however, was not apparent at lower temperatures, $10/15 \text{ }^\circ\text{C}$ day/night.

Herbicide applications in controlled conditions often do not address various issues pertaining to field applications. Edaphic factors, weather and timing of application can all influence herbicide efficacy. It is well known that many herbicides behave differently depending on the soil type, amount of organic matter, moisture and humidity (Johnson and Young 2002; McCullough and Hart 2006; McCurdy et al. 2008). Several studies have been conducted to elucidate whether or not putting green construction, temperature or application timing influence methiozolin efficacy. Sand-based putting greens appear to require more applications or higher rates of methiozolin in a single application to achieve acceptable levels of control as opposed to soil-based putting greens. Research performed by Brosnan et al. (2013a) investigated two types

of putting greens, sand-based and soil-based in both Tennessee and Texas. Despite a lack of statistical support, it appeared that methiozolin was more efficient on soil-based putting greens. When applied at 500 g ha⁻¹ control of annual bluegrass on a soil-based root zone ranged from 72 to 80% whereas control on a sand-based root zone ranged from 57 to 64% (Brosnan et al. 2013a). Due to the lack of organic matter on sand-based putting greens, potential for limited movement of methiozolin away from the root zone exists, which could assist in explaining the apparent lack of control on sand-based root zones (Brosnan et al. 2013a; Flessner et al. 2014). Flessner et al. (2014) found that methiozolin is more mobile in sand with an R_f value of 0.464 versus 0.02 in sandy loam soil. Based on research on creeping bentgrass rooting during flooded conditions performed by Jiang and Wang (2006), it was also proposed that annual bluegrass could be better controlled on soil-based rootzones because rooting is shallower, thus exposing more tissue for herbicide absorption, (Brosnan et al. 2013b; McCullough et al. 2013). Koo et al. (2013) reported that root absorption of methiozolin is the major means of herbicide uptake, and leaf absorption is not, lending some support to results obtained by Brosnan et al. (2013b) and McCullough et al. (2013).

Temperature can sometimes impact herbicide efficacy and turfgrass injury. Bispyribac-sodium will severely injure turfgrass when applied at temperatures less than 13 °C and above 29 °C (McCullough and Hart 2005). McCullough et al. (2013) recommended the best time to apply methiozolin on creeping bentgrass putting greens, from a safety standpoint, is beginning in February/March. Based on laboratory studies with ¹⁴C-methiozolin, McCullough et al. (2013) found that methiozolin applied at lower temperatures (10 C) resulted in 50% injury to annual bluegrass at 1.3 kg ai ha⁻¹, but applications at 20 °C and 30 °C injured annual bluegrass 50% at 1.0 kg ai ha⁻¹. Control of annual bluegrass was 80 to 100% when methiozolin was applied in the

fall, and creeping bentgrass injury was acceptable, but control decreased to 60% in spring with no creeping bentgrass injury (Koo et al. 2013). Brosnan et al. (2013b) found that established roots (5 to 15 cm depth) are injured uniformly by methiozolin, with reductions of root-length density of 9 to 25% and 0 to 5% in sand- and soil-based root zones, respectively. Different application programs are being developed for use in different areas of the country to achieve the least disruptive removal of annual bluegrass possible. Although methiozolin typically controls annual bluegrass over several weeks, it may interact with environmental factors and cause annual bluegrass to decline rapidly, leaving voids in the putting surface (McNulty et al. 2011; Venner et al. 2012). When voids occur on greens, superintendents strive to repair the area quickly through proper cultivar selection, various fertility products, seeding, sodding, etc. (Jones and Christians 2012; Lee 2012; Rossi and Grant 2009; Walker et al. 2003). Although these scenarios could possibly result from methiozolin use, no peer-reviewed research has investigated the effects of unintentional rapid annual bluegrass removal with methiozolin.

Safety of methiozolin to several other warm- and cool-season turfgrass species has been documented. Koo et al. (2013) documented injury to zoysiagrass at 6 weeks after treatment (WAT), between 13% and 27% at 2 and 4 kg ai ha⁻¹, respectively. No injury was observed at 1000 g ai ha⁻¹. Both perennial ryegrass and tall fescue responded similarly. Hoisington et al. (2014) investigated the tolerance of various bentgrass species and cultivars to methiozolin applications and found that creeping bentgrass was the most tolerant to applications of methiozolin, both 50% growth reduction and 25% injury were achieved at 1.9, 2.4 and 3.2 kg ai ha⁻¹, whereas colonial bentgrass and velvet bentgrass only tolerated rates ranging from 0.2 to 0.9 kg ai ha⁻¹. Out of eight popular cultivars examined, 'Penn A-4' was found to be the most tolerant to methiozolin up to 2.6 kg ai ha⁻¹ (Hoisington et al. 2014). Annual bluegrass is

considerably more sensitive than other turfgrass species when mature plants are subjected to field or greenhouse applications (Askew and McNulty 2014; Koo et al. 2013; Yu and McCullough 2014) but treatments to germinating turfgrass or root exposure in hydroponic or aeroponics systems tends to injure all turfgrass species similarly (McCullough and Gomez de Barreda 2012; Koo et al. 2013; Venner et al. 2012).

RESEARCH OBJECTIVES

The objectives of the research are to 1) determine which cultural practices influence the recovery of a voided putting green surface following rapid annual bluegrass removal as a result of methiozolin applications, 2) determine the role aeration plays in the potential antagonistic interaction between methiozolin and ethephon, 3) determine whether or not methiozolin influences the incorporation of labeled sugars into the plant cell wall and 4) determine whether or not methiozolin inhibits tyrosine aminotransferase activity in four grass species, including annual bluegrass.

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Chapter 2. Improving Creeping Bentgrass Recovery on Golf Greens Treated with Methiozolin

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Methiozolin is a new herbicide manufactured by Moghu Research Center, LLC, for the safe and selective removal of annual bluegrass from creeping bentgrass putting greens. Studies were conducted in 2013 and 2014 to assess fertility programs to aid putting green canopy recovery when methiozolin controls annual bluegrass. In all cases, adding 7 kg ha⁻¹ of N, P, and K from fertilizer or biostimulant biweekly to greens sped turfgrass recovery time by 1 to 3 weeks compared to the standard greens fertility program alone. Methiozolin increased turfgrass recovery time at one location where a severe drought occurred but not at another location that did not experience drought stress. These data indicate that it will be important to avoid turfgrass stressors, like drought, when using methiozolin programs to control annual bluegrass on greens. Creeping bentgrass treated with biostimulants recovered equivalent to or faster than synthetic fertilizer (SF) in all cases. In the presence of methiozolin treatments, trinexapac-ethyl reduced time to 90% recovery (T90) by 0.25 to 0.5 weeks at two locations and increased T90 recovery time by 0.1 weeks at one location. Otherwise, SF plus trinexapac-ethyl treated plots were equivalent to SF only plots. These results suggest that turf managers should increase fertilizer

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treatments but will not necessarily need to discontinue trinexapac-ethyl to maximize creeping bentgrass recovery following annual bluegrass control with methiozolin.

NOMENCLATURE: creeping bentgrass (*Agrostis stolonifera* L. AGSST), annual bluegrass (*Poa annua* L. POAAN), methiozolin (5{2, 6-difluorobenzyl}oxymethyl-5-methyl-3, 3{3-methylthiophen-2-yl}-1, 2-isoxazoline), trinexapac-ethyl (ethyl 4-[cyclopropyl(hydroxy)methylidene]-3,5-dioxocyclohexane-1-carboxylate)

KEYWORDS: biostimulant, fertility, turfgrass quality, turfgrass recovery, void

INTRODUCTION

The putting green is arguably, the most important turf on a golf course and requires a smooth, unbroken surface to fulfill its role in the game of golf (Bulson et al. 2009; Fagerness et al. 2000). When the surface is broken by edaphic conditions, disease, animal activity, careless players or other circumstances, voids can appear in the canopy resulting in a possible disruption of ball roll (Baldwin et al. 2008). When voids in the turfgrass surface appear, it is important that they are repaired as quickly as possible to maintain an optimal putting surface (Schmitz et al. 2005; Walker et al. 2003).

Annual bluegrass is a problematic winter annual, grassy weed that regularly infests creeping bentgrass putting greens. Annual bluegrass invades putting greens and ultimately outcompetes creeping bentgrass, especially in cool, wet climates that are conducive to annual bluegrass competitiveness (Beard 1973; Harivandi et al. 2008). Annual bluegrass can produce more tillers than creeping bentgrass under putting green management but, is shallow rooted and sensitive to a variety of biotic and abiotic stress. Summer stress or disease often results in plant death and voided turfgrass canopies where putting greens are highly infested with annual bluegrass (Beard 1973; Bogart and Beard 1973; Harivandi et al. 2008; Lyons et al. 2011; Miltner et al. 2004; Murphy et al. 1994; Smiley et al. 2005; Whener and Watschke 1981).

There are few herbicides on the market that successfully control annual bluegrass POST in cool-season turfgrass, and none are labeled for use on creeping bentgrass putting greens. Amicarbazone and bispyribac-sodium are labeled for annual bluegrass control in cool-season turfgrass, but there are concerns over turfgrass sensitivity and temperature restrictions for use (Anonymous 2012; Jeffries et al. 2013; McCullough et al. 2010). Ethofumesate is another

product commonly utilized for annual bluegrass control. In order to control annual bluegrass, ethofumesate must be applied multiple times, but typically the rates required for turfgrass safety are too low to adequately control annual bluegrass, and control is erratic as a result (Coats and Krans 1986; Meyer and Branham 2006; Woosley et al. 2003). Methiozolin is a new herbicide manufactured by Moghu Research Center, LLC, in Daejeon, South Korea for the safe and selective removal of annual bluegrass from desirable turfgrass swards (Koo et al. 2013; Askew and McNulty et al. 2014). Although methiozolin typically controls annual bluegrass over several weeks, it may interact with environmental factors and cause annual bluegrass to decline rapidly, leaving voids in the putting surface (McNulty et al. 2011; Venner et al. 2012).

When voids occur on greens, superintendents strive to repair the area quickly through proper cultivar selection, various fertility products, seeding, and sodding (Jones and Christians 2012; Lee 2012; Rossi and Grant 2009; Walker et al. 2003). Most commonly, superintendents use soluble N fertilizers that are ammonia- or nitrate-based. These products are available as both water insoluble and soluble sources and account for approximately 80% of fertilizer use (Erisman et al. 2008). Addition of soluble N fertilizers increases turfgrass clipping yields and turfgrass color, which are both dependent on N source ($\text{NO}_3\text{-N}$ or $\text{NH}_4\text{-N}$) (Schlossberg and Schmidt 2007). Since plants require less energy to process NH_4 than NO_3 , increased growth and improved color may occur more rapidly with NH_4 -containing products (Schlossberg and Schmidt 2007). Another common practice of superintendents is to add micronutrient supplements to fertility programs to improve color and overall quality. Biostimulants are a group of products that are derived from natural sources like seaweed, humic acids and other organic sources (Kahn et al. 2009; Schmidt et al. 2003). These products, not only contain nitrogen, phosphorus and potassium, but a vast array of other compounds like auxins and cytokinins; hormones important

for mediating plant growth (Butler et al. 2012, Xu and Huang 2010; Zhang et al. 2003, 2013; Zhang and Ervin 2004).

Biostimulants have been shown to increase percent creeping bentgrass cover nearly equivalent to commercial fertility programs following aeration (Bigelow et al. 2010). When applied to creeping bentgrass plants under summer stress, Xu and Huang (2010) found that plots treated with biostimulant products had between 22 and 100% higher turfgrass quality than the non-treated checks and plots sprayed with the plant growth regulator, trinexapac-ethyl (TE). Biostimulants and TE maintained up to 76% and 56% more chlorophyll content, respectively, in leaves compared to non-treated turf (Xu and Huang 2010). Cytokinins, such as those found in seaweed extracts, have been shown to improve turfgrass vigor (Zhang and Ervin 2008; Xu and Huang 2010).

Golf course superintendents have several options when choosing fertility sources, and to date, no peer reviewed literature exists investigating the options for putting green recovery following rapid annual bluegrass removal with methiozolin. The objectives of this research were to, 1) determine the influence of increased fertility programs from synthetic fertilizers alone or with trinexapac-ethyl or from biostimulants on speed of putting green recovery following rapid annual bluegrass removal with methiozolin or on creeping bentgrass removed via core cultivation, and 2) to determine if methiozolin interacts with various fertility programs to influence speed of creeping bentgrass recovery on golf greens following core cultivation.

MATERIALS AND METHODS

Two separate studies were conducted at various locations in Virginia to assess fertility programs in conjunction with methiozolin on golf greens. In one study, voids were created in the putting surface via rapid removal of annual bluegrass using methiozolin. This study will be referred to as the annual bluegrass removal (ABR) study. In the second study, voids in the putting green canopy were created using a core-cultivator (Greens Aerator; The Toro Company, 8111 Lyndale Avenue S, Bloomington, MN 55420, USA) to remove approximately 30% of a pure creeping bentgrass canopy. This study will be referred to as the core-cultivation (CC) study.

Annual Bluegrass Removal Study. The ABR study was conducted at the Virginia Tech Golf Course on three sites between 2013 and 2014 on two separate greens, both constructed in the 1950's and originally seeded to 'Pennlu' and 'C-19 Congressional'. Studies were initiated at two sites (ABR1 and ABR2) on March 22, 2013 and one site (ABR3) on March 27, 2014. All greens were maintained at 0.4 cm mowing height. The purpose of the ABR study was to create voids in the putting green canopy via rapid control of annual bluegrass to allow assessment of creeping bentgrass recovery rate as influenced by fertility, biostimulant, and PGR programs. Visual assessments of initial annual bluegrass cover averaged 48, 47, and 65% at ABR1, ABR2, and ABR3, respectively with standard deviations of 5 to 7% depending on location. Rapid removal of annual bluegrass was achieved by treating the entire study area with methiozolin at 3000, 500 and 500 g ai ha⁻¹ at initiation, 2 wk and 4 wk after initiation, respectively. Annual bluegrass at all sites was completely controlled by this methiozolin program within 45 days, with no apparent injury to creeping bentgrass.

In order to assess creeping bentgrass recovery as influenced by cultural treatment programs, a randomized complete block study was initiated at each of the three sites. Four

treatments were replicated three times at each site. A more detailed description of fertility programs is provided in Table 1, but generally treatments can be described as 1) increased fertility using synthetic fertilizer, 2) increased fertility via synthetic fertilizer plus trinexapac-ethyl applied every 200 growing degree day with a base of 32 °F (GDD₃₂) (0 °C), 3) increased fertility via biostimulants and 4) no additional cultural treatment.

“Increased fertility” treatments are additional treatments added to the superintendent’s standard fertility program on each green (Table 1). Six Virginia golf course superintendents, representing a range of golf course designs and budgets and one company sales representative, were consulted to determine an appropriate amount of macronutrient increase assuming a 40% loss of putting green turf cover. Based on these discussions and previous research, the macronutrient increase in each treatment, regardless of source, was biweekly treatments of 7 kg N ha⁻¹, 7 kg P ha⁻¹ and 7 kg K ha⁻¹ (Bigelow et al. 2010; Wang et al. 2013). These treatments were repeated until complete turf recovery and included 5 treatments at ABR1 and ABR2 and 4 treatments at ABR3. Increased fertility treatments were initiated at first sign of canopy loss, which occurred on April 14, 2013 at ABR1 and ABR2 and May 2, 2014 at ABR3. Digital images were taken biweekly with an 8.0 megapixel Canon Digital Rebel XT (Rebel XT; Canon U.S.A., Inc., One Canon Park, Melville, NY 11747, USA) at the following settings: F16, ISO100, white balance set to fluorescent, and two-second shutter speed. The camera was mounted to a wooden 68 cm x 51 cm x 68 cm box which functioned to exclude all natural light. The interior of the box was equipped with four 13-watt compact fluorescent light bulbs to ensure image uniformity. Images were analyzed for percent green pixels using SigmaScan Pro 5.2 (SigmaScan Pro 5.2; Systat Software, Inc. 2107 North First Street, Suite 360, San Jose, CA 95131 USA) which optimizes green color detection (Karcher 2003). Normalized difference

vegetation index (NDVI) was assessed using a multispectral analyzer (GeoScout GLS-400; Holland Scientific, Inc., 6001S. 58th Street, Suite D, Lincoln, NE 68516, USA) on each rating date for the duration of the study.

To standardize the influence of varying annual bluegrass population levels, percentage cover of green pixels was converted to a percentage recovery of green turf using the following equation:

$$Y = (P_i - C_i)/(100 - C_i) \quad [1]$$

where y is the percentage recovery of voided turf on a given date, P_i is the observed percentage green pixels in plot i on a given date and C_i is the observed percentage of green pixels in plot i on the initial observation date. To control for variance structure in measurements taken over time, percentage recovery data from each plot were subjected to the hyperbolic function using PROC NLIN in SAS 9.2.

$$Y = (iX)/(1 + \left(\frac{iX}{a}\right)) \quad [2]$$

where Y is the percentage recovery of voided turf, X is time in weeks, i is an estimated parameter that approximates the rate of recovery as time approaches zero, and a is an estimated parameter that approximates the upper asymptote of percentage turf recovery. Using the estimated parameters, the hyperbolic function was used to determine the time required in weeks to reach 75% (T_{75}) and 90% (T_{90}) recovery. These T_{75} and T_{90} values and NDVI data were determined to be normal using the NORMAL option in PROC UNIVARIATE and Shapiro-Wilk statistic and homogeneity was assessed by visually inspecting plotted residuals. Data were subjected to ANOVA using PROC GLM with sums of squares partitioned to reflect the effect of cultural treatments and trial sites, which were considered random. Mean squares associated with trial interactions were used to test for significance of treatment effects (McIntosh 1983). If significant trial interactions occurred, data were presented separately by trial, otherwise, data

were pooled over trial. Mean responses of T_{75} , T_{90} , and NDVI were separated with Fisher's Protected LSD test at $P \leq 0.05$.

