12-2014

Ecological, Physiological, and Molecular Characterization of Annual Bluegrass (Poa annua L.) Herbicide Resistance and Its Control on Golf Courses

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ECOLOGICAL, PHYSIOLOGICAL, AND MOLECULAR CHARACTERIZATION OF ANNUAL BLUEGRASS (*Poa annua* L.) HERBICIDE RESISTANCE AND ITS CONTROL ON GOLF COURSES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Plant and Environmental Science

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December 2014

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ABSTRACT

Annual bluegrass (*Poa annua* L.) is one of the most pervasive, adaptable, and variable plant species in the world and is the most problematic winter annual weed in managed turfgrass. Its prolific seedhead production regardless of mowing height, clumping growth habit, and lack of tolerance to stress reduces turfgrass aesthetic quality and playability on golf courses. Its ability to germinate almost year-round influences cultural practices and herbicide use.

Herbicides are an integral part of a weed control program for all weeds that invade a desirable turfgrass stand. However, inappropriate use of herbicides results in herbicide resistance issues. In recent years, the number of annual bluegrass populations on golf courses resistant to herbicides has increased. Currently, this weed is resistant to 9 mechanisms of action worldwide. Most recently, resistance has evolved to acetolactate synthase (ALS) inhibitors and glyphosate, both of which are integral herbicides for annual bluegrass control. Therefore, the purpose of this research was to investigate ecological, physiological, and molecular characteristics of resistance to these herbicides in order to increase the understanding of annual bluegrass herbicide resistance. Studies included diagnosing resistant biotypes, assays of enzyme activity, DNA sequencing, and simulation modeling. Beyond the issue of herbicide resistance, control remains difficult in all commercial turfgrass situations. Additional studies investigated annual bluegrass control in creeping bentgrass putting greens and bermudagrass fairways overseeded with

perennial ryegrass with current and experimental herbicides and plant growth regulators to determine best management practices for controlling annual bluegrass.

To diagnose resistance to ALS-inhibiting herbicides in biotypes from South Carolina and Georgia, dose-response experiments and ALS activity assays were conducted on mature annual bluegrass plants using trifloxysulfuron, foramsulfuron, and bispyribac-sodium. For dose-response experiments, I_{50} values for susceptible (S) biotypes were 13.6 g ai ha^{-1} for trifloxysulfuron, 7.0 g ai ha^{-1} for foramsulfuron, and 38.3 g ai ha^{-1} for bispyribac-sodium. Fifty percent shoot biomass reduction was not observed in either the South Carolina (CI) or Georgia (FP) biotypes at eight times the labeled field rate of all ALS-inhibiting herbicides tested. For *in vivo* tests of ALS activity, the CI biotype yielded I_{50} values 3650, 3290, and 13 times S biotypes following treatment with trifloxysulfuron, foramsulfuron, and bispyribac-sodium, respectively. Similarly, I_{50} values for the FP biotype were 316, 140 and 64 times greater than S biotypes following the same herbicide treatments. This confirms high levels of annual bluegrass resistance to multiple ALS-inhibiting herbicides in South Carolina and Georgia.

Further investigations into enzyme and growth characteristics of ALS-resistant annual bluegrass were conducted with a biotype from Alabama (GN) containing a mutation in the ALS gene resulting in a Trp_{574} to Leu amino acid substitution. Compared to the susceptible (VS) biotype, the GN biotype exhibited a 27- and 10-fold resistance to trifloxysulfuron at the whole plant level and under *in vitro* conditions, respectively. No significant differences were observed in K_m (pyruvate) or extractable ALS activity between biotypes, but the V_{max} was higher for the GN biotype. The feedback inhibition of

ALS activity by the branched-chain amino acids was higher for the GN biotype than the VS biotype, with leucine, valine, and isoleucine inhibiting ALS activity 20, 6, and 4% more in the R biotype, respectively. The GN biotype produced more inflorescences and seeds per plant in comparison with the VS biotype, but relative growth rates between biotypes were similar at all harvest intervals. This provides baseline information regarding ALS enzyme response, vegetative growth, and reproduction characteristics of annual bluegrass biotypes resistant and susceptible to ALS-inhibiting herbicides.

Glyphosate is used in the transition zone to control annual bluegrass in fully dormant warm-season grasses. A suspected resistant (CN) biotype of annual bluegrass was identified on a golf course in South Carolina after at least 10 consecutive years of glyphosate application. Resistance was confirmed after 4.4 times more glyphosate was required to reduce growth 50% compared to a standard susceptible (VS) biotype. Further studies were conducted to determine the mechanism conferring glyphosate resistance in the CN biotype. Leaf discs of both biotypes accumulated shikimate in response to increasing glyphosate concentration, but the I_{50} for EPSP synthase inhibition in the CN biotype was 3.5-fold higher than the S biotype. At the whole plant level, similar levels of shikimate accumulation were observed between biotypes at 6 and 24 hours after treatment (HAT) with glyphosate, but greater shikimate accumulation occurred in the VS biotype at 72, 120, and 168 HAT. Shikimate levels decreased in the CN biotype after 72 HAT. There were no differences in ^{14}C-glyphosate uptake between biotypes. However, more ^{14}C-glyphosate translocated out of the treated leaf in the CN biotype and into root tissues over time compared to the VS biotype. Partial sequencing of the EPSP synthase

gene revealed a heterozygous mutation at Pro_{106} which resulted in a substitution of Ala. These results represent the first documentation of a Pro_{106} to Ala substitution as the mechanism of glyphosate resistance in annual bluegrass and the first report of glyphosate-resistant annual bluegrass in South Carolina.

A basic simulation model was developed for the evaluation of herbicide resistance evolution in golf course populations of annual bluegrass, to understand key biological parameters of annual bluegrass which result in high resistance risks, to evaluate several annual bluegrass management programs for golf course fairways and their relative risks for selecting resistance, and to compare two herbicides (i.e., glyphosate and ALS-inhibitors) to determine how their respective characteristics influence resistance evolution in turfgrass systems. Annual bluegrass biological characteristics, typical turfgrass weed management strategies, and several genetic parameters were used in the simulations. Values and ranges for parameters were determined via review of the literature and field observations. In these simulations, the first population with evolved herbicide resistance was predicted after 5 and 9 yr of annual use of ALS-inhibitors and glyphosate, respectively. Several herbicide use strategies were subsequently simulated to assess their potential for managing resistance and included using alternate mechanisms of action in rotation or using these herbicides in combination with PRE or POST herbicides for early annual bluegrass control. The most effective use strategy for glyphosate was rotating mechanism of action. In comparison, the most effective resistance management strategy for ALS-inhibitors was applying a PRE or POST herbicide for early control followed by rotating ALS-inhibitors with an alternate mechanism of action in late winter. Regardless

of which strategy was used, >90% resistance risk was predicted for either herbicide after 40 years. Biological parameters including seed bank density, annual germination proportion, and seed removal had significant influence on resistance evolution. These simulations suggest annual bluegrass population dynamics contribute to its propensity to develop herbicide resistance and highlight the need for integrated control programs to manage resistance.

Field trials in creeping bentgrass putting greens revealed the best currently labeled annual bluegrass management options is multiple applications of paclobutrazol in fall and spring. This provided <60% annual bluegrass control after two years, but suppressed seedheads >70%. All methiozolin treatments provided >70% control after two years at two locations, but this herbicide is not currently registered for use. When it becomes registered, the best program in the transition zone will be multiple applications at low rates during the fall and spring. All other treatments failed to provide satisfactory control or seedhead suppression. In overseeded ryegrass, methiozolin and amicarbazone either did not consistently control annual bluegrass or damaged ryegrass beyond an acceptable threshold. Single herbicide applications in overseeded ryegrass will not provide acceptable control. The best program consists of a preemergence herbicide, an early postemergence application with a sulfonylurea herbicide, and a postemergence application with either ethofumesate or bispyribac-sodium.

In summary, annual bluegrass remains as the most problematic weed for managed turfgrasses. Herbicide resistance to many mechanisms of action increases the difficulty of control of this weed. There are only three labeled mechanisms of action for turfgrass

where annual bluegrass has not evolved resistance. Thus, turfgrass managers must use integrated programs to prevent resistance from developing. Where resistance to a single herbicide exists, it is of utmost importance to develop sound herbicide use practices such that multiple resistance will not evolve. Future research should continue to investigate herbicide resistant populations of annual bluegrass, management programs which are best suited to delay or prevent resistance, and new herbicides which provide alternative mechanisms of action for its control.

DEDICATION

I dedicate this work to the memory of Mr. and Mrs. Robert Billings Cross, Sr., and Mr. Henry Moore Young, and the honor of Mrs. Nancy Young Bartlett, all lovers of the game of golf.

ACKNOWLEDGMENTS

The highest acknowledgment and most sincere appreciation belongs to Dr. Bert McCarty, my major professor. It is an honor to work under someone whose reputation in the turfgrass management business is unsurpassed. I especially thank him for the opportunity to return to Clemson to pursue further education under his guidance. Working under him has given me the opportunity to further develop professionally by learning other aspects of the turf management industry, including research and teaching. I would also like to thank him for funding my assistantship and research.

I also greatly appreciate Dr. Ted Whitwell, Dr. Nishanth Tharayil, Dr. Billy Bridges, and Dr. Scott McElroy for serving on my graduate committee. Dr. Whitwell provided me with my first teaching assistant experience, and also some much needed personal advice. Dr. Tharayil was instrumental in the laboratory portion of this research by aiding the development of the herbicide resistance bioassays for annual bluegrass. He graciously allowed the use of his laboratory and many supplies for this work. Dr. Bridges' statistical advice and help with analyzing data was critical to the success of this project. Dr. McElroy gave of his time and resources at Auburn University, allowing me to spend several days with his staff to learn PCR teachniques and provided invaluable advice with his knowledge of annual bluegrass herbicide resistance.

I am grateful for the friendships I developed with my colleagues while working on this degree. I would especially like to thank Alan Estes for his expertise with field trials and advice throughout the duration of this project. In addition, thank you to Dr. Jeff

Atkinson, Gib Bethea, Nate Gambrell, and Brad Shaver for sharing the graduate school experience with me over the last few years.

I would also like to express my deepest thanks to my father and mother, Bob and Melissa Cross, and sister, Meredith, for their constant support and advice throughout this process. Their love throughout my life has shaped me into who I am today.

My greatest personal acknowledgement is for my wife, Meredith, for her support through the entire process of returning to Clemson University to continue my education. Her faithfulness and selflessness allowed me to pursue something that I love. And for my son, Davis, who made it a joy to come home everyday during my last few years of graduate school.

Finally, I would like to thank my Lord and Savior, Jesus Christ, whose grace and mercy is the ultimate reason that I was able to complete this work.

TABLE OF CONTENTS

Page

Table of Contents (continued)

Page

LIST OF TABLES

Table Page

List of Tables (continued)

Table Page

List of Tables (continued)

Table Page

List of Tables (continued)

Table Page

List of Tables (continued)

Table Page

LIST OF FIGURES

Figure Page

List of Figures (continued)

Figure Page

List of Figures (continued)

Figure Page

List of Figures (continued)

Figure Page

CHAPTER ONE

INTRODUCTION

Annual Bluegrass

Introduction. Annual bluegrass (*Poa annua* L.) is one of the most remarkable plant species on earth. Native to Europe, it is now widely distributed throughout the world, including being found in subtropical climates of Florida, near the equator at higher elevations, and is one of the few species of non-native higher plants to colonize Antarctica (Beard et al., 1978; Hemp, 2008; Molina-Montenegro et al., 2012). As a species, annual bluegrass is highly variable, displaying characteristics of both annual and perennial life cycles (Beard, 1970). This extreme variation has made annual bluegrass a model organism for ecological research (Mao and Huff, 2012).

In addition to its ecological importance, annual bluegrass has singlehandedly transformed weed management in commercial turfgrass. The ability to grow and thrive in stressful areas, produce abundant seed at all mowing heights, and germinate year-round under ideal conditions makes this weed the most problematic winter annual in managed turfgrasses (McCarty, 2011). Continual attention and strategic planning of cultural practices and herbicide applications are required of turfgrass managers for acceptable annual bluegrass management.

Annual bluegrass is generally considered to be a weed because of several characteristics it poses to highly maintained turf. First, it has a light green color and produces whitish seedheads that reduce the aesthetic quality of turf stands. Second, its growth habit and seedhead production produce uneven putting surfaces that affect ball

roll. Third, annual bluegrass is highly intolerant of environmental stresses such as drought, heat, and disease. During these stressful occurrences, it quickly dies out leaving voids in desirable turfgrasses that take time to heal (McCarty, 2011).

Before the 1950s, annual bluegrass was simply considered a component of many turf stands. However, through the years, expectations for sports turf and golf courses increased, and efforts in research and management have since been focused on its control (Gibeault, 1974). Unfortunately, the success of annual bluegrass as a weed has resulted in its evasion of most attempts to provide control thus far. For this reason, many turf managers in areas with climatic conditions suitable for annual bluegrass growth have chosen to maintain it as a desirable turf species (Bogart and Beard, 1972).

Annual bluegrass is identified by its clumpy growth habit and yellow-green color. It has smooth (hairless) alternate leaves with a folded sheath around the main axis. New leaves are folded (vernation) when emerging, and leaf tips are mainly pointed, but can be boat-shaped. There are often distinct lines on each side of the midrib when held to light. Ligules on annual bluegrass are membranous, pointed, and typically 0.8 to 3 mm long. Seedheads can be visible throughout its life cycle and appear as light green or white spikelets with dense/open flower clusters (McCarty et al., 2008; Vargas and Turgeon, 2004).

Biotype Variation and Genetics. Annual bluegrass biotypes are often separated into annual and perennial life cycles (Beard, 1970). The annual biotype (*Poa annua* var. *annua* L.) is characterized by bunch-type growth that is nonstoloniferous (noncreeping). This biotype behaves as a true winter annual and germinates in the fall, continues growth

through the winter, produces prolific seed in the spring, and dies shortly thereafter. When temperatures reach the low-30s ^{o}C for several consecutive days, plants quickly die (McCarty, 2011). Bogart (1972) reported discoloration (browning) of roots occurred at 27 ^{o}C or above, and beyond this temperature, plants reach maturity more rapidly. He also noted direct high temperature kill of individual plants could occur at temperatures as low as 40 ^{o}C. This rapid dieback as temperatures increase in the spring creates voids in desirable turfgrasses as these species are more heat tolerant than annual bluegrass.

The perennial biotype (*Poa annua* var. *reptans* L.) is more commonly found on closely mowed, highly fertilized, and frequently irrigated golf greens, and is economically important in the golf industry as a desirable putting surface (Huff, 2003; Johnson et al., 1993). It is characterized by a darker green color (compared to the annual biotype), a creeping (stoloniferous) prostrate growth habit, ability to root at nodes, seed with no dormancy factor, and seedhead production that is less than the annual biotype (Beard, 1970; Gibeault, 1974; McCarty et al., 2008). Beard (1970) also suggested the presence of stolons in the perennial biotype is important in the propagation and spread of the plant. Youngner (1959) demonstrated plants of the perennial biotype could exist for several years on golf greens.

Genetic variation in annual bluegrass is a major contributor to its wide adaptability and morphological differences, and its evolutionary origin can provide some explanation. It is characterized as an allotetraploid species, meaning it contains two copies of each chromosome from two different genomes (Vargas and Turgeon, 2004). The diploid parent species were first proposed by Nannfeldt (1937) as supina bluegrass

(*Poa supina* Schrad.) and weak bluegrass (*Poa infirma* Kunth). However, an ultimate conclusion from this research could not be made because Nannfeldt was unsuccessful at crossing these species (Vargas and Turgeon, 2004). When interspecific hybridization (crossing) of *P. supina* and *P. infirma* is successful, a sterile hybrid results. The resulting sterile hybrid is dihaploid, meaning it has two sets of nonhomologous chromosomes – seven from *P. supina* and seven from *P. infirma* (Vargas and Turgeon, 2004).

Tutin (1952) further supported Nannfeldt's hypothesis of the evolutionary origin of annual bluegrass. Annual bluegrass was crossed with *P. infirma* and the resulting progeny that was not sterile was triploid with 21 chromosomes. He concluded *P. infirma* was one of the annual bluegrass parents because one of the sets of chromosomes from annual bluegrass was homologous with a set from *P. infirma* (Mao and Huff, 2012; Tutin, 1952; Vargas and Turgeon, 2004). Later, Tutin (1957) proposed multiple hybridizations between *P. supina* and *P. infirma* contributed to its morphological variability with *P. supina* serving as the female parent.

Koshy (1968) suggested *P. supina* and *P. infirma* are not the parental species of annual bluegrass. Karyotypic analysis of annual bluegrass and its putative parental species revealed a lack of correspondence in chromosome structure. He concluded either one of *P. supina* or *P. infirma* was not a parent of annual bluegrass, or that chromosomal modification had occurred since the origin of the annual bluegrass species.

Recently, Mao and Huff (2012) provided further evidence through morphological and genetic studies that *P. supina* and *P. infirma* are the parental species of the allotetraploid annual bluegrass. They also concluded *P. infirma* and not *P. supina* is the

female parent of annual bluegrass, as originally indicated by Tutin (1957). *P. supina* is a cool-season (C_3) perennial species native to the cool, moist European Alpine region, while *P. infirma* is a cool-season annual species native to dry, Mediterranean climates in Europe (Mao and Huff, 2012). These genetic characteristics of annual bluegrass explain its continuum from annual to perennial subspecies, wide geographical distribution and morphological variability, and may provide some explanation of the inconsistencies in control of this troublesome weed.

Reproductive Characteristics. When day/night temperatures are consistently in the mid-20s/mid-10s ^{o}C for several consecutive days, annual bluegrass germination begins (McCarty, 2011). Full sun is optimal for maximum seed germination, therefore, turf stands that are weak/thin allow sunlight to penetrate the surface and tend to have the earliest germination. Wet and shady areas that tend to stay cooler may also experience early germination. Temperature is a very important factor in annual bluegrass seed germination. Bogart (1972) reported minimal differences in germination in the constant temperature range of 4 to 21 ^{o}C, but germination declined rapidly at 27 and 32 ^{o}C. However, research has demonstrated alternating temperatures are very significant in seed germination. For example, in mid-winter when days are warm and nights are cold, a flush of annual bluegrass germination can occur. Engel (1967) reported alternating day and night temperatures of 30 and 20 ^{o}C, respectively, resulted in higher annual bluegrass seed germination. Hovin (1957) also reported alternating high (21 ^{o}C) and low (10 ^{o}C) temperatures promoted seed germination. Beard et al. (1978) reported >60% germination at alternating low temperatures of 13 ^{o}C (day) and 2 ^{o}C (night).

After germination, annual bluegrass continues to grow (mostly vegetative) until late winter. At this point, it shifts to reproductive growth and begins forming numerous seedheads regardless of mowing height. Beard (1973) estimated annual bluegrass plants are capable of producing up to 360 seeds each when mowing heights are as low as 0.5 cm although higher seed production occurs at higher mowing heights. The annual biotype generates enormous amounts of seed, capable of producing up to 2,250 seeds in a season (Holm et al., 1997). Renney (1964) estimated the surface layer of soil could contain up to 7,500 seeds m^{-2} where the plant had been growing, and Watschke et al. (1979) estimated up to 185,000 seeds m^{-2} in an infested fairway.

Annual bluegrass has a panicle inflorescence that emerges from enclosing leaf sheaths made up of multiple spikelets with three to eight flowers. The number of flowers per inflorescence is dependent upon multiple factors, but mainly biotype genetics and plant density. In low density populations, flower production is typically higher than in high density populations. For example, Johnson et al. (1993) determined flower number per spikelet was higher (3.1 to 4.2) in low density populations than high density populations (2.2 to 3.5) based on six different biotypes studied. Annual bluegrass is known to flower throughout the growing season, but flowering in the southeastern United States typically occurs during the spring and early summer before plant death. Induction of flowering occurs by either low temperatures or photoperiod length, with low temperatures being more important for annual biotypes. However, annual biotypes flower sooner (often throughout the season) than perennial biotypes and have higher seed production (Johnson et al., 1993; Johnson and White, 1997; Law et al., 1977).

Annual bluegrass has both male and female flowers on the same plant, thus giving it the ability to self-pollinate or cross-pollinate a nearby plant (McCarty, 2011). Self-pollination is more common due to inflorescences being compact and close to the ground and pollen release occurring in early morning hours from 3:00 to 8:00 AM (Darmency and Gasquez, 1983; Johnson et al., 1993; Tutin, 1957). Koshy (1969) determined annual bluegrass is capable of producing viable seeds very soon after pollination and suggested the effectiveness of its reproductive system has been a contributing factor to its success as a weed. As few as 44 days and as many as 149 days are required before the plant forms seedheads from the time a seed germinates (McCarty, 2011). This is largely dependent upon biotype variability, where the annual biotype requires fewer days to flower. Gibeault (1970) estimated annual biotypes produced seedheads in 50 days compared to 81 days required for perennial biotypes. Beard (1970) reported seed of the annual biotype has a dormancy factor allowing it to stay viable in the soil for more than one year. Seed can remain viable in the soil for more than 6 years (McCarty, 2011; Roberts and Feast, 1973). This seed dormancy factor gives the plant the ability to escape stresses associated with its environment until conditions are optimal for its growth, thus contributing to its success as a weed (Beard, 1973).

Cultural Practices and Management. Visual evidence suggests annual bluegrass is capable of growing on highly compacted, poorly aerated soils in which creeping bentgrass (*Agrostis stolonifera* L.) and other desirable turf species cannot successfully compete. Under these conditions, annual bluegrass roots are shallow which allows the plant to tolerate a lack of oxygen (McCarty, 2011). This compact and poorly aerated

condition is commonly found on golf greens, tees, and fairways where traffic is an issue. The lack of tolerance of these conditions for desirable turfgrasses creates weak stands allowing the encroachment of annual bluegrass in these areas (Beard, 1970). In addition, annual bluegrass competes well on bentgrass greens, especially if perennial biotypes are present. The high input requirements of bentgrass greens (e.g., fungicides, fertilization, irrigation/syringing) allow annual bluegrass to survive through the summer months, especially with the increasing number of fans used to manage bentgrass in warmer climates.

High fertility encourages the growth and competition of annual bluegrass. Beard (1970) reported 9.8 to 49 kg N ha^{-1} per growing month is ideal for annual bluegrass, and multiple studies have found increasing nitrogen applications encourage annual bluegrass encroachment (Engel, 1974; Rieke and Bay, 1976). High phosphorus availability is also linked to an increase in annual bluegrass growth and density in bentgrass (Goss et al., 1975).

Mowing practices are important in attempting to control annual bluegrass populations. Clippings must be managed to reduce annual bluegrass spread and occurrence. With its prolific seed production, mowing can spread seed to non-infested areas if clippings are not collected (McCarty, 2011). Beard (1973) reported a stand of Kentucky bluegrass (*Poa pratensis* L.) with a mowing height of 2.5 cm contained 30% annual bluegrass contamination when clippings were not removed. He also determined the same turf stand and mowing height contained only 1% annual bluegrass contamination when clippings were removed. Although seed production can occur at all

mowing heights, the height of cut is also very important in annual bluegrass growth and occurrence. Annual bluegrass turf density has been found to increase with decreasing mowing height (Beard, 1973). Youngner (1959) reported densities were much higher in a lower mowed bermudagrass (*Cynodon* spp.) turf (1.3 cm) compared with a higher mowed turf (7.6 cm).

Annual Bluegrass Control

Annual bluegrass has historically been one of the most discussed and researched weeds in turfgrass science and management. For the first part of the past century, it was accepted as one of the grass species in turf stands. However, in the 1950s, expectations for highly maintained turf increased for sports fields and golf courses. Much attention in research has since been given to annual bluegrass control (Gibeault, 1974). Herbicides, plant growth regulators, biological controls, and sound cultural practices have all served as components of annual bluegrass control programs. Unfortunately, results have mostly been inconsistent at best, or at least only successfully control annual bluegrass for a short period of time. This may be in part due to genetic variability present in this species. As previously discussed, there are two main biotypes of annual bluegrass. However, within these ends of the spectrum, it is possible hundreds or even thousands of genetically different types of annual bluegrass exist. Some of these display the characteristics of the perennial biotype, and some more closely favor the annual biotype (Christians, 1996). It is not uncommon to see genetic variability among populations within certain areas on golf courses (e.g., tees, greens, fairways). Lush (1989) reported differences in most of the

traits measured between populations on golf greens and golf fairways and roughs. She also stated that due to this genetic variability, the different populations may have little to do with maintaining each other's existence. Distances as short as 3 m have been reported as separating genetically different annual bluegrass populations (Law et al., 1977; McNeilly, 1981; Warwick and Briggs, 1978). Therefore, this wide genetic variability provides some explanation of the history of inconsistent chemical control of annual bluegrass and the challenge for future control options.

Reports of attempts to control annual bluegrass date to the 1920s where mechanical means such as knives and cup cutters were used to remove annual bluegrass plants from putting greens (Alexander, 1922). This was prior to selective herbicide development, although the emergence of arsenates, the first selective herbicides for annual weeds, were soon discovered (Christians, 1996). Arsenate herbicides were among the most successful annual bluegrass controls ever developed largely due to their immobility and long life in the soil. Unfortunately, the arsenates were removed from the market in the 1970s when arsenic was found to be extremely toxic to humans (Christians, 1996; Freeborg and Daniel, 1974). Growth regulators emerged in the 1960s as a control strategy for annual bluegrass (Christians, 1996), and by the mid-1970s, bensulide, benefin, and DCPA had been introduced as possible preemergence options. Each had their benefits, but as more research was conducted, results became inconsistent (Turgeon, 1974). Problems such as short-term toxicity, injury to desirable turf species, and the need for multiple applications in a year were documented (Kaufman, 1972; Perkins, 1968). As all of these options did not provide complete control, some turf managers decided to

integrate and maintain annual bluegrass as a desirable turf species. With its extreme intolerance to stress, however, areas with climatic conditions not conducive to annual bluegrass growth could not use this as an option (Christians, 1996).

Numerous chemical control options exist for today's turfgrass managers. These are simply options as no single program or combination of herbicides has produced complete annual bluegrass control. Results will likely differ due to differences in climatic conditions from region to region as well as the high genetic diversity within the annual bluegrass species. Regardless, much effort in research continues with the goal of providing satisfactory control of this troublesome weed.

Herbicide Resistance

Herbicides are an integral part of a weed control program not only for annual bluegrass, but for all weeds invading desirable turfgrass stands. However, inappropriate use of herbicides can result in herbicide resistant weed species. The first case of documented herbicide resistance was that of common groundsel (*Senecio vulgaris* L.) to atrazine in 1968 (Ryan, 1970). Resistance has since been reported to 21 other modes of action in at least 433 plant species (Heap, 2014). Undoubtedly, the number of species and biotypes that are herbicide resistant will continue to rise with the large quantity of herbicide active ingredients used in today's agricultural systems.

Herbicide resistance is the inheritable ability of a plant to withstand a normally lethal herbicide dose and subsequently survive and reproduce (HRAC, 2014). This differs from herbicide tolerance where a plant naturally survives and reproduces following a

herbicide application (HRAC, 2014). For example, many crop species are naturally tolerant of acetolactate synthase (ALS) inhibitors due to rapid metabolism of herbicide active ingredients. This is not an inherited mechanism, but is inherent to the tolerant plant species. Therefore, genes conferring herbicide resistance must already be present in susceptible wild populations (Jasieniuk et al., 1996). This is likely the result of a previous gene mutation not related to the application of a herbicide. Typical mutation rates have been reported as 10^{-5} or 10^{-6} gametes per locus per generation for resistant gene inheritance (Gressel and Segel, 1982; Jasieniuk et al., 1996; Maxwell et al., 1990).

Herbicide applications confer herbicide resistance by applying selection pressure on a weed population. Individuals susceptible to the herbicide are removed giving the competitive advantage to resistant plants. These plants are able to survive and reproduce thereby increasing the percentage of resistant individuals over time. This increase will continue as long as the initial herbicide with the same mode of action is used or until the selection pressure is removed by a change in herbicide mode of action or completely abstaining from herbicide use.

To achieve acceptable annual bluegrass control, multiple herbicide applications are required each year contributing to the recent increase of resistant populations on golf courses. Resistance has been confirmed to 9 mechanisms of action worldwide including photosystem II inhibitors (e.g., simazine), photosystem I inhibitors (e.g., diquat), very long chain fatty acid inhibitors (e.g., ethofumesate), ALS inhibitors (e.g., trifloxysulfuron), 5-enolpyruvate shikimate-3-phosphate synthase (e.g., glyphosate), and

mitosis inhibitors (e.g., prodiamine) (Binkholder et al., 2011; Brosnan et al., 2012a; Cross et al., 2013; Heap, 2014; Isgrigg et al., 2001, Kelly et al., 1999, McElroy et al., 2013).

Herbicide resistant annual bluegrass populations will only make control of this troublesome weed more challenging as successful options are already limited. It is important for turf managers to implement integrated weed management programs when it comes to controlling annual bluegrass and not rely on a signle herbicide or group of herbicides with the same mode of action. Exclusive use of a single mechanism of action for several years is likely to result in herbicide resistant weed populations, minimizing the chemicals available for weed control. Integrated weed management programs involve careful selection of herbicides, but also includes using the best cultural practices of fertilization, water management, and mowing to limit populations.

Summary and Purpose

All of these characteristics make annual bluegrass one of the most enigmatic and elusive plant species in the world. Despite significant efforts, it has continually evaded all attempts for complete control in commercial turfgrass. This requires a continual effort on the part of research to evaluate issues posing a threat to its management in the future. Therefore, the purpose of this research was to evaluate the growing problem of annual bluegrass herbicide resistance on golf courses including characterization of suspected resistant biotypes, evaluation of enzyme functionality and growth characteristics, elucidating mechanisms of resistance, modeling the evolution of resistant populations, and determining alternative control options for resistant populations. An additional goal

of this research was to continue evaluating control options for annual bluegrass including new and experimental herbicides on creeping bentgrass putting greens and bermudagrass fairways overseeded with perennial ryegrass (*Lolium perenne* L.).

CHAPTER TWO

ANNUAL BLUEGRASS RESISTANCE TO ACETOLACTATE SYNTHASE-INHIBITING HERBICIDES

Introduction

ALS-Inhibiting Herbicides

Over 50 years ago, the role of the ALS enzyme (EC 2.2.1.6) in branched-chain amino acid (BCAA) biosynthesis was discovered (Umbarger and Brown, 1958). A great deal of research has since focused on this enzyme, especially with the discovery of the first ALS-inhibiting herbicides, the sulfonylureas, in the late 1970s (Duggleby et al., 2008). This class of herbicides played a major role in the advancement of herbicide technology because of their effectiveness at extremely low rates (Ray, 1984). In 1982, the first sulfonylurea (chlorsulfuron) was commercialized for use in cereal crops. Presently, ALS-inhibitors are widely used in numerous agronomic situations and make up the second largest class of herbicides (Cobb and Reade, 2010). The success and widespread use of these herbicides can be attributed to their high efficacy, metabolism-based selectivity, and environmental safety (low use rates and mammalian toxicity).