Core-Cultivation Study. The CC study was conducted at the Turfgrass Research Center in Blacksburg, VA, on a USGA specification 'L-93' green that is maintained at 0.32 cm. The study was initiated on March 22, 2013 and March 27, 2014. The purpose of the CC study was to digitally assess creeping bentgrass recovery rate following approximately 30% canopy reduction via aggressive core-cultivation as influenced by methiozolin application and fertility, biostimulant and PGR programs. Visual annual bluegrass cover averaged 3% in 2013 and 7% in 2014.

In order to assess creeping bentgrass recovery as influenced by methiozolin and cultural treatment programs, a split-plot study was initiated at each site, designated CC1 and CC2. Treatments were replicated three times at each site. Main plots consisted of fertility treatments, and sub-plots consisted of two rates of methiozolin. Sub-plots contained either no methiozolin or methiozolin applied at 500 g ai ha^{-1} 6 times at 2 week intervals beginning on March 23, 2013 and March 27, 2014. Fertility programs were the same as outlined in the ABR Study, and can be found in Table 1. Superintendent-administered fertility programs applied to the entire green can be found in Table 2.

Fertility treatments were repeated at 2 week intervals beginning immediately prior to core-cultivation on April 14, 2013 and May 2, 2014 and lasted until turfgrass was completely recovered and included 6 treatments at CC1 and 7 treatments at CC2. Digital images, NDVI and cover ratings were taken as outlined in the Annual Bluegrass Removal Study. Statistical analyses were also performed as outlined previously, with the exception that sums of squares

were further partitioned to evaluate effects of main plots, subplots, and interactions of these with each other and with trial.

RESULTS & DISCUSSION

Annual Bluegrass Removal Study. The interaction of location by treatment was significant for T_{75} ($P < 0.0001$) and T_{90} ($P < 0.0001$). Therefore, data are presented separately by location (Table 3). The interaction was probably caused by inconsistencies in the performance of both biostimulant and SF plus trinexapac ethyl. For example, the biostimulant treatment had the least T_{75} in ABR2 and ABR3 but was equivalent to SF in ABR1 (Table 3). The T_{90} of SF plus TE was equivalent to SF alone at ABR1 and ABR2 but required more time at ABR3 (Table 3). Despite these differences, some consistencies in responses between locations were noted. For example, biostimulant and SF treatments reduced 75 and 90% recovery times compared to the no additional fertility check at all locations (Table 3).

The T_{75} times ranged from 2.5 to 7.3 weeks depending on treatment and location (Table 3). In general T_{75} required more time in 2013 than in 2014. Biostimulant treatments required significantly less time than all other treatments at two of three locations. Synthetic fertilizer was equivalent to SF plus TE at all locations except ABR3 where the two differed in T_{75} recovery time by about 3 days (Table 3). In 2013, all additional fertility treatments decreased T_{75} recovery time by at least 2.4 weeks at ABR1 and 1.8 weeks at ABR2 (Table 3). The biostimulant program reduced T_{75} recovery time by 2.5 to 2.8 weeks at these two locations compared to the no additional fertility program (Table 3). Biostimulants are fertility products containing proprietary mixtures of plant hormones and other additives, such as micronutrients, phyto-hormones, and amino acids (Ervin et al. 2004; Zhang and Ervin 2004). Although macronutrient concentrations

were equivalent between biostimulant and SF programs, biostimulants likely provided plants with various hormones and micronutrients that elicited immediate growth responses versus the SF program that provided necessary nutrients but required time to produce such hormones (Gao and Li 2012; Zhang et al. 2009). Biostimulants have been shown to improve overall plant stress response when utilized as a conditioning agent, particularly during drought (Zhang and Ervin 2004; Zhang et al. 2003). Biostimulant treated plants were shown to have between 21 and 68% increased root mass following biostimulant application and subsequent drought stress (Zhang and Ervin 2004). In treatments that did not receive stress, quality was higher in plants that were treated with biostimulants versus the control, 8.4 to 8.7 and 7.9, respectively (Zhang and Ervin 2004). The addition of biostimulants increases the amount of available amino acids, cytokinins, auxins and other antioxidant-type compounds within the plant. Increased cytokinin concentrations have been shown to negatively influence the effects of ethylene production, thereby reducing overall plant senescence. In creeping bentgrass, an ethylene inhibitor and synthetic cytokinin were applied to turfgrass and subjected to heat stress at 35 °C, and after 21 days of heat stress, production of ethylene was reduced 25 and 11%, respectively (Xu and Huang 2009). Under high (350 to 400 kg ha⁻¹) and low (140 to 160 kg ha⁻¹) base fertility regimes turfgrass quality was greater under high fertility than low, and all biostimulant treatments had greater turfgrass quality than the control (Zhang et al. 2003). Biostimulant treatments increased visual quality of an immature putting green, and increases were sustained. It was inferred, however, that increases in turfgrass quality were not related to fertility imparted by biostimulants, but were due to decreases in localized dry spot and heat stress deleterious effects (Mueller and Kussow 2005). Increased fertility imparted by biostimulant products could speed

turfgrass recovery by increasing turfgrass rooting, allowing more shoots to be produced, thereby increasing cover more rapidly (Bigelow et al. 2010; Tucker et al. 2006).

When plots reached T_{90} , actual putting green canopy coverage was approximately 96% and commercially acceptable (data not shown). Some differences were noted between trends in T_{90} response times and those of T_{75} . For example, biostimulant and SF programs were equivalent for T_{90} times and generally averaged about 6 to 6.5 weeks in 2013 and 4.5 weeks in 2014 (Table 4). These two treatments reduced the time required for 90% recovery by over 2 weeks compared to the no additional fertility check at two sites in 2013 and about 1 week at one site in 2014 (Table 4). Adding trinexapac-ethyl to SF did not increase recovery time at two of three locations. Trinexapac-ethyl reduces cellular elongation and may increase the time required to sufficiently mask voids in turfgrass canopies due to reduced vertical plant growth (Ervin and Zhang 2007; Rademacher 2000). In low heights of cut like those on a putting green, vertical growth rate is of minimal benefit in masking canopy voids and trinexapac-ethyl has been shown to have minimal impact on lateral recovery in some studies due to increased levels of cytokinin production leading to increased tiller production after several sequential applications (Ervin et al. 2002; Ervin and Zhang 2007). In shade studies, tiller density of zoysiagrass was increased under shaded conditions with applications of trinexapac-ethyl (Ervin et al. 2002). Divot recovery on putting greens does not appear to be influenced by the addition of trinexapac-ethyl to normal maintenance programs (Stier and Steinke 2003). Trinexapac-ethyl does not consistently increase tillering in turfgrass species, but consistently increases chlorophyll content and cell density (Ervin et al. 2002; Ervin and Zhang 2007). Variability in recovery time between sites could be partially attributed to turfgrass cultivar as has been demonstrated for creeping bentgrass and bermudagrass (Jones and Christians 2009; Karcher et al. 2005).

At 1 WAIT, differences in NDVI between fertility treatments were not evident (Table 4). Plots that received no additional fertility had lower NDVI than all plots that did at 6 WAIT and at ABR1 and ABR2 at 8 WAIT (Table 4). Biostimulant plots were damaged by mower scalping at ABR3 and had poorer quality than plots receiving no additional fertility. These damaged plots probably caused the location interaction at 8 WAIT (Table 4). Increased visual quality of plots receiving additional fertility has been quantified in several studies. Addition of biostimulants to creeping bentgrass has improved quality, particularly during periods of stress. Further, addition of nitrogen fertility increases root-to-shoot ratio and improves NDVI (Christians et al. 1979; Ervin et al. 2002; Schlossberg and Karnok 2001).

Core-Cultivation Study. The interaction of location by methiozolin by treatment was significant for T₇₅ ($p < 0.0001$) and T₉₀ ($p < 0.0001$). Therefore data are presented separately by methiozolin treatment and location. The interaction between CC1 and CC2 could be attributed to a mid-season irrigation malfunction on the CC2 putting green which lasted for approximately 4 weeks. Two out of four irrigation heads surrounding the putting green failed, and were not immediately noticed, as the green was aggressively core cultivated and topdressed to meet study objectives. It was not until heat stress became evident that the issue was discovered and corrected. As a result of this severe stress, turf quality in most plots fell to below 3 on a 1 to 9 scale where 6 is considered minimally acceptable (data not shown). Methiozolin treated plots recovered more slowly than those not treated with methiozolin at CC2, a phenomenon that did not occur at CC1 (Table 5). Despite issues with irrigation, trends in 75 and 90% recovery as influenced by fertility treatments were generally consistent across locations (Table 5).

Recovery times for T₇₅ ranged from 2.2 to 4.7 weeks depending on treatment, location and methiozolin rate (Table 5). All fertility and PGR treatments recovered more quickly than the

no additional fertility treatment at all sites. When methiozolin was applied, 75% creeping bentgrass recovery required an additional 1.5 weeks on average at CC2 but no additional time at CC1 (Table 5). Methiozolin is immobile in all but pure sand putting green soils (Flessner et al. 2013). Variable root depth at or below 5.5 cm did not influence creeping bentgrass response to methiozolin (Brosnan et al. 2013). Although the exact depth of methiozolin distribution on putting greens has not been reported, the aforementioned studies suggest methiozolin does not move vertically in most putting green soils. Thus, substantial root loss is likely required to elicit creeping bentgrass injury from methiozolin in most cases. The combined effects of core cultivation and drought at CC2 could have caused temporary root loss and methiozolin may have prevented new root development. Following core-cultivation, it is important to maintain adequate moisture and fertility while the entire putting green recovers. Additionally, the act of cultivating a putting green damages turfgrass shoots and decreases rooting following cultivation. Murphy et al. (1993) observed a 16% decrease in root weight density following cultivation, most likely due to root severing and trauma to the plant. Further, when soil has reduced moisture, roots are more susceptible to injury than when soil is adequately moist (Murphy et al. 1993). At CC1 where drought stress did not occur, methiozolin had no impact on creeping bentgrass recovery rate (Tables 5 and 6).

Creeping bentgrass treated with SF reached T_{75} equivalent to biostimulant at both locations regardless of methiozolin use (Table 5). Trinexapac-ethyl influence on creeping bentgrass T_{75} recovery time was independent of methiozolin use but dependent on location. At CC1, trinexapac-ethyl plus SF improved creeping bentgrass T_{75} recovery time equivalent to the best performing treatments (Table 5). At CC2 where the drought injury occurred, trinexapac-ethyl programs were inferior to SF in the absence of methiozolin and both SF and biostimulant in

the presence of methiozolin (Table 5). The location dependency of fertility programs could be attributed to the increased amount of plant hormones contained in biostimulant products and growth regulation imparted by trinexapac-ethyl application. Although commonly reported in warm-season turfgrass species like bermudagrass, increases in root growth as a direct result of trinexapac-ethyl application are inconsistent (Beasley et al. 2005; McCann and Huang 2007; Wherley and Sinclair 2009). Wherley and Sinclair (2009) and Beasley et al. (2005) did not observe changes in creeping bentgrass or Kentucky bluegrass root growth following applications of trinexapac-ethyl, but postulated that rooting increases are more likely directly related to nitrogen application. Trinexapac-ethyl has been found to increase tiller density in Kentucky bluegrass under traffic stress, improving quality, but rooting was not affected (Ervin and Koski 2001). Biostimulant products have been observed to increase tillering in creeping bentgrass, when applied at seeding (Butler et al. 2007). When investigated side-by-side in creeping bentgrass affected by etiolating bacteria, biostimulants did not influence turfgrass quality relative to the non-treated, whereas trinexapac-ethyl applied at weekly and biweekly intervals improved quality at several rating dates, including area under the etiolation progress curve (AUEPC) (Roberts et al. 2015). These results can be explained by the reduced turfgrass growth and subsequent increase in cell density imparted by trinexapac-ethyl, thus increasing quality by reducing etiolation (Rademacher 2000; Roberts et al. 2015).

Although recovery trends were evident at T₉₀ and canopy coverage had reached approximately 95%, differences between sites became more evident, particularly at CC2 with methiozolin (Table 6). All fertility treatments recovered between 1.3 and 3.3 weeks more rapidly than the no additional fertilizer treatment. When biweekly treatments of methiozolin were applied, biostimulant and SF treatments improved T₉₀ recovery time by approximately 0.5

weeks compared to SF plus trinexapac ethyl (Table 6). At CC2 where the drought stress occurred, biostimulant treated plots reach 90% recovery 0.7 weeks faster than SF and 0.4 weeks faster than SF plus trinexapac ethyl (Table 6). When methiozolin was not applied, biostimulant and SF treated plots reached 90% recovery at equivalent times but 1 to 3 weeks faster than plots that did not receive additional fertility (Table 6). The spectrum analyzer used at the CC1 and CC2 locations had a calibration error that compromised data integrity. Thus, NDVI from CC1 and CC2 will not be presented.

In all cases, increasing fertility increased turfgrass recovery relative to not increasing fertility over the standard program. These data suggest that turfgrass managers can improve recovery time of creeping bentgrass greens by 1 to 3 weeks by adding 7 kg ha⁻¹ of N, P, and K to existing greens fertility programs. Methiozolin reduced turfgrass recovery time at one location where a severe drought occurred. It will be important to avoid turfgrass stress when using methiozolin programs to control annual bluegrass on greens. Creeping bentgrass treated with biostimulants recovered equivalent to or faster than SF in all cases. In the presence of methiozolin treatments, trinexapac ethyl reduced T₉₀ recovery time by 0.25 to 0.5 weeks at two locations and increased T₉₀ recovery time by 0.1 weeks at one location. Otherwise, SF plus trinexapac ethyl treated plots were equivalent to SF only plots. Further research needs to be conducted to better explain how methiozolin may affect root recovery following stress-induced root loss.

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Table 1. Products utilized in annual bluegrass removal and core-cultivation studies^a.

Common Name	Trade Name	Rate	Manufacturer	City, State, Website
Synthetic Fertilizer (20-20-20)	Bulldog 20-20-20 with micros	7.03 kg ha ⁻¹	SQM North America	Atlanta, GA, www.sqm.com
Urea (46-0-0)	Southern States Urea 46-0-0	2.34 kg ha ⁻¹	Southern States Cooperative, Inc.	Richmond, VA, www.southernstates.com
Trinexapac-ethyl	Primo® MAXX	0.048 kg ha ⁻¹	Syngenta Crop Protection, LLC.	Greensboro, NC, www.syngentacropprotection.com
Biostimulant (0-0-0)	Astron	19.1 L ha ⁻¹	Floratine Products Group	Collierville, NC, www.floratine.com
Biostimulant (13-0-0)	Per-4-Max	19.1 L ha ⁻¹	Floratine Products Group	Collierville, NC, www.floratine.com
Biostimulant (12-0-0)	Knife Plus	19.1 L ha ⁻¹	Floratine Products Group	Collierville, NC, www.floratine.com

Biostimulant (6-2-3)	Protesyn	9.5 L ha ⁻¹	Floratine Products Group	Collierville, NC, www.floratine.com
Biostimulant (0-22-28)	Power 23-0-0 + Mo	9.5 L ha ⁻¹	Floratine Products Group	Collierville, NC, www.floratine.com
Biostimulant (23-0-0)	Power 0-22-28	9.5 L ha ⁻¹	Floratine Products Group	Collierville, NC, www.floratine.com

^aApplication dates in 2013 included: April 14, May 2, May 14, May 26, and June 5 at ABR1, ABR2, and CC1 and June 19 also at CC1. Application dates in 2014 included: May 2, May 17, June 6, and June 20 at ABR3 and CC2 and July 1, July 11, and July 26 also at CC2.

Table 2. Superintendent administered fertility programs for both annual bluegrass removal and core-cultivation studies during the duration of each study.

Year	Date	Location	Common Name	Nitrogen	
				Rate	Manufacturer, City, State, Website
2013	April 22	ABR1 ^a ,	Nutralene Slow Release	24.4 kg ha ⁻¹	Koch Agronomic Services, LLC., Wichita, KS, www.kochagronomicservices.com
		ABR2	(40-0-0)		
	May 23	ABR1,	Proforma 18-3-6 SRN	4.4 kg ha ⁻¹	Agrilience, LLC., St. Paul, MN, no website
		ABR2	Southern States 46-0-0		
	May 6	CC1	SGN 80	24.4 kg ha ⁻¹	www.southernstates.com
			Southern States 46-0-0		
	May 21	CC1	SGN 80	9.76 kg ha ⁻¹	www.southernstates.com
			Southern States 46-0-0		
June 3	CC1	SGN 80	7.3 kg ha ⁻¹	www.southernstates.com	

			Southern States 46-0-0		Southern States Cooperative, LLC., Richmond, VA,
	June 17	CC1	SGN 80	7.3 kg ha ⁻¹	www.southernstates.com
2014	April 21	ABR3	Nutrite (21-0-16)	48.8 kg ha ⁻¹	Nutrite, Pheonix, AZ, www.nutrite.com
	May 20	ABR3	Proforma 18-3-6 SRN	4.4 kg ha ⁻¹	Agriliance, LLC., St. Paul, MN, no website
	June 10	ABR3	Proforma 18-3-6 SRN	4.4 kg ha ⁻¹	Agriliance, LLC., St. Paul, MN, no website
			Southern States 46-0-0		Southern States Cooperative, LLC., Richmond, VA,
	May 5	CC2	SGN 80	24.4 kg ha ⁻¹	www.southernstates.com
			Bulldog 20-20-20 plus		
	May 19	CC2	Micros	7.3 kg ha ⁻¹	SQM North America, Atlanta, GA, www.sqm.com
			Bulldog 20-20-20 plus		
	June 3	CC2	Micros	7.3 kg ha ⁻¹	SQM North America, Atlanta, GA, www.sqm.com
			Bulldog 20-20-20 plus		
	June 16	CC2	Micros	7.3 kg ha ⁻¹	SQM North America, Atlanta, GA, www.sqm.com
			Bulldog 20-20-20 plus		
	July 1	CC2	Micros	7.3 kg ha ⁻¹	SQM North America, Atlanta, GA, www.sqm.com

		Bulldog 20-20-20 plus		
July 15	CC2	Micros	7.3 kg ha ⁻¹	SQM North America, Atlanta, GA, www.sqm.com
		Bulldog 20-20-20 plus		
July 28	CC2	Micros	7.3 kg ha ⁻¹	SQM North America, Atlanta, GA, www.sqm.com
		Bulldog 20-20-20 plus		
August 5	CC2	Micros	7.3 kg ha ⁻¹	SQM North America, Atlanta, GA, www.sqm.com

^aAbbreviations: ABR1, annual bluegrass removal study location 1; ABR2, annual bluegrass removal study location 2; ABR3, annual bluegrass removal study location 3; CC1, core-cultivation study location 1; CC2, core-cultivation study location 2.

Table 3. Time required to reach 75% (T₇₅) and 90% (T₉₀) recovery of voided putting green turf following rapid removal of annual bluegrass with methiozolin and as influenced by various fertility and plant growth regulator programs at three locations (ABR1, ABR2, and ABR3^a).

Fertility program ^b	T ₇₅			T ₉₀		
	ABR1	ABR2	ABR3	ABR1	ABR2	ABR3
	wk					
No additional fertility	7.3a ^c	6.8a	3.0a	8.8a	8.2a	5.3a
Synthetic fertilizer (SF)	4.7bc	5.0b	2.6b	6.2b	6.5bc	4.4c
SF plus trinexapac ethyl	4.9b	5.1b	3.0a	6.4b	6.6b	5.1b
Biostimulant	4.5c	4.3c	2.5c	6.1b	5.9c	4.5c

^aAbbreviations: ABR1, annual bluegrass removal study location 1; ABR2, annual bluegrass removal study location 2; ABR3, annual bluegrass removal study location 3; SF, synthetic fertilizer; T₇₅, time required in weeks to recover 75% of putting green turf voided by annual bluegrass removal by methiozolin; T₉₀, time in weeks to recover 90% of voided turf.

^bFertility programs were added to standard greens management programs and consisted of additional N, P, and K applied at 7, 7, and 7 kg ha⁻¹ every two weeks starting when canopy voids first appeared on the putting green. Trinexapac ethyl was applied at 0.048 kg ai ha⁻¹ and repeated at 200 growing degree days at base 32 °F (0 °C). More information about products applied is provided in Table 1.

^cMeans within a column, followed by the same letter are not significantly different ($p \leq 0.05$).

Table 4. Influence of fertility and plant growth regulator program following rapid annual bluegrass removal on putting green normalized difference vegetative index (NDVI) averaged over three locations at 1 and 6 weeks after initial fertility treatment (WAIT) and separated by location at 8 WAIT.

Fertility program ^b	1 WAIT	6 WAIT	8 WAIT		
			ABR1	ABR2	ABR3
	0-1.0				
No additional fertility	0.592a ^c	0.734b	0.712b	0.753b	0.751a
Synthetic fertilizer (SF)	0.610a	0.763a	0.746a	0.787a	0.744ab
SF plus trinexapac ethyl	0.607a	0.761a	0.766a	0.791a	0.747ab
Biostimulant	0.617a	0.763a	0.747a	0.787a	0.734b

^aAbbreviations: ABR1, annual bluegrass removal study location 1; ABR2, annual bluegrass removal study location 2; ABR3, annual bluegrass removal study location 3; SF, synthetic fertilizer.

^bFertility programs were added to standard greens management programs and consisted of additional N, P, and K applied at 7, 7, and 7 kg ha⁻¹ every two weeks starting when canopy voids first appeared on the putting green. Trinexapac-ethyl was applied at 0.048 kg ai ha⁻¹ and repeated at 200 growing degree days at base 32 °F (0 °C). More information about products applied is provided in Table 1.

^cMeans within a column, followed by the same letter are not significantly different ($p \leq 0.05$).

Table 5. Time required to reach 75% (T₇₅) recovery of voided putting green turf following core-cultivation as influenced by methiozolin and various fertility and plant growth regulator programs at two sites (CC1 and CC2^a).

Fertility program ^b	With Methiozolin		Without Methiozolin	
	CC1	CC2	CC1	CC2
	————— wk —————			
No additional fertility	3.3a ^c	4.7a*	3.4a	2.9a*
Synthetic fertilizer (SF)	2.4b ^d	3.7c*	2.5b	2.4c*
SF plus trinexapac ethyl	2.2c	4.0b*	2.3c	2.5b*
Biostimulant	2.4bc	3.8c*	2.3bc	2.5bc*

^aAbbreviations: CC1, core-cultivation site 1; CC2, core-cultivation site 2; SF, synthetic fertilizer; T₇₅, time required in weeks to recover 75% of putting green turf that was subject to 30% canopy removal via core-cultivation.

^bFertility programs were added to standard greens management programs and consisted of additional N, P, and K applied at 7, 7, and 7 kg ha⁻¹ every two weeks starting when canopy voids first appeared on the putting green. Trinexapac-ethyl was applied at 0.048 kg ai ha⁻¹ and repeated at 200 growing degree days at base 32 °F (0 °C). More information about products applied is provided in Table 1.

^cMeans within a column, followed by the same letter are not significantly different ($p \leq 0.05$).

^dAn asterik following any mean indicates that methiozolin significantly impacted recovery time ($p \leq 0.05$) within a given fertility program and location.

Table 6. Time required to reach 90% (T_{90}) recovery of voided putting green turf following core-cultivation as influenced by methiozolin and various fertility and plant growth regulator programs at two sites (CC1 and CC2^a).

Fertility program ^b	With Methiozolin		Without Methiozolin	
	CC1	CC2	CC1	CC2
	————— wk —————			
No additional fertility	5.9a ^{*cd}	8.6a [*]	7.9a [*]	5.8a [*]
Synthetic fertilizer (SF)	4.5b	7.3b [*]	4.6b	4.5c [*]
SF plus trinexapac ethyl	4.0c	7.0c [*]	4.1b	4.8b [*]
Biostimulant	4.6b	6.6d [*]	4.6b	4.6bc [*]

^aAbbreviations: CC1, core-cultivation site 1; CC2, core-cultivation site 2; SF, synthetic fertilizer; T_{75} , time required in weeks to recover 90% of putting green turf that was subject to 30% canopy removal via core-cultivation.

^bFertility programs were added to standard greens management programs and consisted of additional N, P, and K applied at 7, 7, and 7 kg ha⁻¹ every two weeks starting when canopy voids first appeared on the putting green. Trinexapac-ethyl was applied at 0.048 kg ai ha⁻¹ and repeated at 200 growing degree days at base 32 °F (0 °C). More information about products applied is provided in Table 1.

^cMeans within a column, followed by the same letter are not significantly different ($p \leq 0.05$).

^dAn asterik following any mean indicates that methiozolin significantly impacted recovery time ($p \leq 0.05$) within a given fertility program and location.

Chapter 3. Influence of Plant Growth Regulators on Creeping Bentgrass Response to Methiozolin

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Annual bluegrass is a problematic, grassy weed that regularly infests creeping bentgrass putting greens. Commonly, golf course managers rely on plant growth regulating compounds (PGRs) to suppress seedhead production and reduce clippings, thus improving overall turfgrass quality. These products do not control annual bluegrass, however. Methiozolin is a new herbicide manufactured by Moghu Research Center, LLC, in Daejeon, South Korea for the safe and selective removal of annual bluegrass from fine turfgrass. To date, no studies have investigated the use of PGRs in conjunction with methiozolin. Studies were conducted investigating safety and efficacy of 5 different PGRs alone and in combination with methiozolin on golf course putting greens. Methiozolin by PGR interactions were not detected. Applied alone, flurprimidol and paclobutrazol reduced annual bluegrass cover, whereas ethephon, mefluidide and trinexapac-ethyl did not. Methiozolin contributed to the highest reductions in annual bluegrass cover, as expected. Although several of the PGR's transiently injured creeping bentgrass, methiozolin did not influence injury. Further investigation into the combination of methiozolin with ethephon was conducted in order to determine whether or not ethephon negatively influences annual bluegrass control with methiozolin as well as whether core-cultivation at the time of sequential methiozolin plus ethephon application posed a risk to overall turfgrass safety. When methiozolin and ethephon are applied together, annual bluegrass control increases as methiozolin rate increases, achieving nearly complete control with methiozolin at 2000 g ai ha⁻¹ twice, indicating

a lack of antagonism between ethephon and methiozolin. Addition of core-cultivation results in severe injury to creeping bentgrass. At 8 WAIT, creeping bentgrass cover in the non-treated averaged 62%, whereas plots with methiozolin at 2000 g ai ha⁻¹ averaged 19% cover. No injury to creeping bentgrass was observed in non-core-cultivated sites. These data suggest that caution should be taken when mixing PGRs with methiozolin, and ethephon applications in conjunction with methiozolin and core-cultivation should be avoided.

NOMENCLATURE: creeping bentgrass (*Agrostis stolonifera* L.); annual bluegrass (*Poa annua* L.); ethephon (2-chloroethylphosphonic acid), methiozolin (5{2,6-difluorobenzyl}oxymethyl-5-methyl-3,3{3-methylthiophen-2-yl}-1,2-isoxazoline)

KEYWORDS: aeration, annual bluegrass control, core cultivation, herbicide interaction, putting green, turfgrass injury

INTRODUCTION

The overall lack of herbicides available for effective and safe annual bluegrass control has led many golf course superintendents to rely on plant growth regulators (PGRs) to assist in the management of annual bluegrass growing on creeping bentgrass putting greens (Branham and Sharp 2007; Haugewood et al. 2013). Plant growth regulators are compounds used routinely on golf putting greens to manage turf quality, clipping production, and annual bluegrass infestations (Ervin and Zhang 2008; Reicher et al. 2015; Watschke et al. 1992). Trinexapac-ethyl, a late gibberellin biosynthesis inhibitor, is used to enhance overall turfgrass color and quality and to improve tolerance to various stresses (Ervin et al. 2002; Ervin and Zhang 2007; Rademacher 2000). Paclobutrazol and flurprimidol are both early gibberellin biosynthesis inhibitors, and are used to selectively suppress annual bluegrass population expansion, resulting in severe regulation of annual bluegrass (Branham and Sharp 2007; Han et al. 1998). Applications, however, must be consistent, as plants will exhibit “rebound” growth once regulation has ceased (Branham and Sharp 2007). Ethephon and mefluidide are used to inhibit annual bluegrass seedhead development via release of intracellular ethylene and cell division inhibition, respectively (Beard and Beard 2005; Cooper et al. 1987; Eggens et al. 1989; Haugewood et al. 2013; Inguagiato et al. 2010; Petrovic et al. 1985). These compounds are commonly applied biweekly, but may be applied weekly, monthly or at various frequencies depending on environmental conditions and desired results (Branham and Danneberger 1989; Kreuser and Soldat 2011; Johnson and Murphy 1996). Since most golf greens are treated regularly with PGR products, any new plant protection product registered for use on greens should be tested for possible interactions with PGRs. A new herbicide, methiozolin, has been developed by Moghu Research Center in Daejeon, South Korea,

to selectively control annual bluegrass with minimal injury to the desired turfgrass sward (Koo et al. 2013). Methiozolin is proposed to act in one of two ways: either as an inhibitor of tyrosine aminotransferase or as an inhibitor of cell wall biosynthesis (Grossman et al. 2011; Lee et al. 2007). When applied at the recommended rates of 0.5 to 1.0 kg ai ha⁻¹, methiozolin has not injured creeping bentgrass putting green turf in several studies (Askew and McNulty 2014; McCullough et al. 2013; Brosnan et al. 2013b). Higher rates of 2.0 kg ai ha⁻¹ or more have caused transient creeping bentgrass injury in some preliminary studies (McNulty et al. 2011; Venner et al. 2012a). When applied in spring, methiozolin is generally considered to be safer to creeping bentgrass than when applied in fall. McCullough et al. (2013) observed 7% creeping bentgrass injury when methiozolin was applied in fall and no injury when applied in spring. In unpublished work by McNulty et al. (2011), spring-applied methiozolin at rates above 2.0 kg ai ha⁻¹ injured creeping bentgrass 40% at one of four locations. The researchers observed excessive heat and wear at the site where injury occurred and proposed that these abiotic stressors could have contributed to the observed creeping bentgrass injury (McNulty et al. 2011). Studies have indicated that methiozolin controls annual bluegrass more effectively on soil-based greens than sand-based greens (Brosnan et al. 2013a). Root depth of both annual bluegrass and creeping bentgrass may decrease on soil-based media, leaving plants more susceptible to environmental stress and herbicides (Jiang and Wang 2006). Brosnan et al. (2013a) did not detect differences in creeping bentgrass response to methiozolin when root depth of treated plants varied between 5 to 15 cm. Methiozolin is relatively immobile in putting green soils (Flessner et al. 2013). Thus, only with root depths of less than 5 cm would one expect to observe creeping bentgrass injury from recommended use rates.

The research discussed herein was conducted in two phases. The goal of the first phase was to compare the five most common PGRs currently marketed for use on putting greens for possible interaction with methiozolin with regards to creeping bentgrass and/or annual bluegrass response. Observations made during these studies led to a second phase of experiments designed to more closely evaluate potential interactions between methiozolin and ethephon. Ethephon is a PGR that is commonly used on putting greens to suppress annual bluegrass seedhead formation. It is thought that the presence of these seedheads disrupts golf ball roll uniformity in addition to overall aesthetics (McCullough et al. 2005). Upon metabolism in plant tissues, ethephon is converted to ethylene, increasing senescence and inhibiting the formation of seedheads (March et al. 2013). Ethephon is considered a class E plant growth regulator, meaning, it can impact rooting, branching or leaf elongation by changing microfibril orientation to make cells grow wider instead of longer, but does not affect tissues already formed (Ervin and Zhang 2008; March et al. 2013). Ethephon is commonly combined with other plant growth regulators like trinexapac-ethyl to improve uniformity of plant regulation on golf course putting greens and increase ethephon safety to desirable turfgrasses (Jiang and Fry 1998; McCullough et al. 2005). Although ethephon can be used on putting greens with some margin of safety to the turfgrass, McCullough et al. (2005) observed a decrease in turfgrass quality at 14 and 21 days after treatment (DAT) with one application of ethephon. Although ethephon reduces creeping bentgrass quality when applied alone, turfgrass quality was either improved or not impacted when ethephon was mixed with trinexapac-ethyl (McCullough et al. 2005; Stier et al. 2000).

In some instances, ethephon can cause turfgrass crowns to rise, resulting in scalping injury during mowing. Dernoeden and Pigati (2009) found that treatments containing ethephon (applied alone or in combination with trinexapac-ethyl) caused severe scalping injury for 35 to

56 days after the second application. In addition to concerns surrounding delayed scalping injury from applications of ethephon, root loss can be a concern on creeping bentgrass putting greens (McCullough et al. 2005). When ethephon is applied at 3.8 kg ai ha⁻¹ at a three week interval, root mass decreased 35% at 9 WAIT. After 3 weeks, root length decreased 28% (McCullough et al. 2005). Similarly, in perennial ryegrass, ethephon has been found to reduce root length density by 25 and 28% at 0 to 10 cm and 10 to 20 cm depths, respectively after 2 applications at 3.3 kg ai ha⁻¹ (Jiang and Fry 1998).

The objectives of these putting green studies were 1) to investigate potential interactions between methiozolin and the five commonly used PGRs ethephon, flurprimidol, mefluidide, paclobutrazol, and trinexapac-ethyl for effects on creeping bentgrass and annual bluegrass visually-estimated cover, injury, and quality and normalized difference vegetative index (NDVI); and 2) to determine the effects of three methiozolin rates alone or mixed with ethephon at sites that were core cultivated during treatment programs and sites where core cultivation was withheld during the treatment program for effects on creeping bentgrass visually-estimated cover, injury, and quality and NDVI.

MATERIALS AND METHODS

PGR Admixtures with Methiozolin. Studies were initiated on April 10, 2011 at the Glade Road Research Facility at Virginia Tech in Blacksburg, VA and on May 5, 2012 at Draper Valley Golf Course near Draper, VA. In 2011, the trial was established on a research green that consisted of A-4 creeping bentgrass maintained at 3.4 mm with less than 5% infestation of annual bluegrass. This green comprised a 30-cm deep profile of United States Golf Association specification sand (USGA specified) over native soil entrenched with perforated drainage. In 2012, the trial was

established on a golf course practice putting green that consisted of L-93 creeping bentgrass maintained at 3.3 mm with approximately 40% infestation of annual bluegrass. The green was made of native soil mixed with sand ("pushup" style) and also included subterranean tile drainage. Studies were conducted as randomized complete block designs with three replications. Treatments were arranged in a factorial with two levels of methiozolin (none and 1000 g ai ha⁻¹) and six levels of PGR (Table 1). All possible combinations of methiozolin and PGR included 12 treatments and these were applied once and turf responses were evaluated thereafter.