Branched-chain Amino Acid Biosynthesis

The BCAAs leucine, isoleucine, and valine contain branched aliphatic (non-aromatic) side chains. Plants, among other organisms, synthesize BCAAs, but animals do not. Thus, inhibition of enzymes involved in BCAA biosynthesis provides an excellent opportunity for herbicide use and development (McCourt and Duggleby, 2006). All three BCAAs are synthesized from pyruvate, but leucine requires acetyl-CoA, and isoleucine

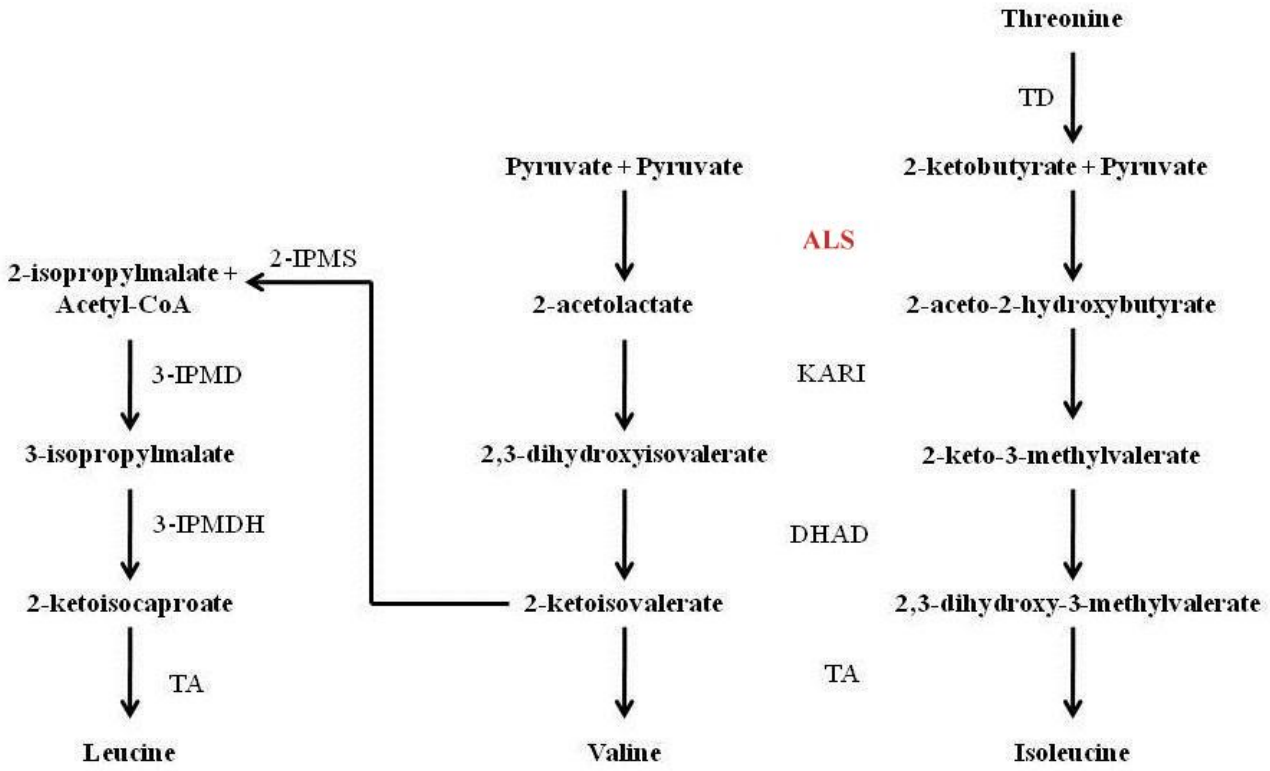


Figure 2.1. The biosynthetic pathway of branched-chain amino acids. The acetolactate synthase (ALS) enzyme, the active site of ALS-inhibiting herbicides, is shown in red. TD, threonine deaminase; KARI, ketol-acid reductoisomerase; DHAD, dihydroxyacid dehydratase; TA, transaminase; 2-IPMS, 2-isopropylmalate synthase; 3-IPMD, 3-isopropylmalate dehydratase; 3-IPMDH, 3-isopropylmalate dehydrogenase (McCourt and Duggleby, 2006).

requires 2-ketobutyrate (Duggleby et al., 2008). A simplified diagram of the BCAA biosynthetic pathway is presented in Figure 2.1.

In the isoleucine biosynthetic pathway, 2-ketobutyrate (2-oxobutyrate) is derived from the deamination of L-threonine via the enzyme threonine deaminase. ALS catalyzes the synthesis of 2-aceto-2-hydroxybutyrate, where one molecule of pyruvate is decarboxylated, yielding an acetaldehyde that reacts with 2-ketobutyrate, resulting in 2-

aceto-2-hydroxybutyrate (Duggleby et al., 2008). 2,3-dihydroxy-3-methylvalerate (the precursor to isoleucine) is converted from 2-aceto-2-hydroxybutyrate via ketol-acid reductoisomerase (KARI), and this step requires the presence of a magnesium ion (Dumas et al., 2001). Dihydroxyacid dehydratase (DHAD) catalyzes the conversion of 2,3-dihydroxy-3-methylvalerate to 2-keto-3-methylvalerate, which is converted to isoleucine by a transaminase (TA).

Leucine and valine are initially derived from two pyruvate molecules which are used in the synthesis of 2-acetolactate by the activity of ALS. KARI catalyzes the reaction of the conversion of 2-acetolactate to 2,3-dihydroxyisovalerate, and DHAD converts this intermediate to 2-ketoisovalerate. At this point in the pathway, either valine is synthesized by the action of a TA from 2-ketoisovalerate, or the biosynthetic pathway for leucine continues. 2-isopropylmalate synthase is the first enzyme that is unique to the leucine synthesis pathway. It catalyzes the conversion of 2-ketoisovalerate to 2-isopropylmalate. Acetyl-CoA is required in this reaction as an acetyl group is transferred to 2-ketoisovalerate. From 2-isopropylmalate, the intermediate 3-isopropylmalate is formed via the action of 3-isopropylmalate dehydratase, and subsequently 2-ketoisocaproate is formed via 3-isopropylmalate dehydrogenase. Again, a TA catalyzes the synthesis of leucine from 2-ketoisocaproate (Duggleby et al., 2008).

Therefore, four enzymes are common to the BCAA biosynthetic pathway: ALS, KARI, DHAD, and TA. Although ALS has received the most attention to date for herbicide activity and development, KARI and DHAD are also potential target enzymes. It is worth noting TA enzymes are likely not suitable for a target site for herbicide action.

These enzymes are found in animals, and multiple different TA enzymes are capable of catalyzing the final step in the synthesis of each of the BCAAs (Cobb and Reade, 2010).

Acetolactate Synthase

Acetolactate synthase is a member of a family of enzymes that are thiamine diphosphate (ThDP) dependent. Carbon dioxide is the product of a reaction between ThDP and pyruvate, and the intermediate hydroxyethyl-ThDP, is formed. This intermediate reacts with either 2-ketobutyrate or pyruvate and forms either 2-acetolactate or 2-aceto-2-hydroxybutyrate (depending upon which branch the pathway proceeds down). The regulation of the two branches of the pathway results from the competition for hydroxyethyl-ThDP between pyruvate and 2-ketobutyrate. It is known ALS has a higher affinity for 2-ketobutyrate than pyruvate, which compensates for the higher concentration of pyruvate within the cell and allows for similar fluxes down both branches of the BCAA biosynthetic pathway (Duggleby et al., 2008). All members of this enzyme family also require a divalent metal ion cofactor. In the case of ALS, the ion cofactor is typically Mg^{2+} (Umbarger and Brown, 1958), and this anchors ThDP to ALS. A third cofactor, a flavin adenine dinucleotide (FAD) molecule, is required by ALS, which is uncommon to other members of the ThDP-dependent enzyme family.

The ALS enzyme is composed of two subunits – a main ‘catalytic’ subunit and a secondary ‘regulatory’ subunit (Duggleby et al., 2008). The catalytic subunit activity is greatly stimulated by the secondary subunit, but its activity is not dependent upon this subunit. However, the secondary subunit is required for ALS inhibition. The three

cofactors previously discussed are required by the catalytic subunit, where ThDP is the main determinant of ALS activity.

The Chemistry and Mode of Action of ALS-Inhibiting Herbicides

There are currently five classes of ALS-inhibiting herbicides: sulfonylureas, imidazolinones, triazolopyriminidines, sulfonylaminocarbonyltriazolines, and pyrimidinyloxybenzoates (Cobb and Reade, 2010). Of the five classes, the sulfonylureas and imidazolinones are the most important, with the sulfonylureas being the largest group on an active ingredient basis.

Figure 2.2 shows the basic structure of a sulfonylurea herbicide molecule. Optimal herbicidal activity occurs when the sulfur atom attached to the aromatic ring has an *ortho*-substitution (R_1 position in Figure 2.2). A *meta*-di-substituted (R_3 and R_4 positions in Figure 2.2) triazine or pyrimidine ring is attached to the urea portion of a

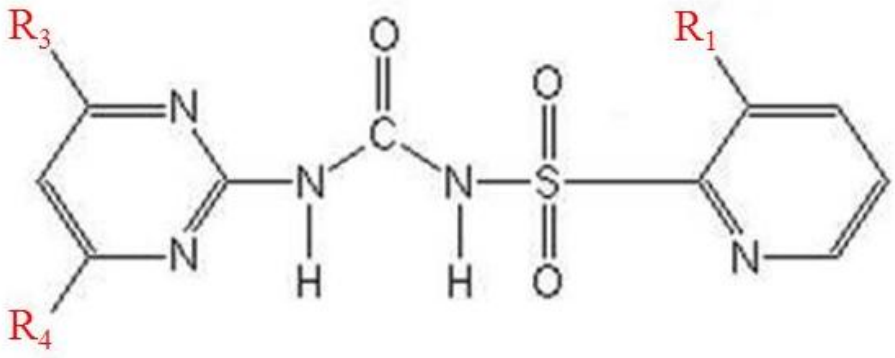


Figure 2.2. The basic chemical structure of sulfonylurea herbicides. High variability in the positions shown in red characterize the sulfonylurea herbicide class.

connecting sulfonylurea bridge. Greatest ALS inhibition by the sulfonylureas occurs when there are larger *ortho* substituents on the aromatic ring and the heterocyclic ring contains small, symmetrical *meta* substituents (Duggleby et al., 2008). The structures of sulfonylureas are highly variable with substitutions differing on both sides of the connecting bridge (Cobb and Reade, 2010; Duggleby et al., 2008; McCourt and Duggleby, 2006; Stidham, 1991). Figure 2.3 shows the chemical structure of two sulfonylurea herbicides commonly used in turfgrass.

The name of the ALS-inhibitors describes the mode of action of these herbicides – the inhibition of the ALS enzyme – which is the first common enzyme in the BCAA biosynthetic pathway (Umbarger, 1978). Inhibition of ALS after the application of herbicides can occur within several minutes (Duggleby et al., 2008). In plants, sulfonylureas bind to ALS on amino acid residues, and at least 16 residues are thought to be involved (Duggleby et al., 2008).

The mechanism of herbicide binding was elucidated by crystallizing the structure of yeast (*Saccharomyces cerevisiae*) and mouse-ear cress [*Arabadopsis thaliana* (L.) Heynh.] ALS with and without bound ALS-inhibiting herbicides (McCourt et al., 2006; Pang et al., 2002, 2003). The active site of ALS-inhibitors is a hydrophobic tunnel which blocks ThDP access subsequently inhibiting the binding of the substrate pyruvate. The heterocyclic ring of a sulfonylurea herbicide molecule is inserted into the tunnel while the aromatic ring projects toward the surface of the protein (Duggleby et al., 2008; McCourt et al., 2005). There are multiple amino acid side chains that interact and bind the herbicide molecule. Thus, mutations resulting in amino acid substitutions at many of

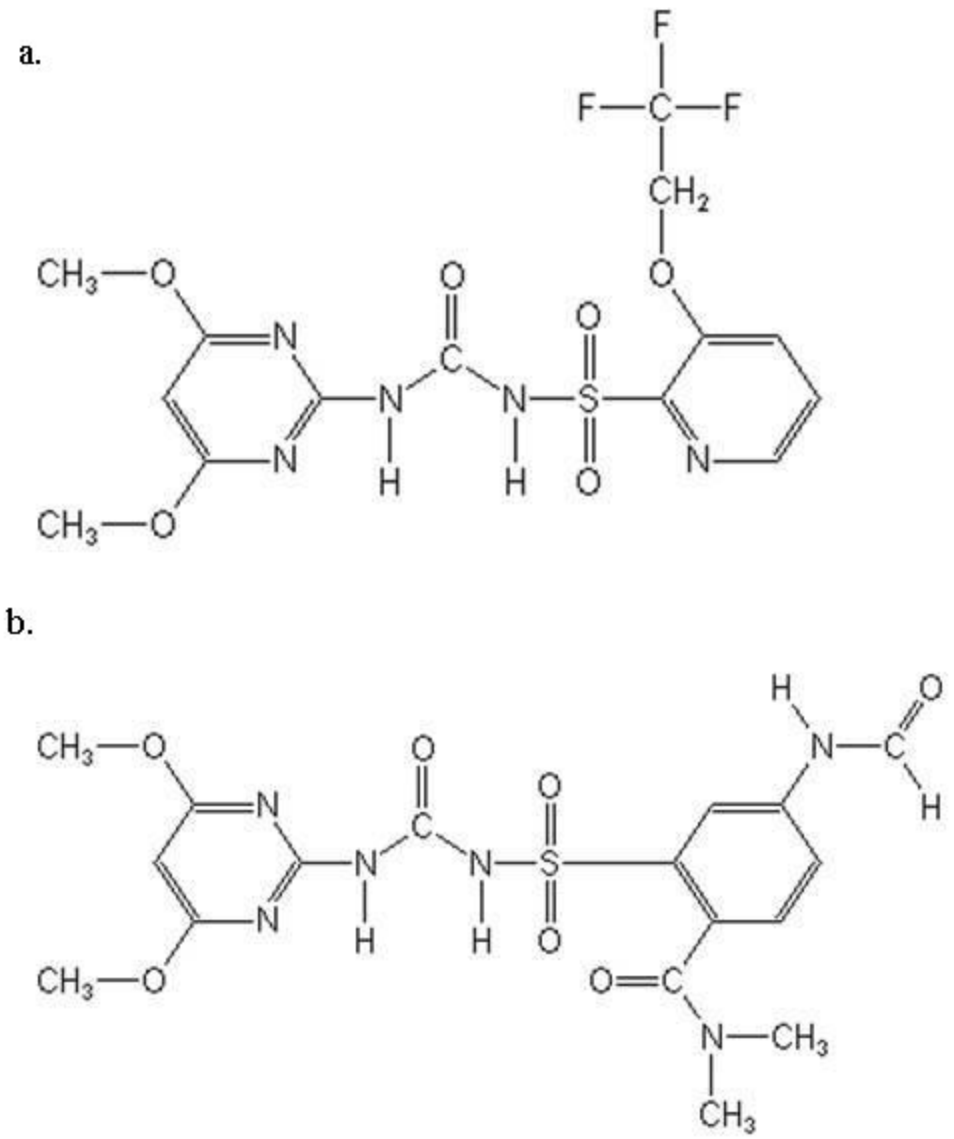


Figure 2.3. Chemical structure of two sulfonylurea herbicides commonly used in turfgrass weed management: (a) trifloxysulfuron, with an *ortho*-substitution on the aromatic ring, and (b) foramsulfuron, with an *ortho*- and *meta*-substitution on the aromatic ring. Both sulfonylurea molecules contain a *meta* di-substituted ring (two methoxy groups) on the pyrimidine ring on the opposite end of the sulfonylurea bridge.

these residues confer resistance to ALS-inhibitors (Duggleby et al., 2003). Upon binding of these herbicides, damage to the ThDP cofactor results, especially when the enzyme is

catalyzing a reaction. If enough undamaged ThDP is available to replenish the damaged cofactor, ALS activity will resume. It is also possible these herbicide molecules bind directly to hydroxyethyl-ThDP, the intermediate that reacts with pyruvate and/or 2-ketobutyrate, to inhibit ALS (LaRossa and Schloss, 1984). Since there is no resemblance in structure between ALS-inhibitors and any required cofactors or substrates of the enzyme, ALS is noncompetitively inhibited with respect to pyruvate (Pang et al., 2002). Sulfonylurea herbicides are very strong inhibitors of ALS and are much more potent (approximately 100 times) than the imidazolinones (Duggleby et al., 2008). The high activity of ALS inhibition by sulfonylureas can be attributed to the ability to make greater than 50 hydrophobic connections and hydrogen bonds with multiple amino acid residues on the ALS enzyme. There are 16 known amino acids that are directly involved in the binding of sulfonylureas, with the Trp_{574} residue being the most important in plants (Duggleby et al., 2008).

Resistance to ALS-Inhibiting Herbicides

Currently, 144 weed species worldwide are reported resistant to ALS-inhibitors, more than any other mechanism of action (Heap, 2014). The first ALS-inhibitor, chlorsulfuron, was introduced in the early 1980s. Five years later, the first case of ALS-inhibitor resistance was reported in two weed species – prickly lettuce (*Lactuca serriola* L.) (Mallory-Smith et al., 1990) and kochia [*Kochia scoparia* (L.) Schrad.] (Primiani et al., 1990). Subsequent evolution of other resistant species occurred at an exponential rate,

and by 1998, more weed species were resistant to ALS-inhibitors than all other mechanisms of action (Heap, 2014; Tranel and Wright, 2002).

The propensity of this mechanism of action to evolve herbicide resistance is attributed to several characteristics. These herbicides are highly efficacious on susceptible individuals providing strong selection of resistant alleles, have a high specificity of target site, and relatively long soil residual. The frequency of ALS-inhibitor resistant genes present in the wild is unknown, but it is likely their frequency is 10^{-6} or less (Haughn and Somerville, 1987; Saari et al., 1994). Initial frequency depends upon the ploidy level, gene dominance, and the number of genes involved (Gressel and Segel, 1982). The resistant ALS alleles are dominant over susceptible alleles adding to rapid development and high occurrence of resistance cases. The gene encoding for ALS is a nuclear gene, and thus, follows a normal Mendelian inheritance pattern. Therefore, the resistant gene can be transferred in both pollen and seed (McCourt and Duggleby, 2006; Tranel and Wright, 2002).

Plant fitness is often defined as the ability to survive and produce viable offspring (Silvertown, 1987). It has been accepted the adaptation to a new environment will result in some type of fitness cost for the adapting organism (Purrington, 2000; Strauss et al., 2002). In many, but not all resistance cases, some type of fitness cost has been observed in resistant individuals (Vila-Aiub et al., 2009). Research indicates ALS-resistant crop species do not suffer fitness costs (Saari et al., 1994). For example, no negative effects could be attributed to the changes in sensitivity of the ALS enzyme in resistant flax (*Linum usitatissimum* L.), canola (*Brassica napus* L.), corn (*Zea mays* L.), and tobacco

(*Nicotiana tabacum* L.). Parameters comparing susceptible and resistant varieties included, among others, yield, disease tolerance, and growth properties, and these parameters were similar between varieites (McHughen and Holm, 1991; Newhouse et al., 1991; Swanson et al., 1989). In addition, numerous studies have compared traits of sulfonylurea resistant and susceptible weed biotypes to understand what, if any, fitness costs are associated with resistance. Alcocer-Ruthling et al. (1992a, 1992b) and Mallory-Smith (1992) demonstrated seed germination of resistant biotypes of prickly lettuce germinated faster than susceptible biotypes in all cases. Similar results were observed with kochia but only at cooler temperatures (Dyer et al., 1993). Research with prickly lettuce has also shown biomass production and growth rates are higher in susceptible biotypes when compared to resistant biotypes, but the competitive abilities of both biotypes were nearly equal (Alcocer-Ruthling et al., 1992a, 1992b). Studies of seed production and seed viability in resistant versus susceptible biotypes of prickly lettuce and kochia concluded seed output and longevity were nearly equal between the biotypes (Alcocer-Ruthling et al., 1992a, 1992b; Mallory-Smith et al., 1992; Thompson and Thill, 1992). Based on these reports, it is possible, but not certain, there is some fitness cost associated with weed resistance to ALS-inhibitors.

Mechanisms of ALS-Inhibitor Resistance

Herbicide molecules have specific active sites, typically enzymes, which are targeted to disrupt some type of process essential to normal plant function. If these target sites undergo some type of structural change (e.g., amino acid substitution), herbicides

may no longer be useful in disrupting their function (Cobb and Reade, 2010). In most reported cases of ALS-inhibitor resistant weeds, mutations in the ALS gene subsequently creating an altered amino acid sequence on the protein account for resistance (Tranel and Wright, 2002). Substitutions at most amino acid residues that bind ALS-inhibitors have been linked to resistance (Duggleby et al., 2008). Some variability exists in the ALS enzyme between species and even within species, but of the known ALS amino acid sequences, most of the amino acids (substitutions) involved in resistance have been found at the same positions. One specific substitution, Trp_{574}, can result in resistance to numerous ALS-inhibitor classes, but especially the sulfonylureas, because of the strong binding interaction between this residue and the heterocyclic ring of sulfonylurea molecules. This substitution would also alter the structure of the enzyme reducing the fit of the herbicide molecule into the ThDP access channel (Duggleby et al., 2008). The heterocyclic ring of sulfonylurea molecules makes contact with Gly_{121}, Met_{124}, Met_{570}, and Val_{571}, and mutations in any of these residues would result in resistance to sulfonylurea herbicides. Mutations at Ala_{122}, Pro_{197}, Ala_{205}, or Asp_{376} also confer resistance to sulfonylurea herbicides because these amino acids either bind the aromatic ring or interact with the *ortho* substituent (Cobb and Reade, 2010; Duggleby et al., 2008; Tranel and Wright, 2002). Interestingly, other mutations on the ALS enzyme have been reported to confer ALS resistance that are not known to be involved in sulfonylurea herbicide binding. For example, His_{352}, Asp_{375}, and Phe_{578} confer resistance to sulfonylurea herbicides. Research which crystallized five bound sulfonylurea herbicides to mouse-ear cress or yeast ALS revealed none of these three mutations are involved in

binding sulfonylurea herbicides (Duggleby et al., 2008; Pang et al., 2002, 2003). On the contrary, Ser_{653} is involved in binding sulfonylureas, but mutations at this position more commonly confer resistance to the imidazolinones (Duggleby et al., 2008).

Enhanced herbicide metabolism and detoxification is often a mechanism attributed to herbicide resistance, although this is likely not important in weed species resistant to ALS-inhibitors. In contrast, the most important mechanism in natural tolerance to ALS-inhibitors is the rapid metabolism of the herbicide resulting in their high selectivity (Saari et al., 1994; Tranel and Wright, 2002). In most cases, the active ingredient is detoxified to other compounds that are not harmful to plant processes, preventing the herbicide molecule from reaching the active site. The cytochrome P_{450} monooxygenase family of enzymes are important in the selectivity of many herbicide active ingredients and probably play an important role in sulfonylurea detoxification. The most common reaction for detoxification is the hydroxylation of the aryl or aliphatic groups on the ALS-inhibiting herbicide molecules (Brown, 1990; Cobb and Reade, 2010; Saari et al., 1994).

Annual Bluegrass Control with ALS-Inhibiting Herbicides

ALS-inhibitors (specifically the sulfonylureas) have steadily increased in popularity for turfgrass weed management over the past 15 years. These are often used to remove cool-season overseeding species (e.g., perennial ryegrass) from bermudagrass to speed spring transition or for weed control in desirable turf species. However, sulfonylureas must be used with caution because of variation within this herbicide class.

For example, cool-season grasses are tolerant to halosulfuron, but severely damaged by foramsulfuron and trifloxysulfuron (Murphy et al., 2004).

Many of the sulfonylureas are effective for controlling annual bluegrass. Their short soil residual relative to preemergence herbicides allows them to be used for early postemergence control of annual bluegrass before overseeding bermudagrass (McElroy et al., 2011). Sulfonylureas cannot be used for postemergence control of annual bluegrass in established cool-season grasses, however, are an important tool for control in non-overseeded bermudagrass. Toler et al. (2007) reported >95% control with four sulfonylurea herbicides applied in February.

The effectiveness of sulfonylureas for annual bluegrass control and their selectivity in warm-season grasses has resulted in exclusive use of this mechanism of action for annual bluegrass control in many cases. This has quickly selected for resistant individuals and the failure of these herbicides for annual bluegrass control, especially in the southeastern United States. Therefore, the purpose of these studies was to detect resistance to ALS-inhibiting herbicides of several annual bluegrass biotypes obtained from South Carolina and Georgia, to investigate *in vitro* responses of ALS in a resistant biotype of annual bluegrass with two distinct ALS gene copies, to compare vegetative and reproductive growth characteristics of susceptible and resistant biotypes for initial indications of how herbicide resistance may influence these parameters, and to investigate alternate control options for turfgrass managers where resistant biotypes are present.

Materials and Methods

Detecting Annual Bluegrass Resistance to ALS-Inhibitors

Plant Material. Plants of suspected resistant annual bluegrass biotypes were collected from 'Tifway' bermudagrass golf course fairways at Calawassie Island Golf Club (CI; Okatie, SC) and the Ford Plantation (FP; Richmond Hill, GA) in 2010 using a golf course cup cutter. These areas had been treated with ALS-inhibiting herbicides for at least eight consecutive years (B. Bagwell, N. Caron, personal communication). Additional annual bluegrass plants were obtained from a site where no known previous herbicide applications had been made (CU; Clemson, SC) and seeds were obtained from a commercial source (VS; Valley Seed Service, Fresno, CA). These biotypes were susceptible to ALS-inhibitors and included for comparison. Plants were potted in a commercial potting mix (Fafard 3B Mix, Conrad Fafard Inc., Agawam, MA) and grown to seed in an isolated greenhouse to prevent cross-pollination. Mature seed was harvested from all populations and stored in a -20 ^{o}C freezer until further tests were conducted.

Whole Plant Dose Response. Annual bluegrass seeds were sown in 3.8 cm x 21 cm polypropylene containers filled with a mixture of sand and peat (85:15 by volume). Plants were grown under greenhouse conditions to tillering stage (>3 tillers per plant) and treated with three ALS-inhibiting herbicides: trifloxysulfuron (Monument® Herbicide, Syngenta Crop Protection Inc., Greensboro, NC) or foramsulfuron (Revolver® Herbicide, Bayer CropScience, Research Triangle Park, NC) at 0, 3.5, 7, 14, 28, 56, 112 and 224 g ai ha^{-1} or bispyribac-sodium (Velocity® Herbicide, Valent U.S.A. Corporation, Walnut Creek, CA) at 0, 9.3, 18.5, 37, 74, 148, 296, and 592 g ai ha^{-1}. Labeled rates for

trifloxysulfuron and foramsulfuron are 28 g ai ha^{-1} compared to 74 g ai ha^{-1} for bispyribac-sodium (Anonymous, 2007, 2009, 2010). Each treatment included a nonionic surfactant (Induce®, Helena Chemical Company, Collierville, TN) at 0.25% v v^{-1}. Each container consisted of a single annual bluegrass plant. Herbicides were applied using an enclosed spray chamber (DeVries Manufacturing, Hollandale, MN) calibrated to deliver 374 L ha^{-1} through an 8001E flat fan nozzle (Tee Jet Spraying Systems Co., Wheaton, IL).

Following herbicide application, plants were maintained in a greenhouse under an approximately 14 h photoperiod of natural light (average intensity 700 µmol m^{-2} s^{-1}) with day/night temperatures maintained near 29/18 ^{o}C. Twenty-one days after treatment (DAT), surviving plants were removed from containers and washed free of soil. Above- and below-ground biomass was separated, dried at 80 ^{o}C for 72 hours, and weighed.

***In vivo* ALS Activity Assay**. Annual bluegrass seeds were sown in a commercial potting mix and grown to maturity (>10 tillers) in a greenhouse growth room with a 12 h photoperiod under 400 µmol m^{-2} s^{-1} and day/night temperatures of 24/15 ^{o}C. Plants were watered every 48 to 72 h to prevent moisture stress and fertilized monthly with 4.9 g N m^{-2}.

Determination of ALS activity was conducted according to procedures of Gerwick et al. (1993) with some modifications. Tissue from the apical portion of annual bluegrass leaves (300 mg) was removed from a plant, weighed, and transferred to a 100 mL petri plate for incubation (Gerwick et al., 1993). The incubation solution consisted of 5 mL of 25% Murashige and Skoog salt media, 500 µM 1,1-cyclopropanedicarboxylic

acid (CPCA), 50 mM L-alanine, and 0.1% v v^{-1} nonionic surfactant (Triton X-100) (all materials obtained from Sigma Aldrich, St. Louis, MO). Trifloxysulfuron, foramsulfuron, or bispyribac-sodium (commercial formulations) were added to the incubation solution at 0, 0.001, 0.01, 0.1, 1, 10, 100, and 1000 µM concentrations. An incubation solution containing neither CPCA nor ALS-inhibiting herbicides was used as the reference absorbance. To quantify ALS activity in relation to herbicides, the nontreated control was a solution which contained CPCA but no ALS-inhibiting herbicide. Incubations were conducted in a growth chamber for 16 to 20 hours under 350 µmol m^{-2} s^{-1} of constant light and ambient atmospheric conditions (Uchino et al., 1999). After removal from the growth chamber, plates were wrapped with Parafilm (Bemis Company, Inc., Neenah, WI) and stored in a freezer at -80 ^{o}C until analyzed.

For analysis, contents of plates were transferred to 15 mL culture tubes and acidified using H_2SO_4 to a final concentration of 0.5% (25 µL). Tubes were placed in a water bath and heated to 60 ^{o}C for 30 minutes. Acetoin was quantified according to the procedure described by Westerfeld (1945) with modifications (Gerwick et al., 1993; Kuk et al., 2003). A 2 mL aliquot was taken from each sample and transferred into a clean culture tube. A reagent consisting of 1-naphthol and creatine monohydrate dissolved in 2.5 N NaOH was added to each sample for a final concentration of 20 mg mL^{-1} 1-naphthol and 2 mg mL^{-1} creatine monohydrate. Tubes were heated to 37 ^{o}C in a water bath for 30 minutes for color development. Samples were measured in a spectrophotometer at 530 nm, and a standard curve was produced to quantify acetoin. A high concentration of acetoin results in a red/pink color upon addition of the reagent to

the solution. Thus, solutions with a high absorbance (red/pink color) in the presence of ALS-inhibiting herbicides indicate a resistant annual bluegrass biotype.

Statistical Design and Analysis. The experimental design for both whole plant dose-response and *in vivo* studies was completely randomized with three replications. Treatments consisted of the factorial combination of three ALS-inhibiting herbicides (trifloxysulfuron, foramsulfuron, and bispyribac-sodium), four annual bluegrass biotypes (CU, VS, CI, FP), and eight herbicide rates or concentrations. Two replications of the whole plant dose-response and ALS activity assay were conducted with data from each subjected to analysis of variance (ANOVA).

Nonlinear regression analysis was used in the dose-response component of each study to estimate GR_{50} or I_{50} values (as a percentage of nontreated control). The formula used was

$$y = C + (D\text{-}C)/(1+10^{x\text{-}\log I_{50}}) \quad [2.1]$$

proposed by Seefeldt et al. (1995) where, for the dose-response study, y was shoot biomass, x was herbicide rate (g ai ha^{-1}), D was the upper bound of y, C was the lower bound of y, and GR_{50} was the rate of herbicide which reduced shoot biomass 50%. For the *in vivo* ALS activity assay, y was ALS activity, x was the herbicide concentration (µM), D was the upper bound of y, C was the lower bound of y, and I_{50} was the concentration of herbicide which reduced ALS activity 50%. When ANOVA suggested a significant effect of biotype, GR_{50}/I_{50} comparisons between susceptible and resistant annual bluegrass biotypes were conducted using Student's *t*-test at $P < 0.05$. All ANOVA

and *t*-test calculations used JMP version 9.0 (SAS Institute Inc., Cary, NC) and nonlinear response modeling used Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

Comparison of Enzyme and Growth Characteristics in ALS-Inhibitor Susceptible and Resistant Annual Bluegrass Biotypes

Plant Material. Research was conducted with a known resistant (GN) annual bluegrass biotype containing a Trp_{574} to Leu amino acid substitution obtained from Grand National Golf Course in Opelika, AL (McElroy et al., 2013). The VS biotype described previously was used as a susceptible comparison. This biotype was used in previous research studies with ALS-inhibiting herbicides and is a true annual biotype selected for seed production (McElroy et al., 2002). Annual bluegrass seed was stored at -20 ^{o}C until sowing.

Whole Plant Dose-Response. Annual bluegrass seeds from both biotypes were sown in polypropylene containers containing a commercial potting mix and maintained in a greenhouse with average day/night temperatures of 21/15 ^{o}C under natural light (average intensity of 700 µmol m^{-2} s^{-1}). After germination, plants were watered every 48 to 72 h to prevent moisture stress. At 2-3 tiller stage, annual bluegrass plants were treated with trifloxysulfuron (commercial formulation) containing 0.25% v v^{-1} nonionic surfactant. Trifloxysulfuron rates were 3.5 to 224 g ai ha^{-1} and 28 to 1,792 g ai ha^{-1} for VS and GN biotypes, respectively, including a nontreated control. Previous research indicated GR_{50} (trifloxysulfuron dose reducing shoot dry weight 50%) values >224 g trifloxysulfuron ha^{-1} for two ALS-resistant biotypes (Cross et al., 2013). For this reason, higher rates were applied to GN plants to obtain the lower asymptote and accurately define the GR_{50}.

Herbicide application was conducted in an enclosed spray chamber calibrated to deliver 374 L ha^{-1} through an 8001E flat fan nozzle.

Following trifloxysulfuron application, plants were immediately returned to the greenhouse and maintained under the same growth conditions as previously described. Twenty-one DAT, above-ground biomass was harvested, dried at 80 ^{o}C for 72 h, and weighed.

***In vitro* ALS Assays**. To provide sufficient biomass for assays, 20 annual bluegrass seeds of each biotype were sown in pots as previously described. Plants were maintained in a growth chamber with day/night temperatures of 18/10 ^{o}C and a 10 h photoperiod (500 µmol m^{-2} s^{-1}). *In vitro* assays were conducted according to methods of Yu et al. (2010) with minor modifications. At 35 to 42 days after germination (DAG), all above-ground tissue from one pot (20 plants) was harvested, cut into small sections, and thoroughly mixed. Five to six g fresh tissue was immediately frozen in liquid nitrogen and ground to a fine powder. Ground tissue was added to 30 mL extraction buffer (pH 7.5) containing 0.1 M K_2HPO_4, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 10 µM FAD, 10 mM sodium pyruvate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10% v v^{-1} glycerol, and 0.5% w v^{-1} polyvinylpyrrolidone. The homogenate was thoroughly mixed and kept on ice for 10 min, filtered through four layers of cheesecloth, and centrifuged at 27,000 *g* for 15 min. Seven mL 100% (5.8 M) ammonium sulfate solution was added to 7 mL supernatant and centrifuged at 27,000 *g* for 20 min to precipitate protein. The supernatant was poured off and remaining protein was dissolved in a reaction buffer (pH 7.5) containing 50 mM HEPES, 200 mM sodium pyruvate, 20 mM

$MgCl_2$, 2 mM TPP, and 20 µM FAD. All steps after tissue grinding were conducted at 4 ^oC.

Reactions were started immediately with addition of either 100 µL herbicide, amino acid, or sodium pyruvate solutions to 100 µL protein and incubated at 37 ^oC for 1 h. To stop the reaction, 40 µL 6 N H_2SO_4 was added and samples were incubated at 60 ^oC for 15 min to convert acetolactate formed during the reaction to acetoin. For color development, 190 µL each of 0.55% creatine and 5.5% 1-naphthol were added to samples with an additional incubation at 60 ^oC for 15 min. Activity of ALS was determined by measuring absorbance at 530 nm and acetoin concentrations were determined by developing a standard curve with acetoin. To determine potential aceotin formation via other enzymes, 40 µL 4 N NaOH was added to stop reactions instead of H_2SO_4 (Forlani et al., 1999; Muhitch, 1988; Pornprom et al., 2005; Tanaka, 2003; Yu et al., 2010). Background absorbance was determined by adding H_2SO_4 or NaOH to the reaction prior to addition of protein. Protein was quantified using the Bradford assay with bovine serum albumin standard.

In vitro assays were conducted to determine ALS response to trifloxysulfuron, K_m (substrate concentration at one-half maximum rate of enzyme activity), V_{max} (maximum rate of enzyme activity), extractable ALS activity, and feedback inhibition by BCAAs. To determine the response of ALS to trifloxysulfuron, technical grade herbicide material was dissolved in 20% acetone such that acetone concentration was not greater than 0.4% v v^{-1} in the reaction mixture. Trifloxysulfuron concentrations of 0, 0.001, 0.01, 0.1, 1, 10, 100 and 1,000 µM were used. For evaluation of ALS feedback inhibition by BCAAs,

isoleucine, leucine, and valine were dissolved in water at concentrations of 0, 0.1, 1, 10, and 100 mM and added separately to reactions. To determine K_m and V_{max}, sodium pyruvate was removed from extraction and reaction buffers and included in reactions at concentrations of 0, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 mM (Yu et al., 2010).

Vegetative Growth and Reproductive Comparison. Annual bluegrass seeds were sown as described for whole plant dose-response studies and placed in a growth chamber with day/night temperatures of 21/10 ^{o}C and a 12 h photoperiod (500 µmol m^{-2} s^{-1}). Upon germination, seedlings were thinned to contain one plant per pot. No supplemental fertilizer was applied and plants were watered every 48 to 72 h and periodically rotated in the growth chamber to minimize environmental differences.

For growth rate studies, four randomly selected plants of each biotype were removed from the growth chamber weekly for 10 wk beginning 21 DAG. At each harvest, above-ground biomass from each plant was harvested, dried at 80 ^{o}C for 72 h and weighed.

For reproductive studies, the number of DAG for initial visible appearance of a developing inflorescence were counted. Ten wk after the first inflorescence appeared, 10 randomly selected mature inflorescences (characterized by tan/brown seed color and absence of flowers) were removed from each plant and the number of seeds present on the inflorescence were recorded. All additional mature inflorescences were subsequently counted. Five replicate plants were used for each biotype.

Statistical Design and Analysis. For whole plant and *in vitro* response to trifloxysulfuron, the GR_{50} and I_{50} (trifloxysulfuron dose/concentration inhibiting shoot

dry weight or ALS activity 50%, respectively) were estimated using equation 2.1 where *y* was shoot dry weight or ALS activity, *x* was trifloxysulfuron dose/concentration, *D* was the upper bound of *y*, and *C* was the lower bound of *y*. K_m (pyruvate) and V_{max} values were determined using the nonlinear Michaelis-Menten equation

$$v = VS/(K_m + S) \quad [2.2]$$

where *v* was the velocity of the reaction at any pyruvate concentration, *V* was the maximal reaction velocity at substrate saturation, and *S* was the concentration of pyruvate. Comparisons of best-fit values for nonlinear regression parameters were conducted using the extra-sum-of-squares *F*-test. Feedback inhibition by BCAAs was determined by subjecting data to ANOVA to determine main effects of biotype, amino acid, concentration, and appropriate interactions on mean ALS activity. Each *in vitro* assay contained three technical replicates and was repeated using at least two independent protein extractions from separate pots.

Relative growth rates (RGR) were determined at selected harvest intervals using linear regression. As is typical with plant growth studies, shoot dry weight data were ln-transformed. Estimates of RGR between biotypes were compared using Student's *t*-test.

For each analysis, assumptions such as equality of variance and normal distribution were evaluated and adjusted if necessary. Significant differences and effects were based on $P < 0.05$. Calculations were performed using PROC GLM in SAS version 9.3 (SAS Institute, Inc., Cary, NC) or Prism 6.0.

Field Study. A field study was conducted during 2011 and 2012 at Calawassie Island Golf Club in Okatie, SC to assess annual bluegrass control in 'Tifway' bermudagrass under field conditions with ALS-inhibiting herbicides and herbicides of various other modes of action. The golf course superintendent reported poor control of annual bluegrass using ALS-inhibiting herbicides which had been previously applied yearly for at least 10 years (B. Bagwell, personal communication). Herbicide treatments and rates are presented in Table 2.1. Applications were made using a CO_2 pressurized backpack sprayer calibrated to deliver 187 L ha^{-1} through 8003 flat fan nozzles. Treatments were applied on 25 Feb. 2011 and 17 Feb. 2012.

Ratings were taken 21 and 42 DAT and included annual bluegrass density, annual bluegrass phytotoxicity, and bermudagrass spring greenup. Annual bluegrass density was visually estimated using a scale of 0 to 100% (100% = complete control). Control was also evaluated using the line intersect method and a 1 x 1 m grid containing 36 squares. The grid was placed in the center of the plot and the number of squares containing annual bluegrass plants were recorded. Visual and line-intersect estimates were converted to percentage annual bluegrass control using the formula

$$[1-(\text{observed}/\text{initial})] \times 100 \qquad [2.3]$$

where observed was the visual/line-intersect value at the given rating date and initial was the visual/line-intersect value at the initiation of the study. Annual bluegrass phytotoxicity was visually evaluated on a scale of 0 to 100% (100% = no green annual

Table 2.1. Herbicide treatments, formulations, and rates applied in 2011 and 2012 at Calawassie Island Golf Club (Okatie, SC) to suspected ALS-resistant annual bluegrass.

Treatment[a]	Formulation	Rate
		---kg ai ha^{-1}---
Nontreated	---	---
Trifloxysulfuron	75 WDG	0.029
Foramsulfuron	0.19 SC	0.029
Glyphosate	4 L	0.56
Simazine	4 L	1.12
Rimsulfuron	25 WSP	0.035
Diquat	2 L	0.56
Pronamide	50 WP	1.68

[a] Treatments were applied on 25 Feb. 2011 and 17 Feb 2012.

bluegrass tissue) and bermudagrass spring greenup was evaluated on a scale of 0 to 100% (100% = green actively growing bermudagrass).

Greenhouse Study. A study was conducted in 2012 at the Clemson University Greenhouse Complex to assess additional control options for annual bluegrass biotypes with confirmed resistance to ALS-inhibiting herbicides. Seeds from the four biotypes listed previously (CU, VS, CI, FP) were sown in polypropylene containers measuring 10 cm in diameter and 12 cm deep filled with sand and peat (85:15 by volume). Plants were grown to maturity (3-5 tillers) in a greenhouse under a 14 h photoperiod with day/night temperatures maintained at 29/18 ^{o}C. Plants were watered every 48 to 72 h, fertilized with 2.4 g N m^{-2} every two wk to encourage establishment, and clipped to 25 mm prior to herbicide application. Herbicides were applied using a spray chamber calibrated to deliver 374 L ha^{-1} through an 8001E flat fan nozzle. Following herbicide application, plants were returned to the greenhouse and watered every 48 to 72 h to prevent moisture

Table 2.2. Herbicide treatments, formulations, and rates for the annual bluegrass control study in the Clemson University greenhouse complex.

Treatment	Formulation	Rate
		---kg ai ha^{-1}---
Nontreated	---	---
Trifloxysulfuron	75 WDG	0.029
Foramsulfuron	0.19 SC	0.029
[a]Bispyribac-sodium	17.6 WDG	0.074
fb[b] bispyribac-sodium	17.6 WDG	0.074
Glyphosate	4 L	0.56
Diquat	2 L	0.56
Simazine	4 L	2.24
Pronamide	50 WP	1.68
Glufosinate	1 SC	1.12
Methiozolin fb methiozolin	2 EC	1.68
Ethofumesate fb ethofumesate	4 SC	1.68

[a] Sequential applications were applied 21 days after initial treatment.
[b] Abbreviation: fb, followed by.

stress, but fertility and clipping ceased. Herbicide treatments and rates are presented in Table 2.2.

Annual bluegrass control was visually evaluated weekly after herbicide application using a scale of 0 to 100% (100% = dead annual bluegrass plant). Forty-two DAT, surviving annual bluegrass plants were counted.

Statistical Design and Analysis. The experimental design for the field study was a randomized complete block with three replications. Plots were 2 x 3 m in 2011 and 1 x 1 m in 2012. Data were subjected to ANOVA to evaluate treatment, year, and treatment by year interactions. Assumptions such as equality of variance and normal distribution were

evaluated and adjusted if necessary. Mean separation was performed using Fisher's protected LSD.

The greenhouse study was conducted as a completely randomized design in a 4 x 11 factorial treatment arrangement with four annual bluegrass biotypes (CU, VS, CI, FP) and eleven herbicide treatments. There were three replicate plants for each treatment and the study was conducted in two separate greenhouse locations. Annual bluegrass control data were subjected to ANOVA to determine the effect of biotype, herbicide, greenhouse, and their interactions. A mixed model was produced to account for the random effect of greenhouse. Assumptions such as equality of variance and normal distribution were evaluated and adjusted if necessary. Comparisons among treatments were conducted according to Fisher's protected LSD and between biotypes according to Student's *t*-test (susceptible versus resistant biotypes). Survival data were analyzed using chi-square tests for differences between proportion of surviving individuals for each biotype. Where the assumption of expected counts > 5 was not met, Fisher's exact test was employed. All calculations for field and greenhouse studies used JMP version 9.0 or SAS version 9.3. Significant differences and effects were based on $P < 0.05$.

Results and Discussion

Detecting Annual Bluegrass Resistance to ALS-Inhibitors

In both experiments, responses of each susceptible (VS, CU) annual bluegrass biotype to increasing doses of trifloxysulfuron, foramsulfuron, and bispyribac-sodium were not significantly different (data not shown). Thus, these biotypes were pooled and

Table 2.3. ANOVA for whole plant dose-response and *in vivo* ALS activity studies for detecting annual bluegrass resistance to ALS-inhibitors.

Source	df	Whole Plant Dose-Response	*In vivo* ALS Activity
Herbicide	2	*	*
Biotype	2	*	*
Dose/Concentration	7	*	*
Herbicide*Biotype	4	NS[a]	*
Herbicide*Dose/Concentration	14	NS	NS
Biotype*Dose/Concentration	14	*	*
Herbicide*Biotype*Dose/Concentration	28	NS	NS
Experiment	1	*	NS
Herbicide*Biotype*Dose/Concentration* Experiment	28	NS	NS
Error	475		

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

used for comparison to CI and FP biotypes and are further referred to as "S" biotypes. No treatment-by-experiment interactions were detected for either study; therefore, data from both experiments were combined. The effects of herbicide, biotype, dose/concentration, run, and appropriate interactions are presented in Table 2.3.

Whole Plant Dose-Response. GR_{50} (± standard error) values for S biotypes were 13.57 (± 1.16) g ai ha^{-1} for trifloxysulfuron, 7.04 (± 0.57) g ai ha^{-1} for foramsulfuron, and 38.29 (± 4.85) g ai ha^{-1} for bispyribac-sodium (Table 2.4, Figure 2.4). All three herbicides were applied up to eight times labeled field rates, but 50% shoot biomass reductions were not achieved for CI and FP biotypes. Thus, the GR_{50} values for these biotypes were concluded to be greater than 224 g ai ha^{-1} for trifloxysulfuron and foramsulfuron and 592 g ai ha^{-1} for bispyribac-sodium, which indicate high levels of resistance.

McElroy et al. (2013) reported minimal activity of four ALS-inhibiting herbicides on an annual bluegrass biotype from Alabama. No visible injury and minimal (<23%) decrease in above-ground biomass were observed 28 DAT with trifloxysulfuron, foramsulfuron, bispyribac-sodium, and imazaquin at 32, 50, 300, and 980 g ai ha^{-1}, respectively. Similarly, sulfometuron-methyl provided little control of a rigid ryegrass (*Lolium rigidum* Gaud.) population at rates as high as 64 g ai ha^{-1} (Christopher et al., 1992). Even higher rates were required to achieve 50% above-ground biomass reduction in an accession of monochoria [*Monochoria vaginalis* (Burm. f.) Kunth], where the GR_{50} for imazosulfuron was 1,586 g ai ha^{-1} (Kuk et al., 2003).

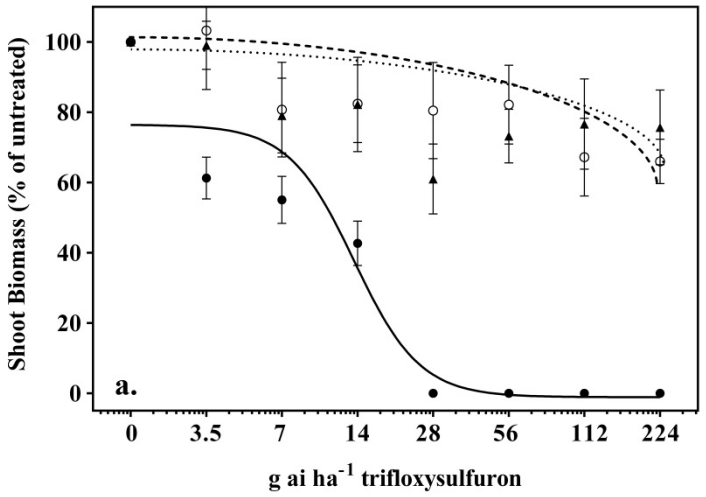
***In vivo* ALS Activity Assay**. This *in vivo* ALS activity assay was previously optimized for resistance diagnosis in numerous weed species (Gerwick et al., 1993; Kuk et al., 2003; Uchino et al., 1999), but never for annual bluegrass. With cases of annual bluegrass resistance to ALS-inhibiting herbicides on the rise, the ability to confirm resistance with multiple methods would be beneficial.

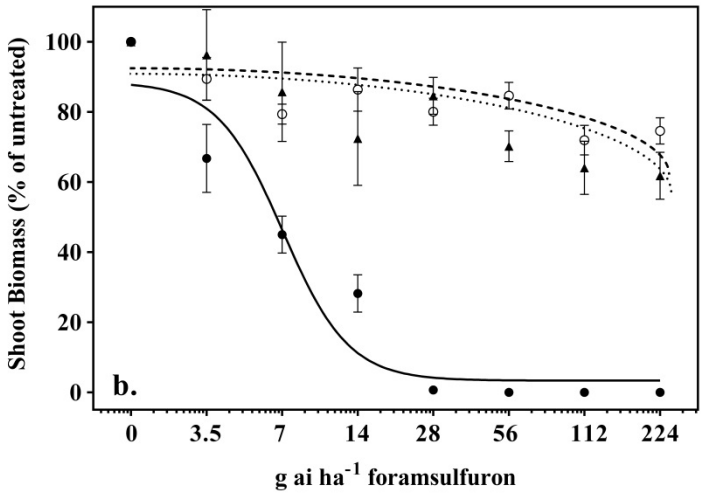
Based on the dose-response analysis from the *in vivo* ALS activity assay, I_{50} (± standard error) values for ALS activity in S biotypes were 0.003 (± 0.0008), 0.004 (± 0.0009), and 0.009 (± 0.003) µM for trifloxysulfuron, foramsulfuron, and bispyribac-sodium, respectively (Table 2.4, Figure 2.5). Acetolactate accumulated in the CI and FP biotypes regardless of ALS-inhibiting herbicide or concentration. The CI biotype exhibited I_{50} (± standard error) values for ALS activity of 10.95 (± 4.96) µM for trifloxysulfuron, 13.16 (± 6.2) µM for foramsulfuron, and 0.12 (± 0.06) µM for bispyribac-sodium. In comparison, the FP biotype exhibited I_{50} (± standard error) values

of 0.95 (± 0.39) µM for trifloxysulfuron, 0.56 (±0.26) µM for foramsulfuron, and 0.57 (± 0.3) µM for bispyribac-sodium. Thus, resistance factors for the CI biotype were 3650 for trifloxysulfuron, 3290 for foramsulfuron, and 13 for bispyribac-sodium (Table 2.4). For the FP biotype, resistance factors were 316, 140, and 64 for trifloxysulfuron, foramsulfuron, and bispyribac-sodium, respectively. These were the herbicide concentrations required to reduce acetolactate accumulation (absorbance), and in theory, *in vivo* ALS activity, by 50% of the nontreated control. These values cannot be assumed to represent the true herbicide concentrations inhibiting ALS activity *in vivo* because nothing is known about this assay concerning herbicide uptake, translocation, and metabolism. However, the values do provide clear evidence ALS enzymes for the CI and FP biotypes are still active in the presence of high concentrations of ALS-inhibiting herbicides.

This *in vivo* assay was optimized for use with annual bluegrass as a rapid diagnostic procedure for resistance to ALS-inhibiting herbicides and allows for the determination of *in vivo* activity of the ALS enzyme in the presence of ALS-inhibiting herbicides. This is accomplished by inhibiting KARI which is downstream of ALS in the leucine/valine branch of BCAA biosynthetic pathway (Figure 2.6). The inhibition of this enzyme prevents the conversion of acetolactate to 2,3-dihidroxyisovalerate which ultimately results in the accumulation of acetolactate.

Cyclopropane dicarboxylic acid is a known inhibitor of KARI (Gerwick et al., 1993). In this study, annual bluegrass tissue was incubated in a solution with a 500 µM concentration of CPCA for inhibition of KARI. Other studies have applied CPCA to





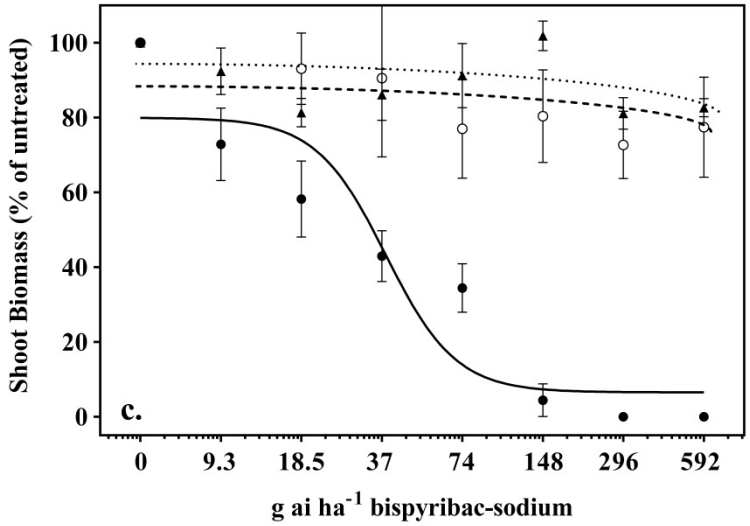
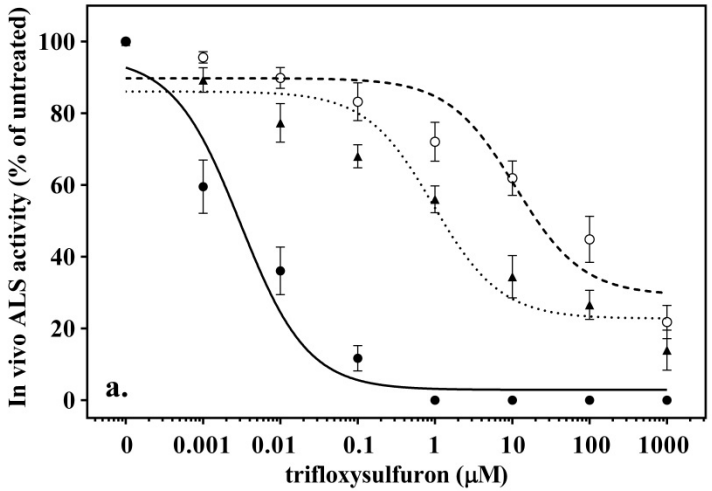
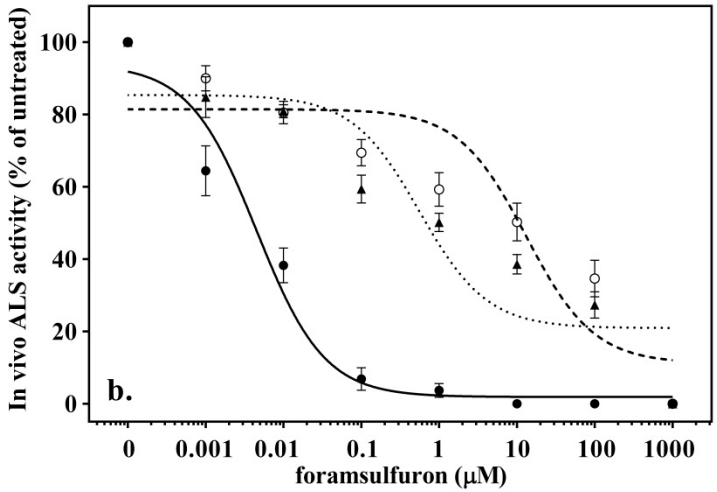


Figure 2.4. Effect of (a) trifloxysulfuron, (b) foramsulfuron, and (c) bispyribac-sodium on shoot biomass of susceptible (S; closed circles, solid lines) and resistant annual bluegrass biotypes from South Carolina (CI; open circles, dashed lines) and Georgia (FP; closed triangles, dotted lines). Vertical bars represent standard errors of the mean. Shoot biomass values were calculated as a percentage of the nontreated and analyzed using the nonlinear regression equation $y = C + (D\text{-}C)/(1+10^{x\text{-}\log I_{50}})$. I_{50} estimates for the S biotypes were 13.57 g trifloxysulfuron ha^{-1}, 7.04 g foramsulfuron ha^{-1}, and 38.29 g bispyribac-sodium ha^{-1}. Fifty percent shoot biomass was not achieved for CI or FP biotypes, thus, I_{50} values were concluded to be > 224 g trifloxysulfuron/foramsulfuron ha^{-1} and 592 g bispyribac-sodium ha^{-1}.





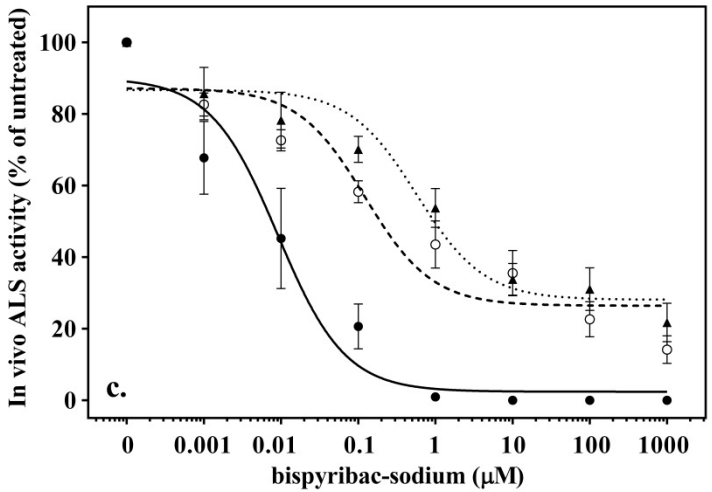


Figure 2.5. Effect of (a) trifloxysulfuron, (b) foramsulfuron, and (c) bispyribac-sodium on *in vivo* ALS activity of susceptible (S; closed circles, solid lines) and resistant annual bluegrass biotypes from South Carolina (CI; open circles, dashed lines) and Georgia (FP; closed triangles, dotted lines). Vertical bars represent standard errors of the mean. ALS activity values were calculated as a percentage of the nontreated and analyzed using the nonlinear regression equation $y = C + (D\text{-}C)/(1+10^{x\text{-}\log I_{50}})$. I_{50} estimates for S, CI, and FP biotypes, respectively, were 0.003, 10.95, and 0.95 µM trifloxysulfuron, 0.004, 13.16, and 0.559 µM foramsulfuron, and 0.009, 0.122, and 0.574 µM bispyribac-sodium.

foliage prior to harvesting plant tissue, but incubating the tissue in a solution with the inhibitor is more logical for this purpose (Simpson et al., 1995). In the presence of CPCA alone, both resistant and susceptible biotypes accumulate acetolactate. When an ALS-inhibitor is added to the incubation solution, a susceptible ALS enzyme will be inhibited, thus preventing the conversion of two pyruvate molecules to acetolactate (Figure 2.6). However, if acetolactate accumulation occurs even in the presence of an ALS-inhibitor, the ALS enzyme is resistant to this mode of action and activity resumes resulting in continued production of leucine, valine, and isoleucine.

The total amount of acetolactate produced by annual bluegrass tissue in this assay can be quantified by the decarboxylation of acetolactate to produce acetoin. This reaction

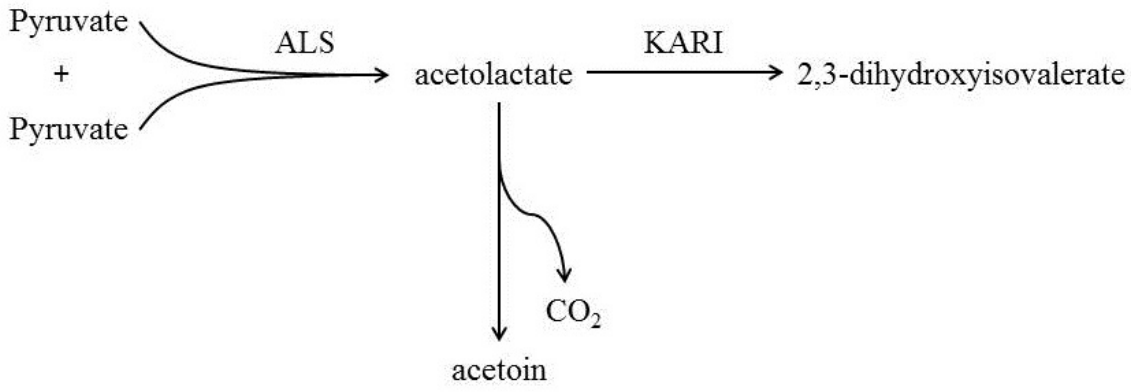


Figure 2.6. The conversion of two pyruvate molecules to acetolactate and 2,3-dihydroxyisovalerate via acetolactate synthase (ALS) and ketol acid reductoisomerase KARI), respectively, in the leucine/valine branch of the branched-chain amino acid biosynthetic pathway. The inhibition of KARI allows for the accumulation of acetolactate, and subsequent decarboxylation of acetolactate to acetoin allows for the quantification of ALS enzyme activity.

readily occurs when the solution is acidified and heated via the enzyme acetolactate decarboxylase (Davies, 1964). A reagent containing 1-naphthol and creatine monohydrate can be added to solutions containing acetoin for colorimetric analysis (Westerfeld, 1945). A high concentration of acetoin results in a red/pink color upon addition of the reagent to the solution. Thus, in the presence of ALS-inhibiting herbicides in the incubation solution, resistant annual bluegrass biotype solutions would turn a distinct red/pink color (Figure 2.7). When no acetoin is present, solutions remain yellow/brown upon addition of the reagent, indicative of a susceptible ALS enzyme.

Most cases of resistance to ALS-inhibiting herbicides in other weed species have been the result of ALS enzyme mutations because of numerous amino acid connections involved in binding ALS-inhibiting herbicides (Duggleby et al., 2008). A second mechanism of resistance to ALS inhibitors is enhanced metabolism and detoxification of the herbicide (Tranel and Wright, 2002). This bioassay is an enzyme activity assay, and thus, limitations for determining resistance to ALS-inhibiting herbicides are possible. However, because living annual bluegrass tissue is used to conduct the assay, both target site and non-target site resistance could be detected. For example, if an ALS-inhibiting active ingredient did not reach the target site due to metabolism/detoxification, the ALS enzyme would remain active and results would indicate a resistant annual bluegrass plant, assuming the detoxification occurred in the leaf tissue being used for analysis. Further research, including *in vitro* ALS enzyme assays and ALS gene sequencing, would provide further insight for the detection of resistance using the *in vivo* assay.

Figure 2.7. Color development in the absence (left) and presence (right) of acetoin upon the addition of a reagent containing 1-napththol and creatine monohydrate, where the deeper red color indicates a higher level of acetoin. Acetoin concentrations can be quantified by measuring the absorbance at 530 nm in a spectrophotometer.

Without further studies, a conclusion cannot be made concerning the mechanisms conferring resistance in the CI and FP biotypes. However, high resistance factors indicate the possibility a nucleotide mutation in the ALS gene has led to an amino acid substitution conferring resistance to multiple ALS-inhibiting herbicides (Table 2.4).

Substitutions at Trp_{574} in plants, the most commonly observed substitution conferring ALS resistance, results in high levels of resistance to multiple chemical classes of ALS inhibitors (Duggleby et al., 2008; Tranel and Wright, 2002). In yeast, a substitution at Trp_{586} (equivalent to Trp_{574} residue in plants) results in resistance factors of approximately 10^4 to multiple sulfonylurea herbicides (Duggleby et al., 2003). Similarly, Yu et al. (2010) reported a high resistance factor (>1,333) for a rigid ryegrass population with a Trp_{574} substitution. Other amino acid substitutions on the ALS enzyme confer varying levels of resistance, and may differ in their resistant characteristics to other ALS-

Table 2.4. I_{50} values and resistance factors (RF) for whole plant dose-response and *in vivo* ALS (acetolactate synthase) enzyme activity assays as affected by annual bluegrass biotype and ALS-inhibiting herbicide.