Assessments included visually-estimated creeping bentgrass and annual bluegrass cover, visually-estimated annual bluegrass control, visually-estimated creeping bentgrass injury, and NDVI assessed with a multispectral analyzer (GeoScout GLS-400; Holland Scientific, Inc., 6001S, 58th Street, Suite D, Lincoln, NE 68516, USA). All assessments were taken at 0, 1, 2, 4, and 8 weeks after treatment. To control for variance structure in data collected over time, all data were converted to the area under the progress curve (AUPC) as in other studies (Askew and McNulty 2014; Inguagiato et al. 2010). Data were tested for normality and homogeneity prior to analysis of variance. Sums of squares were partitioned to reflect the main effects and interactions of methiozolin and PGR factors, which were considered fixed, and the effect of trial, which was considered random. Mean squares associated with fixed effects were tested using the mean square associated with the interaction of a given fixed effect with trial (McIntosh 1983).

Methiozolin Interaction with Ethephon. Studies were conducted on four sites in Blacksburg, Virginia from 2012 to 2014. In 2012, the study was conducted at the Blacksburg Country Club (BCC), Blacksburg VA on a push-up style putting green originally seeded to 'Penncross' creeping bentgrass. In 2013, the study was conducted on the Virginia Tech Golf Course (VTG),

Blacksburg VA on a push-up style putting green originally seeded to ‘C-19 Congressional’ creeping bentgrass. At both the BCC and VTG, over- and interseeding had occurred over many years, and the predominant variety on the greens is unknown. In 2014, the study was conducted at two sites, one at the VTG on a USGA-specified creeping bentgrass putting green originally seeded to a 50/50 mix of ‘C-19 Congressional’ and ‘C-1 Arlington’ and the other at the Turfgrass Research Center (TRC), Blacksburg, VA on a push-up style putting green originally seeded to ‘Penneagle’ creeping bentgrass. All sites were infested with natural stands of mixed annual and, to a lesser extent, perennial biotypes of annual bluegrass, ranging from 25 to 80% cover across the trial area. Plot sizes were 1 by 3 m at all locations. Mowing heights for each site were between 2.9 and 3.4 mm. Soil moisture was maintained at acceptable levels with both manual and automatic irrigation, depending on the site. Sites were fertilized by the golf course superintendent under their normal fertility regime.

Treatments were applied using a CO₂ powered backpack sprayer calibrated to deliver 280 L ha⁻¹ at 262 kPa using a hooded sprayer equipped with two TeeJet 6502 flat fan spray tips to deliver a spray span 71 cm (TeeJet; Spraying Systems Co., P. O. Box 7900, Wheaton, IL 60187, USA). All treatments were applied twice at a 4 week interval beginning at 50 growing degree day, base temperature 50 °F (GDD₅₀) (March 13, 2012, April 13, 2013 and at two sites on April 12, 2014). Treatments included methiozolin (MRC-01; Moghu Research Center Ltd., BVC 311, KRIBB, Yuseong, Daejeon, 305-333, South Korea) applied alone at 500, 1000 and 2000 g ai ha⁻¹ or with ethephon (Proxy; Bayer Environmental Science, 2 T. W. Alexander Drive, Research Triangle Park, NC 27709, USA) at 3818 g ai ha⁻¹. An untreated check and comparison treatment consisting of methiozolin applied at 1000 g ai ha⁻¹, ethephon at 3818 g ai ha⁻¹ and trinexapac-ethyl (Primo MAXX; Syngenta Crop Protection, LLC, P. O. Box 18300, Greensboro, NC 27419,

USA) at 48 g ai ha⁻¹ were also included. Two of the sites were core cultivated in conjunction with the second application. The core cultivator (Greens Aerator; The Toro Company, 8111 Lyndale Avenue S, Bloomington, MN 55420, USA) had 12 tines that were spaced 5.1 cm apart and removed 1.3 cm² surface area per tine. Each hole was approximately 7.6 cm deep. Following core cultivation, the putting green was topdressed with sand and watered to encourage green recovery. Core-cultivation was withheld from the other two sites during the experimental duration. Treatment area was evaluated for visual injury, percent cover and percent control, where 0 is completely dead turfgrass and 100 is healthy turfgrass. Normalized difference vegetative index (NDVI) was included to quantify plot quality as a response to herbicide applications (Leinauer et al. 2014). Annual bluegrass cover was converted to a percentage reduction based on the following equation:

$$Y = \frac{(i - o)}{i} * 100 \quad \text{[eq.1]}$$

Where *i* = initial visually-estimated annual bluegrass cover and *o* = visually estimated annual bluegrass cover observed at given evaluation time. Statistical analysis was performed in SAS (SAS Institute, Inc., 100 SAS Campus Drive, Cary, NC 27513, USA).

RESULTS AND DISCUSSION

PGR Admixtures with Methiozolin. The trial by PGR and trial by methiozolin interactions were significant ($P < 0.05$) for both annual bluegrass and creeping bentgrass cover AUPC (Table 2). The trial interaction was likely caused by disparity between annual bluegrass infestation levels between 2011 (<5%) and 2012 (approximately 40%) (data not shown). The main effects of PGR ($P = <0.0001$) and methiozolin ($P = <0.0001$) were each significant for the AUPC of visually-estimated annual bluegrass control. The main effect of PGR was significant for creeping

bentgrass injury ($P = < 0.0001$) and the main effect of methiozolin was significant for the AUPC of NDVI ($P = 0.0458$). For simplicity, the main effects of both PGR and methiozolin are shown in Table 2 for all measured responses because mean separation procedures were in agreement with analysis of variance and properly represent insignificant effects in all cases.

Both flurprimidol and paclobutrazol in 2012 and paclobutrazol in 2011 significantly reduced annual bluegrass cover AUPC compared to no PGR (Table 2). Ethephon, mefluidide, and trinexapac-ethyl did not decrease annual bluegrass cover AUPC either year (Table 2). Paclobutrazol and flurprimidol have been shown to transiently reduce annual bluegrass cover in other studies (Baldwin and Brede 2011; Johnson and Murphy 1995) and are primary ingredients of market-leading products for annual bluegrass suppression on U.S. greens. In addition, methiozolin likely contributed much of the observed reduction in annual bluegrass cover AUPC (Table 2). Trends in creeping bentgrass cover AUPC also varied between trials (Table 2). In 2011, mefluidide and paclobutrazol slightly reduced creeping bentgrass AUPC compared to no PGR. In 2012, the no PGR level had equivalent to the lowest creeping bentgrass cover AUPC (Table 2) presumably due to higher annual bluegrass cover in these plots. Although significant, the creeping bentgrass cover AUPC reductions in 2011 only represent a 7 and 10% change in the estimated average daily creeping bentgrass cover. The transient creeping bentgrass injury caused by mefluidide and paclobutrazol may have resulted in a significant reduction in cover AUPC (Table 2). Methiozolin had no impact on creeping bentgrass cover AUPC in 2011 and increased creeping bentgrass cover AUPC in 2012 (Table 2). These differences are probably influenced by higher annual bluegrass cover in 2012, which was partially controlled by methiozolin, allowing creeping bentgrass cover to expand.

One of the most interesting observations in this study was the reduced annual bluegrass control in ethephon programs (Table 2). Control reduction with ethephon is reflected in annual bluegrass control AUPC that is considerably less than all other levels of PGR. Methiozolin is responsible for most of the annual bluegrass control as evidenced by an annual bluegrass control AUPC of 719 averaged over the six levels of PGR when methiozolin was not included. When methiozolin was included, the average AUPC was three times higher (Table 2). The fact that the average annual bluegrass control AUPC by ethephon was only 169, suggests that ethephon may have antagonized annual bluegrass control by methiozolin. It was this observation that led to subsequent studies on the interaction between methiozolin and ethephon.

Methiozolin did not influence creeping bentgrass injury AUPC as evidenced by a lack of significant effect between levels of methiozolin and a creeping bentgrass injury AUPC of only 9 when no PGR was applied (Table 2). Flurprimidol, paclobutrazol, and mefluidide all caused transient injury that resulted in an AUPC between 136 and 160. If estimated as a daily average, those values would equate to about 3% injury per day. The actual injury observed by these treatments ranged from 5 to 35% and occurred between 1 and 4 weeks after treatment with peak injury observed at 2 weeks after treatment (data not shown). Flurprimidol, paclobutrazol, and mefluidide have been shown to transiently injure creeping bentgrass in other studies (Branham and Sharp 2007; Cooper et al. 1987; Han et al. 1998). The slight and transient injury by some PGR products was not reflected in the NDVI AUPC (Table 2). Only methiozolin influenced NDVI, causing a significant reduction in NDVI AUPC of 2%. This reduction in NDVI AUPC was probably the result of annual bluegrass control by methiozolin as NDVI is assessed for the whole plot.

These data suggest that common PGR products used on golf greens will have minimal influence on the activity of methiozolin with the exception of ethephon, which appeared to decrease annual bluegrass control. Other researchers have reported apparent reductions in annual bluegrass control when annual bluegrass suppression programs that employed paclobutrazol products were utilized in conjunction with multi-application methiozolin programs (SJ Koo, personal communication). To date, there is no information in peer-reviewed literature as to how methiozolin may interact with any PGR products.

Methiozolin Interaction with Ethephon. When data from the four trial sites were included in a combined analysis of variance, trial by rate interactions for annual bluegrass cover reduction were detected at 4, 6 and 8 WAIT ($p < 0.0108$, 0.0001 and 0.0001 , respectively). Two sites were core cultivated within two days of the second treatment and core cultivation was withheld from the other two sites. When sites were analyzed separately based on inclusion or exclusion of core-cultivation, trial interactions were not detected. The influence of core-cultivation cannot be separated from overall site effects, and therefore cannot be compared statistically. However, pooled data from the two sites that were core-cultivated will be presented separately from that of the two sites where core-cultivation was excluded as this distinction appeared to contribute strongly to the observed trial interactions.

The main effect of methiozolin rate was significant for annual bluegrass percent cover reduction at 6 ($P < 0.0001$) and 8 ($P < 0.0001$) WAT. The lack of ethephon effect on annual bluegrass percent cover reduction, led us to reject our hypothesis that ethephon would antagonize annual bluegrass control by methiozolin based on the previous study (Table 2). It is possible that ethephon may negatively impact methiozolin activity on annual bluegrass but applying two treatments of methiozolin in this study compared to only one application in the PGR admixtures

study may have overcome any potential antagonism. When averaged over levels of ethephon, methiozolin increased annual bluegrass cover reduction rapidly at sites that were core cultivated, reaching an asymptote of over 90% at between 1000 and 1500 g ai ha⁻¹ (Figure 1). In contrast, the response of annual bluegrass cover reduction to methiozolin rates was almost linear at sites that were not core cultivated (Figure 1). At 8 WAIT, trends in annual bluegrass cover reduction at sites that were core cultivated were similar to that observed at 6 WAT (Figure 2). At sites that were not core cultivated, annual bluegrass cover reduction had increased at 8 WAIT compared to 6 WAIT suggesting that annual bluegrass control by methiozolin may be more rapid when methiozolin treatments are applied in close proximity to core cultivation. Although no peer-reviewed literature exists on the effects of core-cultivation and methiozolin treatment on annual bluegrass, it is documented that in order to avoid stress, particularly in summer, annual bluegrass should be core-cultivated only during times of active growth, particularly following spring green-up and seed production (Vargas and Turgeon 2004). Core cultivation stress can also damage root systems and aggressive spring root growth will typically overcome this stress (Fagerness and Yelverton 2001; Murphy and Reike 1994). Root inhibition by methiozolin, however, could limit stress recovery following core cultivation. Trends in these studies suggest more research is needed to statistically compare the effects of core cultivation on annual bluegrass response to methiozolin.

For creeping bentgrass cover, a trial by rate by ethephon interaction was detected ($p < 0.0285$). As with annual bluegrass data, when analyzed separately by sites that included or excluded core-cultivation, trial interactions were no longer detected. Pooled data are presented separately by core-cultivation class, but cannot be compared statistically between core-cultivation classes. The nature of the interaction seemed to be strongly dependent on trial site, in

that core cultivated sites had severe creeping bentgrass injury only in the presence of ethephon (Figure 3). At 8 WAT, non-core-cultivated creeping bentgrass cover increased from 50 to as much as 80% in a curvilinear fashion as methiozolin rates increased (Figure 3a) suggesting that methiozolin did not injure creeping bentgrass but allowed it to expand as annual bluegrass was controlled. Addition of ethephon did not influence creeping bentgrass response at sites that were not core cultivated. At core-cultivated sites without the addition of ethephon, creeping bentgrass cover responded similarly to that of non-cultivated sites with a curvilinear response of increasing creeping bentgrass cover between 50 to 70% (Figure 3b). Conversely, when ethephon was added, creeping bentgrass cover was reduced to 19% by 2000 g ai ha⁻¹ methiozolin.

At 6 WAT and 8 WAT, a rate by ethephon interaction for NDVI was detected at the aerated site (Table 3). At 2000 g ai ha⁻¹ methiozolin, NDVI was 0.55 and 0.65 at 6 and 8 WAT, respectively, when ethephon was not applied and significantly more than 0.47 and 0.57 at 6 and 8 WAT, respectively, in plots that did receive ethephon treatment. At sites that were not core cultivated, ethephon did not affect NDVI regardless of methiozolin rate (Table 3). Reductions in NDVI as a result of ethephon application could be attributed to the ability of ethephon to reduce overall turfgrass quality. Cullough et al. (2005) observed decreased turfgrass quality for 9 weeks when ethephon was applied every three weeks during the study duration. Further, quality decreased as ethephon rate increased (McCullough et al. 2005). Addition of stress, like core-cultivation can reduce turfgrass quality and NDVI significantly, simply by the reduction in the total amount of tissue present for reflectance.

Murphy and Reike (1994) found that using hollow tine cultivation on a compacted putting green reduced root weight of 'Penncross' creeping bentgrass at the 0- to 50- mm and 50- to 100- mm depth, indicating more damage to surface root systems following cultivation.

Wiecko et al. (1993) observed similar results in hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* (Burt-Davis)) in August 1987, but the opposite in June 1988, with a 13% increase in surface rooting (0- to 10- cm) when cultivated with hollow tines following cultivation in April. Since ethephon has also been reported to decrease turfgrass rooting (McCullough et al. 2005), it could be more damaging to creeping bentgrass and annual bluegrass on core-cultivated sites. Previous research has demonstrated the potential for antagonistic/synergistic relationships between ethephon and other herbicides. For example, Hinshalwood and Kirkwood (1988) found decreased accumulation of ¹⁴C-labeled asulam in the rhizome meristems of bracken fern (*Pteridium aquilinum* (L.) Kuhn) when applied with ethephon thereby reducing control at 7DAT. This could be due to the fact that ethephon is hindering the ability of asulam to reach the target site within the fern plants. In research conducted by Lawrie and Clay (1993), addition of ethephon to fluzifop-butyl increased herbicide efficacy on quackgrass (*Elymus repens* (L.) Gould), when applied within 4 days of herbicide application via an increase in herbicide availability to growing points. Researchers also observed some inconsistency in herbicide efficacy, possibly as a direct result of variable ethephon activity within plant tissues (Lawrie and Clay 1993).

These data represent the first report of possible interactions between methiozolin and PGR products in turf. Negative interactions were observed only for ethephon and only for methiozolin rates that are two to four times that recommended by the developing company (SJ Koo, personal communication). Despite the fact that most PGR products did not interact with methiozolin, the increased creeping bentgrass injury caused by mixtures with ethephon suggest caution should be practiced when using PGR products with methiozolin. Although statistical comparisons between sites that were core cultivated versus those not cultivated could not be

made, the consistency between multiple trials within these site types and apparent desperate responses between the two site types indicate further research is needed. Future research should attempt to better characterize the influence of core cultivation on creeping bentgrass and annual bluegrass response to methiozolin.

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Table 1. Products utilized PGR admixtures study^a.

Common Name	Trade Name	Rate	Manufacturer	City, State, Website
Methiozolin	PoaCure™	1000 g ai ha ⁻¹	Moghu Research Center, LLC	Daejeon, South Korea www.moghu.com
Ethephon	Proxy®	1910 g ai ha ⁻¹	Bayer Environmental Science	Research Triangle Park, NC www.backedbybayer.com
Trinexapac-ethyl	Primo® MAXX	48 g ai ha ⁻¹	Syngenta Crop Protection, LLC.	Greensboro, NC, www.syngentacropprotection.com
Mefluidide	Embark®	70 g ai ha ⁻¹	PBI Gordon Corporation	Kansas City, MO, www.pbigordon.com
Flurprimidol	Cutless™	280 g ai ha ⁻¹	SePro Corporation	Karmel, IL, www.sepro.com

Paclobutrazol Trimmit 210 g ai ha⁻¹ Syngenta Crop Protection,
LLC. Greensboro, NC,
www.syngentacropprotection.com

^aApplication dates included: April 10, 2011 at Glade Road Research Facility, Virginia Tech, Blacksburg VA and May 5, 2012 at Draper Valley Golf Club near Draper, VA.

Table 2. Main effect of plant growth regulator (PGR) averaged over two levels of methiozolin and main effect of methiozolin averaged over six levels of PGR on area under the progress curve^a (AUPC) of annual bluegrass and creeping bentgrass cover separated by trial and annual bluegrass control, creeping bentgrass injury, and turf normalized difference vegetative index (NDVI) averaged over two trials.