Biotype[a]	Herbicide	Whole plant dose-response		*In vivo* ALS activity	
		I_{50}	RF[b]	I_{50}	RF
		g ai ha^{-1}		µM	
S	Trifloxysulfuron	13.57	---	0.003	---
	Foramsulfuron	7.04	---	0.004	---
	Bispyribac-sodium	38.29	---	0.009	---
CI	Trifloxysulfuron	>224*	>17	10.95*	3650
	Foramsulfuron	>224*	>32	13.16*	3290
	Bispyribac-sodium	>592*	>15	0.122*	13
FP	Trifloxysulfuron	>224*	>17	0.948*	316
	Foramsulfuron	>224*	>32	0.559*	140
	Bispyribac-sodium	>592*	>15	0.574*	64

[a]Susceptible (S) biotpyes were obtained from a commercial source (Fresno, CA) and a wild-type population where no previous herbicide applications had been made (Clemson, SC) and pooled for comparison to resistant biotypes from South Carolina (CI) and Georgia (FP).

[b]Resistance factor (RF) is a ratio comparing I_{50} values of resistant to susceptible annual bluegrass biotypes quantifying the level of resistance to an individual ALS-inhibiting herbicide.

*Indicates significantly different than the S biotypes at $P < 0.05$ according to Student's *t*-test.

inhibiting chemical classes. For example, four rigid ryegrass populations with different substitutions at Pro_{197} resulted in resistance factors ranging from 95 to 241 for sulfometuron, but these plants were controlled with imidazolinone herbicides (Yu et al., 2010).

A Trp_{574} to Leu substitution was recently confirmed in an annual bluegrass biotype in Alabama and, like other weed species, appears to be the most likely target site mutation conferring resistance to ALS-inhibiting herbicides in annual bluegrass (McElroy et al., 2013). Within a biotype, variation in resistance factors is likely due to structural differences in the active ingredient molecules. Duggleby et al. (2008) noted significant variation in the potency of ALS inhibition within the sulfonylurea chemical class. The researchers surmised this variability was from the numerous connections between herbicide molecules and the ALS enzyme as well as general fit of the molecule into the active site.

Results from the ALS enzyme activity assay, which indicated ALS activity in the presence of ALS-inhibiting herbicides in two annual bluegrass biotypes, support evidence from the whole plant dose-response experiment. Thus, this rapid diagnostic procedure is useful for determining and quantifying annual bluegrass resistance to ALS-inhibiting herbicides. Results can be obtained in as quickly as 72 h providing an additional method which can be used in confirming annual bluegrass resistance to ALS-inhibitors.

Whole Plant Dose-Response. The whole plant dose-response study revealed GR_{50} values for VS and GN biotypes of 46.8 and 1,261.9 g ai ha^{-1}, respectively, resulting in a resistance factor of 27 (Figure 2.8). A 95% confidence interval for the GR_{50} estimate is 14.1 to 133.2 g ai ha^{-1} for the VS biotype and 904.1 to 1,791.2 g ai ha^{-1} for the GN biotype. McElroy et al. (2013) applied trifloxysulfuron at 32 g ai ha^{-1} to the GN biotype and measured a 0.4% increase in above-ground biomass 28 DAT. The previous study indicated GR_{50} values > 224 g trifloxysulfuron ha^{-1} for two resistant annual bluegrass biotypes from Georgia and South Carolina, but their specific mechanism of resistance was unknown. Higher rates (up to 1,792 g trifloxysulfuron ha^{-1}) were applied to the GN biotype so as to obtain the lower asymptote to accurately define the GR_{50}. Resistance-conferring mutations resulting in a Trp_{574} to Leu substitution are commonly observed in plants and result in high levels of resistance at the whole plant level to multiple chemical classes of ALS-inhibitors (Duggleby et al., 2008; Tranel and Wright, 2002). This substitution thus confers high levels of resistance at the whole plant level to sulfonylurea herbicides in annual bluegrass.

***In vitro* ALS Assays**. The inhibition of ALS activity in response to trifloxysulfuron *in vitro* was 10-fold higher (P = 0.0017) in the GN biotype (I_{50} = 0.014 µM) compared to the VS biotype (I_{50} = 0.0014 µM; Figure 2.9). A 95% confidence interval for the I_{50} estimate is 0.0008 to 0.002 µM for the VS biotype and 0.005 to 0.039 µM for the GN biotype. Other weed species with the Trp_{574} to Leu amino acid substitution generally have

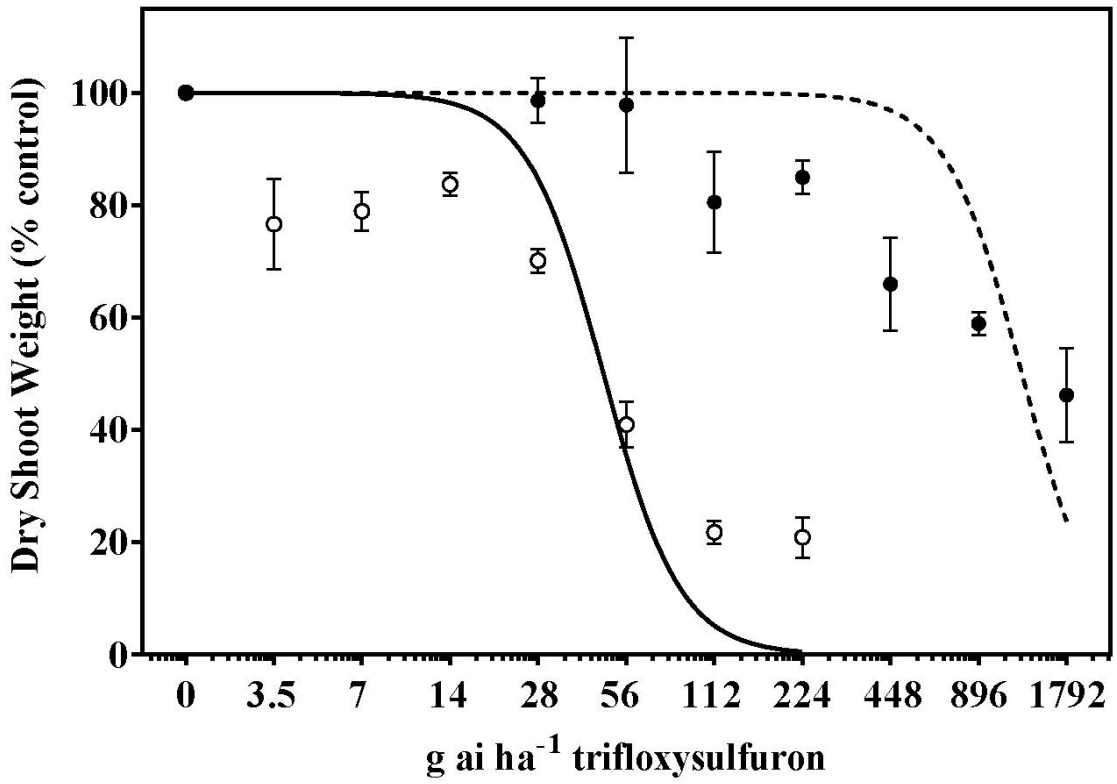


Figure 2.8. Effect of trifloxysulfuron on above-ground biomass of ALS-inhibitor susceptible (VS; solid line, open circles) and resistant (GN; dashed line, closed circles) annual bluegrass biotypes. Vertical bars represent standard errors of the means at each trifloxysulfuron dose. Above-ground biomass values were calculated as a percentage of the nontreated control. Nonlinear regression analysis was performed using the equation $y = C + (D-C)/(1+10^{x-\log GR_{50}})$ where y was above-ground biomass, x was trifloxysulfuron dose (g ai ha^{-1}), D was the upper bound of y, C was the lower bound of y, and GR_{50} was the trifloxysulfuron dose (g ai ha^{-1}) reducing above-ground biomass 50%. Mean and 95% confidence interval estimates for the GR_{50} were 46.8 and (14.1, 133.2) g ai ha^{-1} for the VS biotype and 1,261.9 and (904.1, 1791.2) g ai ha^{-1} for the GN biotype. Comparisons of GR_{50} values between biotypes were conducted according to the extra-sums-of-squares F-test ($P < 0.0001$).

a very high level of resistance *in vitro*. For example, Yu et al. (2010) reported a resistance factor >1,333 for a population of rigid ryegrass with a Trp_{574} to Leu substitution in

response to sulfometuron *in vitro*. Our results contrast with the above study as the ALS activity of the GN biotype was only 10 times less sensitive to trifloxysulfuron *in vitro*. The GN biotype lost 27 and 50% of ALS activity at trifloxysulfuron concentrations of

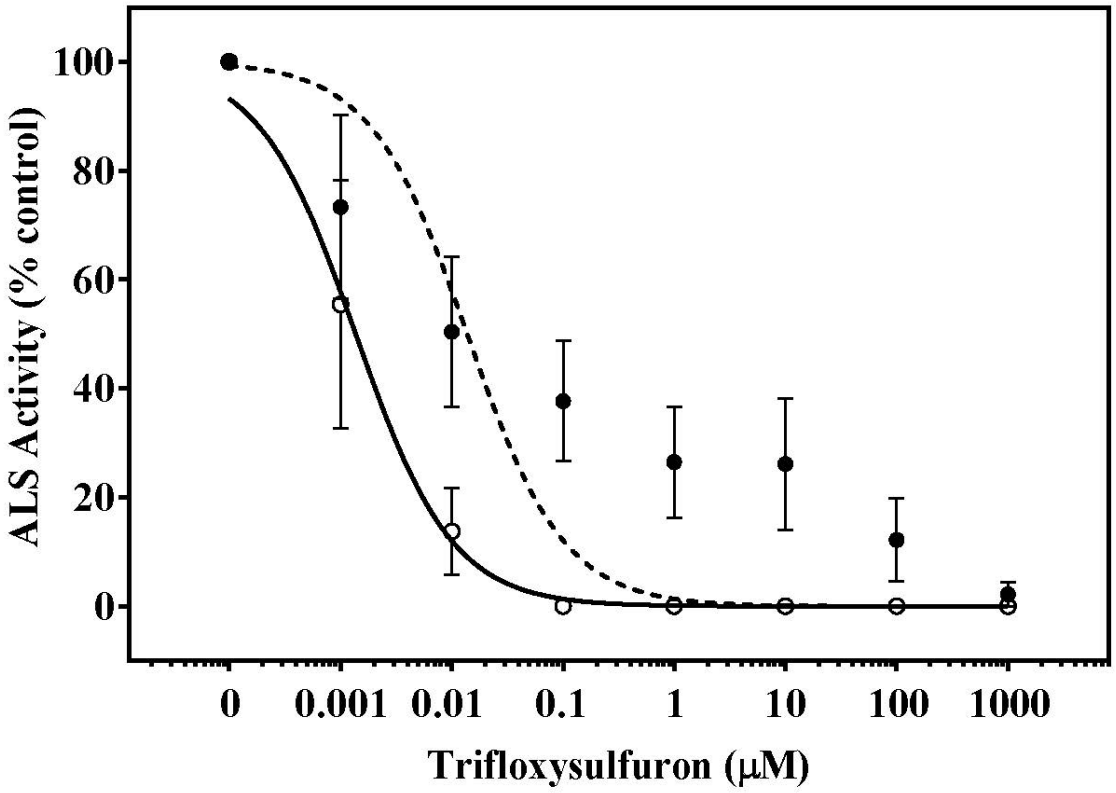


Figure 2.9. Effect of trifloxysulfuron on *in vitro* ALS activity of ALS-inhibitor susceptible (VS; solid line, open circles) and resistant (GN; dashed line, closed circles) annual bluegrass biotypes. Vertical bars represent standard errors of the means at each trifloxysulfuron concentration. ALS activity values were calculated as a percentage of the control. Nonlinear regression analysis was performed using the equation $y = C + (D-C)/(1+10^{x-\log I_{50}})$ where y was ALS activity, x was trifloxysulfuron concentration, D was the upper bound of y, C was the lower bound of y, and I_{50} was the trifloxysulfuron concentration (µM) reducing *in vitro* ALS activity 50%. Parameters C and D were constrained to 0 and 100%, respectively. Mean and 95% confidence interval estimates for the I_{50} were 0.0014 and (0.0008, 0.002) µM for the VS biotype and 0.014 and (0.005, 0.039) µM for the GN biotype. Comparisons of I_{50} values between biotypes were conducted according to the extra-sums-of-squares F-test (P = 0.0017).

0.001 and 0.01 µM, respectively (Figure 2.9). Despite this initial rapid inhibitory effect of trifloxysulfuron on ALS activity in the GN biotype, further increase in the herbicide concentration had lower influence in reducing activity. For example, while a 10-fold increase in trifloxysulfuron concentration from 0.001 to 0.01 µM caused a 25% reduction in ALS activity of the GN biotype, a further reduction of 25% required a 1,000-fold increase of trifloxysulfuron to 10 µM. This response seems to model the effect of two isoforms (i.e., a wild-type and mutant) of ALS present in the GN biotype. The portion of ALS activity initially lost could be attributed to the inhibition of the wild-type enzyme, but a mutant enzyme that is less inhibited could maintain ALS activity even at higher levels of herbicide. This poses an interesting question of the relative expression of wild-type and mutant ALS genes in this resistant biotype. As evident from whole plant dose-response studies above, expression of the mutant enzyme is sufficient enough to sustain plant growth at high doses of ALS-inhibitor. Differential ALS expression patterns have been reported in canola which, like annual bluegrass, is allotetraploid (Ouellet et al., 1992).

Similar levels of extractable ALS activity were observed between VS (0.5 ± 0.09 µmol acetoin mg^{-1} protein h^{-1}) and GN (0.4 ± 0.06 µmol acetoin mg^{-1} protein h^{-1}) biotypes (Table 2.5). In contrast, Yu et al. (2010) and Li et al. (2013) reported increased and decreased extractable ALS activity in resistant rigid ryegrass and wild radish (*Raphanus raphanistrum* L.), respectively, both with a Trp_{574} to Leu substitution. In general, difficulty in isolating and maintaining ALS activity in susceptible versus resistant plants has been observed for many resistance-conferring amino acid

substitutions (Yu et al., 2010). Here, we suspect the presence of wild-type and mutant ALS isoforms in the GN biotype is a partial contributor to similar extractable ALS activities between biotypes.

Enzyme kinetic constants investigated in this study were the maximum rate of enzyme activity (V_{max}, obtained when all active sites are occupied by substrate) and the Michaelis constant (K_m, substrate concentration at one-half V_{max}) (Dowd and Riggs, 1965). Together, these estimates predict enzymatic function and efficiency in living organisms, and comparisons between susceptible and resistant plants may indicate differences in enzyme functionality as a result of resistance. K_m values between VS (4.9 ± 1.2 mM) and GN (3.6 ± 0.27 mM) biotypes were similar suggesting pyruvate binding is not affected by resistance in the GN biotype (Table 2.5; Figure 2.10). This observation is consistent with numerous studies in other ALS-resistant weed species where minimal effect on K_m values for different resistance-conferring substitutions has been reported

Table 2.5. K_m (pyruvate), V_{max}, and extractable ALS activity ± standard error of ALS-inhibitor susceptible (VS) and resistant (GN) annual bluegrass biotypes.

Parameter	VS	GN[a]	p-value
K_m (mM pyruvate)	4.9 ± 1.2	3.6 ± 0.27	0.1928[b]
V_{max} (µmol acetoin mg^{-1} protein h^{-1})	0.21 ± 0.01	0.26 ± 0.005	0.0038
Extractable ALS activity (µmol acetoin mg^{-1} protein h^{-1})	0.5 ± 0.09	0.4 ± 0.06	0.4392[c]

[a] The GN annual bluegrass biotype contained a Trp_{574} to Leu amino acid substitution (McElroy et al., 2013).
[b] Comparisons of K_m and V_{max} between VS and GN biotypes were conducted according to the extra-sums-of-squares *F*-test at $P < 0.05$.
[c] Comparisons of extractable ALS activity were conducted according to Student's *t*-test at $P < 0.05$.

(Eberlein et al., 1997; Tanaka, 2003; Yu et al., 2010). The crystallization of yeast ALS in complex with several sulfonylurea herbicides confirmed these herbicides do not bind directly to the active site of ALS, but rather block substrate access by binding within the substrate access tunnel (McCourt et al., 2005; McCourt and Duggleby, 2006; Pang et al., 2003). While binding of ALS herbicides are greatly affected by these resistance-conferring substitutions, the active site remains unchanged, providing some explanation

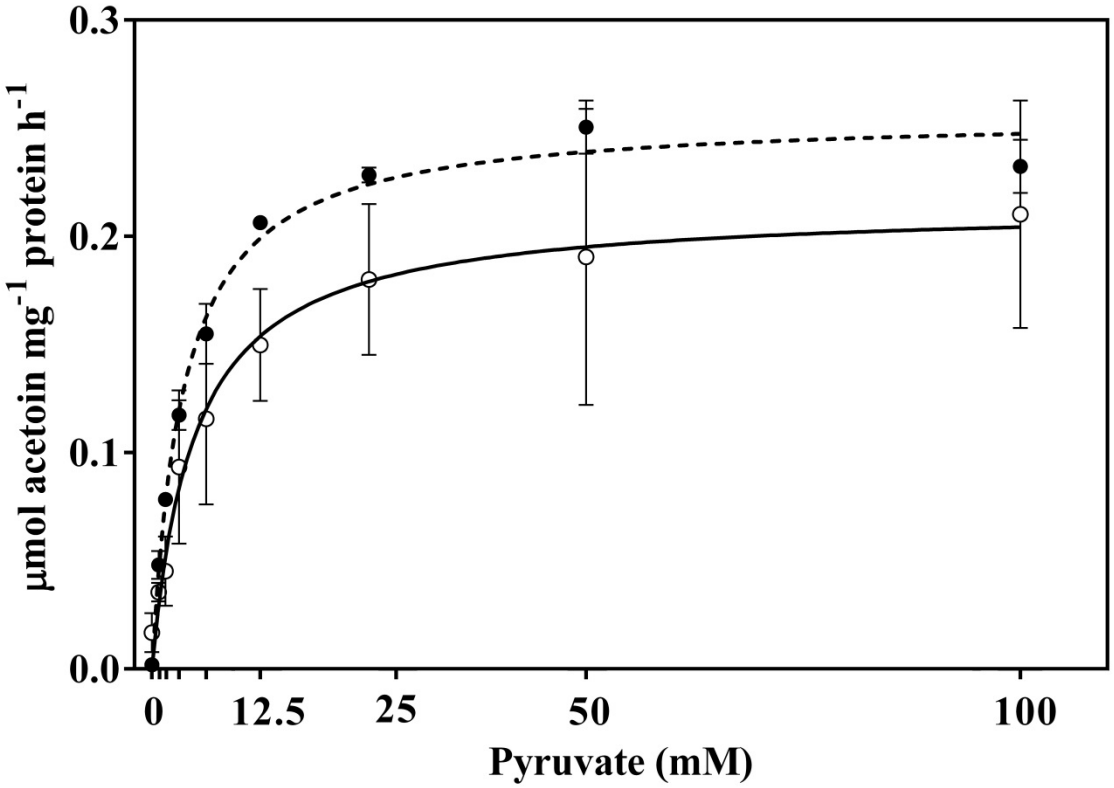


Figure 2.10. Michaelis-Menten kinetics of susceptible (VS; solid line, open circles) and resistant (GN; dashed line, closed circles) annual bluegrass biotypes. Vertical bars represent standard errors for the means at each pyruvate concentration. K_m and V_{max} estimates were determined using the nonlinear Michaelis-Menten equation $v = VS/(K_m+S)$. K_m estimates were 4.9 ± 1.2 mM and 3.6 ± 0.27 mM for VS and GN biotypes, respectively (P = 0.1928). V_{max} estimates were 0.21 ± 0.01 and 0.26 ± 0.005 µmol acetoin mg^{-1} protein h^{-1} for VS and GN biotypes, respectively (P = 0.0038).

of the typically unchanged K_m values with respect to ALS resistance (Boutsalis et al., 1999; Yu et al., 2010). The co-occurrence of a wild-type ALS in the GN biotype could also partly contribute to similar K_m estimates observed in our study.

The V_{max} was higher in the GN biotype (0.26 ± 0.005 µmol mg^{-1} protein h^{-1}) compared to the VS biotype (0.21 ± 0.01 µmol mg^{-1} protein h^{-1}; Table 2.5). Since K_m estimates were similar, this would suggest ALS in the GN biotype can achieve a higher rate of product formation at K_m, and thus, a higher catalytic efficiency. However, levels of extractable ALS activity were not different, thus, further research with more susceptible biotypes would be required to clarify this interaction. An ALS-resistant population of rigid ryegrass that contained a Trp_{574} to Leu substitution exhibited higher V_{max} compared to susceptible populations, but this was accompanied by higher extractable ALS activity (Yu et al., 2010).

Regulation of the BCAA biochemical pathway in bacteria, fungi, and plants may involve several mechanisms, but plants have demonstrated regulation through ALS feedback inhibition by BCAAs (McCourt and Duggleby, 2006). There were significant main effects of type and concentration of amino acid, and biotype on feedback inhibition (Table 2.6). ALS in both VS and GN biotypes was feedback inhibited by all BCAAs at concentrations greater than 0.1 mM (Figure 2.11). Regardless of concentration or biotype, ALS activity was inhibited 34% of the control by valine, 31% of the control by leucine, and 20% of the control by isoleucine. These results are consistent with observations in other studies where valine is typically the most effective inhibitor of ALS (McCourt and Duggleby, 2006). Additionally, the BCAAs do not completely inhibit ALS

activity (McCourt and Duggleby, 2006). At BCAA concentrations of 100 mM, 33 to 44% ALS activity remained compared to the control regardless of annual bluegrass biotype (Figure 2.11). When all three BCAAs were included in the reaction, ALS activity was quickly inhibited in both biotypes (Figure 2.12). While high levels of ALS activity in both biotypes were observed at 0.1 mM concentrations of single BCAAs, inclusion of all three BCAAs in the reaction at 0.1 mM each resulted in >50% inhibition of ALS activity regardless of biotype. As these concentrations were increased, ALS of both biotypes maintained approximately 35% ALS activity. This explains ALS activity in annual bluegrass is at least partially regulated by BCAA concentrations.

Across all amino acids and concentrations, ALS in the VS biotype was feedback inhibited less (76% of control) compared to the GN biotype (69% of control) (Table 2.7).

Table 2.6. ANOVA for acetolactate synthase (ALS) feedback inhibition by branched chain amino acids.

Source	df	ALS Feedback Inhibition
Biotype	1	*
Amino Acid	2	*
Concentration	3	*
Biotype*Amino Acid	2	*
Biotype*Concentration	3	*
Amino Acid*Concentration	6	*
Biotype*Amino Acid* Concentration	6	NS[a]
Run	1	NS
Run*Biotype*Amino Acid* Concentration	6	NS
Error	113	

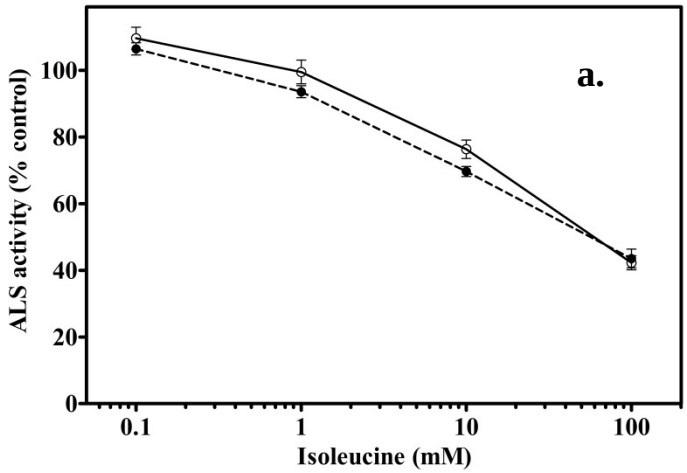
[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

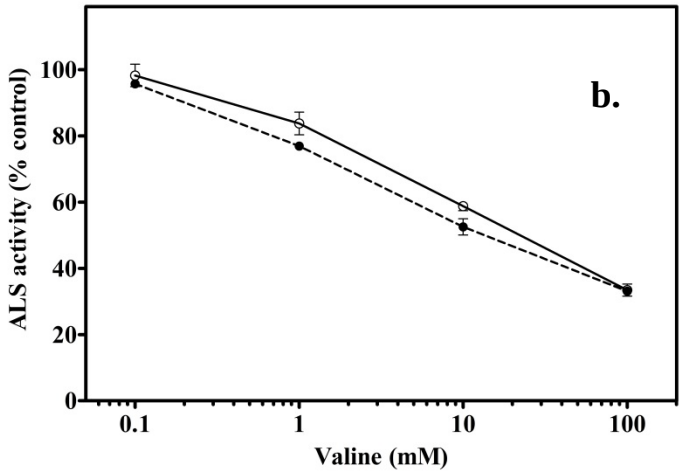
Feedback inhibition was increased in the GN biotype compared to the VS biotype for all three BCAAs, with leucine (20%) exhibiting the greatest increase, followed by valine (6%) and isoleucine (4%) (Figure 2.11; Table 2.7). These observations are interesting for two reasons. First, herbicide resistance-conferring amino acid substitutions occur on the catalytic ALS subunit while feedback inhibition is controlled by the regulatory ALS subunit (Duggleby et al., 2008). The interaction between subunits in plant ALS is unclear, but an understanding of plant ALS structure would be beneficial to further clarify this interaction, especially for allotetraploid species such as annual bluegrass (Yu et al., 2010). Second, research conducted on ALS feedback inhibition with respect to herbicide resistance-conferring mutations has generally resulted in decreased ALS sensitivity to BCAAs in resistant weed species (Eberlein et al., 1997; Preston et al., 2006;

Table 2.7. Mean acetolactate synthase (ALS) activity of susceptible (VS) and resistant (GN) annual bluegrass biotypes as affected by the branched-chain amino acids.

	Biotype			
Amino Acid	VS	GN[a]	p-value[b]	Amino Acid Mean[c]
	---ALS Activity (% control)---			---ALS activity (% control)---
Isoleucine	81.9 ± 5.6	78.3 ± 5.1	0.0438	80.1 ± 3.7
Leucine	76.2 ± 5.9	61.0 ± 5.4	<0.0001	68.6 ± 4.1
Valine	68.5 ± 5.3	64.6 ± 5.0	0.0274	66.6 ± 3.6
Biotype Mean	75.6 ± 3.3	67.9 ± 3.1	<0.0001	$LSD_{0.05}$ = 2.2

[a] The GN annual bluegrass biotype contained a Trp_{574} to Leu amino acid substitution (McElroy et al., 2013).
[b] Mean comparisons between biotypes were conducted according to Student's *t*-test at P < 0.05.
[c] Mean comparisons among amino acids were conducted according to Fisher's protected LSD at P < 0.05.





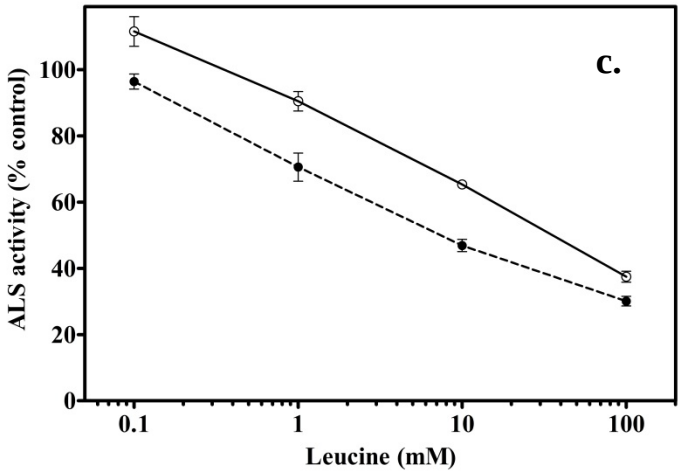


Figure 2.11. Feedback inhibition of ALS activity by (a) isoleucine, (b) valine, and (c) leucine for ALS-inhibitor susceptible (VS; solid line, open circles) and resistant (GN; dashed line, closed circles) annual bluegrass biotypes. Vertical bars represent standard errors of the mean at each amino acid concentration. ALS activity values were calculated as a percentage of the control. Lines are presented to show trends.

Yu et al., 2010). For example, only a substitution of Pro_{197} to Arg in rigid ryegrass resulted in increased ALS inhibition by BCAAs while substitutions of Pro_{197} to Ala, Pro_{197} to Ser, and Trp_{574} to Leu resulted in decreased inhibition (Yu et al., 2010). It is possible altered feedback inhibition of ALS as a result of herbicide resistance mutations could change amino acid pool concentrations (Yu et al., 2010). Tranel and Wright (2002) suggested reduced feedback inhibition could be advantageous as BCAA concentrations

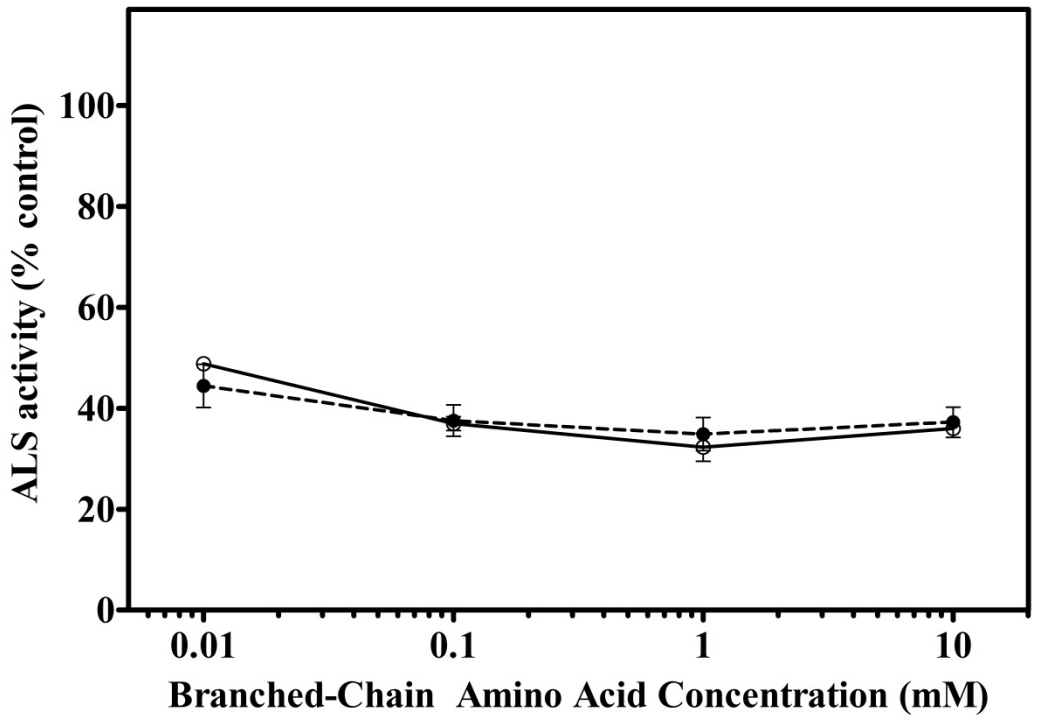


Figure 2.12. Feedback inhibition of ALS activity by branched-chain amino acids for ALS-inhibitor susceptible (VS; solid line, open circles) and resistant (GN; dashed line, closed circles) annual bluegrass biotypes. Vertical bars represent standard errors of the mean at each amino acid concentration. Isoleucine, leucine, and valine were included in reactions at 0.01, 0.1, 1, and 10 mM each. ALS activity values were calculated as a percentage of the control. Lines are presented to show trends.

would be higher. This has been associated with faster kochia seed germination in resistant versus susceptible populations (Dyer et al., 1993). To determine the overall effect of these differential feedback inhibitions in resistant and susceptible biotypes of annual bluegrass, further investigations are needed.

Vegetative Growth and Reproductive Comparison. It is generally assumed a competitive advantage under selective conditions would lead to a disadvantage in the absence of selection. To provide some initial perspective of this concept with respect to the two biotypes in this study, vegetative and reproductive growth characteristics of these two annual bluegrass biotypes were compared. While vegetative and reproductive growth of annual bluegrass is ecotypically and genetically variable (Johnson and White, 1997; McElroy et al., 2002), annual bluegrass biotypes resistant to ALS-inhibitors have not been previously characterized based on these parameters.