	Annual bluegrass cover		Creeping bentgrass cover		Annual bluegrass control	Creeping bentgrass injury	Turf NDVI
	2011	2012	2011	2012			
	————— % AUPC —————				0-1 AUPC		
No PGR	160 ab	1763 a	5392 ab	3828 b	1006 a	9 c	19.8 a
Ethephon (1910 g ai ha ⁻¹)	183 ab	1574 ab	5437 a	4026 ab	169 c	20 c	19.8 a
Flurprimidol (280 g ai ha ⁻¹)	180 ab	1061 b	5169 bc	4540 a	2295 a	136 b	19.7 a
Mefluidide (70 g ai ha ⁻¹)	202 a	1336 ab	4853 d	4264 ab	1949 a	280 a	19.7 a
Paclobutrazol (210 g ai ha ⁻¹)	117 b	1090 b	5008 cd	4475 a	2252 a	160 b	19.7 a

Trinexapac ethyl (96 g ai ha ⁻¹)	210 a	1561 a	5305 ab	4039 ab	988 b	41 c	19.8 a
Methiozolin main effect ^{bd}							
No methiozolin	165 a	1579 a	5235 a	3966 a	712 b	96 a	19.9 a
Methiozolin (1000 g ha ⁻¹)	185 a	1082 b	5132 a	4519 b	2174 a	119 a	19.6 b

^aArea under the progress curve was calculated based on six assessments taken over a 56-day period following single treatments of PGR products and methiozolin alone or as tank mixtures applied on April 10, 2011 and May 5, 2012 on greens of two Virginia golf courses. Turf NDVI was assessed by scanning a 0.5 by 2.0 m area of each plot using a multispectral analyzer. All other assessments were visually estimated.

^bMain effects of both PGR and methiozolin were significant ($P < 0.05$) but the interaction of these two factors was insignificant ($P > 0.05$). In the case of annual bluegrass and creeping bentgrass, both methiozolin and PGR factors interacted with trial and are presented separately by trial. In all cases, means representing a PGR effect are pooled over two levels of methiozolin and means representing a methiozolin effect are pooled over six levels of PGR.

^cMeans for a given level of PGR are different from other levels of PGR if followed by a different letter ($P = 0.05$).

^dMeans for the two levels of methiozolin are different if followed by a different letter ($P = 0.05$).

Table 3. Interaction of methiozolin rates and ethephon on turf normalized difference vegetative index (NDVI) 6 and 8 weeks after initial treatment (WAIT) averaged over two sites that were core cultivated within 2 days of the second treatment and two sites that were not core cultivated during the study duration.

Methiozolin _b	Ethephon	NDVI ^a 6 WAIT		NDVI ^a 8 WAIT	
		Cultivated sites	Noncultivated sites	Cultivated sites	Noncultivated sites
Index 0-1					
0.5 g ai ha ⁻¹	None	0.6054 a	0.6043 a	0.6814 a	0.7807 a
1.0 g ai ha ⁻¹	None	0.5674 b*	0.6254 a	0.6656 b	0.7674 b
2.0 g ai ha ⁻¹	None	0.5503 c*	0.5696 b	0.6493 c*	0.7338 c
0.5 g ai ha ⁻¹	3.8 kg ai ha ⁻¹	0.6028 a	0.5771 a	0.6967 a	0.7899 a
1.0 g ai ha ⁻¹	3.8 kg ai ha ⁻¹	0.5470 b*	0.5678 b	0.6595 b	0.7702 a
2.0 g ai ha ⁻¹	3.8 kg ai ha ⁻¹	0.4716 c*	0.5680 b	0.5669 c*	0.7532 b

^aNormalized difference vegetative index was assessed using a multispectral radiometer adjusted to scan a 0.5 by 2.0 m area in the center of each plot.

^bMethiozolin and ethephon were applied initially at 50 GDD₅₀ and again one month later based on typical recommendation for annual bluegrass seedhead suppression with ethephon. Initial treatments were applied on March 13, 2012 and April 12, 2014 at the two sites that were core cultivated. Initial treatments were applied on April 13, 2013 and April 12, 2014 at the two sites that were not

Figure 1. Effect of methiozolin rate averaged over two levels of ethephon on annual bluegrass cover at 6 weeks after the initial treatment (WAIT) as the average response of two trials that received core cultivation within two days of the second treatment and the average response of two trials that were not core cultivated for the study duration. Means were subjected to the hyperbolic function as $y = (0.4077*x)/(1+(0.4077*x)/111)$; $R^2 = 0.90$ for core cultivated sites and $y = (0.0696*x)/(1+(0.0696*x)/200)$; $R^2 = 0.83$ for noncultivated sites.

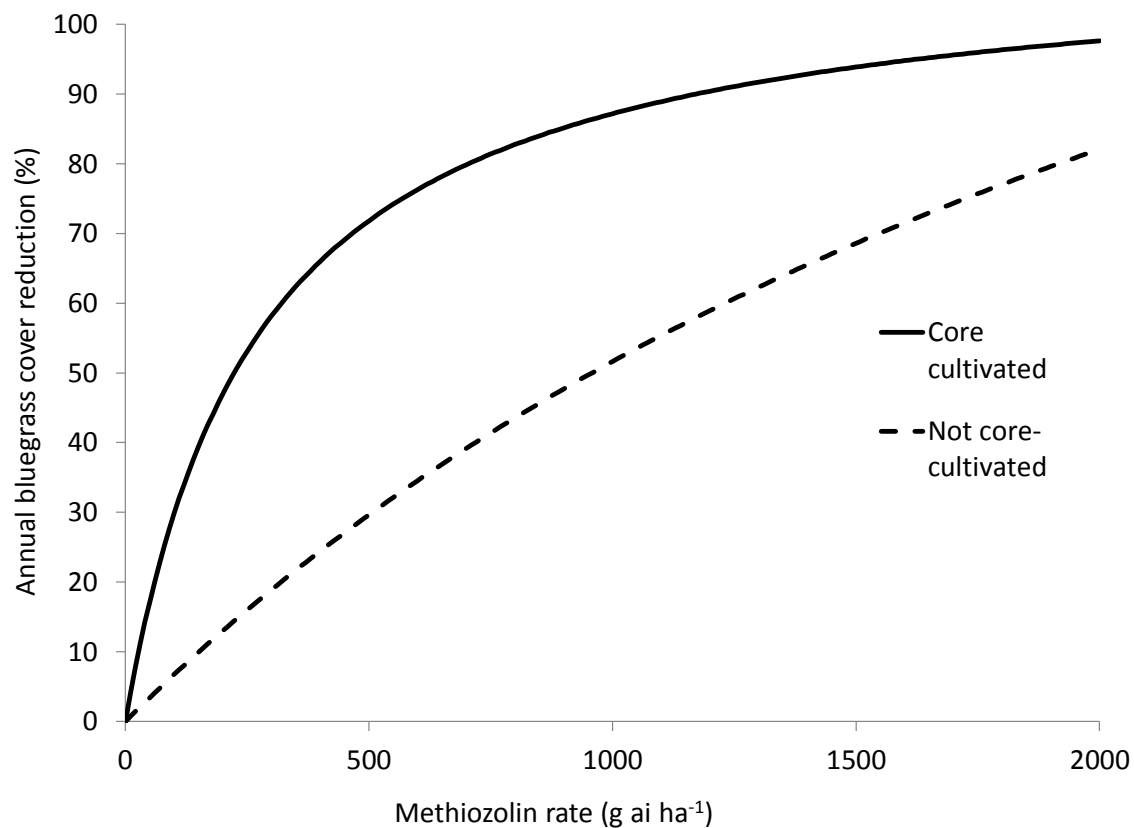


Figure 2. Effect of methiozolin rate averaged over two levels of ethephon on annual bluegrass cover at 8 weeks after the initial treatment (WAIT) as the average response of two trials that received core cultivation within two days of the second treatment and the average response of two trials that were not core cultivated for the study duration. Means were subjected to the hyperbolic function as $y = (0.3377*x)/(1+(0.3377*x)/107)$; $R^2 = 0.89$ for core cultivated sites and $y = (0.0908*x)/(1+(0.0908*x)/200)$; $R^2 = 0.97$ for noncultivated sites.

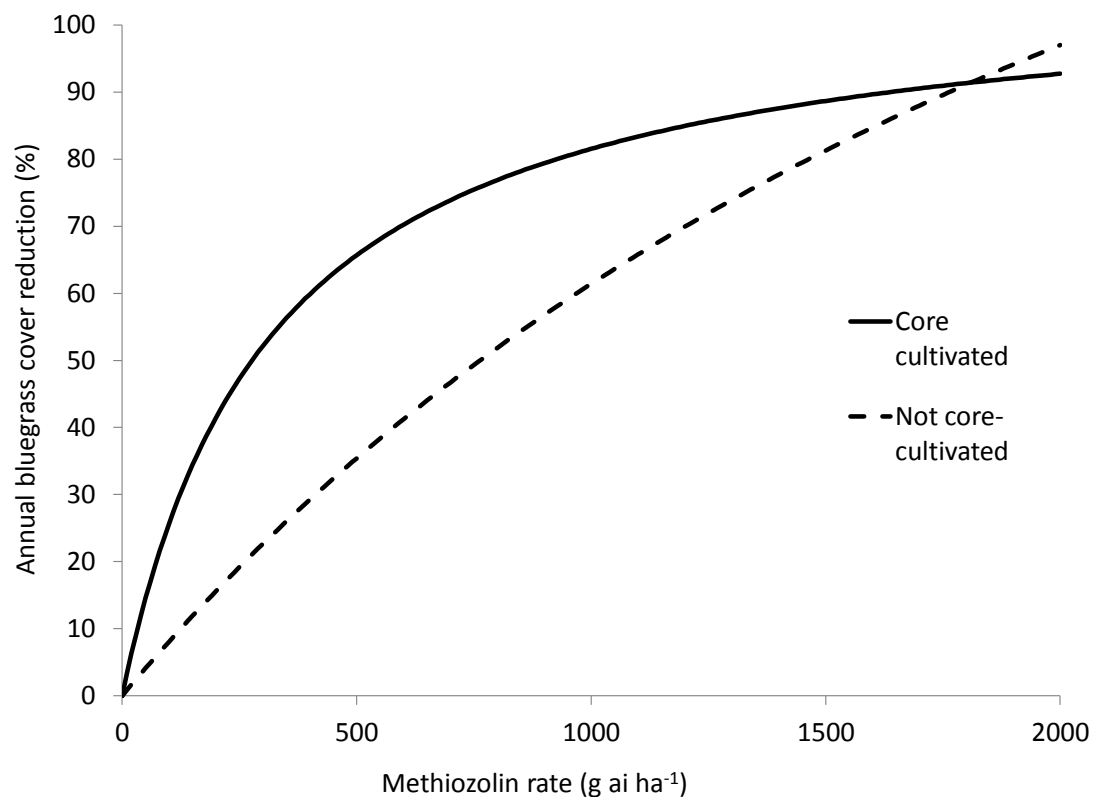
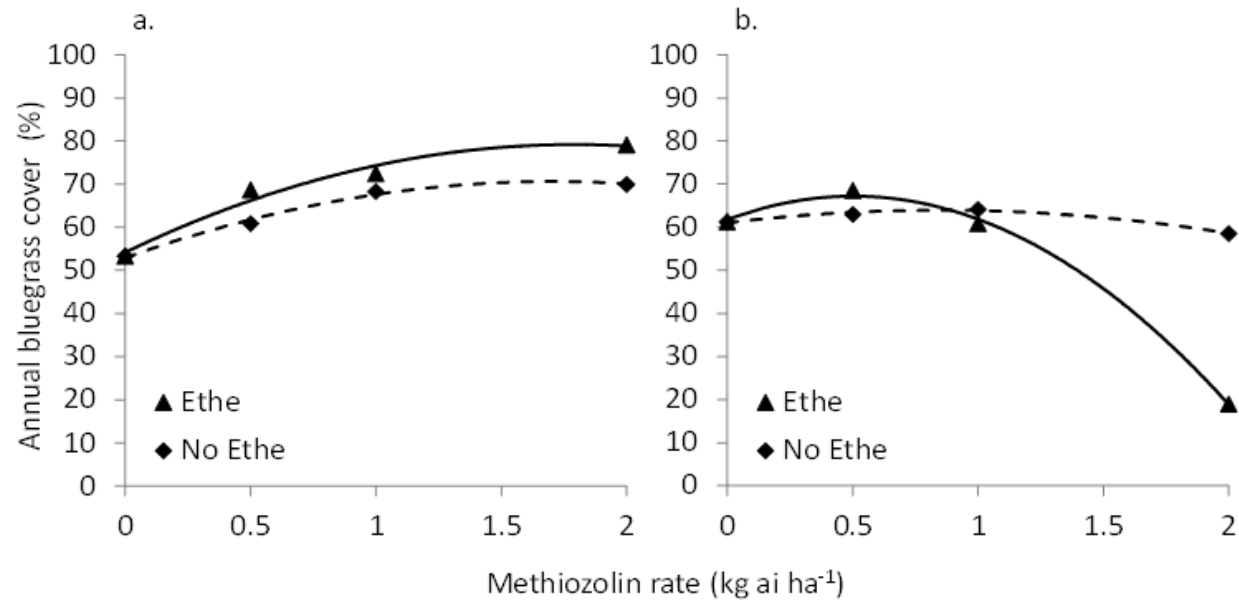


Figure 3. Interaction of methiozolin rates and ethephon on creeping bentgrass cover at 8 WAT averaged over two sites that were not core cultivated for the study duration (A) and an additional two sites that received core cultivation within two days of the second treatment (B). Means in (A) were subjected to quadratic equation as $y = 7.79x^2 + 27.9x + 54.2$, $R^2 = 0.97$ for no ethephon (No Ethe) effects and $y = -6.08x^2 + 20.7x + 52.9$; $R^2 = 0.99$ for ethephon (Ethe) effects. Means in (B) were subjected to the quadratic equation as $y = -21.6x^2 + 21.7x + 61.8$; $R^2 = 0.99$ for no ethephon (No Ethe) effects and $y = -3.98x^2 + 6.72x + 61.1$; $R^2 = 0.98$ for ethephon (Ethe) effects.



Chapter 4. Investigating Tyrosine Aminotransferase as a Potential Target Site for Annual Bluegrass Control by Methiozolin

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Methiozolin is a new herbicide used primarily for annual bluegrass and roughstalk bluegrass control in a variety of turfgrasses. A few previous publications have speculated on the potential mode of action of methiozolin based on responses of model plant species such as *Arabidopsis thaliana*, lesser duckweed, and maize. Researchers proposed tyrosine aminotransferase (TAT) inhibition as a putative target site of methiozolin based on in vitro inhibition of an *Arabidopsis* TAT isoenzyme and altered response of lesser duckweed due to exogenous 4-HPP feeding. Since TAT inhibition and associated symptoms do not appear to explain field observations of shoot response and potent root inhibition of methiozolin on annual bluegrass, the primary targeted weed, studies were conducted to compare the response of annual bluegrass and desirable turfgrass species to methiozolin alone or with exogenous 4-HPP. Plants were exposed to 0.2 μM methiozolin and 10 μM 4-HPP axenically in hydroponic solution. Lesser duckweed canopy cover was reduced with applications of methiozolin, and addition of 4-HPP to cultures partially alleviated herbicide symptoms at 7 and 10 DAT. Canopy cover and root length of all four turfgrasses at 7 DAT was reduced by methiozolin but not affected by exogenous 4-HPP. Methiozolin reduced secondary root density of annual and Kentucky bluegrass but did not affect that of creeping bentgrass or perennial ryegrass. Addition of 4-HPP did not influence secondary root density of annual bluegrass, Kentucky bluegrass, or creeping bentgrass but had variable effect on secondary root density of perennial ryegrass. The length of secondary roots for all species was reduced by methiozolin but not affected by 4-HPP. The differing response to

exogenous 4-HPP between lesser duckweed and the four tested grass species, suggest that either potential TAT inhibition in targeted weeds is not a primary mechanism of action for methiozolin or exogenous 4-HPP in hydroponic solution is less biologically available to the tested grasses compared to lesser duckweed.

NOMENCLATURE: tyrosine aminotransferase (TAT), 4-hydroxyphenyl pyruvate (4-HPP), annual bluegrass (*Poa annua* L. POAAN), barnyardgrass (*Echinochloa crus-galli* P.B. var. *aristata* S.F.GRAY ECHCG), lesser duckweed (*Lemna aequinoctialis* Welw. LEMPA), maize (*Zea mays* L. ZEAMD), methiozolin (5{2, 6-difluorobenzyl}oxymethyl-5-methyl-3, 3{3-methylthiophen-2-yl}-1, 2-isoxazoline)

KEYWORDS: annual bluegrass, differential response, digital image analysis, mode of action

INTRODUCTION

Methiozolin is a new herbicide developed by Moghu Research Center, Ltd., for selective control of annual bluegrass in turfgrass areas (Koo et al. 2014). Methiozolin does not typically injure desirable species like creeping bentgrass, perennial ryegrass or Kentucky bluegrass in field settings (Koo et al. 2014). Methiozolin is a member of the isoxazoline chemical family, which encompasses chemistries including anti-parasitic agents, corrosion inhibitors and one herbicide registered in the United States, pyroxasulfone (Gassel et al. 2014; Koo et al. 2013; Lee et al. 2007; Yildirim and Cetin 2008). Pyroxasulfone is a very long chain fatty acid elongase (VLCFAE-) inhibitor (Tanetani et al. 2009). Methiozolin was reported to have no effect on very-long-chain fatty acid synthesis based on toluidine-blue staining of cress (*Lepidum savitum* L.) hypocotyls (Grossman et al. 2011), however, the thin, broad, violet cotyledons on of barnyardgrass seedlings by Lee et al. (2007) would not exclude this mechanism of action.

To date, two possible modes of action for methiozolin have been proposed (Grossmann et al. 2011; Lee et al. 2007). Lee et al. (2007) proposed that cell wall biosynthesis could be a primary or secondary mode of action based on a reduction in ¹⁴C-labeled cellulose and hemicellulose (2'-O-(4-O-methyl- α -D-glucopyranosyluronic acid)-D-xylose (methylglucuronoxyllose [GAX1]) and 2'-O-(4-O-methyl- α -D-glucopyranosyluronic acid)-D-xylobiose (methylglucuronoxyllobiose [GAX2])) in maize root tips. The researchers found that although ¹⁴C-glucose incorporation in acid-insoluble fractions of cell walls was inhibited 42 to 80% by 0.1 μ M methiozolin, root growth was inhibited before detectable reductions in ¹⁴C-glucose assimilation. This apparent lag between root growth inhibition and detectable reductions in ¹⁴C-glucose assimilation in cell wall fractions suggests methiozolin effects on cell wall

biosynthesis in corn root tips may be secondary. Barnyardgrass roots and shoots were inhibited by methiozolin similar to dichlobenil, butachlor and pendimethalin, but morphology of roots and coleoptiles were distinct. The effects of methiozolin on cell walls of annual bluegrass, the primary targeted weed of methiozolin, are not known.

More recently, Grossmann et al. (2011) proposed inhibition of tyrosine aminotransferase (TAT), an enzyme responsible for the catalysis of L-tyrosine to 4-hydroxyphenyl pyruvate (4-HPP), as the primary mode of action of methiozolin. Under stressful conditions or during plant aging, plants require more tocopherols in order to reduce the number of reactive oxygen species present in the cells. In order to produce tocopherols in the amounts needed, TAT activity in the plastids increases, thus raising the amounts of the tocopherol precursor, homogentisic acid via 4-HPP (Hollander-Cytko et al. 2005; Rippert et al. 2009). Aminotransferase enzymes, including TAT, are important for the interconversion of amino acids and their related 2-oxoacids via transamination (Lee and Facchini 2011; Liepman and Olsen 2004; Prabhu and Hudson 2010). Despite the fact that required rates for 50% TAT inhibition were over 2000 times that needed to control annual bluegrass with methiozolin, Grossman et al. (2011) implicated methiozolin as a putative inhibitor of TAT on the assumption that other isoenzymes may be more sensitive, along with more supporting evidence. The most compelling evidence implicating TAT inhibition by biologically-realistic methiozolin rates was related to methiozolin effects on lesser duckweed growth (Grossmann et al. 2011). Lesser duckweed plants were treated with methiozolin and subsequently exposed to either L-tyrosine, homogentisic acid, or 4-HPP. As TAT is required to catalyze the reaction forming 4-HPP from L-tyrosine, addition of L-tyrosine was not effective at reversing symptoms, and neither was homogentisic acid. Re-addition of exogenous 4-HPP, however, appeared to alleviate symptoms of methiozolin application in lesser duckweed, further

supporting TAT as a potential target site for methiozolin (Grossmann et al. 2011). The effects of methiozolin on annual bluegrass TAT are unknown.

To date, there have been no further studies published in peer-reviewed literature investigating the mode of action of methiozolin, and no studies to determine differences between susceptible and tolerant grass species on a molecular level. Only one study has been published investigating an apparent differential selectivity to methiozolin between annual bluegrass and creeping bentgrass. McCullough et al. (2013) found that, when absorbed through the roots at warmer temperatures, annual bluegrass translocates more ¹⁴C-labeled methiozolin to the shoots than creeping bentgrass. This difference, however, was not apparent at lower temperatures and there is no known link between this slight difference in translocation and differential species response.

Our aim was not only to reproduce experimental conditions of the study by Grossman et al. (2011) on lesser duckweed, but also to measure effects of 4-HPP feeding and methiozolin on annual bluegrass and three turfgrass species that differ in sensitivity to methiozolin. If methiozolin mode of action is primarily dependent on TAT inhibition, we expect to see similar growth responses in both duckweed and annual bluegrass. The objectives of these studies are to determine if TAT inhibition plays a role in methiozolin activity in annual bluegrass and several other turfgrass species and whether or not annual bluegrass and desirable, cool-season turfgrass species are differentially affected by methiozolin alone or in the presence of exogenous 4-HPP.

MATERIALS AND METHODS

Feedback Study. Four laboratory experiments were conducted between 2013 and 2015. All experiments were conducted as randomized complete block designs with four replicates that were spatially arranged in growth chambers. In three of the experiments, annual bluegrass, creeping bentgrass, Kentucky bluegrass, lesser duckweed and perennial ryegrass were investigated. These experiments were initiated on October 23, 2013 and in two separate growth chambers on January 10, 2015. In an additional experiment initiated on January 7, 2014, only annual bluegrass and lesser duckweed were included. Treatments were arranged in a five by two by two factorial with up to five levels of species, two levels of methiozolin and two levels of 4-HPP. Methiozolin (MRC-01; Moghu Research Center Ltd., BVC 311, KRIBB, Yuseong, Daejeon, 305-333, South Korea) was added to nutrient solution at 0.2 μM or 0 μM and 4-HPP (4-HPP; Sigma-Aldrich, Co., 3050 Spruce Street, St.Louis, MO 63103, USA) was included at 10 μM or 0 μM . Seeds of annual bluegrass, creeping bentgrass, Kentucky bluegrass and perennial ryegrass were sterilized according to methods outlined by Zhang et al. (2001). Seeds were incubated at room temperature for 1 minute in 190 proof ethanol (Ethanol 190 Proof; Decon Labs, Inc., 460 Glennie Circle, King of Prussia, PA 19406, USA) followed by 20 minutes in 2% v/v sodium hypochlorite solution (Bleach; The Kroger Co., 1014 Vine St. Cincinnati, OH 45202, USA). Seed were germinated over the course of several days in sterile, 0.25x Hoagland's modified basal salt mixture (Hoagland's Modified Basal Salt Mixture; MP Biomedicals, LLD., 29525 Fountain Parkway, Solon, OH 44139, USA) in solution with ultra-pure water (Barnstead Smart2Pure; Thermo Electron LED GmbH, Trobert-Bosch-Straße 1, D-63505 Langenselbold, Germany). Lesser duckweed was maintained in aseptic culture on 0.5x Schenk and Hildebrandt media (Schenk and Hildebrandt Basal Salt medium; MP Biomedicals, 3 Hutton Center, Suite 100, Santa Ana, CA 92707, USA) with 0.5% w/v sucrose, as recommended by the supplier, Rutgers

Duckweed Stock Cooperative (Rutgers 2015). When grass seedling leaves reached approximately 2 cm in length, three to five seedlings of each grass species and 10 fronds of lesser duckweed were aseptically transferred to 9.6 cm² 6-well culture plates (Falcon Tissue Culture Plates; Corning Life Sciences, One Riverfront Plaza, Corning, NY 14831, USA) containing 0.25x Hoagland's modified basal salt mixture in solution with ultra-pure water, methiozolin, and 4-HPP as appropriate for each treatment. Plants remained in the treatment solution for the duration of the experiment.

Photographs were taken at 0, 5, 7, and 10 days after treatment (DAT) and canopy green pixels were analyzed with SigmaScan 5.2 (SigmaScan Pro; Systat Software, Inc., 2107 North First Street, Suite 360 San Jose, CA 95131, USA) software. Images were analyzed at hue: 35-100 and saturation: 35 to 349, in order to optimize green pixel analysis (Karcher et al. 2003). Digital images were taken with an 8.0 megapixel Canon Digital Rebel XT (Rebel XT; Canon U.S.A., Inc., One Canon Park, Melville, NY 11747, USA) at the following settings: F16, ISO100, white balance set to fluorescent, two-second shutter speed. Roots were photographed with a Canon M-1 macro lens set to 2 X magnification on a Canon EOS 5D Mark II digital camera set to 21.1 megapixel resolution and digitally magnified to approximately 30X magnification. Secondary root density was counted as the number of secondary roots cm⁻¹ of visible primary root, with 4 sub-samples per image in order to account for variance between seedlings in each plate. Secondary root length was measured from digital images using Adobe Photoshop to measure pixel length and converting to distance based on scale relations from a metric rule photographed under the same experimental conditions as roots. Primary root length was measured to the nearest mm by removing plants from axenic culture at study conclusion and placing on a metric rule.

Data were analyzed in SAS 9.1 (SAS Institute, Inc., 100 SAS Campus Drive, Cary, NC 27513, USA). These data were determined to be normal using the NORMAL option in PROC UNIVARIATE and Shapiro-Wilk statistic and homogeneity was assessed by visually inspecting plotted residuals. Data were subjected to ANOVA using PROC GLM with sums of squares partitioned to reflect the main effects and interactions of methiozolin and 4-HPP and the effects of trial, which were considered random. Mean squares associated with trial interactions were used to test for significance of fixed effects (McIntosh 1983). If significant trial interactions occurred, data were presented separately by trial, otherwise, data were pooled over trial. Mean responses of species were separated with Fisher's Protected LSD test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Trial interactions were insignificant ($P > 0.05$), for all measured responses and data were pooled over trial. The interaction of methiozolin by 4-HPP was significant for lesser duckweed canopy cover at 7 and 10 DAT ($P = 0.0192$ and 0.0362 , respectively) while the main effect of methiozolin was significant at 5 DAT ($P = <0.0001$). Methiozolin at $0.2 \mu\text{M}$ significantly decreased lesser duckweed canopy cover at 5, 7, and 10 DAT (Table 1). A significant improvement in lesser duckweed canopy cover was observed when exogenous 4-HPP was added to methiozolin at 7 and 10 DAT while 4-HPP had no influence on canopy cover in the absence of methiozolin (Table 1). These observations are similar to that reported by Grossman et al. (2011) although the impact of 4-HPP on lesser duckweed canopy cover 10 DAT was greater in that study. When averaged over four trials, the cover of 4-HPP plus methiozolin treated plant canopies was 65 and 50% of the nontreated canopies at 7 and 10 DAT, respectively compared to an average of 96% canopy preservation in one trial in the Grossman et al. (2011) study. In our

study, lesser duckweed canopy response varied in magnitude between trials although treatment trends were consistent. Mean responses of canopy preservation 10 DAT were 98, 84, 30, and 90% in trials 1, 2, 3, and 4, respectively (data not shown). Regardless of magnitude of the 4-HPP effect, our observations agree with that of Grossman et al. (2011) that lesser duckweed growth inhibition by methiozolin can be substantially mitigated by exogenous 4-HPP. Sandorf and Hollander-Czytko (2002) found that TAT activity can be induced by several octadecanoids, and plant stress, thereby influencing the amount of tocopherols found in the plant. A lack of TAT activity would result in reduced plant growth and injury from the stressor (Lee et al. 1997). Thus, TAT inhibition by methiozolin is plausible given the response of lesser duckweed. Further, fluctuations in TAT activity influence the amount of 4-hydroxyphenylpyruvate dioxygenase, another important downstream enzyme in synthesis of carotenoids via phytoene desaturase (Norris et al. 1995). Our objective was to determine if the same responses occur in annual bluegrass and other species that will be exposed to methiozolin.

The interaction of methiozolin and 4-HPP was insignificant ($P > 0.05$) and main effect of methiozolin significant for canopy cover of annual bluegrass ($P = 0.0075$), creeping bentgrass ($P = 0.0320$), Kentucky bluegrass ($P = 0.0093$), and perennial ryegrass ($P = 0.0006$) 7 DAT (Table 2). Canopy cover and primary root length was reduced by methiozolin an average of 67 and 89%, respectively, across all turfgrass species (Table 2). Lesser duckweed root growth was not quantified, as the length could not be assessed accurately. Root length did not exceed 17 mm for methiozolin treated plants, whereas non-treated roots ranged from 43 to 81 mm, depending on species (Table 2). Previous research has characterized methiozolin as a potent root inhibitor by evaluating primary root growth of corn seedlings and associated inhibition of cell wall constituents in corn root tips (Lee et al. 2007).

The interaction of species by methiozolin by HPP was significant for both secondary root density and length ($P < 0.05$). Creeping bentgrass secondary root density was unaffected by any treatment, but secondary root length was reduced 44% by methiozolin alone (Table 3). Creeping bentgrass secondary root length when treated with methiozolin plus 4-HPP was not different from the secondary root length of non-treated plants or plant treated only with 4-HPP. This similarity in secondary root length could indicate possible symptom alleviation by 4-HPP although the comparison across levels of 4-HPP within methiozolin treated creeping bentgrass was insignificant. Perennial ryegrass was the only species with significant effects of 4-HPP on secondary root density but the effect was inconsistent between presence or absence of methiozolin (Table 3). When methiozolin was not added, 4-HPP significantly increased perennial ryegrass root density but when methiozolin was added, 4-HPP significantly decreased root density (Table 3), the opposite of effects observed in lesser duckweed. Annual bluegrass and Kentucky bluegrass secondary root density and length were strongly reduced by methiozolin and not affected by 4-HPP. These results support field observations that methiozolin can inhibit root growth when roots are exposed to the herbicide. It is recommended to avoid planting grass seed into methiozolin-treated areas for at least two weeks following application, as reductions in germination were observed in creeping bentgrass, perennial ryegrass and tall fescue (McCullough and Gomez de Barreda 2012). Annual bluegrass is controlled both PRE and POST with methiozolin, indicating sensitivity of annual bluegrass at all stages of growth (Askew and McNulty 2014; Flessner et al. 2013; Koo et al. 2014; Moghu 2015). The apparent tolerance of desirable turfgrass species to methiozolin in field studies is well characterized, but the reason for observed field selectivity has not been evaluated (Hoisington et al. 2014; Koo et al. 2014). Differences in secondary root density and length could be the result of methiozolin acting

secondarily on cell wall biosynthesis as proposed by Lee et al. (2007). In aeroponics studies, secondary root production in annual bluegrass and creeping bentgrass was altered by low rates of methiozolin. Symptoms presented as an altered, swollen morphology of secondary roots, and overall reduction in root growth. Further, creeping bentgrass roots regenerated more rapidly than those of annual bluegrass when grown in the same chamber exposed to methiozolin in nutrient solution (Venner et al. 2012). The apparent difference between secondary root inhibition of creeping bentgrass and other grass species, suggest a possible mechanism of selectivity but likely of minor effect. The primary mechanism of selectivity is likely associated with differential root exposure in the field as hydroponic, aeroponics, and seedling emergence studies indicate that interspecific responses to methiozolin are minimal (Koo et al. 2014; McCullough and Gomez de Barreda 2012; Venner et al. 2012). In this study, exogenous 4-HPP did not influence methiozolin effects on annual bluegrass or turfgrasses as was observed in lesser duckweed. These results indicate that either possible TAT inhibition by methiozolin is of minor importance to annual bluegrass response or grasses such as annual bluegrass, creeping bentgrass, Kentucky bluegrass, and perennial ryegrass are not able to utilize exogenous 4-HPP as efficiently as lesser duckweed.

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Table 1. Interaction of exogenous 0.2 μM methiozolin and 10 μM 4-HPP on lesser duckweed canopy cover at three assessment times averaged over four trials^a.

Methiozolin ^b	4-HPP	Lesser duckweed green pixel cover		
		5 DAT	7 DAT	10 DAT
		%		
None	None	19a	29a	38a
None	10 μM	18a	29a	40a
0.2 μM	None	8b	11b*	13b*
0.2 μM	10 μM	9b	19b*	19b*

^aLesser duckweed canopy cover was assessed on October 30, 2013, January 14, 2014, and in two separate growth chambers on January 17, 2015.

^bDifferences between levels of methiozolin within a given level of 4-HPP are significant if means are followed by a different letter. If means are followed by an asterisk, the difference between levels of 4-HPP within a given level of methiozolin are significant. Statistical comparisons are based on Fisher's Protected LSD test ($p \leq 0.05$).

Table 2. Effect of 0.2 μM methiozolin averaged over trials^s and exogenous 10 μM 4-HPP^b treatment on canopy cover and root length of four grass species grown in hydroponic solution.

Species	Green pixel cover 7 DAT		Primary root length 7 DAT	
	0.2 μM methiozolin	Without methiozolin	0.2 μM methiozolin	Without methiozolin
	————— % —————		————— mm —————	
Annual bluegrass	6b ^c	17a	10b	81a
Creeping bentgrass	6b	15a	17b	73a
Kentucky bluegrass	5b	16a	7b	74a
Perennial ryegrass	3b	12a	17b	43a

^aAll species were assessed for canopy cover on October 30, 2013 and in two separate growth chambers on Jan 17, 2015. Annual bluegrass and lesser duckweed were also evaluated in a fourth trial on Jan 14, 2014. Lesser duckweed canopy cover was dependent on the interaction of methiozolin and 4-HPP (Table 1). Lesser duckweed roots could not be accurately measured and were not assessed.

^bInteractions with 4-HPP were not significant ($P > 0.05$) for the four grass species and data were pooled over two levels of 4-HPP (none and 10 μM 4-HPP).

^cDifferences between levels of methiozolin within a given species are significant if means are followed by a different letter based on Fisher's Protected LSD test ($p \leq 0.05$).

Table 3. Interaction of exogenous methiozolin and 4-HPP on density and length of root hairs from four grass species 10 DAT averaged over two trials^a.

Methiozolin _b	4-HPP	Average ^b secondary root density				Average ^b secondary root length			
		AGSST	LOLPE	POAAN	POAPR	AGSST	LOLPE	POAAN	POAPR
		no. cm ⁻¹				mm			
None	None	41a	40a*	42a	43a	4.2a	2.5a	3.3a	3.7a
None	10 μM	37a	46a*	42a	42a	3.3a	3.1a	3.0a	3.5a
0.2 μM	None	37a	44a*	32b	30b	1.8b	1.1b	0.6b	0.8b
0.2 μM	10 μM	37a	33b*	35b	33b	2.1a	0.9b	0.7b	0.8b

^aRoot measurements were taken from trials in two separate growth chambers on January 20, 2015.

^bMeasurements were taken from four randomly chosen sections of root on digital images viewed at approximately 30 times magnification.

^bDifferences between levels of methiozolin within a given level of 4-HPP are significant if means are followed by a different letter.