The average time for inflorescence initiation was 48.1 ± 0.6 d for the GN biotype and 85.4 ± 2.8 d for the VS biotype (Table 2.8). While the number of seeds per inflorescence was lower in the GN biotype (178.5 ± 3.0 seeds inflorescence^{-1}) compared to the VS biotype (238.9 ± 7.4 seeds inflorescence^{-1}), the GN biotype produced nearly double the inflorescences per plant than the VS biotype (102.2 ± 2.1 versus 56.8 ± 6.9 inflorescences plant^{-1}, respectively). This resulted in higher estimates of total seed production per plant for the GN biotype (Table 2.8).

Relative growth rates of GN and VS biotypes were not different within any harvest intervals, including the growth periods from 28-49, 49-70, 70-91, and 28-91 DAG (Table 2.9; Figure 2.13). Additionally, while RGR were not different between biotypes,

Table 2.8. Reproductive parameter (inflorescence and seed production) means ± standard error of ALS-inhibitor susceptible (VS) and resistant (GN) annual bluegrass biotypes.

Parameter	VS	GN[a]	p-value[b]
Days to first inflorescence	85.4 ± 2.8	48.1 ± 0.6	<0.0001
Inflorescences plant^{-1}	56.8 ± 6.9	102.2 ± 2.1	<0.0001
Seeds inflorescence^{-1}	238.9 ± 7.4	178.5 ± 3.0	<0.0001
Seeds plant^{-1}	13,570 ± 631	18,243 ± 1130	<0.0001

[a] The GN annual bluegrass biotype contained a Trp_{574} to Leu amino acid substitution (McElroy et al., 2013).
[b] Mean comparisons of parameters between biotypes were conducted according to Student's *t*-test at $P < 0.05$.

above-ground biomass data were analyzed and biotype differences were not observed (data not shown).

The rapid evolution of resistance to ALS-inhibitors across numerous weed species is attributed to, among other characteristics, high efficacy against susceptible individuals, long soil residual activity, genetics of ALS resistance (e.g., complete dominance of resistance allele, nuclear-encoded gene), and numerous target-site mutations that confer resistance (Tranel and Wright, 2002). However, biological characteristics of annual bluegrass potentially enhance selection of resistant individuals. For example, annual bluegrass seed banks on golf courses can be extensive (up to 185,000 seeds m^{-2}) and seeds can germinate year-round under favorable conditions, thus exposing a large number of individuals to herbicide application and selection (McCarty, 2011; Watschke et al., 1979). Differences in these measurements cannot be directly attributed to the resistance-conferring mutation in the GN biotype as many other factors are involved in these parameters. For example, annual bluegrass biotypes evolving in similar locations tend to have similar growth and reproductive characteristics (McElroy et al., 2002). The

Table 2.9. Mean relative growth rates (RGR) ± standard error of ALS-inhibitor susceptible (VS) and resistant (GN) annual bluegrass biotypes at selected time intervals.

Interval[a]	VS	GN[b]	p-value[c]
DAG[d]	----------RGR d^{-1}----------		
28-49	0.12 ± 0.01	0.1 ± 0.01	0.1783
49-70	0.093 ± 0.007	0.1 ± 0.009	0.3274
70-91	0.06 ± 0.005	0.05 ± 0.008	0.5387
28-91	0.091 ± 0.003	0.086 ± 0.003	0.2713

[a] Above-ground biomass of four randomly selected plants from each biotype were harvested at each time interval, dried for 72 h at 80 ^{o}C, and weighed. The slope of the line of ln-transformed dry weights for two harvests constitutes the RGR for that time interval.
[b] The GN annual bluegrass biotype contained a Trp_{574} to Leu amino acid substitution (McElroy et al., 2013).
[c] Comparisons of RGR between biotypes were conducted using PROC GLM in SAS and associated Student's *t*-tests at $P < 0.05$.
[d] Abbreviation: DAG, days after germination.

susceptible biotype in this study was from seed grown in Oregon, which probably contributes to differences observed in these measurements. However, initial indications from these comparisons suggest the GN biotype produces plentiful seed and grows at normal rates compared to a true annual susceptible biotype selected for seed production. These characteristics could contribute to rapid evolution of resistance. To determine if differences in these growth characteristics can be attributed directly to the herbicide resistance allele would require fitness studies controlling for genetic differences at other loci. These studies would also be important to elucidate the maintenance of the resistance allele in the absence of ALS-inhibitor selection.

The presence of two ALS isoforms in the GN biotype presents a complex situation for resistance to ALS-inhibitors. Future studies on ALS gene expression in this biotype would help to further clarify interactions observed in our study. A wild-type and

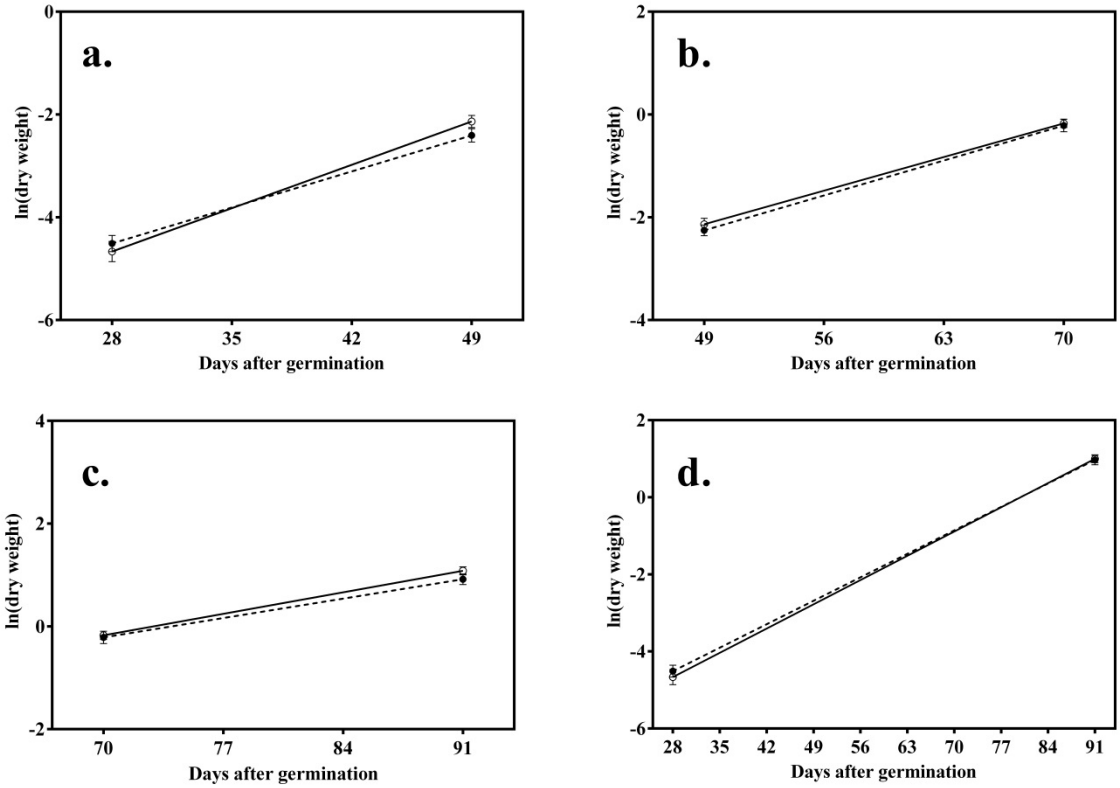


Figure 2.13. Relative growth rate (RGR) comparisons of susceptible (VS; solid line, open circles) and resistant (GN; dashed line, closed circles) at harvest intervals from (a) 28 to 49 days after germination (DAG), (b) 49 to 70 DAG, (c) 70 to 91 DAG, and (d) 28 to 91 DAG. Vertical bars represent standard errors of the means. Above-ground biomass of four randomly selected plants from each biotype were harvested at each time interval, dried for 72 h at 80 ^{o}C, and weighed. The slope of the line of ln-transformed dry weights for two harvests constitutes the RGR for that time interval. Comparisons of RGR between VS and GN biotypes were conducted using PROC GLM in SAS and associated Student's *t*-tests at $P < 0.05$.

mutant ALS enzyme could potentially provide an advantage in both the presence (herbicide resistance from mutant) and absence (normal enzyme function from wild-type) of ALS-inhibitors. These initial observations also suggest heavy seed production and normal growth rates for this resistant biotype in comparison with a standard susceptible

biotype. If other resistant annual bluegrass biotypes contain the same mechanism, these results could partially explain of the increased cases of resistance currently being observed on golf courses. Annual bluegrass continues to evolve resistance to multiple herbicide mechanisms of action. Considering the genetic variability and biological characteristics of this weed, turfgrass managers should evaluate their herbicide programs to maintain the efficacy of other mechanisms of action and prevent the development of multiple-resistant populations.

Evaluating Alternative Control Options for Annual Bluegrass Populations Resistant to ALS-Inhibitors

Field Study. ALS-inhibiting herbicides had been used on the study site for at least 10 consecutive years for annual bluegrass control, and the golf course superintendent reported unsatisfactory results with these herbicides prior to the study. To compare results of laboratory and greenhouse studies for the CI biotype, a field study was initiated to evaluate field control with ALS-inhibiting herbicides and to investigate additional options for control with several alternative mechanisms of action. The effects of treatment and year on annual bluegrass control and bermudagrass greenup are presented in Table 2.10. Where ANOVA indicated a significant treatment-by-year interaction, data are presented separately by year. Correlations between visual and line-intersect control ratings were significant (Table 2.10), thus, only visual control data are presented. Trifloxysulfuron provided 4% annual bluegrass control 42 DAT and was not different from the nontreated control, while foramsulfuron and rimsulfuron provided no

Table 2.10. ANOVA and correlation between visual and line-intersect control ratings for field study investigating alternative control options for an annual bluegrass population resistant to ALS inhibitors at Calawassie Island Golf Club (Okatie, SC) during 2011 and 2012.

Source	df	Control				Greenup	
		Visual		Line-Intersect			
		21 DAT[a]	42 DAT	21 DAT[a]	42 DAT	21 DAT	42 DAT
Treatment	7	*	*	*	*	*	*
Year	1	*	NS	*	NS	*	*
Treatment*Year	7	*	NS	NS	NS	*	*
Block	2	NS	NS	NS	NS	NS	NS

	21 DAT		42 DAT
	2011	2012	
Pearson Correlation Coefficients[b]	0.9507*	0.8677*	0.9859*

[a] Abbreviations: DAT, days after treatment; NS, not significant.
[b] Correlation coefficients for 21 DAT ratings separated by year due to significant treatment-by-year interaction for visual ratings.
*Indicates a significant effect at $P < 0.05$.

control (Table 2.11). Greater than 95% control with several sulfonylurea herbicides after February applications was observed by Toler et al. (2007). Therefore, these results support the superintendent's claim of poor annual bluegrass control and confirm results from the *in vivo* diagnostic assay and whole plant dose-response studies above using the CI biotype. ALS-inhibiting herbicides are no longer effective at Calawassie Island and other herbicides must be considered.

Other postemergence herbicides investigated for alternate control options included glyphosate, simazine, diquat, and pronamide. Complete control of annual bluegrass with glyphosate was obtained 21 DAT in 2012 and 42 DAT in both years

Table 2.11. Annual bluegrass control in 'Tifway' bermudagrass 21 and 42 days after treatment (DAT) at Calawassie Island Golf Club (Okatie, SC) during 2011 and 2012.

Treatment[a]	Rate	Annual Bluegrass Control[b]		
		21 DAT		42 DAT
		2011	2012	2011-2012
	---kg ai ha^{-1}---	----------%----------		
Nontreated	---	0 ± 0	0 ± 0	0 ± 0
Trifloxysulfuron	0.029	0 ± 0	8.3 ± 8.3	4.2 ± 4.2
Foramsulfuron	0.029	0 ± 0	0 ± 0	0 ± 0
Rimsulfuron	0.035	0 ± 0	0 ± 0	0 ± 0
Glyphosate	0.56	78.3 ± 7.9	100 ± 0	100 ± 0
Simazine	1.12	5.6 ± 5.6	0 ± 0	45.7 ± 14.2
Diquat	0.56	17.8 ± 9.7	63.3 ± 8.8	15.0 ± 9.6
Pronamide	1.68	0 ± 0	16.7 ± 16.7	11.1 ± 8.2
$LSD_{0.05}$		14.5	21.9	19.7

[a] Treatments were applied on 25 February 2011 and 17 February 2012.
[b] Annual bluegrass control was evaluated visually on a 0-100% scale (100% = complete control).

(Table 2.11). Toler et al. (2007) reported 93% annual bluegrass control with glyphosate alone applied in February. Bermudagrass must be completely dormant when glyphosate applications are made to prevent undesirable injury. However, Calawassie Island Golf Club is located in a coastal area where the subtropical climate rarely allows bermudagrass to go completely dormant. In both years, green bermudagrass tissue was visible at the time of application due to late February applications. Thus, bermudagrass greenup was significantly less than the nontreated control both 21 and 42 DAT in both years (Table 2.12). If glyphosate is to be used as an alternative control option for ALS-resistant annual bluegrass at Calawassie Island, applications should be made earlier in the year and only when there is no green tissue visible.

Table 2.12. Percent bermudagrass greenup 21 and 42 days after treatment (DAT) for herbicide treatments at Calawassie Island Golf Club (Okatie, SC) in 2011 and 2012.

Treatment[a]	Rate	Bermudagrass Greenup[b]			
		21 DAT		42 DAT	
		2011	2012	2011	2012
	---kg ai ha^{-1}---	----------%----------			
Nontreated	---	75.0 ± 0	6.7 ± 1.7	100 ± 0	46.7 ± 3.3
Trifloxysulfuron	0.029	75.0 ± 0	33.3 ± 3.3	100 ± 0	63.3 ± 3.3
Foramsulfuron	0.029	75.0 ± 0	33.3 ± 3.3	100 ± 0	63.3 ± 3.3
Rimsulfuron	0.035	26.7 ± 3.3	36.7 ± 3.3	96.7 ± 3.3	66.7 ± 3.3
Glyphosate	0.56	0 ± 0	3.3 ± 1.7	13.3 ± 3.3	16.7 ± 3.3
Simazine	1.12	75.0 ± 0	30.0 ± 5.8	100 ± 0	60.0 ± 5.8
Diquat	0.56	45.0 ± 15.0	17.8 ± 9.7	100 ± 0	17.8 ± 9.7
Pronamide	1.68	75.0 ± 0	30.0 ± 0	100 ± 0	60.0 ± 0
$LSD_{0.05}$		16.3	9.7	5.0	10.6

[a] Treatments were applied on 25 Feb. 2011 and 17 Feb. 2012.
[b] Bermudagrass greenup was evaluated visually on a 0-100% scale (100% = green, actively growing bermudagrass).

Other herbicides did not provide satisfactory control 42 DAT. Simazine provided 48% annual bluegrass control but did not significantly delay bermudagrass greenup compared to the nontreated (Tables 2.11 and 2.12). Control was likely poor in this study for two reasons. First, a low rate of simazine was applied (1.12 kg ai ha^{-1}), and 2.24 kg ai ha^{-1} is a rate more likely to provide adequate control. Second, mature annual bluegrass is more difficult to control, thus, applications should be made earlier than late February. Toler et al. (2007) observed reduced annual bluegrass control with 2.24 kg simazine ha^{-1} when applications were made in February (79%) compared with December applications (86%). Similarly, diquat did not provide adequate annual bluegrass control 42 DAT (Table 2.11). Control was 63% 21 DAT in 2012, but only 15% control was observed 42 DAT. Diquat is minimally translocated, and thus, mature plants are quickly burned down

but have enough energy reserves to regrow. As with glyphosate, diquat is nonselective and caused a delay in bermudagrass greenup during both years 21 DAT and in 2012 42 DAT (Table 2.12). Pronamide was safe on bermudagrass, but control was <20% regardless of rating date or year (Tables 2.11 and 2.12). For most effective results on annual bluegrass with pronamide, applications should be made early postemergence (mid-fall) with repeat applications if needed (McCarty, 2011). The late application date probably led to decreased levels of control with pronamide. Additionally, pronamide is a mitosis-inhibiting herbicide, and the superintendent has reported poor preemergence control with mitosis-inhibitors after many years of applying these herbicides prior to overseeding (B. Bagwell, personal communication). It is possible this population may have tolerance or resistance to this mechanism of action, but further research would be required to determine this.

Other options are available but were not investigated in this study. These include preemergence herbicides such as mitosis inhibitors (e.g., prodiamine, dithiopyr), cellulose biosynthesis inhibitors (e.g., indaziflam), and PPO inhibitors (e.g., oxadiazon). Other postemergence herbicides include flumioxazin (PPO inhibitor) and glufosinate (glutamine synthetase inhibitor). As this population evolved resistance to ALS-inhibiting herbicides after at least 10 years of consecutive use, all alternative options must be used judiciously to prevent multiple resistance. This is especially a concern with the use of simazine as it is one of the few postemergence options for golf courses where bermudagrass does not readily go dormant. Triazine resistance in annual bluegrass has been observed for over 30 years (Darmency and Gasquez, 1981; McCarty, 2011) and

Hutto et al. (2004) reported widespread annual bluegrass resistance to simazine in Mississippi with 44% of biotypes tested being resistant. This is probably less of a concern for glyphosate at Calawassie Island as continuous selection is not likely from repeated applications due to the warm climate.

Greenhouse Studies. Responses of respective susceptible (CU and VS) and resistant (CI and FP) annual bluegrass biotypes were not significantly different (data not shown). Thus, for data analysis and presentation, susceptible and resistant biotypes were pooled and are hereafter referred to as S and R biotypes, respectively. ANOVA revealed no significant interaction between herbicide and greenhouse at either rating time, thus, data are pooled across both greenhouses for further comparison (Table 2.13).

Table 2.13. ANOVA for greenhouse study investigating control options for annual bluegrass biotypes susceptible and resistant to ALS-inhibiting herbicides.

Source	df	Annual Bluegrass Control	
		21 DAT	42 DAT
Biotype[a]	1	NS[b]	*
Herbicide	10	*	*
Greenhouse	1	NS	NS
Biotype*Herbicide	10	*	*
Biotype*Greenhouse	1	NS	NS
Herbicide*Greenhouse	10	NS	NS
Biotype*Herbicide*Greenhouse	10	NS	*
Error	220		

[a] Similar responses between susceptible (VS and CU) and resistant (CI and FP) biotypes were observed. The two susceptible and two resistant biotypes were pooled for further analysis.
[b] Abbreviations: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

With respect to control with ALS-inhibiting herbicides, results from the greenhouse control study are consistent with the whole plant dose-response studies and *in vivo* ALS assay for CI and FP biotypes as well as field trials at CI. ALS-inhibiting herbicides provided no annual bluegrass control of R biotypes 21 or 42 DAT (Tables 2.14 and 2.15) and 100% of these plants survived all ALS-inhibitor treatments (Table 2.16). The sulfonylureas provided complete control of S biotypes while bispyribac-sodium controlled S biotypes 70% 42 DAT.

To compare the control of various active ingredients in S versus R biotypes, the VS and CU biotypes (confirmed susceptible) were pooled and compared to CI and FP biotypes (confirmed resistant). At 21 DAT, control across all treatments was greater for the S biotypes due to >87% control with trifloxysulfuron and foramsulfuron compared to no control in the R biotypes with these treatments (Table 2.14). As expected, bispyribac-sodium provided higher control of the S biotypes, but control was 42%. This is expected as two applications are typically required to control mature annual bluegrass with this herbicide. Methiozolin and ethofumesate provided greater control of the R biotype after one application, but control was ≤33%. Three herbicides controlled annual bluegrass >90% regardless of biotype and included simazine, glyphosate, and glufosinate. Similar observations were observed 42 DAT with greater control regardless of treatment observed for the R biotypes (Table 2.15). Complete control of S biotypes was obtained with the sulfonylureas and 70% with bispyribac-sodium compared to no control of R biotypes with these treatments. Differences between biotypes were observed with ethofumesate (66%) and methiozolin (72%) providing greater control of R biotypes

Table 2.14. Control (± standard error) of ALS-suscetpible (S) and resistant (R) annual bluegrass biotypes 21 days after treatment with herbicides under greenhouse conditions.

Treatment	Rate	Biotype[a]		
		S	R	S vs. R
	---kg ai ha^{-1}---	----------%----------		
Nontreated	---	0 ± 0	0 ± 0	NS
Trifloxysulfuron	0.029	87.1 ± 4.5	0 ± 0	*
Foramsulfuron	0.029	94.2 ± 1.8	0 ± 0	*
[b]Bispyribac-sodium fb[c] bispyribac-sodium	0.074 0.074	41.7 ± 9.4	0 ± 0	*
Glyphosate	0.56	92.1 ± 2.3	100 ± 0	NS
Diquat	0.56	12.5 ± 8.8	24.2 ± 9.2	NS
Simazine	2.24	100 ± 0	100 ± 0	NS
Pronamide	1.68	67.7 ± 5.6	65.8 ± 6.1	NS
Glufosinate	1.12	91.7 ± 4.9	99.6 ± 0.4	NS
Methiozolin fb methiozolin	1.68	15.0 ± 2.9	33.3 ± 7.0	*
Ethofumesate fb ethofumesate	1.68	0 ± 0	23.3 ± 4.7	*
$LSD_{0.05}$	---	13.6	11.7	
Biotype Mean	---	54.6 ± 3.7	40.7 ± 3.8	*

[a] Similar responses between susceptible (VS and CU) and resistant (CI and FP) biotypes were observed. The two susceptible and two resistant biotypes were pooled for analysis.
[b] Sequential applications were applied 21 days after initial treatment.
[c] Abbreviation: fb, followed by; NS, not significant.
*Indicates significant difference between S and R biotypes according to Student's *t*-test at $P < 0.05$.

compared to S biotypes (≤33% with either treatment). In contrast with 21 DAT, glufosinate provided complete control of R biotypes but 37% of S biotypes. At 42 DAT, glyphosate and simazine provided complete control of S and R biotypes, while pronamide provided ≥88% control.

With the exception of ALS-inhibiting herbicides, differences in control are not necessarily related to resistance, but probably to maturity and vigor of plants at the time

of treatment. At time of application, S plants were more mature and had more vigor compared to R biotypes. This is especially true of herbicides which are minimally translocated such as glufosinate and diquat. For glufosinate, >90% control of S biotypes was observed 21 DAT, but this reduced to 37% 42 DAT. Additionally, 75% of S individuals survived glufosinate application (Table 2.16). Annual bluegrass plants were thus mature enough to regrow following initial burndown with glufosinate. Similarly,

Table 2.15. Control (± standard error) of ALS-suscetpible (S) and resistant (R) annual bluegrass biotypes 42 days after treatment with herbicides under greenhouse conditions.

Treatment	Rate	Biotype[a]		
		S	R	S vs. R
	---kg ai ha^{-1}---	----------%----------		
Nontreated	---	0 ± 0	0 ± 0	NS
Trifloxysulfuron	0.029	100 ± 0	0 ± 0	*
Foramsulfuron	0.029	100 ± 0	0 ± 0	*
[b]Bispyribac-sodium fb[c] bispyribac-sodium	0.074 0.074	70.0 ± 9.0	0 ± 0	*
Glyphosate	0.56	100 ± 0	100 ± 0	NS
Diquat	0.56	8.3 ± 8.3	0 ± 0	NS
Simazine	2.24	100 ± 0	100 ± 0	NS
Pronamide	1.68	87.5 ± 6.3	98.3 ± 1.7	NS
Glufosinate	1.12	36.7 ± 13.6	100 ± 0	*
Methiozolin fb methiozolin	1.68	33.3 ± 8.1	71.7 ± 4.9	*
Ethofumesate fb ethofumesate	1.68	18.3 ± 7.3	65.8 ± 4.3	*
$LSD_{0.05}$	---	18.8	5.7	---
Biotype Mean	---	59.5 ± 3.9	48.7 ± 4.0	*

[a] Similar responses between susceptible (VS and CU) and resistant (CI and FP) biotypes were observed. The two susceptible and two resistant biotypes were pooled for analysis.
[b] Sequential applications were applied 21 days after initial treatment.
[c] Abbreviation: fb, followed by; NS, not significant.
*Indicates significant difference between S and R biotypes according to Student's *t*-test at $P < 0.05$.

diquat provided initial burndown of both biotypes, but ≥92% of plants survived diquat application regardless of biotype.

The vast majority of plants (≥92%) also survived applications of ethofumesate and methiozolin (Table 2.16). Ethofumesate is typically applied to less mature annual bluegrass plants for optimal control, so this result is not surprising. However, resistant

Table 2.16. Percent survival of ALS-susceptible (S) and resistant (R) annual bluegrass biotypes 42 days after treatment with herbicides under greenhouse conditions.

Treatment	Rate	Biotype[a]		
		S	R	S vs. R[b]
	---kg ai ha^{-1}---	----------%----------		
Nontreated	---	100	100	NS
Trifloxysulfuron	0.029	0	100	*
Foramsulfuron	0.029	0	100	*
[c]Bispyribac-sodium fb[d] bispyribac-sodium	0.074 0.074	58	100	*
Glyphosate	0.56	0	0	NS
Diquat	0.56	92	100	NS
Simazine	2.24	0	0	NS
Pronamide	1.68	42	8	NS
Glufosinate	1.12	75	0	*
Methiozolin fb methiozolin	1.68	100	92	NS
Ethofumesate fb ethofumesate	1.68	100	100	NS
Biotype Mean[e]	---	52	64	*

[a] Similar responses between susceptible (VS and CU) and resistant (CI and FP) biotypes were observed. The two susceptible and two resistant biotypes were pooled for analysis.
[b] To satisfy assumptions, comparisons between S and R biotypes were conducted according to Fisher's exact test at $P < 0.05$.
[c] Sequential applications were applied 21 days after initial treatment.
[d] Abbreviation: fb, followed by; NS, not significant.
[e] Comparisons between biotypes across all treatments were conducted according to the chi-square test at $P < 0.05$.
*Indicates a significant difference at $P < 0.05$ according to corresponding test.

populations of ALS-inhibitors are often found on overseeded golf courses, thus, this herbicide remains an important tool for postemergence control in these situations. Methiozolin is a slow herbicide and typically requires multiple applications to control annual bluegrass at putting green height.

These results suggest control of ALS-resistant populations can be obtained with glyphosate, simazine, pronamide, and possibly glufosinate. For best control, these herbicides should be applied to less mature and vigorous annual bluegrass plants. Simazine and pronamide will remain the most viable postemergence options for coastal areas such as CI and FP due to bermudagrass which rarely goes fully dormant.

Conclusions

These studies served to confirm annual bluegrass resistance to ALS-inhibiting herbicides and expand the understanding of how resistance affects plant growth and function. Both CI and FP biotypes have high levels of resistance to trifloxysulfuron, foramsulfuron, and bispyribac-sodium. Whole plant dose-response and *in vivo* ALS activity assays were used to confirm resistance to these herbicides. The exact mechanism of resistance of CI and FP biotypes was not investigated but is probably a target-site mutation conferring resistance. Further research would be required to confirm this. Regardless, alternate mechanisms of action will be required for annual bluegrass control at these sites.

Investigations into enzyme function and growth characteristics of the GN biotype revealed interesting results. ALS activity was only 10-fold higher in the GN biotype in

response to trifloxysulfuron *in vitro* which is much lower than what is typically expected for plants with a Trp_{574} to Leu amino acid substitution. K_m (pyruvate) remained the same for both biotypes, but a higher V_{max} was observed for the GN biotype which may indicate a more efficient enzyme. However, feedback inhibition was higher for the GN biotype. Further investigations would be needed to see if there are any negative effects associated with this increased inhibition. Seed production was higher in the GN biotype while growth rates between VS and GN biotypes were similar at all harvest intervals. Although more research would be needed to determine if the resistance allele is the cause for these differences, initial indications suggest annual bluegrass with this amino acid substitution can aggressively grow and produce copious seed with minimal effect on enzyme functionality. Due to the presence of a wild type and mutant enzyme, an advantage could be gained through the resistance allele in the presence and absence of ALS herbicides.

Control studies in the field and greenhouse suggest glyphosate, pronamide, simazine, and glufosinate are the most effective postemergence options for ALS-resistant populations. For coastal areas such as CI and FP, simazine and pronamide would be the safest alternatives because bermudagrass rarely goes dormant. For areas where bermudagrass is fully dormant, glyphosate and glufosinate can be added options. Preemergence herbicides were not investigated in these studies, but should be part of an integrated annual bluegrass control program. Regardless of which herbicide is used, it is important for turf managers with resistant biotypes to rotate or tank-mix mechansisms of action to prevent multiple resistance from evolving.

CHAPTER THREE

ANNUAL BLUEGRASS RESISTANCE TO GLYPHOSATE

Introduction

Glyphosate

The discovery of glyphosate (*N*-phosphonomethyl glycine) for use as a herbicide changed the face of weed control in agriculture. First described in 1971 (Baird et al., 1971), glyphosate is a non-selective postemergence herbicide used to control nearly all problematic weeds that exist today and is often used where total vegetation control is required (Franz et al., 1997; Sikorski and Gruys, 1997). The herbicide was first available commercially in 1974 when Monsanto received a patent for phosphoric acid derivative herbicides (Duke and Powles, 2008). The success of glyphosate can be attributed to its broad spectrum weed control with minimal non-target organism toxicity (Duke and Powles, 2008). Glyphosate is highly systemic in plants, allowing for control of perennial weeds and even woody shrubs and trees that are often difficult to control with other herbicide compounds. It has very little soil residual activity, largely due to the rapid microbial degradation of the compound, eventually yielding ammonia, inorganic phosphate, and carbon dioxide (Reuppel et al., 1977).

As of 2003, glyphosate was the most widely used active ingredient (by sales) in the United States than any other pesticide. A significant increase of glyphosate usage has occurred since 1996 and the introduction of genetically modified glyphosate-resistant crops (Roundup Ready) which are widely used in North and South America (Powles and Preston, 2006). According to Cobb and Reade (2010), glyphosate sales nearly reached

three billion dollars, almost ten times greater than the second highest used compound, paraquat.

Use of glyphosate in turf management situations is limited because it is nonselective. Typically, the herbicide is used for postemergence weed control in areas where turf cover is not desired such as natural areas and landscape beds on golf courses and sports turf complexes. In addition, glyphosate is used when complete vegetation control is desired prior to renovation or preplant applications before sprigging, seeding, or sodding desirable turfgrasses. Use in bermudagrass turf is common during periods of complete winter dormancy to control winter annual weeds, especially annual bluegrass, where glyphosate can be applied to bermudagrass with minimal impacts to desirable turfgrass.

The Shikimate Pathway

The aromatic amino acids (phenylalanine, tyrosine, and tryptophan) are formed via the shikimate pathway through which nearly 20% of all carbon fixed by plants passes. In addition to the aromatic amino acids, numerous secondary plant metabolites are products of this pathway including vitamins, lignins, quinones, alkaloids, and some phenolic compounds (e.g., flavonoids) (Dewick, 1998; Kishore and Shah, 1988). The precursors of this pathway are the carbohydrates erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). Chorismate is the precursor to the aromatic amino acids and its production leads to the branching that results in their biosynthesis (Herrmann and Weaver, 1999).

The shikimate pathway has several branches, the main branch ending with the production of chorismate. The first step of the main branch combines PEP and E4P to yield the inorganic phosphate 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). This reaction is catalyzed by DAHP synthase, which, like most enzymes of the shikimate pathway, has been most highly studied in *Escherichia coli* (Herrmann and Weaver, 1999). Removal of the phosphate group in DAHP yields 3-dehydroquinate (DHQ) and is catalyzed by DHQ synthase. The production of 3-dehydroshikimate (DHS) is the third step in the shikimate pathway and results from the dehydration of DHQ by the activity of the bifunctional enzyme DHQ dehydratase-shikimate dehydrogenase. This enzyme also catalyzes the fourth step of the pathway, where DHS is reduced to shikimate. Shikimate-3-phosphate, which forms a herbicide-enzyme complex with glyphosate, is produced by the action of shikimate kinase from the phosphorylation of shikimate. At this point, an additional PEP is combined with shikimate-3-phosphate (S3P) to yield 5-enolpyruvylshikimate 3-phosphate (EPSP). This reaction is catalyzed by EPSP synthase (EC 2.5.1.19), the enzyme which glyphosate inhibits to disrupt the shikimate pathway. The reaction catalyzed by EPSP synthase is reversible. Finally, a reaction catalyzed by chorismate synthase yields chorismate by the removal of a phosphate from EPSP. Here, the pathway branches to the further production of phenylalanine, tryptophan, and tyrosine, and other secondary metabolites (Bentley, 1990; Herrmann and Weaver, 1999). Figure 3.1 outlines the main branch of the shikimate pathway.

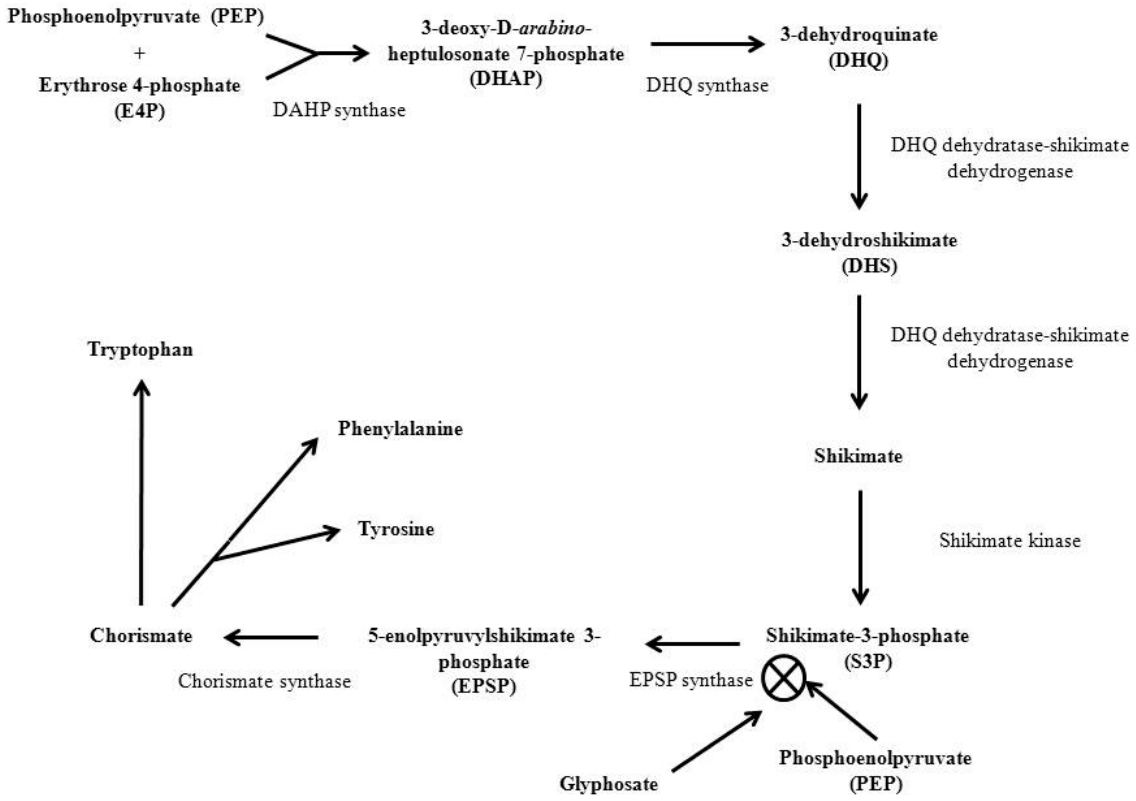


Figure 3.1. The shikimate pathway and the site of EPSP synthase inhibition by glyphosate.

Mode of Action of Glyphosate

The first attempt to identify the site of action of glyphosate was by Jaworski (1972) whose studies of gibbous duckweed (*Lemna gibba* L.) revealed negative effects of glyphosate application could be alleviated by addition of phenylalanine. Further study led to the discovery that glyphosate inhibited conversion of shikimate to chrosimate (Amrhein et al., 1980). Upon determining glyphosate induces accumulation of shikimic acid in treated plants, it was learned EPSP synthase is the only enzyme inhibited by

glyphosate in the shikimate pathway, and confirmed as the target enzyme of glyphosate (Steinrucken and Amrhein, 1980). No other herbicides are known to target EPSP synthase. The enzyme is found in the chloroplast, but is synthesized in the cytoplasm prior to its activity in the chloroplast (Cobb and Reade, 2010). Glyphosate is active on the enzyme only when it is present in the chloroplast. Therefore, for the herbicide to be effective, the compound must reach the chloroplast in a high enough concentration to inhibit the enzyme. Denis and Delrot (1993) discovered glyphosate has a carrier protein that plays a role in its crossing the plasma membrane, thus aiding its ability to reach the chloroplast for activity.

Glyphosate does not bind directly to EPSP synthase (it binds away from the active site), and thus acts as a non-competitive inhibitor of the enzyme. The herbicide molecule binds to an enzyme-S3P complex inhibiting binding of the substrate PEP (Herrmann and Weaver, 1999). In the absence of glyphosate, S3P and PEP are combined to yield EPSP via the enzyme EPSP synthase, and thus, glyphosate acts as a competitive inhibitor of PEP (Figure 3.1).

EPSP synthase has two distinct domains which have been described as moving in a ‘screw-like’ pattern, causing a conformational change in the enzyme and revealing its active site. This conformational change is apparently caused by the binding of S3P. The true active site of EPSP synthase is unknown, but is probably located where the two domains of the enzyme meet (Cobb and Reade, 2010). It is likely the glyphosate molecule interacts with the active site of EPSP synthase which binds PEP (Kishore and Shah, 1988).

Interestingly, light indirectly influences the activity of glyphosate. The conformational change that reveals the active site of EPSP synthase creates an electropositive charge, thus attracting the anionic forms of its substrates (i.e., PEP). Cyclic electron flow in photosynthesis creates a proton motive force for ATP production. As a result, the chloroplast stroma experiences an increase in pH during light conditions. EPSP synthase is synthesized in the cytoplasm but is active in the chloroplast stroma. The activity of the enzyme is much higher above pH 7, thus during high light. Additionally, glyphosate is unique in that it is zwitterionic (i.e., possesses positively and negatively charged groups). At physiological pH, glyphosate is anionic, thus providing greater inhibition and affinity for the electropositive binding site of the EPSPS-S3P complex during light (Duke and Powles, 2008).

The shikimate pathway is involved in the production of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. Numerous secondary plant metabolites are also synthesized as a result of this pathway, including vitamins, lignins, and numerous phenolics. Approximately 20% of carbon fixed by plants passes through the shikimate pathway (Cobb and Reade, 2010). The true cause of plant death in glyphosate-treated plants is not entirely known because of numerous metabolic processes glyphosate influences. The starvation of the plant for the aromatic amino acids needed to sustain protein synthesis is certainly a cause of plant death supported by slow activity of glyphosate. However, it is possible increased carbon flow to the shikimate pathway leads to changes in carbon allocation from photosynthesis and subsequent carbon shortages from other pathways (Servaites et al., 1987; Siehl, 1997).

Resistance to Glyphosate

In contrast to the sulfonylurea herbicides where resistance was very fast to evolve (i.e., five years after initial usage), glyphosate resistance in weeds has developed at a much slower pace (Bradshaw et al., 1997). In fact, it was first presumed evolved resistance to glyphosate would be highly unlikely due to high fitness costs of altered EPSP synthase being involved in synthesizing numerous compounds (Bradshaw et al., 1997; Jasieniuk, 1995; Waters, 1991). Glyphosate, the world's most important herbicide active ingredient, has been widely used since 1974. In a review of glyphosate resistance in 1997, there were no known reports of weed resistance to glyphosate (Bradshaw et al., 1997). However, one year later, glyphosate resistance was confirmed in rigid ryegrass in Australia (Powles et al., 1998; Pratley et al., 1999), and since, resistance to glyphosate has risen with reports of resistance in at least 30 weed species worldwide (Heap, 2014).

The major contributing factor to glyphosate resistance is the rise in use of genetically modified crops tolerant to glyphosate. The development of glyphosate resistance in crop species was largely unsuccessful until the discovery of the *CP4* gene from *Agrobacterium* species. This gene encodes a glyphosate resistant form of EPSP synthase (Padgette et al., 1996). Genome insertion of the *CP4* gene plus a promoter into certain crop species generates high levels of glyphosate resistance. Six genetically modified glyphosate resistant crop species are currently deregulated for use in the United States. Of these, only corn, canola, soybean (*Glycine max*), and cotton (*Gossypium* spp.) are currently being grown (Duke and Powles, 2008). In 2006, 90% of soybean, 75% of canola, and 70% of cotton grown in the United States were glyphosate resistant (Duke

and Powles, 2008; Gianessi, 2005). Owing to its nonselective activity, glyphosate has almost extensively become the sole herbicide used for weed control in these crops, and this extreme selection for glyphosate resistant individuals, in addition to adoption of zero tillage, has increased the rate of resistance development. The majority of reports of evolved glyphosate resistance in weeds thus far have come from sites using glyphosate resistant crop species. This makes glyphosate resistance in annual bluegrass on golf courses a unique and alarming discovery.

Mechanisms of Glyphosate Resistance

Multiple mechanisms of resistance to glyphosate have been identified in various weed species which can be separated into target site and nontarget site mechanisms. These include reduced glyphosate translocation, target-site amino acid substitutions, EPSP synthase gene duplication, or glyphosate metabolism (Sammons and Gaines, 2014). Glyphosate's excellent broad spectrum, non-selective efficacy can be attributed to its systemic activity and high mobility in plants (Powles and Preston, 2006). Typically, glyphosate accumulates in plant sink tissues (e.g., roots, rhizomes) following patterns of photosynthate translocation (Arnaud et al., 1994; Bromilow et al., 1993). Alteration of normal glyphosate translocation patterns has resulted in glyphosate-resistant weeds. In fact, the first case of glyphosate resistance was due to alteration of glyphosate translocation in an Australian population of rigid ryegrass (Powles et al., 1998; Pratley et al., 1999). Lorraine-Colwill et al. (2002) determined glyphosate applied to susceptible plants accumulated in lower plant parts compared to resistant plants, where glyphosate

accumulated in leaf tips with minimal root translocation. Additional studies of four other rigid ryegrass populations revealed a similar resistance mechanism (Wakelin et al., 2004). Additional evidence of reduced translocation as the resistance mechanism in rigid ryegrass came from experiments showing shikimate accumulation in glyphosate treated plants (Lorraine-Colwill et al., 1999). Both susceptible and resistant plants accumulated shikimate, indicating EPSP synthases of resistant plants were susceptible to inhibition by glyphosate and glyphosate was rapidly moved away from the target site. Populations of horseweed [*Conyza canadensis* (L.) Cronq.] have also been confirmed to resist glyphosate by a reduced translocation mechanism. Experiments revealed no differences in shikimate accumulation after glyphosate applications or glyphosate absorption. However, translocation of glyphosate out of the treated leaf into roots was significantly reduced (Feng et al., 2004; Koger et al., 2005; Koger and Reddy, 2005; Mueller et al., 2003). An additional nontarget site glyphosate resistance mechanism in horseweed was recently identified (González-Torralva et al., 2012). Rates of glyphosate metabolism in a resistant horseweed biotype were very fast, with nearly all applied glyphosate being converted into glyoxylate, sarcosine, and aminomethylphosphonic acid within four days of treatment. In contrast, nearly all glyphosate applied to a susceptible biotype was recovered without being metabolized into other compounds.

Several target site mutations of the EPSP synthase enzyme are known to cause resistance. In all reported cases, a mutation at the Pro_{106} amino acid residue has resulted in glyphosate resistance, where Ser, Thr, Ala, or Leu substitutions can confer resistance. This was first reported in goosegrass (*Eleusine indica* L.) in Malaysia (Lee and Ngim,

2000), but has since been confirmed in other weed speices. These target site mutations do not result in extremely high levels of glyphosate resistance (Powles and Yu, 2010). In addition, overexpression of the EPSP enzyme can result in glyphosate resistance. Gruys et al. (1999) reported an increase in gene transcription resulted in twice the normal amount of EPSP synthase which has conferred glyphosate resistance. Other documentation has shown up to 40 times the normal amount of EPSP synthase can exist from a 100-fold gene expression (Gaines et al., 2011). Palmer amaranth (*Amaranthus palmeri* S. Watson) is a significant problem in the southeastern United States in cotton, peanut (*Arachis hypogaea*), and soybean crops (Culpepper et al., 2006) and is the most widespread glyphosate resistance problem in South Carolina (Heap, 2014). Overexpression of the EPSP synthase gene is likely the resistance mechanism of Palmer amaranth, and confers a higher level of resistance than the target site mutations at Pro_{106}.

There is currently no evidence to suggest any higher plant species with EPSP synthase enzymes having natural resistance (i.e., tolerance/selectivity) to glyphosate. Some plant species, and even biotypes within a species, differ in their level of susceptibility to glyphosate, but these are due to physiological or biochemical mechanisms other than the EPSP synthase enzyme (Duke and Powles, 2008). For example, control of morningglory (*Ipomoea* spp.) with glyphosate is often poor and highly variable (Burke et al., 2009; Norsworthy et al., 2001; Shaw and Arnold, 2002). Additionally, Bryson and Will (1985) demonstrated differing susceptibilities to glyphosate of several bermudagrass biotypes.

Although glyphosate usage in established turfgrass stands is limited due to its nonselectivity, populations of annual bluegrass resistant to glyphosate in Tennessee (Brosnan et al., 2012a) and Missouri (Binkholder et al., 2011) have been reported. Several golf course superintendents in South Carolina have recently reported poor control of annual bluegrass with glyphosate after many years of consecutive applications. The mechanism of glyphosate resistance has been extensively studied in other weed species, but never in annual bluegrass. Thus, the purpose of this study was to confirm glyphosate resistance in an annual bluegrass biotype from a South Carolina golf course, quantify the level of resistance in this biotype, and use shikimate accumulation assays, ^{14}C-glyphosate uptake and translocation, and EPSP synthase gene sequencing to determine the mechanism conferring resistance.

Materials and Methods

Plant Material. Plants of a suspected glyphosate resistant annual bluegrass biotype were collected from common bermudagrass golf course fairways at Cherokee National Golf Club (CN; Gaffney, SC) in 2011 using a golf course cup cutter. These areas had been treated with glyphosate for at least ten consecutive years (T. Roberson, personal communication). A susceptible annual bluegrass biotype was obtained from a commercial source (VS; Valley Seed Service, Fresno, CA) and used for comparison. This biotype was used in previous research studies investigating resistance to ALS-inhibiting herbicides and is a true annual biotype selected for seed production (McElroy et al., 2002). Suspected resistant plants were potted in a commercial potting mix and grown to

seed in an isolated greenhouse to prevent cross-pollination. Mature seed (characterized by brown/tan seed color and absence of flowers) was collected and stored at -20^{o} C until sowing.

Whole Plant Dose-Response. Annual bluegrass seeds from both biotypes were sown in polypropylene containers containing a commercial potting mix and maintained in a greenhouse with average day/night temperatures of 21/15^{o} C under natural light (average intensity of 700 µmol m^{-2} s^{-1}). After germination, plants were watered every 48 to 72 h to prevent moisture stress. At 2-3 tiller stage, annual bluegrass plants were treated with glyphosate (Roundup ProMax® Herbicide, Monsanto Company, St. Louis, MO). No additional surfactant was added. Glyphosate rates were 0, 0.026, 0.0525, 0.105, 0.21, 0.42, 0.84, 1.68, 3.36 and 6.72 ka ae ha^{-1}. The typical field rate of glyphosate for annual bluegrass control in dormant bermudagrass is 0.42 kg ae ha^{-1}. Herbicide application was conducted in an enclosed spray chamber calibrated to deliver 187 L ha^{-1} through an 8001E flat fan nozzle.

Following glyphosate application, plants were immediately returned to the greenhouse and maintained under the same growth conditions as previously described. Twenty-eight DAT, above-ground biomass was harvested, dried at 80^{o} C for 72 h, and weighed. Each treatment contained three replicate plants of each biotype and the study was repeated.

Leaf Segment Shikimate Accumulation Acid Assay. The effect of glyphosate on shikimate accumulation in annual bluegrass was investigated according to the methods of Shaner et al. (2005) with modifications. Annual bluegrass seeds of each biotype were

sown as described for whole plant dose-response studies and maintained in a growth chamber with day/night temperatures of 21/12 ^{o}C and a 12 h photoperiod (500 µmol m^{-2} s^{-1}). Plants were thinned to one per pot. At the 1-2 tiller stage, the first and second leaves of each tiller were removed, cut into small sections (~0.5 cm), and pooled. Approximately 100 mg fresh tissue were placed into 20 mL glass vials containing 1 mL 10 mM $NH_4H_2PO_4$ (pH 4.4) and 0.1% v v^{-1} polysorbate surfactant (Tween^{TM} 80, Thermo Fisher Scientific Inc., Waltham, MA). Glyphosate (commercial formulation) was added to vials at concentrations of 0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1,000 µM. Vials were incubated in a growth chamber at 25 ^{o}C for 16 h under 500 µmol m^{-2} s^{-1}. After incubation, 250 µL 1.25 N HCl was added to each vial (final concentration of 0.25 N), then stored at -20^{o} C for approximately 24 h. Shikimate accumulation was subsequently determined according to the methods of Cromartie and Polge (2000) with modifications. Tubes were removed from the freezer and thawed at room temperature for 30 min. A 50 µL aliquot from each vial was pipetted into a 2 mL microcentrifuge tube and 200 µL periodic acid and sodium metaperiodate (0.25% w v^{-1} each) was added to each tube. Tubes were incubated at room temperature for 90 min and 200 µL 0.6 N sodium hydroxide and 0.22 M sodium sulfite was added to each tube. Samples were measured in a spectrophotometer at 380 nm within 30 min. The reference absorbance was vials not containing glyphosate. A standard curve was developed using known concentrations of shikimate. Each glyphosate concentration contained three replicate plants of each biotype and the study was repeated.

Whole Plant Shikimate Accumulation Assay. The effect of glyphosate on shikimate accumulation over time on annual bluegrass was evaluated according to the methods of Perez-Jones et al. (2007). Annual bluegrass seeds were sown as previously described and maintained in a growth chamber with day/night temperatures of 21/12 ^{o}C and a 12 h photoperiod (900 µmol m^{-2} s^{-1}). Plants were thinned to one per pot. At the 2-3 tiller stage, plants were treated with glyphosate at 0.42 kg ae ha^{-1} using an enclosed spray chamber calibrated as previously described. Plants were immediately returned to the growth chamber. At 6, 24, 72, 120, and 168 hours after treatment (HAT), the first and second leaves of each tiller were harvested, cut into small sections (~0.5 cm), and pooled. Approximately 100 mg tissue was placed into 20 mL glass vials containing 1 mL 0.25 N HCl plus 0.1% v v^{-1} polysorbate surfactant. Vials were stored at -20 ^{o}C for approximately 24 h. Shikimate accumulation was subsequently determined as described for the leaf segment assay. The reference absorbance was tissue from a nontreated plant at each harvest time. A standard curve was developed using known concentrations of shikimate. Each harvest time contained five replicate plants of each biotype and the study was repeated.

^{14}C-glyphosate Uptake and Translocation. Annual bluegrass seeds of both biotypes were sown in polypropylene containers with river sand and maintained in a growth chamber with day/night temperatures of 21/12 ^{o}C and a 14 h photoperiod (500 µmol m^{-2} s^{-1}). Pots were fertilized by sub-irrigation containing 3.8 mL L^{-1} hydroponic fertilizer (Grow Big® Hydroponic, FoxFarm Fertilizer, LLC, Samoa, CA) at 2 and 4 weeks after germination. Additional irrigation was supplied every 48 to 72 h to prevent moisture

stress. ^{14}C-glyphosate (phosphonomethyl-^{14}C; American Radiolabeled Chemicals, Inc., St. Louis, MO) treatment solution was prepared by combining nonlabeled and ^{14}C-glyphosate such that the nonlabeled glyphosate concentration was 2.25 mg ml^{-1} and ^{14}C-glyphosate concentration was 1 MBq ml^{-1}. This simulated a spray droplet from a 0.42 kg ae ha^{-1} application at 187 L ha^{-1}. At the 3-5 tiller growth stage, plants were pretreated with nonlabeled glyphosate at 0.42 kg ae ha^{-1} using an enclosed spray chamber (previously described). Thirty minutes following application, plants were transferred to a greenhouse and two 2.5 µL drops of treatment solution were applied to a mature, fully expanded leaf halfway between the leaf tip and base. At 24, 72, and 168 HAT, the treated leaf was removed and placed in a 15 mL centrifuge tube containing 5 mL water:methanol (1:1 v v^{-1}) solution. The tube was shaken for 30 s to remove any unabsorbed radioactivity. A 3 mL aliquot was transferred to a 20 mL scintillation vial containing 15 mL scintillation fluid (Optiphase HiSafe 3, PerkinElmer, Waltham, MA). Plants were further separated into nontreated shoots and roots. All samples were dried at 50 ^{o}C for 96 h, combusted in a OX-500 biological oxidizer (R.J. Harvey Instrument Corp., Tappan, NY), and analyzed using scintillation spectrometry. Following treatment with ^{14}C-glyphosate, plants were maintained under a 12 h photoperiod (450 µmol m^{-2} s^{-1}) with artificial high-pressure sodium light bulbs (HPS400, Sunlight Supply, Inc., Vancouver, WA). Day/night temperatures were 23/19 ^{o}C with 50% relative humidity. Each harvest time had five replicate plants of each biotype and the study was repeated.

EPSP Synthase Gene Sequencing. Total RNA was extracted from fresh tissue of mature (3-5 tiller) CN and VS plants using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA).

RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription kit (Qiagen). Polymerase chain reaction (PCR) was conducted to aplify a 374 bp section of the EPSPS gene using forward (5`TGTCCGAGGGAACAACTGTG-3`) and reverse (3`-ACGAACAGGTGGGCAGTTAG-5`) primers designed based on the annual bluegrass EPSP synthase mRNA sequence (S. Chen, personal communication). PCR reactions were conducted using an Arktik Thermal Cycler (Thermo Fisher Scientific, Inc., Waltham, MA) in a 50 µL volume with the Phusion High-Fidelity PCR Kit (New England Biolabs Inc., Ipswich, MA). Each reaction contained 50-100 ng cDNA, 10 mM dNTP mix, 10 µL 5x Phusion HF buffer, 0.5 µM each primer, and 1 unit Phusion DNA polymerase. The cycling program consisted of an initial denaturation step of 98 ^{o}C for 30 s, 35 cycles of 10 s at 98 ^{o}C, 30 s at 58 ^{o}C, and 30 s at 72 ^{o}C, with a final extension step at 72 ^{o}C for 7.5 min. PCR product was purified using PCR Kleen Spin Columns (BioRad Laboratories Inc., Hercules, CA) and sequenced by the Clemson University Genomics Institute using a the ABI 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA). Nucleotide sequences were analyzed using nucleotide (blastn) and amino acid (blastx) searches in the National Center for Biotechnology Information Basic Local Alignment Search Tool. As a means of replication, RNA was extracted from 5 nontreated VS plants and 5 CN plants surviving 0.42 kg ae ha^{-1} glyphosate application.

Statistical Design and Analysis. With the exception of EPSP synthase gene sequencing, the experimental design for all studies was a completely randomized design. Data from each study were subjected to ANOVA to evaluate main effects and interactions of

factors. Whole plant dose-response data were fit to a three parameter log-logistic equation:

$$y = C + (D-C)/(1+10^{x-\log GR_{50}}) \quad [4.1]$$

where y was shoot dry weight, x was glyphosate dose, D was the upper bound of y, and C was the lower bound of y, and GR_{50} was the glyphosate concentration inhibiting dry weight 50%. Leaf segment shikimate accumulation data were fit to a three parameter sigmoidal regression equation

$$y = D/(1+e^{[-(x-I_{50})/b]}) \quad [4.2]$$

where y was shikimate concentration, D was the upper bound of y, x was glyphosate concentration, b was the slope of the curve around I_{50}, and I_{50} was the glyphosate concentration resulting in 50% EPSP synthase inhibition as a result of shikimate accumulation. For each analysis, assumptions such as equality of variance and normal distribution were evaluated and adjusted if needed. Comparisons between biotypes at different harvest times were conducted according to Student's *t*-test. Best-fit values of nonlinear regression parameters were compared using extra-sum-of-squares *F*-tests. Significant effects and differences were based on $P < 0.05$. Nonlinear response modeling used Prism version 6.0 and ANOVA and *t*-tests used SAS version 9.3.

Results and Discussion

Whole Plant Dose-Response. ANOVA revealed no treatment-by-experiment interaction, thus, data were combined across experiments and further analyzed using nonlinear regression (Table 3.1). The whole plant dose-response study revealed GR_{50} values of 0.18

Table 3.1. ANOVA for whole plant dose-response to glyphosate.

Source	df	Dry Weight (% of nontreated)
Experiment	1	NS
Biotype	1	*
Rate	9	*
Experiment*Biotype	1	*
Experiment*Rate	9	NS
Biotype*Rate	9	*
Experiment*Biotype*Rate	9	NS
Block	2	NS
Error	79	

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

kg ae glyphosate ha^{-1} for the VS biotype and 0.81 kg ae glyphosate ha^{-1} for the CN biotype ($P < 0.0001$; Figure 3.2). This resulted in a 4.4-fold resistance at the whole plant level. This level of glyphosate resistance is less than other glyphosate-resistant annual bluegrass biotypes. Brosnan et al. (2012a) indicated a 12-fold level of resistance in a biotype from Tennessee while a biotype from Missouri exhibited a 5.2-fold resistance (Binkholder et al., 2011). This suggests mechanisms of resistance could differ amongst these annual bluegrass populations. Other monocotyledonous weed species range from 2- to 20-fold resistant to glyphosate, but it should be noted that differing mechanisms of resistance result in variable levels of resistance. Target-site mutations generally conferr a weaker level of resistance (e.g., 2 to 5-fold) compared to reduced translocation (e.g., 8 to 12-fold) (Sammons and Gaines, 2014). A typical field rate of glyphosate for annual bluegrass control in dormant warm-season turfgrasses is 0.42 kg ae ha^{-1}. At this rate, dose-response curves revealed dramatic reductions in dry weight of the S biotype (78%), while only a 17% reduction was observed in the R biotype. Furthermore, the R biotype

consistently survived applications of 3.36 kg ae ha^{-1} 28 DAT while all S plants were controlled with rates above 0.42 kg ae ha^{-1}. These results confirm glyphosate resistance in the R biotype.

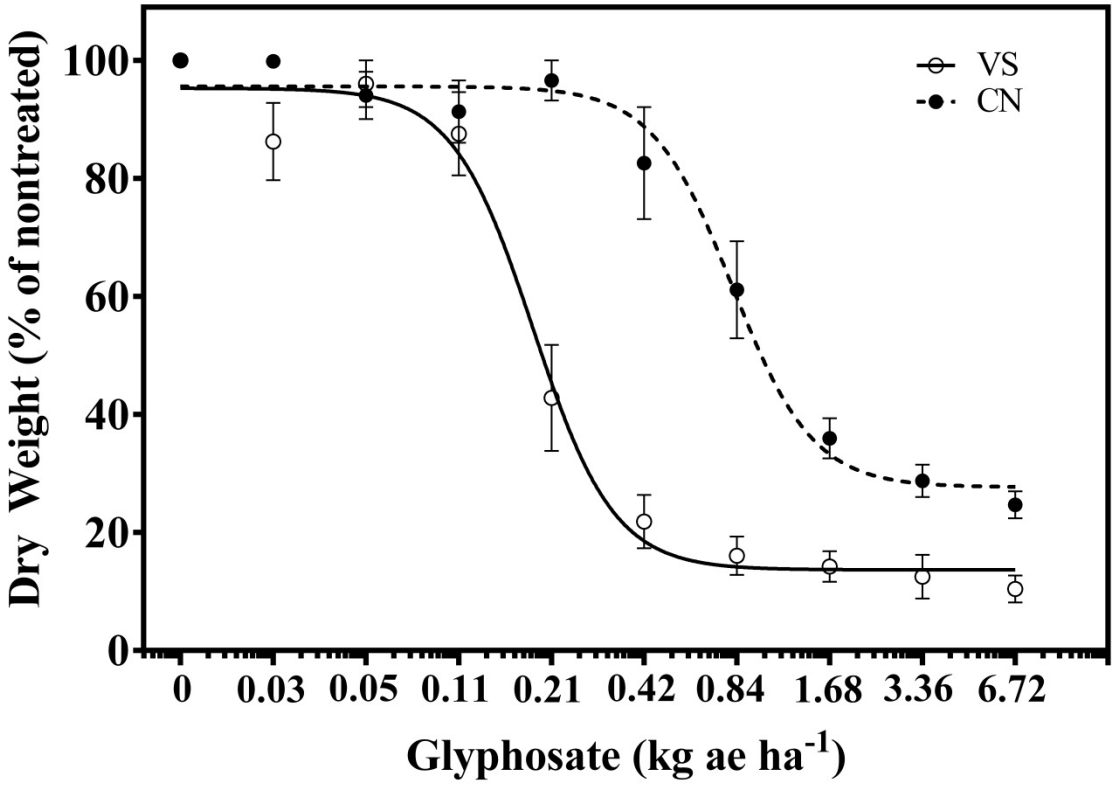


Figure 3.2. Effect of glyphosate on dry weight of glyphosate-susceptible (VS; solid line, open circles) and resistant (CN; dashed line, closed circles) annual bluegrass biotypes. Vertical bars represented standard errors of the mean (n=6). Nonlinear regression analysis was performed using the equation $y = C + (D\text{-}C)/(1+10^{x\text{-}\log GR_{50}})$, where y was dry weight (% of nontreated), x was glyphosate dose (kg ae ha^{-1}), D was the upper bound of y, C was the lower bound of y, and GR_{50} was the glyphosate dose reducing dry weight 50%. Mean and 95% confidence interval estimates for the GR_{50} were 0.18 and (0.16, 0.21) kg ae glyphosate ha^{-1} for the VS biotype and 0.81 and (0.68, 0.97) kg ae glyphosate ha^{-1} for the CN biotype. GR_{50} estimates between biotypes were significantly different ($P < 0.0001$) according to the extra sum-of-squares F-test.

Leaf Segment Shikimate Accumulation Assay. ANOVA revealed no significant treatment-by-experiment interaction, thus, data were combined across experiments for nonlinear regression analysis (Table 3.2). Both VS and CN biotypes accumulated shikimate as glyphosate concentration increased (Figure 3.3). The extra-sum-of-squares

Table 3.2. ANOVA for shikimate accumulation in leaf segment assay.

Source	df	Shikimic acid (mg g^{-1} fresh weight)
Biotype	1	*
Concentration	8	*
Experiment	1	NS[a]
Biotype*Concentration	8	*
Biotype*Experiment	1	*
Concentration*Experiment	8	*
Biotype*Concentration*Experiment	8	NS
Error	72	

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

Table 3.3. Shikimate accumulation (± standard error) in the leaf segment assay as affected by glyphosate concentration for glyphosate-susceptible (VS) and resistant (CN) annual bluegrass biotypes.

Glyphosate Concentration (µM)	VS	CN	VS vs. CN
	---shikimate (mg g^{-1} fresh weight)---		
3.9	0.009 ± 0.006	0.014 ± 0.007	NS[a]
7.8	0.010 ± 0.005	0.014 ± 0.008	NS
15.6	0.031 ± 0.009	0.026 ± 0.012	NS
31.25	0.036 ± 0.014	0.053 ± 0.024	NS
62.5	0.135 ± 0.011	0.082 ± 0.013	*
125	0.223 ± 0.022	0.116 ± 0.024	*
250	0.291 ± 0.016	0.145 ± 0.017	*
500	0.306 ± 0.023	0.187 ± 0.019	*
1000	0.332 ± 0.013	0.209 ± 0.030	*

[a] Abbreviations: NS, not significant.
*Indicates a significant difference between VS and CN biotypes according to Student's *t*-test at $P < 0.05$.