If means are followed by an asterisk, the difference between levels of 4-HPP within a given level of methiozolin are significant. Statistical comparisons are based on Fisher's Protected LSD test ($p \leq 0.05$).

Chapter 5. Effect of Methiozolin on ¹³C-Glucose Accumulation in Annual Bluegrass and Three Turfgrasses

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Methiozolin is a new herbicide developed by Moghu Research Center, Ltd., for the selective control of annual bluegrass in a variety of turfgrasses. Although the mode of action of methiozolin is unknown, a few previous publications suggested inhibition of tyrosine aminotransferase (TAT) and/or cell wall biosynthesis. Studies were conducted to first determine if differential sugar incorporation to cell walls, as observed in corn by previous researchers, would also be prevalent in annual bluegrass and three turfgrass species. Exogenous feeding of 4-hydroxyphenyl pyruvate (4-HPP) has been demonstrated to alleviate methiozolin effects on lesser duckweed in other studies. Subsequent experiments were conducted to determine if exogenous 4-HPP would have any impact on potential cell wall inhibition by methiozolin in annual bluegrass and three turfgrasses. If tyrosine aminotransferase (TAT) inhibition is the primary mode of action of methiozolin, as has been proposed by other researchers, alleviating possible TAT inhibition via 4-HPP supplement should alleviate annual bluegrass injury by methiozolin. This possibility will be elucidated in a separate report. If cell wall biosynthesis inhibition by methiozolin is a secondary effect of TAT inhibition, we expect to see differences in isotopic enrichment of pentose and hexose sugars when 4-HPP is added to methiozolin-treated annual

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bluegrass and other turfgrasses. Annual bluegrass, creeping bentgrass, perennial ryegrass and Kentucky bluegrass all exhibited decreased ^{13}C incorporation into arabinose, xylose, galactose and glucose in response to 0.01 or 1.0 μM methiozolin or 0.7 nM indaziflam, a known cell wall biosynthesis inhibitor, when fed with uniformly labeled glucose ($[\text{U-}^{13}\text{C}_6]$ -glucose), confirming inhibitory activity of these herbicides on cell wall biosynthesis. However, there were no differential responses among the tested grasses. Indaziflam at 0.7 nM and methiozolin at 0.01 μM inhibited ^{13}C isotopic enrichment in all sugars equivalently and less than methiozolin at 1.0 μM in all cases except galactose. Methiozolin inhibited xylose incorporation to cell walls to a lesser degree the presence of 10 μM 4-HPP. Exogenous 4-HPP had no other influence on methiozolin-mediated inhibition of cell wall synthesis. The differential inhibition of xylose incorporation in the presence of 4-HPP suggests TAT may be associated with the mechanism of action of methiozolin but is unlikely to be the only target site or the driving mechanism behind observed cell wall inhibition. Lack of a species by treatment interaction suggests differential sugar incorporation is not a mechanism of interspecific tolerance to methiozolin.

NOMENCLATURE: annual bluegrass (*Poa annua* L. POAAN), tyrosine aminotransferase (TAT), barnyardgrass (*Echinochloa crus-galli* P.B. var. *aristata* S.F.GRAY ECHCG), creeping bentgrass (*Agrostis stolonifera* L. AGSST), Kentucky bluegrass (*Poa pratensis* L. POAPR), perennial ryegrass (*Lolium perenne* L. LOLPE), tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort. FESAR), zoysiagrass (*Zoysia japonica* Steud. ZOYJA)

KEYWORDS: alditol acetates, annual bluegrass, GC-MS, mode of action, stable isotope labeling

INTRODUCTION

Methiozolin (5{2, 6-difluorobenzyl}oxymethyl-5-methyl-3, 3{3-methylthiophen-2-yl}-1, 2-isoxazoline) is a new herbicide manufactured by Moghu Research Center, LLC., in Daejeon, South Korea for the selective removal of annual bluegrass in turfgrass areas. Currently, the mode of action of methiozolin is unknown, but previous research implicates two potential modes of action. Lee et al. (2007) concluded that methiozolin inhibited cell wall biosynthesis (CBI) based on a reduction of ^{14}C -glucose incorporation into cellulose and hemicellulose fractions of corn root cell walls. Methiozolin at 1.0 μM reduced barnyardgrass root length over 90% and was similar to pendimethalin, butachlor and dichlobenil, which were inhibitory at concentrations of 10 μM or higher (Lee et al. 2007). The symptoms observed by Lee et al. (2007) on barnyardgrass did not resemble known CBI herbicides like dichlobenil, and a 6-hr difference between root length inhibition (6 HAT) and inhibition of ^{14}C -glucose incorporation in cell walls (12 HAT) led the authors to conclude that cell wall biosynthesis inhibition could be a secondary effect of some other primary mode of action.

More recently, a study published by Grossman et al. (2011) suggested that methiozolin is a putative inhibitor of tyrosine aminotransferase (TAT). TAT catalyzes the reaction that transforms *L*-tyrosine to 4-hydroxyphenyl pyruvate (4-HPP). Inhibition of TAT disrupts the downstream formation of tocopherols, plastoquinone, and carotenoids, and negatively influences photoprotection of the photosynthetic apparatus (Falk et al. 2002; Munne-Bosch 2005). By adding exogenous 4-HPP to lesser duckweed (*Lemna aequinoctialis* Welw.) cultures, researchers were able to alleviate symptoms caused by methiozolin exposure (Grossmann et al. 2011). Despite requiring 200 μM methiozolin which is over 1000 times the concentration required for

annual bluegrass control, the authors also reported inhibition of an *Arabidopsis thaliana* isoenzyme as support of the TAT target site hypothesis (Grossmann et al. 2011). Additionally, researchers detected an increase in tyrosine accumulation in methiozolin-treated plants and an increase in 3, 4-dihydroxyphenylalanine (DOPA). DOPA is an allelopathic compound that is more active on broadleaf species than grasses (Hachinohe et al. 2004). Although CBI or TAT inhibition appear to contribute to the mode of action of methiozolin in some species investigated, the mode of action in annual bluegrass remains unknown.

Methiozolin selectively controls annual bluegrass with safety to warm- and cool-season turfgrass species, including: zoysiagrass, creeping bentgrass, Kentucky bluegrass, tall fescue, and perennial ryegrass (Koo et al. 2014; McCullough and Gomez de Barreda 2012). The difference in susceptibility to methiozolin between annual bluegrass and these desirable species has not been explained. Although several studies have investigated annual bluegrass control and subsequent safety to cool-season turfgrasses, only one study has investigated the possible mechanism for selectivity between annual bluegrass and creeping bentgrass (Askew and McNulty 2014; Brosnan et al. 2012; Hoisington et al. 2014; Koo et al. 2013; McCullough and Gomez de Barreda 2012; McCullough et al. 2013). When grown at day/night temperatures of 30/25 °C, annual bluegrass absorbed more foliarly applied ¹⁴C-methiozolin than creeping bentgrass, but when temperatures were lowered to 16/10 °C, no differences were observed (McCullough et al. 2013). When applied via a spiked hydroponic solution to the roots, 33% more ¹⁴C-methiozolin was recovered from annual bluegrass shoots than roots. In addition, twice as much ¹⁴C-methiozolin was maintained in creeping bentgrass roots than annual bluegrass (McCullough et al. 2013). According to these data, annual bluegrass and creeping bentgrass could translocate methiozolin differently.

Researchers have utilized ^{14}C -labeled pesticides and metabolites to measure pesticide accumulation and activity of plant processes to propose modes of action (Brabham et al. 2014; Heim et al. 1990; Sabba and Vaughn 1999). Commonly, the incorporation of isotopic tracers in glucose molecules is necessary to determine whether or not the formation of the acid-insoluble fraction of the cell wall (cellulose and hemicellulose) is inhibited (Sabba and Vaughn 1999). Hemicellulose and cellulose form a complex of many different sugars, including xylose, arabinose, glucose and galactose, among others (Fang et al. 2000; Sun et al. 1995). Cellulose is composed of β (1 \rightarrow 4) linked D-glucose subunits (Carpita and Gibeau 1993). Hemicellulose is more intricate, composed of several different sugars, but with a high ratio of arabinose to xylose (Kozlova et al. 2014; Sun et al. 1995; Verbruggen et al. 1995). The mode of action of the CBI dichlobenil was elucidated in several studies using ^{14}C -labeled glucose. When acclimated and grown in 25 μM dichlobenil, tomato cells will incorporate 31 to 63 nmol ^{14}C -glucose whereas plants removed from 25 μM dichlobenil accumulate between 59 to 93 nmol ^{14}C -glucose, indicating an inhibition of sugar deposition into cell walls in the presence of dichlobenil (Shedletzky et al. 1992). Another well-known CBI, isoxaben, was also characterized (Heim et al. 1990). Researchers then compared dichlobenil to isoxaben and found that they similarly inhibit ^{14}C -glucose accumulation in cell walls, but that isoxaben is more active at lower concentrations (Heim et al. 1990). Indaziflam, a new CBI, was evaluated using ^{14}C -glucose feeding. Within one hour after administering ^{14}C -glucose, incorporation into the acid insoluble fraction of the cell wall was completely inhibited (Brabham et al. 2014).

Both ^{13}C and ^{14}C have been used in herbicide mode of action discovery, but ^{13}C offers several advantages (Bromand et al. 2001). Using ^{13}C isotopes facilitates quantifying ^{13}C enrichment in individual compounds as opposed to ^{14}C -labeling in a group of compounds mixed

within fractions (Allen et al. 2007; Bromand et al. 2001). In addition, positional information on ^{13}C enrichment (which carbons are labeled and to what degree) can sometimes be obtained with the use of MS- and/or NMR-based methods. By allowing the plant to take up ^{13}C -labeled CO_2 , Morvan-Bertrand et al. (1999) were able to show that approximately 91% of the carbon found in the basal portion of elongating leaves came from carbon reserves derived from older tissues. New tissues incorporated approximately 1% of ^{13}C CO_2 , which is difficult to separate from background. Greve et al. (1991) utilized ^{13}C -glucose to observe changes in tomato pericarp tissue during the ripening process. By tracing where ^{13}C incorporated in the tomato tissue, investigators determined that specific sugars were incorporated in a curvilinear fashion as fruit ripening increased. In terms of use in herbicide mode of action discovery, utilization of ^{13}C -glucose feeding has not been published in peer reviewed literature. The advantages of using ^{13}C -labeled compounds in probing metabolism offers new possibilities in this field.

Methiozolin is an important new herbicide for the selective control of annual bluegrass in many cool- and warm-season turfgrass species and the mode of action is currently disputed. We aimed to answer specific questions based on reports of previous literature regarding proposed CBI (Lee et al. 2007) and TAT inhibition (Grossmann et al. 2011) by methiozolin. Based on our experiences with annual bluegrass control by methiozolin (Askew and McNulty 2014), we expect annual bluegrass and other turfgrasses to exhibit reduced cell wall biosynthesis in response to methiozolin as has been reported for corn (Lee et al. 2007). If CBI activity by methiozolin is secondary to TAT inhibition, exogenous exposure to 4-HPP will prevent or reduce inhibitory effects of methiozolin on ^{13}C isotopic enrichment of key cell-wall-derived sugars in annual bluegrass. Interspecific responses between mature annual bluegrass and desirable turfgrasses do not occur in seedlings of these species or when the entire root system is treated

with methiozolin in aeroponics (Koo et al. 2013; Venner et al. 2012). As such, differential species responses in cell wall biosynthesis inhibition are not expected to be observed between annual bluegrass and desirable turfgrasses. If cell wall inhibition does differ between species, it would, however, implicate cell wall biosynthesis as an important mechanism behind annual bluegrass control with methiozolin. Our objective is to determine the effect of methiozolin at two rates; alone and in the presence of 4-HPP on ^{13}C incorporation from $[\text{U-}^{13}\text{C}_6]$ -glucose into the cell-wall-derived pentoses and hexoses. The resulting effects will be compared to the effects of indaziflam on ^{13}C isotopic enrichment of these sugars in cell walls in annual bluegrass and three turfgrass species.

MATERIALS AND METHODS

Experimental design was a randomized complete block with treatment arranged in a four-by-five factorial combination with four replications. Factors included turfgrass species (annual bluegrass, creeping bentgrass, Kentucky bluegrass and perennial ryegrass) and herbicide treatment (0, 0.01, 1.0 μM methiozolin (MRC-01; Moghu Research Center Ltd., BVC 311, KRIBB, Yuseong, Daejeon, 305-333, South Korea), 1.0 μM methiozolin + 10 μM 4-hydroxyphenylpyruvate (4-HPP; Sigma-Aldrich, Co., 3050 Spruce Street, St. Louis, MO 63103, USA) and 700 pM indaziflam (indaziflam; Sigma-Aldrich Chemie GmbH, Riedstr. 2 D-89555, Steinheim, 49 732 970, Germany)). Concentrations of methiozolin represent rate minima and maxima for annual bluegrass root inhibition based on preliminary studies (data not shown). The indaziflam rate was reported directly to the authors as the rate minima for annual bluegrass root inhibition (C Brabham, personal communication).

Seed of annual bluegrass, creeping bentgrass, Kentucky bluegrass and perennial ryegrass were sterilized for 1 minute in 190 proof ethanol (Ethanol 190 Proof; Decon Labs, Inc. 460 Glennie Circle, King of Prussia, PA 19406, USA) followed by soaking for 20 minutes in a 2% v/v sodium hypochlorite solution (Bleach; The Kroger Co. 1014 Vine St, Cincinnati, OH, 45202, USA) and germinated in sterile, 0.25x Hoagland's modified basal salt mixture (Hoagland's Modified Basal Salt Mixture; MP Biomedicals, LLC, 29525 Fountain Parkway, Solon, OH 44139) in solution with ultra-pure water (Barnstead Smart2Pure; Thermo Electron LED GmbH, Trobert-Bosch-Straße 1, D-63505 Langenselbold, Germany) (Zhang et al. 2001). Petri dishes were placed in a growth chamber set to constant 26°C with a day/night interval of 10/14 hours of 180 $\mu\text{mol m}^2 \text{s}^{-1}$ PAR via high intensity compact fluorescent lights (Sun Systems Tek light; Sunlight Supply, Inc., 5408 NE 88th St., Bldg. A, Vancouver, WA 98665, USA) to achieve optimal conditions for seed germination (Larsen and Andreasen 2004). Due to the fact that each species germinates at a different rate, seeds were sown daily over the course of several days allowing for selection of seedlings that were at the same growth stage. After growing in solution for approximately 7 days, several plants, separated by species, were aseptically transferred to petri dishes containing the same nutrient and previously mentioned concentrations of herbicides. Plants were then exposed to herbicide for 24 hours. Following treatment, plants were washed with sterile, ultra-pure water to remove any external residues and aseptically transferred to petri dishes containing a 10 μM , ^{13}C -glucose (D-Glucose, U-13C6; Cambridge Isotope Laboratories, Inc., 50 Frontage Road, Andover, MA 01810, USA) solution for an additional 72 hours to allow for ^{13}C -glucose incorporation into hexose and pentose fractions of actively dividing cells. At harvest, plants were washed with pure water to remove any external ^{13}C -glucose from leaf and root surfaces and placed in a -29 °C freezer until processing.

Methods utilized were modified from Allen et al. (2007). After harvest, frozen seedlings were lyophilized and placed with 3-3 mm glass beads in clean 1.5 mL centrifuge tubes. Tubes were shaken for 5-10 minutes until tissue was pulverized. Cell wall constituents were isolated with a biphasic extraction in chloroform and water. All liquid was extracted in such a way that the remaining pellet was not disturbed and the chloroform-water treatment was repeated a total of three times. Upon completion of the biphasic extraction, samples were allowed to air dry at 75°C such that no liquid was visible. Cell walls were hydrolyzed using 2N trifluoroacetic acid (Trifluoroacetic acid; Sigma-Aldrich, Co., 3050 Spruce Street, St. Louis, MO 63103, USA) and incubated for 2 hours at 120 °C (Encina et al. 2001; Pettolino et al. 2012). Samples were then dried under nitrogen gas and stored at -20 °C for further processing.

In order to transform the cell wall sugars into alditol acetates for analysis, equal parts acetic anhydride (Acetic-anhydride; Sigma-Aldrich, Co., 3050 Spruce Street, St. Louis, MO 63103, USA), pyridine (DriSolv Pyridine; VWR International, LLC., P.O. Box 6660, 100 Matsonford Road, Radnor, PA 19087, USA) and methylene chloride (GCSolv MeCl₂; Spectrum Chemical Manufacturing Group, 769 Jersey Avenue, New Brunswick, NJ 08901, USA) were added and samples were incubated for one hour at 75 °C. Reactions were terminated by adding cold water drop-wise into each tube. The samples were then back extracted with ultrapure water and 1 ul of the methylene chloride fraction was analyzed on a 7890A series gas chromatography machine (GC) equipped with a 30-m DB-23 column (0.25mm x 0.25 μM; Agilent Technologies, Santa Clara CA, 95051, USA) coupled to a 5975C series single quadrupole mass spectrometer (MS) (Agilent Technologies, Santa Clara, CA, 95051, USA). For each sample, 1 μL of derivatives was injected in a pulsed splitless mode where the injection port temperature was 250 °C. The GC oven was as follows: hold at 150 °C for 1 min, followed by a temperature increase

at a rate of 5 °C min⁻¹ to 175 °C, followed by another increase at a rate of 4 °C min⁻¹ to 240 °C and a final increase at a rate of 10 °C min⁻¹ to 250 °C. The MS temperature was set to 280 °C, with e-energy of 70 eV, with mass to charge ratio (m/z) range from 100 to 400 in a scan mode. The retention time of six sugars was as follows: rhamnose, 14.9 minutes, arabinose, 17.7 minutes, xylose, 19.7 minutes, mannose, 22.4 minutes, galactose, 23.1 minutes, and glucose, 23.8 minutes. Following initial testing, subsequent runs used m/z from 285 to 310. Data were collected and analyzed by using the Agilent Enhanced Mass Selective Detector ChemStation Software (Agilent Technologies, Santa Clara, CA, 95051, USA).