F-test for comparison of I_{50} between biotypes was not significant (P = 0.1563). However, it was apparent less shikimate accumulated in the R biotype at higher concentrations of glyphosate. Nonlinear regression analysis revealed I_{50} values of 23.15 µM glyphosate for the VS biotype and 80.75 µM glyphosate for the CN biotype, indicating a 3.5-fold resistance level for shikimate accumulation in the CN biotype. Shikimate accumulation

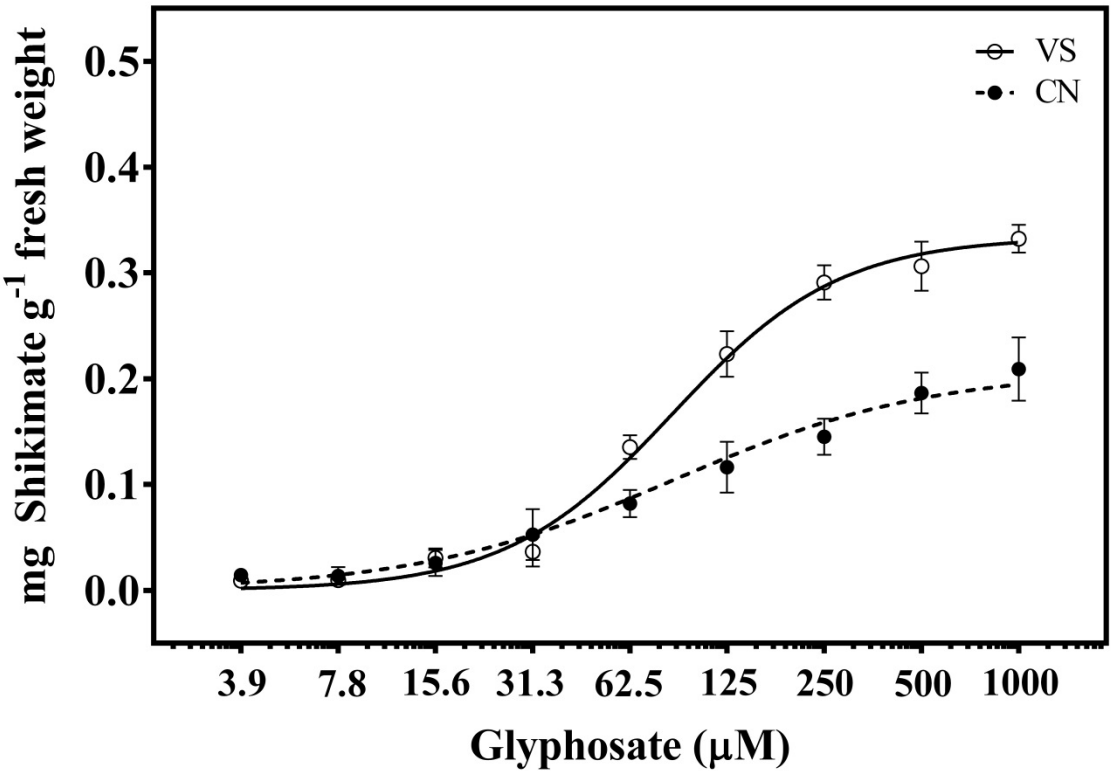


Figure 3.3. Effect of glyphosate concentration on shikimate accumulation in glyphosate-susceptible (VS; solid line, open circles) and resistant (CN; dashed line, closed circles) annual bluegrass biotypes. Vertical bars represented standard errors of the mean (n=6). Nonlinear regression analysis was performed using the equation $y = D/(1+e^{[-(x-I_{50})/b]})$, where *y* was shikimate concentration, *x* was glyphosate concentration, *D* was the upper bound of y, *b* was the slope of the curve around I_{50}, and I_{50} was the glyphosate concentration resulting in 50% EPSP synthase inhibition as a result of shikimate accumulation. Mean I_{50} estimates ± standard error were 23.15 ± 8.4 µM for the VS biotype and 80.75 ± 57.3 µM for the CN biotype. I_{50} estimates were not significantly different (P = 0.1563) according to the extra sum-of-squares *F*-test.

was similar between biotypes for glyphosate concentrations up to 31.25 µM (Table 3.3). However, at concentrations of 62.5 µM and greater, the CN biotype accumulated less shikimate than the VS biotype. This confirms less sensitivity of EPSP synthase in the CN biotype.

Whole Plant Shikimate Accumulation Assay. ANOVA indicated significant main effects of biotype ($P = 0.0011$) and harvest time ($P < 0.0001$), and a significant interaction between biotype and harvest time ($P = 0.0013$; Table 3.4). Shikimate accumulation was similar between biotypes at 6 and 24 HAT, but the VS biotype accumulated more shikimate than the CN biotype at all other harvest times (Table 3.5, Figure 3.4). Regardless of biotype, greatest shikimate accumulation occurred 72 HAT with glyphosate. An increase in shikimate was observed in both biotypes until 72 HAT, but increases were much more subtle in the CN biotype. Although a decrease was observed in the VS biotype between 72 and 120 HAT, there was an additional increase between 120 and 160 HAT. In contrast, shikimate levels consistently decreased in the CN biotype 72 HAT.

Table 3.4. ANOVA for shikimate accumulation in whole plant assay.

Source	df	Shikimic acid (mg g^{-1} fresh weight)
Biotype	1	*
Harvest Time	4	*
Experiment	1	NS[a]
Biotype*Harvest Time	4	*
Biotype*Experiment	1	*
Harvest Time*Experiment	4	*
Biotype*Harvest Time*Experiment	4	NS
Error	76	

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

Table 3.5. Shikimate accumulation (± standard error) in the whole plant assay as affected by harvest time for glyphosate-susceptible (VS) and resistant (CN) annual bluegrass biotypes.

Harvest Time (HAT[a])	VS	CN	VS vs. CN
	--------shikimate (mg g^{-1} fresh weight)--------		
6	0.48 ± 0.04	0.38 ± 0.05	NS
24	0.85 ± 0.15	0.62 ± 0.11	NS
72	1.33 ± 0.20	0.71 ± 0.17	*
120	0.92 ± 0.16	0.43 ± 0.10	*
168	1.11 ± 0.14	0.28 ± 0.10	*

[a] Abbreviations: HAT, hours after treatment; NS, not significant.
*Indicates a significant difference between VS and CN biotypes according to Student's *t*-test at $P < 0.05$.

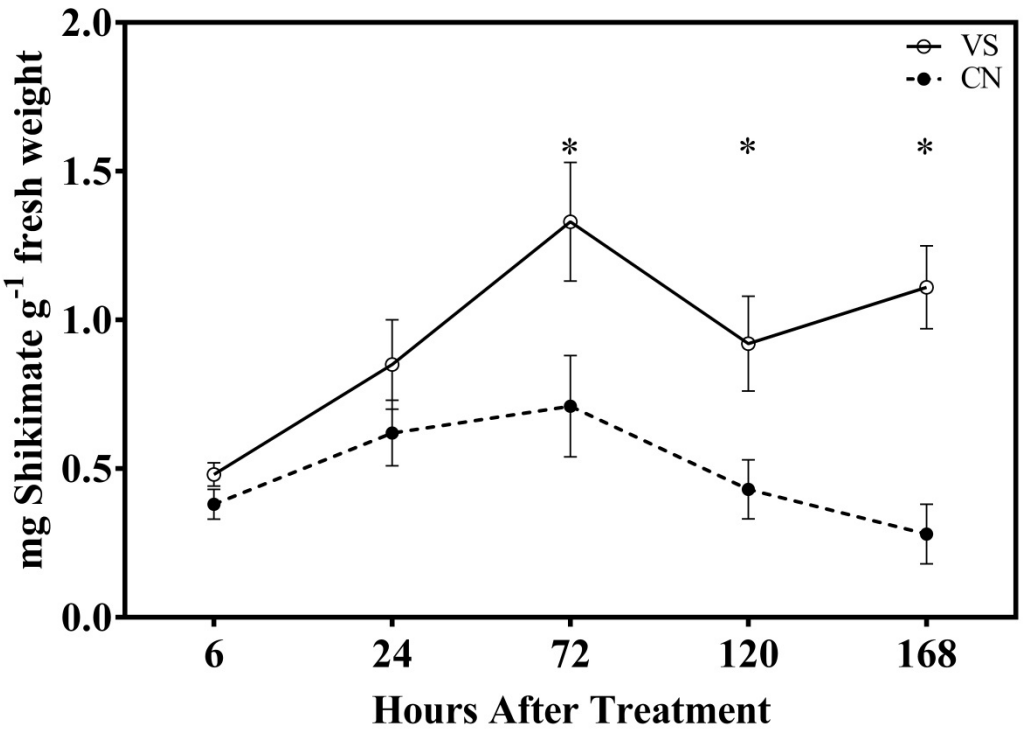


Figure 3.4. Shikimate accumulation of glyphosate-susceptible (VS; solid line, open circles) and resistant (CN; dashed line, closed circles) annual bluegrass biotypes as affected by time. Vertical bars represent standard errors of the mean (n=10). Asterisks indicate a significant difference between biotypes within a harvest time according to Student's *t*-test at $P < 0.05$.

^{14}C-glyphosate Uptake and Translocation. A significant main effect of harvest time on percent ^{14}C-glyphosate leaf uptake was observed, but there was no main effect of biotype or interaction between harvest time and biotype (Table 3.6). Across all biotypes and harvest times, ^{14}C-glyphosate leaf uptake ranged from 24 to 34% of applied radioactivity (Figure 3.5). Regardless of biotype, leaf uptake was greater at 72 and 168 HAT compared to 24 HAT.

Table 3.6. ANOVA for ^{14}C-glyphosate uptake and translocation experiment.

		% ^{14}C-glyphosate			
		Absorption	Translocation		
Source	df		Treated Leaf	Shoots	Roots
Experiment	1	NS	NS	NS	NS
Biotype	1	NS	*	*	*
Harvest Time	2	*	*	*	*
Experiment*Biotype	1	NS	NS	NS	NS
Experiment*Harvest Time	2	NS	NS	NS	NS
Biotype*Harvest Time	2	NS	NS	NS	NS
Experiment*Biotype*Harvest Time	2	NS	NS	NS	NS
Error	48				

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

The patterns of ^{14}C-glyphosate translocation were different between biotypes and harvest times, but there were no significant interactions (Table 3.6). Over time, more ^{14}C-glyphosate was translocated out of the treated leaf and into roots in the CN biotype when compared with the VS biotype (Table 3.7, Figure 3.6). Differences were not observed between biotypes in any plant part 24 HAT (Table 3.7). At 72 HAT, ^{14}C-glyphosate in treated leaves remained similar between biotypes. However, compared to the VS biotype,

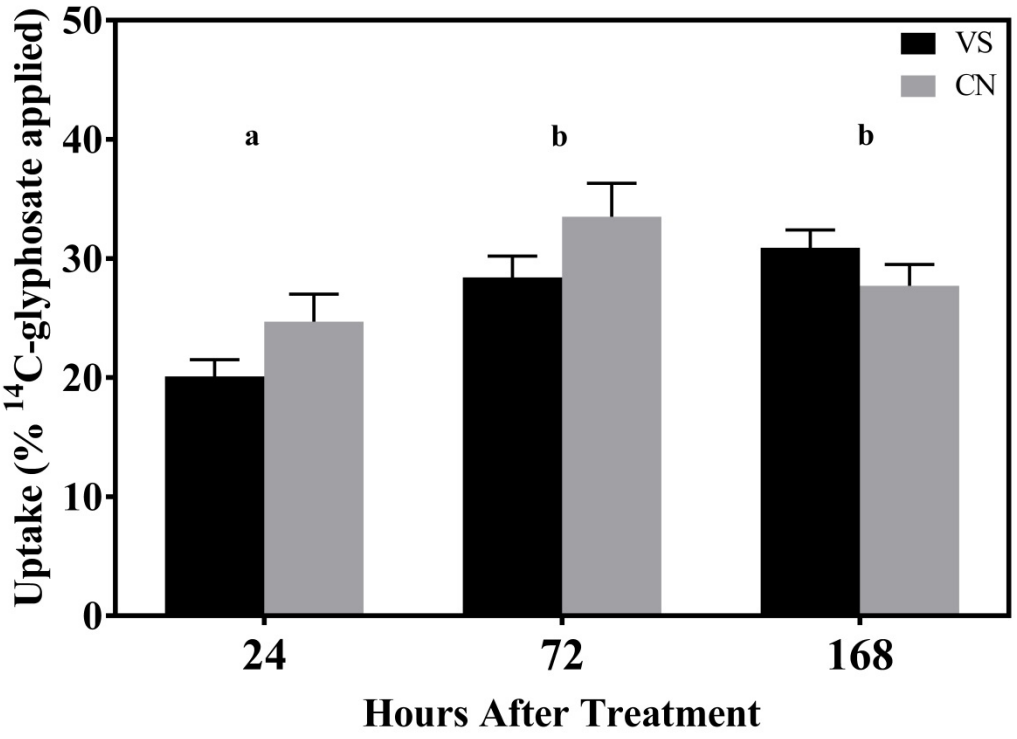
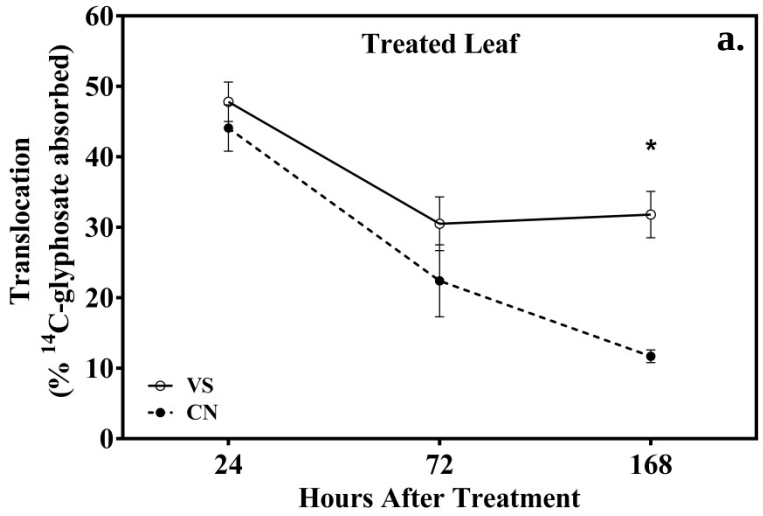
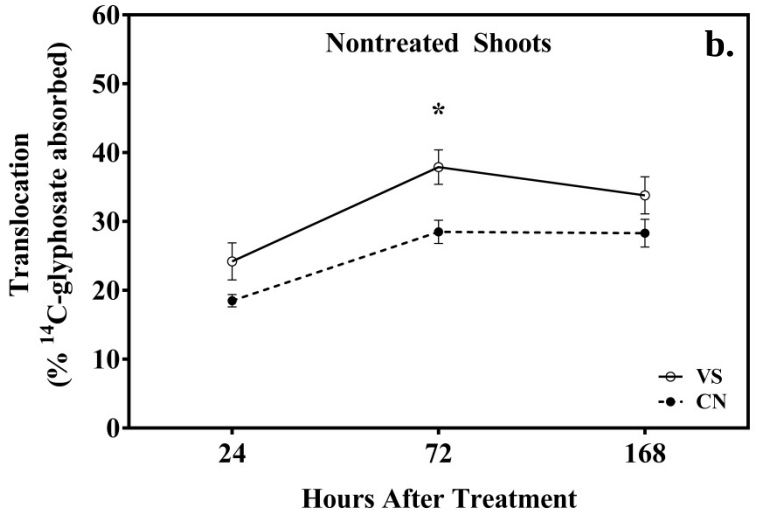


Figure 3.5. Uptake of ^{14}C-glyphosate as a percentage of applied radioactivity for glyphosate susceptible (VS) and resistant (CN) annual bluegrass biotypes. Harvest times not followed by the same letter indicate a significant difference according to Fisher's protected LSD at $P < 0.05$.

less radioactivity was recovered in the nontreated shoots (29 vs. 38%) and more radioactivity was recovered in the roots of the CN biotype (49 vs. 32%). At 168 HAT, similar levels of radioactivity were recovered in the nontreated shoots of both biotypes. However, less radioactivity was detected in the treated leaf (12 vs. 32%) and more radioactivity was recovered in the roots (60 vs. 34%) of the CN biotype. These results indicate translocation of ^{14}C-glyphosate continued (phloem transport to sink tissues) in the CN biotype, whereas translocation diminished in the VS biotype at 72 HAT (Figure 3.6).





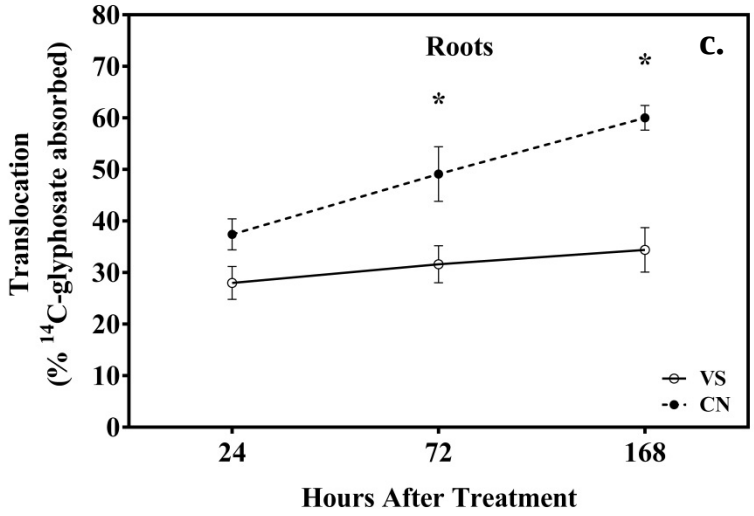


Figure 3.6. ^{14}C-glyphosate translocation in (a) treated leaves, (b) nontreated shoots, and (c) roots for a glyphosate-susceptible (VS; solid line, open circles) and resistant (CN; dashed line, closed circles) annual bluegrass biotypes. Asterisks indicate a significant difference between biotypes at a particular harvest time according to Student's *t*-test at $P < 0.05$.

Table 3.7. ^{14}C-glyphosate translocation and distribution in treated leaves, nontreated shoots, and roots in susceptible (VS) and resistant (CN) annual bluegrass biotypes.

		Harvest Time (Hours After Treatment)		
Plant Part	Biotype	24[a]	72	168
		----------% of absorbed[b]----------		
Treated Leaf	VS	47.8 ± 2.8 a	30.5 ± 3.8 a	31.8 ± 3.3 a
	CN	44.1 ± 3.3 a	22.4 ± 5.1 a	11.7 ± 0.9 b
Nontreated Shoots	VS	24.2 ± 2.7 a	37.9 ± 2.5 a	33.8 ± 2.7 a
	CN	18.5 ± 0.9 a	28.5 ± 1.7 b	28.3 ± 2.0 a
Roots	VS	28.0 ± 3.2 a	31.6 ± 3.6 a	34.4 ± 4.3 a
	CN	37.4 ± 3.0 a	49.1 ± 5.3 b	60.0 ± 2.4 b

[a] Values within a column (harvest time) and row (plant part) not followed by the same letter are significantly different according to Student's *t*-test at $P < 0.05$.
[b] Data are means ± standard error of 5 replicate plants of each biotype over two experiments.

EPSP Snythase Gene Sequencing. A 374 bp region surrounding the Pro_{106} codon on the EPSP synthase gene was amplified to determine if a target-site mutation confers glyphosate resistance in the CN biotype. cDNA from 5 nontreated VS plants and 5 CN plants surviving 0.42 kg ae ha^{-1} glyphosate application were sequenced. Sequences of each plant are presented in Figure 3.7 and compared to annual bluegrass mRNA sequence (S. Chen, personal communication). Sequences were subjected to blastn and blastx searches and aligned with nucleotide and protein sequences of Johnsongrass [*Sorghum halepense* (L.) Pers.] (NCBI accession KC914621.1), Italian ryegrass (*Lolium multiflorum* Lam.; NCBI accession DQ153168.2), and mouse-ear c ress (NCBI accession NM_130093.2) (Figures 3.8 and 3.9). Sequences of VS and CN biotypes were very similar but revealed double peaks at 6 locations (Figure 3.10). Only two of the double peaks had potential to result in missense mutations. At amino acid position 85 (numbered

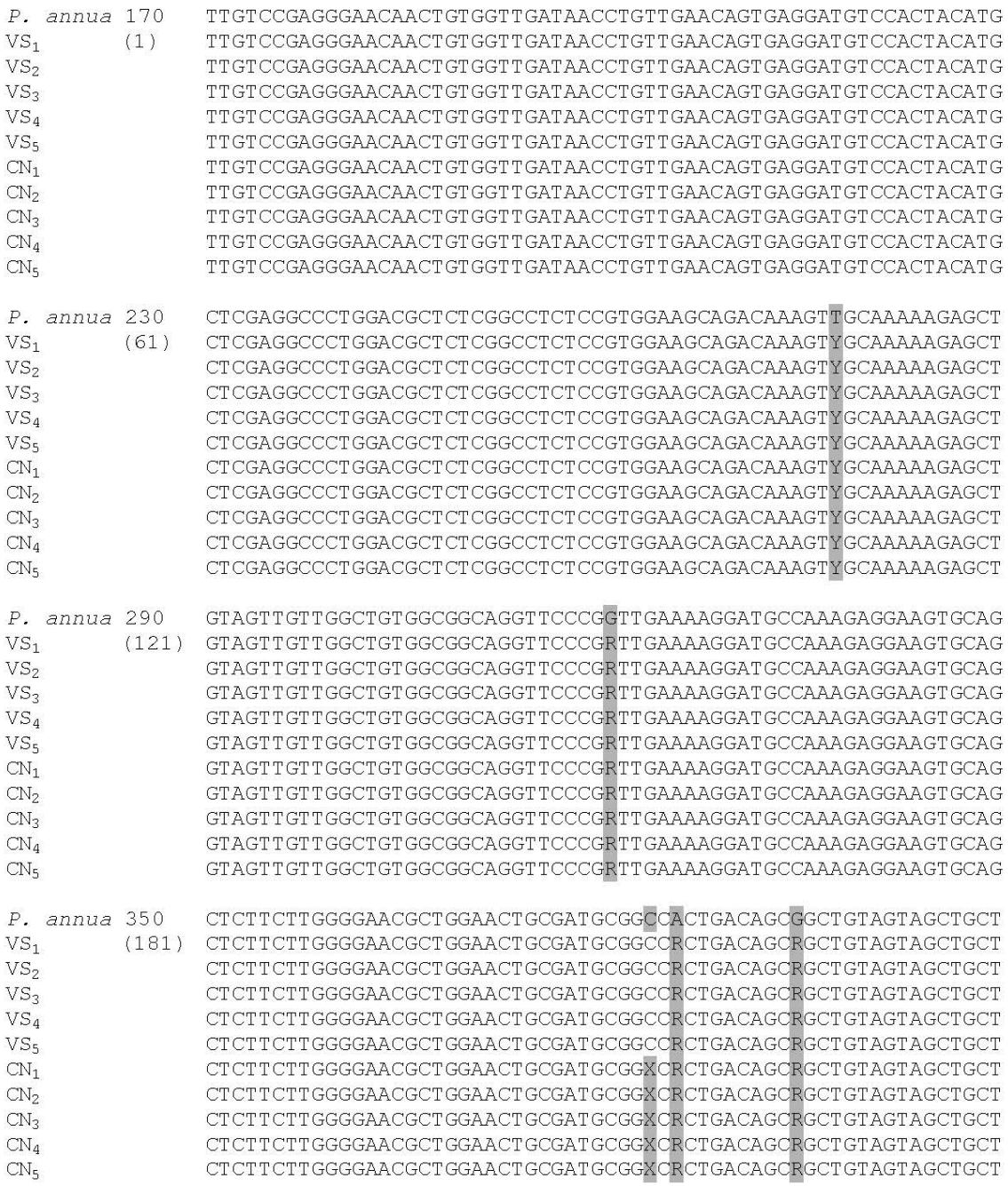


Figure 3.7. Nucleotide sequences of a 374 bp section surrounding the Pro_{106} codon of the EPSP synthase gene of five susceptible (VS) and five resistant (CN) annual bluegrass plants. Sequences were aligned with annual bluegrass EPSP synthase mRNA sequence (S. Chen, personal communication). Shaded regions indicate double peaks in sequences. Numbers not in parentheses correspond to the nucleotide base in the annual bluegrass mRNA sequence and numbers in parentheses correspond to the nucleotide base of the sequenced portion of the EPSP synthase gene. Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; R, A or G; Y, C or T; X, C or G.

```
P. annua 410    GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
VS1      (241)  GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
VS2             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
VS3             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
VS4             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
VS5             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
CN1             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
CN2             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
CN3             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
CN4             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
CN5             GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT

P. annua 470    GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTCGATTGTTTCCTTGGCACTAAC
VS1      (301)  GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
VS2             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
VS3             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
VS4             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
VS5             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
CN1             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
CN2             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
CN3             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
CN4             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
CN5             GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC

P. annua 540    TGCCCACCTGTTCG
VS1      (361)  TGCCCACCTGTTCG
VS2             TGCCCACCTGTTCG
VS3             TGCCCACCTGTTCG
VS4             TGCCCACCTGTTCG
VS5             TGCCCACCTGTTCG
CN1             TGCCCACCTGTTCG
CN2             TGCCCACCTGTTCG
CN3             TGCCCACCTGTTCG
CN4             TGCCCACCTGTTCG
CN5             TGCCCACCTGTTCG
```

Figure 3.7 continued.

according to *A. thaliana*), both VS and CN biotypes contained double peaks with the initial codon predicted as G or A.This amino acid position is thus transcribed by GTT (valine) or ATT (isoleucine). Both *S. halepense* and *L. multiflorum* contain isoleucine at position 85 while *A. thaliana* contains alanine (Figure 3.9). There are no reports of mutations at this amino acid residue conferring glyphosate resistance, and both VS and CN biotypes contain this double peak. In contrast, only the CN biotype contained a

```
VS (P. annua)          TTGTCCGAGGGAACAACTGTGGTTGATAACCTGTTGAACAGTGAGGATGTCCACTACATG
CN (P. annua)          TTGTCCGAGGGAACAACTGTGGTTGATAACCTGTTGAACAGTGAGGATGTCCACTACATG
S. halepense           TTGTCCGAGGGAACAACGGTGGTGGATAACCTGTTGAACAGTGAGGATGTCCACTACATG
L. multiflorum         TTGTCCGAGGGAACAACGGTCGTGGATAACCTGTTGAACAGTGAGGATGTCCACTACATG
A. thaliana      538   CTGTCTGAGGGAACAACTGTAGTGGACAACTTGTTGAATAGCGATGACATCAATTACATG 597

VS (P. annua)          CTCGAGGCCCTGGACGCTCTCGGCCTCTCCGTGGAAGCAGACAAAGTYGCAAAAAGAGCT
CN (P. annua)          CTCGAGGCCCTGGACGCTCTCGGCCTCTCCGTGGAAGCAGACAAAGTYGCAAAAAGAGCT
S. halepense           CTCGAGGCCCTGGATGCTCTCGGACTCTCCGTGGAAGCAGATAAAGTTGCAAAAAGAGCT
L. multiflorum         CTCGAGGCCCTGGACGCCCTCGGGCTCTCTGTGGAAGCAGACAAAGTTGCAAAAAGAGCT
A. thaliana      598   CTTGATGCGTTGAAGAGATTGGGACTTAATGTGGAAACTGACAGTGAAAATAATCGTGCT 657

VS (P. annua)          GTAGTTGTTGGCTGTGGCGGCAGGTTCCCGRTTGAAAAGGATGCCAAAGAGGAAGTGCAG
CN (P. annua)          GTAGTTGTTGGCTGTGGCGGCAGGTTCCCGRTTGAAAAGGATGCCAAAGAGGAAGTGCAG
S. halepense           GTAGTTGTTGGCTGTGGCGGCAGGTTCCCGATCGAAAAGGATGCCAAAGAGGAAGTACAA
L. multiflorum         GTAGTCGTTGGCTGTGGCGGCAGGTTCCCGATTGAAAAGGATGCCAAGGAGGAAGTCAAG
A. thaliana      658   GTAGTTGAAGGATGTGGCGGGATATTCCCAGCTTCCATAGATTCAAAGAGTGATATCGAA 717

VS (P. annua)          CTCTTCTTGGGGAACGCTGGAACTGCGATGCGGCCRCTGACAGCRGCTGTAGTAGCTGCT
CN (P. annua)          CTCTTCTTGGGGAACGCTGGAACTGCGATGCGGXCRCTGACAGCRGCTGTAGTAGCTGCT
S. halepense           CTCTTCTTGGGGAACGCTGGAACTGCAATGCGGCCATTGACGGCTGCGGTAGTAGCAGCT
L. multiflorum         CTCTTCTTGGGCAACGCTGGAACTGCAATGCGGCCATTGACGGCTGCTGTAGTAGCTGCT
A. thaliana      718   CTTTACCTCGGTAATGCAGGAACAGCAATGCGTCCACTTACCGCTGCGGTCACTGCTGCA 777

VS (P. annua)          GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
CN (P. annua)          GGTGGAAATGCAACATATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
S. halepense           GGTGGAAATGCAACTTATGTGCTTGATGGAGTACCAAGAATGAGGGAGCGACCCATTGGT
L. multiflorum         GGTGGAAATGCGACTTATGTTCTTGATGGAGTACCAAGAATGAGGGAGCGACCTACCGGT
A. thaliana      778   GGTGGAAACGCAAGTTATGTGCTTGATGGGGTGCCTCGTATGAGAGAAAGACCTATAGGG 837

VS (P. annua)          GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
CN (P. annua)          GACTTAGTTGTCGGTTTGAAACAACTCGGTGCCGACGTYGATTGTTTCCTTGGCACTAAC
S. halepense           GACTTAGTTGTTGGTTTGAAACAACTCGGTGCAGACGTTGATTGTTTCCTTGGCACTGAC
L. multiflorum         GACTTAGTTGTCGGTTTGAAACAGCTAGGTGCGAATGTTGATTGTTTCCTTGGCACTGAC
A. thaliana      838   GATTTGGTTGTTGGTCTTAAGCAGCTTGGTGCTGATGTTGAATGTACTCTTGGAACTAAC 897

VS (P. annua)          TGCCCACCTGTTCG
CN (P. annua)          TGCCCACCTGTTCG
S. halepense           TGCCCACCCGTTCG
L. multiflorum         TGCCCACCTGTTCG
A. thaliana      898   TGCCCTCCTGTTCG
```

Figure 3.8. Consensus nucleotide sequences of a 374 bp portion of the EPSP synthase gene of glyphosate susceptible (VS) and resistant (CN) annual bluegrass biotypes. Sequences were aligned with *Sorghum halepense* (L.) Pers. (NCBI accession KC914621.1), *Lolium multiflorum* Lam. (NCBI accession DQ153168.2) and *Arabadopsis thaliana* (L.) Heynh. (NCBI accession NM_130093.2). Shaded regions indicate double peaks in annual bluegrass sequences. Nucleotide base numbers correspond to *A. thaliana* EPSP synthase. Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; R, A or G; Y, C or T; X, C or G.

double peak at Pro_{106} in the initial nucleotide position (predicted as C or G) which could result in a missense mutation. CCA transcribes proline while GCA transcribes alanine (Figure 3.10). Pro_{106} is transcribed by CCA for *S. halepense*, *L. multiflorum*, *A. thaliana*, and the VS annual bluegrass biotype (Figures 3.7 and 3.8). Although an additional double peak was observed in the third nucleotide of the Pro_{106} codon in both biotypes (predicted as either A or G), the amino acid transcribed at residue 106 is determined by the first two nucleotide positions. Therefore, either proline or alanine would still be transcribed regardless of which nucleotide is in the third position. This suggests two forms of the EPSP synthase gene in the CN biotype. The Pro_{106} to Ala substitution has been reported

```
                                                                  V/I
VS (P. annua)          LSEGTTVVDNLLNSEDVHYMLEALDALGLSVEADKVAKRAVVVGCGGRFP-EKDAKEEVQ
                                                                  V/I
CN (P. annua)          LSEGTTVVDNLLNSEDVHYMLEALDALGLSVEADKVAKRAVVVGCGGRFP-EKDAKEEVQ
S. halepense           LSEGTTVVDNLLNSEDVHYMLEALDALGLSVEADKVAKRAVVVGCGGRFPIEKDAKEEVQ
L. multiflorum         LSEGTTVVDNLLNSEDVHYMLEALDALGLSVEADKVAKRAVVVGCGGRFPIEKDAKEEVK
A. thaliana      35    LSEGTTVVDNLLNSDDINYMLDALKRLGLNVETDSENNRAVVEGCGGIFPASIDSKSDIE 94

VS (P. annua)          LFLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGADVDCFLGTN
                                   P/A
CN (P. annua)          LFLGNAGTAMR-LTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGADVDCFLGTN
S. halepense           LFLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGADVDCFLGTD
L. multiflorum         LFLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRERPTGDLVVGLKQLGANVDCFLGTD
A. thaliana      95    LYLGNAGTAMRPLTAAVTAAGGNASYVLDGVPRMRERPIGDLVVGLKQLGADVECTLGTN 154

VS (P. annua)          CPPV
CN (P. annua)          CPPV
S. halepense           CPPV
L. multiflorum         CPPV
A. thaliana      155   CPPV
```

Figure 3.9. Amino acid sequence of a portion of the EPSP synthase protein of glyphosate susceptible (VS) and resistant (CN) annual bluegrass biotypes. Sequences were aligned with *Sorghum halepense* (L.) Pers. (NCBI accession KC914621.1), *Lolium multiflorum* Lam. (NCBI accession DQ153168.2) and *Arabadopsis thaliana* (L.) Heynh. (NCBI accession NM_130093.2). Shaded regions indicate potential missense mutations from heterozygous nucleotide bases. Amino acid numbers correspond to *A. thaliana* EPSP synthase.

in a South African *L. rigidum* population (Yu et al., 2007) and three Californian *L. multiflorum* populations (Jasieniuk et al., 2008).