Natural abundance was removed from data by subtracting the % of natural abundance from each sugar from the recorded value of each sample. Natural abundance values were obtained from non-labeled samples (Hayes 2004). Integrals of ion data were used to calculate isotopic enrichments in extracted tissue as a ratio of ¹³C to ¹²C. Isotopic enrichment data were analyzed as initial ratios and also converted to percent change compared to the non-treated samples. These data were determined to be normal using the NORMAL option in PROC UNIVARIATE and Shapiro-Wilk statistic and homogeneity was assessed by visually inspecting plotted residuals. Data were subjected to ANOVA using PROC GLM with sums of squares partitioned to reflect the effect of cultural treatments and experimental locations, which were considered random. Mean squares associated with trial interactions were used to test for significance of treatment effects (McIntosh 1983). If significant trial interactions occurred, data were presented separately by trial, otherwise, data were pooled over trials. Mean responses were separated with Fisher's Protected LSD test at $P \leq 0.05$.

RESULTS AND DISCUSSION

To test whether there were differences in cell wall biosynthesis among experiments, results from all trials involving ^{13}C incorporation from $[\text{U-}^{13}\text{C}_6]$ -glucose into cell-wall-derived pentoses and hexoses were compared. Because trial interactions were not detected for ^{13}C labeling in any of the sugars in any plant species ($P > 0.05$), data from all trials were combined to investigate additional potential interactions between other variables. The interaction of between the herbicide and species was insignificant for ^{13}C labeling in arabinose ($p = 0.6109$), galactose ($p = 0.4875$), glucose ($p = 0.2550$), and xylose ($p = 0.7411$), but the main effect of species was significant for all species ($p = 0.0008$ to 0.0204) and data were pooled for presentation (Table 1). Although ^{13}C labeling in all six cell-wall-derived sugars was quantified, mannose and rhamnose data were not used due to coelution with other sugars (each pentose and hexose gives multiple peaks on chromatograms as alditol acetates and some peaks overlap). Because all these pentoses and hexoses originate from the same carbon precursor and only differ in the orientation of hydroxyl groups, they will have the same degree of ^{13}C labeling in their backbones. A representative pentose and hexose are sufficient to obtain non-redundant ^{13}C labeling information. Glucose is the only exception, as it is derived from both starch and cell walls and it was analyzed alongside another hexose (galactose). Data are reported for two pentose and two hexose sugars: xylose, arabinose, galactose and glucose. The lack of a species by treatment interaction suggests that differential inhibition of cell wall production does not explain interspecific differences in response to methiozolin by annual bluegrass and the three cool-season turfgrasses tested (Table 1). These results are in contrast to studies with other herbicides that indicate differential plant response to herbicide also leads to differential incorporation of ^{14}C glucose in cell walls (Koo et al. 1997; Sabba and Vaughn 1999). In the case of indaziflam, a

differential response in cell wall inhibition between the four species tested in these experiments would not necessarily be expected as indaziflam is not registered for use in cool-season turfgrasses due to injury concerns (Brosnan et al. 2012; Henry et al. 2012; Perry et al. 2011). In the case of methiozolin, annual bluegrass is considerably more sensitive than other turfgrass species when subjected to field or greenhouse applications to mature plants (Askew and McNulty 2014; Koo et al. 2013; Yu and McCullough 2014) but treatments to germinating turfgrass or root exposure in hydroponic or aeroponics systems tends to injure all species similarly (McCullough and Gomez de Barreda 2012; Koo et al. 2013; Venner et al. 2012). Flessner et al (2014) showed that methiozolin mobility in soil is highly limited. Since annual bluegrass roots are shallower than other turfgrasses, differential root exposure in field environments may contribute to interspecific differences in species response rather than physiological differences in cell wall inhibition (Bogart and Beard 1973; Murphy et al. 1994).

Although the trends in hexose and pentose sugar inhibition by herbicide treatments were consistent across species, the magnitude of ^{13}C isotopic enrichment varied by species (Table 1). Creeping bentgrass incorporated more ^{13}C into xylose than Kentucky bluegrass, perennial ryegrass and annual bluegrass (Table 1). Creeping bentgrass and Kentucky bluegrass incorporated the most ^{13}C into arabinose followed by perennial ryegrass and annual bluegrass. Kentucky bluegrass incorporated more ^{13}C into galactose than creeping bentgrass followed by perennial ryegrass and annual bluegrass. Creeping bentgrass incorporated the most ^{13}C into glucose followed by perennial ryegrass. Kentucky bluegrass and annual bluegrass incorporated similar amounts of ^{13}C into glucose. These data indicate that an effect on cell wall biosynthesis does not explain interspecific responses to methiozolin.

The main effect of herbicide was also significant ($P < 0.05$) for both isotopic enrichment and percentage change in isotopic enrichment compared to nontreated controls for all sugars except glucose (Table 2). When averaged over species, herbicide treatments inhibited ^{13}C isotopic enrichment of arabinose, galactose, and xylose between 16 and 32% relative to the untreated controls (Table 2). No treatment inhibited ^{13}C incorporation to glucose more than 9% (Table 2). Interpretation of glucose data is compounded by the inability of our method to completely eliminate starch. Thus, both starch and cellulose contributed to isotopic enrichment of glucose. The high levels of ^{13}C -enriched glucose, as shown in Table 1, were probably due to starch content in the samples and likely contributed to the inability to detect possible differences in ^{13}C -enriched cellulose. Methiozolin is not expected to inhibit ^{13}C enrichment of starch based on previous work with ^{14}C isotopic tracers (Lee et al. 2007). Methiozolin at 1.0 μM inhibited arabinose and xylose more than indaziflam at 0.7 nM or methiozolin at 0.01 μM . Previous research by Lee et al. (2007) demonstrated similar results in that methiozolin reduces incorporation of ^{14}C via ^{14}C -glucose into corn root tip cell wall hemicellulose by 55 and 48%, and cellulose by 70%. The increased levels of inhibition observed by Lee et al. (2007) are likely due to exclusive use of root tips for analysis compared to our use of entire seedlings in the current study. There could also be a difference between the response of corn observed by Lee et al. (2007) and the average response of annual bluegrass and three turfgrass species in this study. Addition of exogenous 4-HPP did not reduce methiozolin inhibition of arabinose ($p = 0.6109$), galactose ($p = 0.4875$), and glucose ($p = 0.2550$), but reduced incorporation of xylose ($p = 0.7411$) by 9% (Table 2). The predominant lack of effect that exogenous 4-HPP has on cell wall inhibition by methiozolin suggests that either exogenous 4-HPP is less biologically available in annual bluegrass than in lesser duckweed (Grossmann et al. 2011) or that possible TAT

inhibition in annual bluegrass does not drive the mechanism responsible for cell wall inhibition. Indaziflam also reduced incorporation of ^{13}C -glucose into all sugars investigated, supporting reports by others that classify it as a cell wall biosynthesis inhibitor (Brabham et al. 2014). Other CBI herbicides reduced incorporation of labeled glucose into cell walls, including cellulose and hemicellulose (Heim et al. 1990; Montezinos and Delmer 1980). Similar to results obtained by Lee et al. (2007), hemicellulose was decreased in plants treated with methiozolin. Verbruggen et al. (1995) noted that most of hemicelluloses extracted were composed of xylose and arabinose in sorghum, and in maize arabinose is the main component of glucuronoarabinoxylan, where it is attached to xylose residues. Grass cell walls are composed mainly of glucuronoarabinoxylan, where 20 to 40% makes up the weight of the primary cell wall, and 40 to 50% makes up the weight of the secondary cell wall (Scheller and Ulvskov 2010). The main function of hemicellulose in plants appears to be strengthening of the cell wall via anchorage of cellulose microfibrils (Scheller and Ulvskov 2010). When cellulose microfibril structure is altered, via mutation or other factors, plants become more brittle (Vogel 2008). Inhibition of cellulose production and deposition results in symptoms of known cell wall biosynthesis inhibitors, including clubbed or truncated roots, and reduced growth (Brabham et al. 2014; Sabba and Vaughn 1999).

These data support the assertion of others that methiozolin has an adverse effect on the synthesis or deposition of hemicellulose and cellulose in plant tissues. Although methiozolin appears to influence the formation of plant cell walls, our data did not aid in elucidating if cell wall inhibition is a primary mode of action for methiozolin. We can conclude that cell wall biosynthesis is inhibited in annual bluegrass and other turfgrasses, but differential cell wall inhibition is not a mechanism of methiozolin selectivity between the target weed, annual

bluegrass, and turfgrass species that have been shown to tolerate methiozolin. We cannot determine from our results if methiozolin inhibits annual bluegrass TAT but the minimal effect of exogenous 4-HPP on pentose and hexose sugar incorporation in cell walls of annual bluegrass and three turfgrasses suggest potential TAT inhibition does not drive methiozolin impacts on cell walls, though the differential methiozolin uptake efficiency among the tested species cannot be ruled out.

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Table 1. Response of four species on proportion of isotopic enrichment of ^{13}C -carbon in cell wall fractions averaged over herbicide treatments.

Turfgrass species	Xylose	Arabinose	Galactose	Glucose
	-----% isotopic enrichment ($^{13}\text{C}/^{12}\text{C}$)-----			
Creeping bentgrass	25 a ^a	26 a	34 b	51 a
Kentucky bluegrass	19 b	26 a	36 a	45 b
Perennial ryegrass	20 b	23 b	32 c	38 c
Annual bluegrass	19 b	21 c	30 d	44 b

^aMeans within a column with a different letter are statistically different based upon Fisher's Protected LSD at $p < 0.05$.

Table 2. Effect of herbicide treatment on percent reduction of ¹³C-carbon incorporation relative to control, averaged over turfgrass species.

Treatments	Xylose	Arabinose	Galactose	Glucose
	-----%-----			
Indaziflam (0.7 nM)	17 bc ^a	20 b	20 a	2 bc
Methiozolin (0.01 μM)	16 c	18 b	18 a	-2 c
Methiozolin (1.0 μM)	32 a	25 a	21 a	9 a
Methiozolin (1.0 μM) + 4-HPP (10 μM)	23 b	24 a	20 a	5 ab

^aMeans within a column with a different letter are statistically different based upon Fisher's

Protected LSD at p<0.05.

APPENDIX A. Supplemental Images.

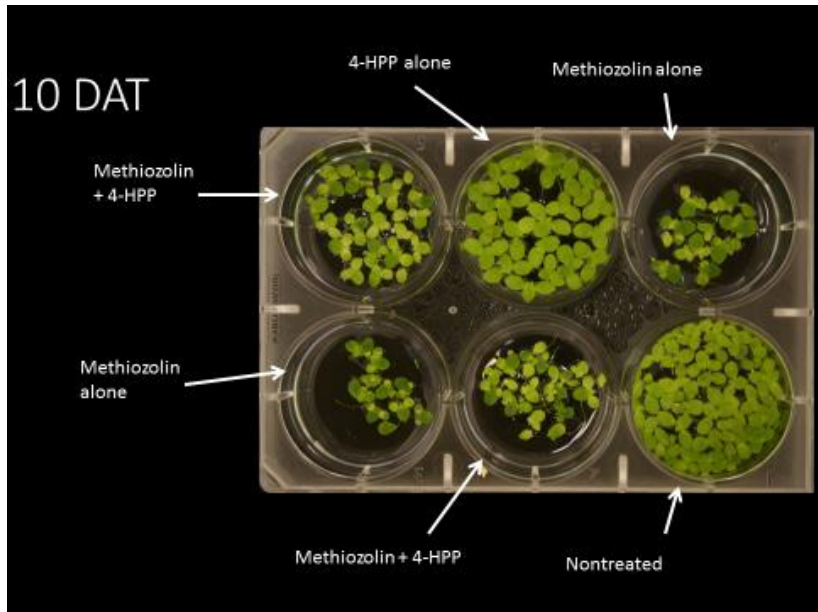


Image 1. Lesser duckweed as affected by methiozolin alone, methiozolin + 4-HPP and 4-HPP alone compared to the control at 10 DAT.

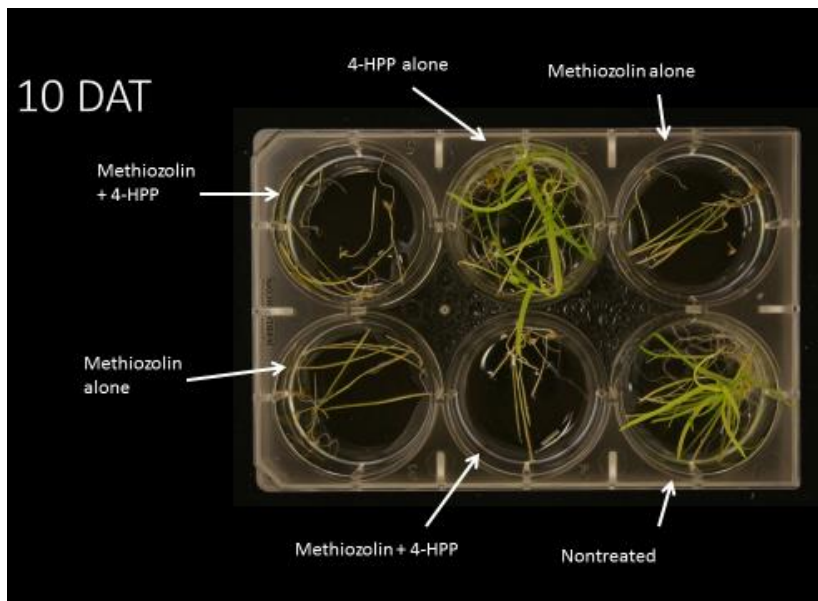


Image 2. Annual bluegrass as affected by methiozolin alone, methiozolin + 4-HPP and 4-HPP alone compared to the control at 10 DAT.

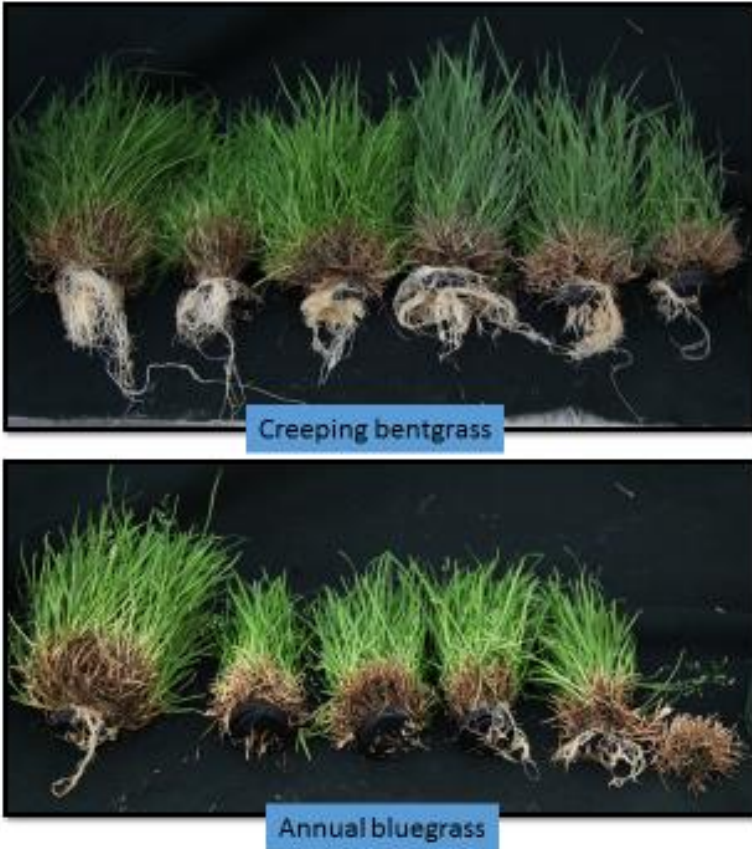


Image 3. Effects on creeping bentgrass and annual bluegrass rooting following extended exposure (2.5 months) to < 10 ppb methiozolin in an aeroponics system.

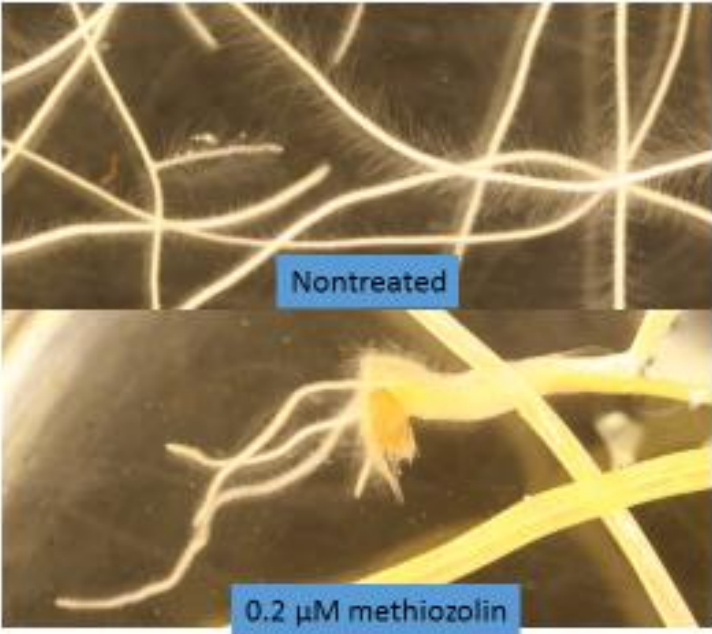


Image 4. Inhibition of rooting and reduction in root hairs of annual bluegrass following methiozolin application at 0.2 μM .