Target-site mutations conferring glyphosate resistance have resulted in amino acid substitutions of Ser, Ala, or Thr (mutations at the first nucleotide) or Leu (a mutation at the second nucleotide) for Pro_{106} (Sammons and Gaines, 2014). Glyphosate acts as a non-competitive inhibitor of EPSP synthase. The herbicide binds to the EPSP synthase/

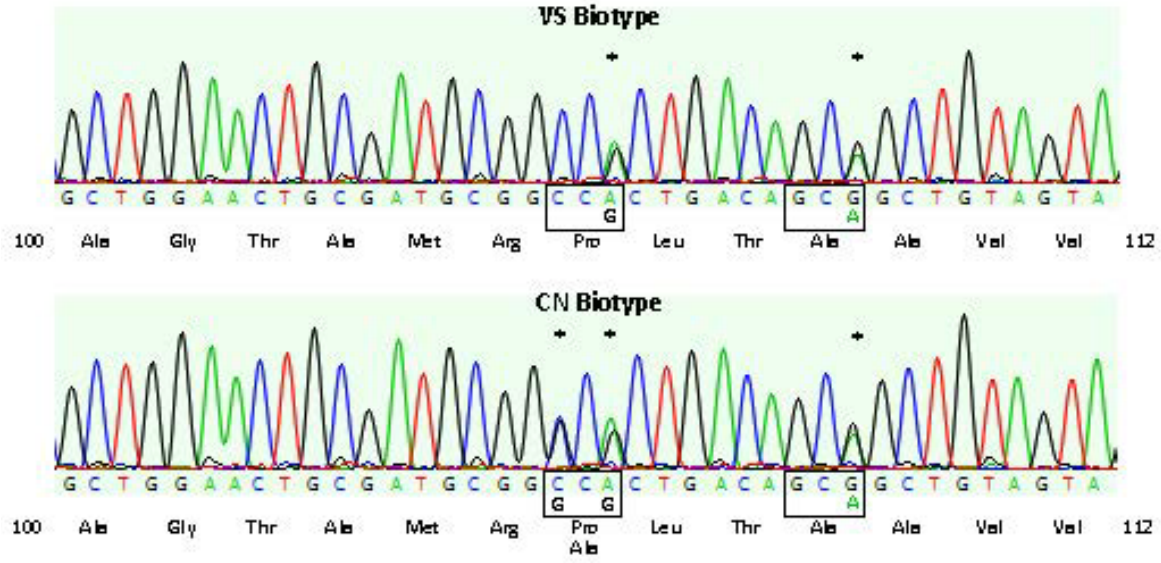


Figure 3.10. Chromatogram of nucleotide sequence surrounding Pro_{106} codon on EPSP synthase gene in glyphosate susceptible (VS) and resistant (CN) annual bluegrass biotypes. Codons with potential heterozygocity are contained in boxes with asterisks above double peaks. A double peak at the initial nucleotide base in the CCA codon (predicting C or G) indicates two EPSP synthase genes are present in the CN biotype. GCA transcribes Ala and would result in a Pro_{106} to Ala amino acid substitution conferring glyphosate resistance.

shikimate-3-phosphate complex inhibiting binding of the substrate PEP (Herrmann and Weaver, 1999). Glyphosate resistance due to target-site mutation was initially thought to be unlikely because alterations of EPSP synthase which reduced glyphosate activity also resulted in reduced enzyme activity (Bradshaw et al., 1997). However, crystallization of *Escherichia coli* EPSP synthase revealed Pro_{106} substitutions change the PEP binding site subtly enough to preserve EPSP synthase activity while reducing glyphosate inhibition such that plants survive normal field rates (Healy-Fried et al., 2007). Other substitutions such as Gly_{101} and Thr_{102} have been investigated in *E. coli*. These result in higher levels of resistance than substitutions at Pro_{106}, but reduce EPSP synthase functionality (Eschenburg et al., 2002; Funke et al., 2009; Powles and Yu, 2010).

Interestingly, most target-site based glyphosate resistance has been reported in monocotyledonous species. Tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer.] was only recently the first dicotyledonous species reported with an altered target-site conferring glyphosate resistance (Bell et al., 2013; Nandula et al., 2013). However, populations of grassy weeds such as rigid ryegrass (Wakelin and Preston, 2006), Italian ryegrass (Perez-Jones et al., 2007), junglerice [*Echinochloa colona* (L.) Link] (Alarcón-Reverte et al., 2013), sourgrass [*Digitaria insularis* (L.) Mez ex Ekman] (de Carvalho et al., 2012) and goosegrass (Molin et al., 2013; Ng et al., 2003) have been reported to evolve target-site glyphosate resistance. In contrast to the many grassy weed species with evolved target-site resistance, many broadleaf weeds have evolved nontarget site glyphosate resistance from reduced glyphosate translocation.

Further investigation of different forms of EPSP synthase in the CN biotype were not performed. Annual bluegrass is an allotetraploid derived from progenitor species supina bluegrass and weak bluegrass (Mao and Huff, 2012) and might explain two forms of the EPSP synthase gene in the CN biotype. McElroy et al. (2013) observed double peaks in the sequence of an ALS-resistant annual bluegrass biotype and cloned the Trp_{574} region of the ALS gene. This resulted in two distinct ALS gene sequences suggesting ALS mRNA is derived from the progenitor genomes of annual bluegrass. Glyphosate resistant and susceptible populations are most likely homozygous for resistance and susceptibility (Lorraine-Colwill et al., 2001), therefore, resistance in the CN biotype is conferred by a mutation in one of the progenitor genomes.

A target-site mutation in the CN biotype can help explain the differences in glyphosate translocation observed between biotypes. In comparison with other glyphosate resistant weed species whose mechanism of resistance is reduced translocation, more ^{14}C-glyphosate remains in the treated leaf of resistant plants with minimal translocation to meristematic regions (i.e., young, actively growing roots and stems). For example, Wakelin et al. (2004) reported four different resistant populations of rigid ryegrass from Australia translocated more ^{14}C-glyphosate to the tips of treated leaves compared to susceptible populations but no differences in any population in the amount translocated to roots. Similar glyphosate translocation patterns have been reported in other glyphosate-resistant weed species including Italian ryegrass (Michitte et al., 2007) and horseweed (Feng et al., 2004).

In this study, greater translocation out of the treated leaf of the CN biotype was observed over time with higher amounts of radioactivity being recovered in the roots (Figure 3.6). These results can be supported by considering the self-limitation of glyphosate translocation. In susceptible plants, inhibition of EPSP synthase by glyphosate results in a diversion of carbon from the Calvin cycle (in the form of E4P) to S3P and an ultimate accumulation of shikimate. Therefore, a decline in photosynthesis can be observed several hours after glyphosate application (Geiger and Bestman, 1990). Although sufficient translocation to meristematic regions for plan control has already occurred, this decline in photosynthesis eventually translates to reduced phloem transport, and thus, reduced glyphosate translocation to sink tissues (Geiger and Bestman, 1990). The reduced inhibition of EPSP synthase by glyphosate as a result of the target site mutation in the CN biotype allows for continued carbon metabolism and allocation, and subsequently, phloem transport. By 168 HAT, only 11% of absorbed ^{14}C-glyphosate remained in the treated leaf of the CN biotype and 60% had translocated to root tissue (Table 3.7, Figure 3.6). Alarcón-Reverte et al. (2013) reported a trend toward greater glyphosate translocation out of treated leaves and into roots in a resistant jungelrice population containing a Pro_{106} to Ser amino acid substitution. Therefore, reduced glyphosate translocation does not contribute to glyphosate resistance in the CN annual bluegrass biotype.

The shikimate accumulation assays also support a target-site mutation as the mechanism of resistance in the CN biotype. When compared with the VS biotype, less shikimate accumulated in the CN biotype in the leaf segment assay at glyphosate

concentrations greater than 31.25 µM (Table 3.3). This indicates an EPSP synthase enzyme that is less susceptible to glyphosate. Although shikimate accumulated in both biotypes at the whole plant level, shikimate levels in the VS biotype were ≥1.9x the levels observed in the CN biotype after 24 HAT (Table 3.5, Figure 3.4). Furthermore, shikimate levels steadily decreased after 72 HAT in the CN biotype while this reduction was not consistently observed in the VS biotype (Figure 3.4). Similar results have been reported in other weed species with target-site mutations. Resistant populations of rigid ryegrass and junglerice with EPSP synthase mutations accumulated shikimate after glyphosate application, but did so in lower amounts compared to susceptible populations (Alarcón-Reverte et al., 2013; Wakelin and Preston, 2006).

There have been several reports of both target-site and non-target site glyphosate resistance mechanisms evolving in the same weed population. However, this was not observed in the CN biotype and provides an explanation of the lower levels of resistance compared to other weeds with the Pro_{106} to Ala amino acid substitution. For example, Yu et al. (2007) reported a rigid ryegrass population from South Africa with a Pro_{106} to Ala substitution was 14-fold resistant to glyphosate, but also exhibited reduced glyphosate translocation. In general, target-site mutations conferring glyphosate resistance result in lower levels of resistance than reduced translocation (Sammons and Gaines, 2014).

Conclusions

This represents the first report of glyphosate-resistant annual bluegrass in South Carolina and the first report of a Pro_{106} to Ala amino acid substitution as conferring

resistance in annual bluegrass. Further research may investigate other glyphosate-resistant annual bluegrass populations to determine if resistance mechanisms are associated with nontarget-site resistance. Few labeled herbicide mechanisms of action remain for annual bluegrass control in commercial turfgrass where resistance has not evolved. The low selection pressure of glyphosate applications in established turfgrass (i.e., one per year) demonstrates the propensity of annual bluegrass to evolve resistance after exposure to herbicides. Therefore, turfgrass managers must integrate both chemical and non-chemical control options to manage resistance evolution in this troublesome weed.

CHAPTER FOUR

MODELING HERBICIDE RESISTANCE IN GOLF COURSE POPULATIONS OF ANNUAL BLUEGRASS

Introduction

Cultural practices and herbicide use patterns for golf courses differ vastly from those for most cropping systems and influence herbicide resistance development. First, turfgrass stands are perennial in nature, and thus, species rotation and large scale soil disturbances (e.g., tillage) are mostly unavailable. In crops, tillage can delay herbicide resistance development, and the adoption of glyphosate-tolerant crops has favored conservation tillage which has been associated with increasing populations of resistant weeds (Neve et al., 2003b, Stanton et al., 2008). Second, differential management practices are required amongst distinct turf areas. For example, the most intensively maintained turf areas on golf courses are putting greens and tees which sustain higher levels of inputs compared to fairways and roughs. Third, other integrated weed management practices are more difficult to control in turfgrass systems. In crops, delayed crop sowing can be used as a weed management component, but this is not available in perennial turfgrass stands (Powles and Matthews, 1996, Neve et al., 2003b). Lastly, annual bluegrass often invades other C_3 grasses severely limiting herbicide options without damaging desirable species and often results in exclusive use of a single mechanism of action (McCarty, 2011).

The recent increase in herbicide resistant annual bluegrass populations on golf courses is alarming, especially when considering glyphosate. Use of glyphosate in

established turfgrass is limited because of its nonselectivity, and thus, can only be applied to desirable warm-season (C_4) turfgrasses during periods of complete winter dormancy. In most years, winter turf dormancy in the southeastern United States occurs in a relatively short time period allowing for only one annual glyphosate application. This is in contrast with glyphosate-tolerant crops (e.g., cotton, corn, soybean) where as many as five yearly glyphosate applications are made for broad-spectrum weed control (Norsworthy et al., 2007).

Herbicides which inhibit ALS, specifically sulfonylureas, have a variety of uses in warm-season turfgrass and control numerous troublesome weeds including annual bluegrass. Toler et al. (2007) reported greater than 95% annual bluegrass control with four sulfonylurea herbicides applied in February. Unfortunately, ALS-inhibitors comprise the greatest number of documented cases of resistance worldwide with confirmed resistance in 144 species (Heap, 2014). High specificity of target site, excellent efficacy against target weed species, and relatively long residual contribute to rapid resistance development (Tranel and Wright, 2002). Unlike glyphosate, bermudagrass and zoysiagrass (*Zoysia* spp.) are tolerant of most ALS-inhibitors which control annual bluegrass, and thus, multiple applications can be made in a single season increasing selection of resistant plants.

Simulation modeling has many practical applications including predicting and assessing risks of herbicide resistance. Such models have been previously produced for troublesome weed species such as Palmer amaranth, blackgrass (*Alopecurus myosuroides* Huds.), and rigid ryegrass to evaluate the effects of cropping practices and herbicide use

on the evolution of herbicide resistance (Cavan et al., 2000; Gustafson, 2008; Neve et al., 2003a, 2003b, 2010, 2011). Simulations provide a method of evaluating resistance risks without the need for field trials which are costly, highly variable, and require large-scale resources and long-term evaluation generally unavailable for turfgrass research applications (Neve et al., 2010; Renton et al., 2014). The value of simulation models depend on parameter values and require some knowledge of the true value of the parameters in field situations. While simulation models cannot solve herbicide resistance issues, they can provide valuable information for reducing risks via implementing alternative herbicide use strategies and/or cultural practices and can reveal needs for future research.

The recent increase in herbicide resistant weed populations in golf course turf, led by annual bluegrass, requires an immediate response to understand and mitigate risks and develop proactive approaches to manage resistance. As turfgrass weed dynamics are vastly different from typical cropping systems, a basic simulation model was developed to evaluate annual bluegrass herbicide resistance evolution. The purpose of the model was to (1) understand key biological parameters of annual bluegrass which result in high resistance risks, (2) evaluate several annual bluegrass management programs for golf course fairways and their relative risks for selecting resistance, and (3) compare two herbicides (i.e., glyphosate and ALS-inhibitors) to determine how their respective characteristics influence resistance evolution in turfgrass systems.

Materials and Methods

Simulation Introduction. The simulations presented here are based upon annual bluegrass biology, general turfgrass weed management strategies, and some genetic parameters which are required to produce estimates of resistance evolution. Other herbicide resistance simulation models were referenced during development (Cavan et al., 2000; Diggle et al., 2003; Neve et al., 2003a, 2010). Here, the parameters considered to have primary influence on evolution of annual bluegrass resistance were soil seed bank density, annual loss of seed viability, annual proportion of seed germination, new seed production, removal rate of newly produced seed, rate of mutation to produce herbicide resistance alleles, and initial frequency of herbicide resistance alleles.

Parameter values or ranges used in the model were determined from a review of the literature and/or field observations and are presented in Table 4.1. These parameters and methods of their incorporation into the simulations are discussed in detail below. Some of these parameters have not been estimated specifically for annual bluegrass in commercial turf scenarios. Where these estimates are unavailable, similar species (i.e., Poaceae) or similar studies (i.e., herbicide resistance simulations) are used to establish estimates. Given the nature of annual bluegrass, most of these parameters will be highly variable in the field, and a review of the literature supports this variability. In general, a mean value of each parameter is established, and where parameter values are uncertain or would naturally vary, a distribution of values is used.

Annual Bluegrass Soil Seed Bank. Areas with colonized populations of annual bluegrass have large and persistent seed banks (Lush, 1988). Estimates of viable annual

Table 4.1. Description of parameters used in annual bluegrass simulation models.

Parameter	Symbol	Value Description[a]
Herbicide Treated Turf Area		12 ha
Initial Seed Bank Density[b]	I	0 – 20,000 seeds m^{-2}
Proportion Loss of Seed Viability	LV_y	0.25 ± 0.05
Proportion New Seed Viable	NSV	0.9
Total Annual Germination Proportion	GP_y	0.1 ± 0.025 (0.01 – 0.2)
Proportion Cohort Germination	GP_{cy}	
Cohort 1		0.75 ± 0.05 (0.6 – 0.9)
Cohort 2		0.2 ± 0.025 (0.15 – 0.25)
Cohort 3		1 – (GP_1 + GP_2)
Natural Mortality	MP_{cy}	
Cohort 1		0.5 ± 0.1 (>0.01)
Cohort 2		0.2 ± 0.05 (>0.01)
Cohort 3		0.05 ± 0.01 (>0.01)
Proportion Seed Removal	PSR_y	0.5 ± 0.05
Mutation Rate[c]	MR_h	
Glyphosate	MR_{Gly}	$1 \times 10^{-9} \pm 5 \times 10^{-8}$
ALS-Inhibitor	MR_{ALS}	$1 \times 10^{-8} \pm 5 \times 10^{-7}$
Initial Resistance Allele Frequency	IF_{Rh}	
Glyphosate	IF_{RGly}	$1 \times 10^{-8} \pm 5 \times 10^{-7}$
ALS-Inhibitor	IF_{RALS}	$1 \times 10^{-7} \pm 5 \times 10^{-6}$

[a] Values for random parameters are means selected from a normal distribution with standard deviations in parentheses unless otherwise specified.
[b] The value for initial seed bank density was randomly selected from a uniform distribution with the specified range.
[c] Values for mutation rate and initial resistance allele frequency parameters were randomly selected from log-normal distributions.

bluegrass seed banks in previous literature have been wide-ranging. Beard et al. (1978) reported approximately 7,500 annual bluegrass seeds m^{-2}, but infested golf course fairways can contain up to 185,000 seeds m^{-2} (Watschke et al., 1979). To encompass the large variability associated with this parameter, a value between 0 and 20,000 seeds m^{-2} was randomly chosen from a uniform distribution to represent initial seed bank density prior to herbicide selection. The total seed bank subjected to selection from herbicide

applications is the product of seed bank density (seeds m^{-2}) and 12 ha, the average turfgrass area of golf course fairways in the United States (GCSAA, 2007).

Seed Germination. Large annual bluegrass seed banks and extended seed viability contribute to the difficulty of predicting seed germination patterns. Lush (1988) reported an annual germination proportion of approximately 0.85 in a creeping bentgrass golf green in Australia, but seed germination is often higher in golf greens because of more intensive management compared to lesser maintained areas such as fairways and roughs (Naylor and Abdalla, 1982; Wu et al., 1987). A data set to model this parameter was not available, but it is assumed competition with established turfgrasses and variability in the depth of burial in the seed bank results in significantly reduced germination. Since the true value of this parameter is unknown and is likely to vary from year to year and among locations, a value is chosen each year from a normal distribution with mean 0.1 and standard deviation 0.025. Upper and lower bounds on this parameter were set to 0.2 and 0.01, respectively.

Annual Bluegrass Cohorts. Annual bluegrass seed has the ability to germinate year-round under optimal conditions, but in the southeastern United States, germination typically occurs from late August to early May. The majority of annual germination occurs during an initial flush when day/night temperatures are consistently around 25/12 ^{o}C. This usually happens in late September or early October in this region (McCarty, 2011). A second flush of germination occurs in early winter when alternating warm/cold temperatures scarifies additional seed (McCarty, 2011). Kaminski and Dernoeden (2007) investigated seasonal annual bluegrass germination patterns in Maryland and determined

75% of germination occurred from late September to mid-November with approximately 25% occurring from November to May. Based on these data, three annual bluegrass cohorts were established. Since the true distribution among cohorts of total germinated seeds is unknown and would vary from year to year and across locations, proportion of germination among cohorts were established using normal distributions. The first cohort consisted of annual bluegrass seeds that germinated in early fall (i.e., late Aug. to early Dec.). A value between 0.6 and 0.9 was chosen from a distribution with mean 0.75 and standard deviation 0.05. The second cohort comprised the mid-winter germination flush (i.e., early Dec. to mid Feb.). The value for cohort 2 was between 0.15 and 0.25 and chosen from a distribution with mean 0.2 and standard deviation 0.025. If the proportion of cohort 1 and cohort 2 was >1, the value for cohort 2 became $(1 - GP_1)$ where GP_1 was the proportion of germinated seeds for cohort 1. The proportion of the third cohort was $[1 - (GP_1 + GP_2)]$. An analysis of 10,000 runs of this submodel revealed the mean germination proportion of cohort 3 was 0.055.

Seedling Survival. In these simulations, plant survival to maturity (i.e., reproductive stage) is dependent upon herbicide applications and natural mortality. The effect of herbicide applications is discussed below. A data set to determine natural annual bluegrass mortality was not available. However, when bermudagrass is overseeded in early fall with perennial ryegrass, seeding rates are generally used to achieve approximately 15,000 surviving plants m^{-2} (McCarty, 2011). This occurs by reducing established turfgrass competition by scalping and/or vertical mowing. It is assumed for these simulations competition from established turfgrasses will decrease the survivability

of germinated seeds, and that natural mortality will increase in later cohorts as other plants have already become established. The seed bank carrying capacity of this model was approximately 180,000 seeds m^{-2} in the absence of selection (data not shown). This value and values of other parameters were used to establish realistic means of plant survival for each cohort. This parameter will remain highly variable from year to year and across locations. These values were randomly selected from normal distributions with mean proportions of 0.5, 0.8, and 0.95 natural mortality and standard deviations 0.1, 0.05, and 0.01, respectively, for cohorts 1, 2, and 3. These values are considered to be density independent in these simulations.

Seed Production. Annual bluegrass is capable of producing seed at all mowing heights typically practiced on golf courses, although plants maintained at higher mowing heights produce more seed. This allows the species to persist in the seed bank and continually regenerate itself. Beard et al. (1978) reported a single annual bluegrass plant could produce greater than 360 seeds per plant, but plants maintained at higher mowing heights are capable of producing from 1,000 to 2,250 seeds per plant (Holm et al., 1997). A data set was not available to estimate this parameter, thus, a density dependent competition function for seed production was implemented similar as Neve et al. (2010):

$$SP_c = sd/[1+(sd/m)] \quad [4.1]$$

where SP_c is the number of seeds produced per plant in cohort c, s is the number of seeds produced per plant as d approaches 0, d is the number of plants m^{-2} surviving to seed production, and m is the maximum number of seeds that can be produced m^{-2}. An artificial data set was established for three annual bluegrass cohorts to account for

Table 4.2. Parameters for the density-dependent seed production submodel for three levels of fecundity corresponding to annual bluegrass cohorts.

	Parameter	
Annual Bluegrass Fecundity[a]	*s*	*M*
High (Cohort 1)	2,000	200,000
Medium (Cohort 2)	1,000	100,000
Low (Cohort 3)	250	25,000

[a] Seed production is determined according to the density-dependent formula $SP_c = sd/[1+(sd/m)]$ where SP_c is the number of seeds produced per plant in cohort *c*, *s* is the number of seeds produced per plant as *d* approaches 0, *d* is the number of plants m^{-2} surviving to seed production, and *m* is the maximum number of seeds that can be produced m^{-2}.

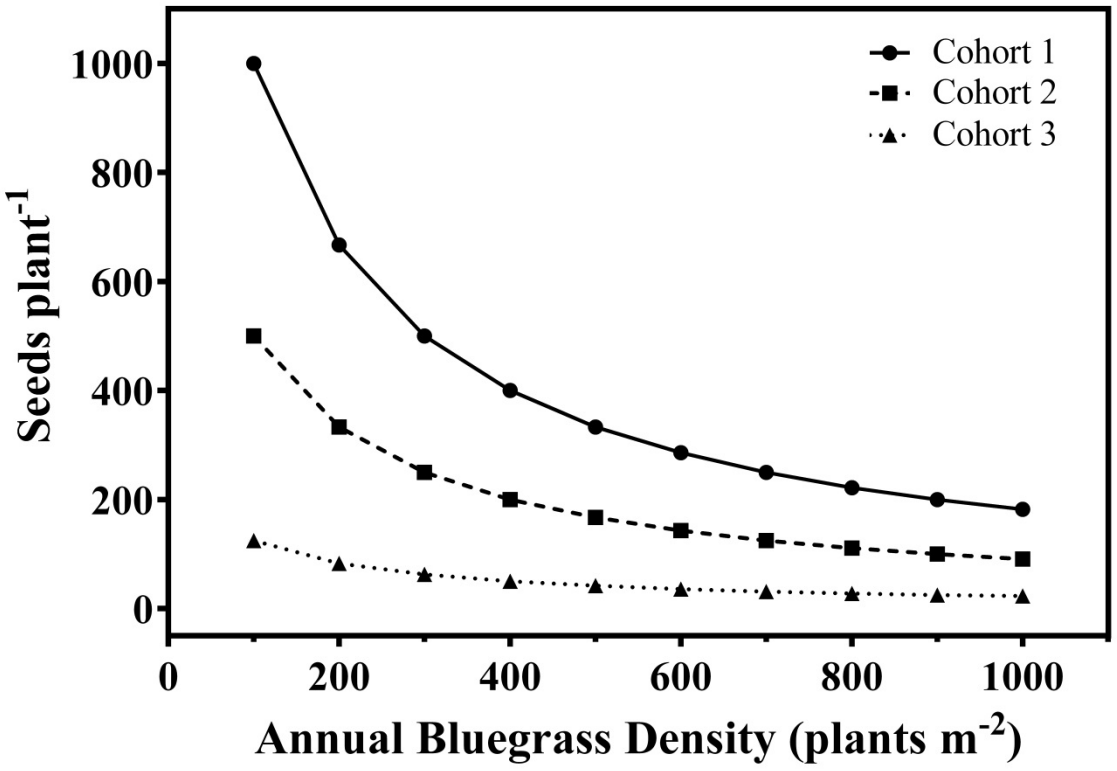


Figure 4.1. Density-dependent annual bluegrass seed production for three levels of fecundity (cohorts 1-3).

reducing fecundity of later cohorts (Table 4.2, Figure 4.1). These parameters allowed for realistic seed productivity per plant based on previous literature reports.

Seed Removal. Weed seed predation is an important ecological factor that can affect plant population dynamics. Generally, insects, birds, and small mammals are responsible for most seed predation, but in turf, mowing, irrigation, and other cultural practices can also contribute to weed seed removal prior to incorporation into the seed bank. Many turf weeds provide important food sources for arthropods (Blubaugh et al., 2011) and research on similar grassy weed species in pastures or crops reveals high seed predation rates. Williams and Bartholomew (2008) reported mean Italian ryegrass predation rates between 26 and 53% in a warm-season grass pasture while 73% monthly predation of Italian ryegrass was observed in a wheat (*Triticum aestivum* L.) field in Australia (Jacob et al., 2006). Given these considerations, and influence of turfgrass cultural practices, it is assumed seed removal rates follow a normal distribution across years and locations. A value for this parameter is selected from a distribution with mean proportion seed removal of 0.5 and standard deviation 0.05.

Loss of Seed Viability. A number of variables are involved in the maintenance of weed seed viability in soil including moisture, temperature, and exposure to light (Taylorson, 1970). As these factors vary depending upon seed location in the soil profile, a proportion of seeds in the soil seed bank lose viability each year. This parameter is highly influenced by the lack of soil disturbance in perennial turfgrass stands. Roberts and Feast (1973) reported annual bluegrass seed may remain viable in the soil for six yr or longer, and estimated a 17 to 21% annual decrease in the number of viable annual bluegrass seeds in

an undisturbed soil. Thus, a value is randomly selected from a normal distribution with mean proportion loss of viability of 0.25 and standard deviation 0.05. This value represents mean viability loss across all soil profile depths. The simulations assume a proportion of 0.9 of newly produced seeds are viable.

Genetic Parameters. Genetic parameters are difficult to estimate in general, but specifically for annual bluegrass because it is an allotetraploid species. Several of these parameters, however, are required for the evaluation of resistance evolution. These simulations consider three genetic parameters including resistance inheritance, mutation rates, and resistance allele frequencies. Relatively little information is available investigating these parameters for annual bluegrass. Herbicide resistance inheritance in these simulations is considered to occur in Mendelian fashion via a single, completely dominant nuclear gene (ALS-inhibitors; Tranel and Wright, 2002) or via a single, incompletely dominant nuclear gene (glyphosate; Lorraine-Colwill et al., 2001).

Rates of gene mutation conferring herbicide resistance are difficult to quantify and are unknown for any weed species, but Maynard Smith (1989) estimated mutation rates were between 1×10^{-6} and 1×10^{-7}. Specific estimates for mutation rates for resistance to ALS-inhibitors in mouse-ear cress and tobacco were reported as 1×10^{-9} and 2.7×10^{-8}, respectively (Harms and DiMaio, 1991; Saari et al., 1994). In comparisons of mouse-ear cress mutants selected for herbicide resistance to ALS-inhibitors and glyphosate, Jander et al. (2003) reported mutations conferring glyphosate resistance occurred less frequently than those conferring ALS resistance. In these simulations,

glyphosate (mean 1×10^{-9}, standard deviation 5×10^{-8}) is associated with a lower mutation rate than ALS-inhibitors (mean 1×10^{-8}, standard deviation 5×10^{-7}).

Initial frequency of herbicide resistance alleles is equally difficult to quantify. Even for relatively high mutation rates, millions of plants would need to be screened to have a reasonable level of confidence of finding one resistant individual (Jasieniuk et al., 1996). It is expected the initial frequencies of resistance will be an order of magnitude higher than mutation rates in the absence of selection according to mutation-selection equilibrium (Jasieniuk et al., 1996; Neve et al., 2010). Therefore, initial resistant frequencies are established by random selection from a lognormal distribution with mean and standard deviation of 1×10^{-8} and 5×10^{-7} for glyphosate and 1×10^{-7} and 5×10^{-6} for ALS-inhibitors. For calculations of initial genotypes present in the soil seed bank, populations of annual bluegrass are assumed to be in Hardy-Weinberg equilibrium. While annual bluegrass is capable of cross-pollinating, studies suggest the majority of annual bluegrass pollination occurs by selfing, and beyond initial genotype calculations, simulations consider only self-pollination when calculating frequencies of genotypes in new seed production (Darmency and Gasquez, 1983).

Herbicide Applications. In these simulations, herbicide applications occur at different times prior to the emergence of cohort 3 (i.e., after cohort 1 or cohort 2). No annual bluegrass seed is produced until after herbicide applications have been made. Herbicide options, timings, and effectiveness against genotypes are presented in Table 4.3. When herbicides were applied at later timings, control of earlier cohorts is assumed to be reduced. Resistance to glyphosate is conferred in an incompletely dominant fashion, thus,

control of the heterozygote is lower in comparison to ALS-inhibitors. Further, Resistance to alternate herbicides in these simulations is not considered.

Model Simulations. Simulations were developed and implemented using SAS version 9.3 (SAS Institute, Inc., Cary, NC) and are represented in the flow chart in Figure 4.2. In the first year of each simulated location, a value for initial seed bank density (*I* seeds m^{-2}) is generated and multiplied by 12 ha to produce a total viable seed bank (*TSB*) across the

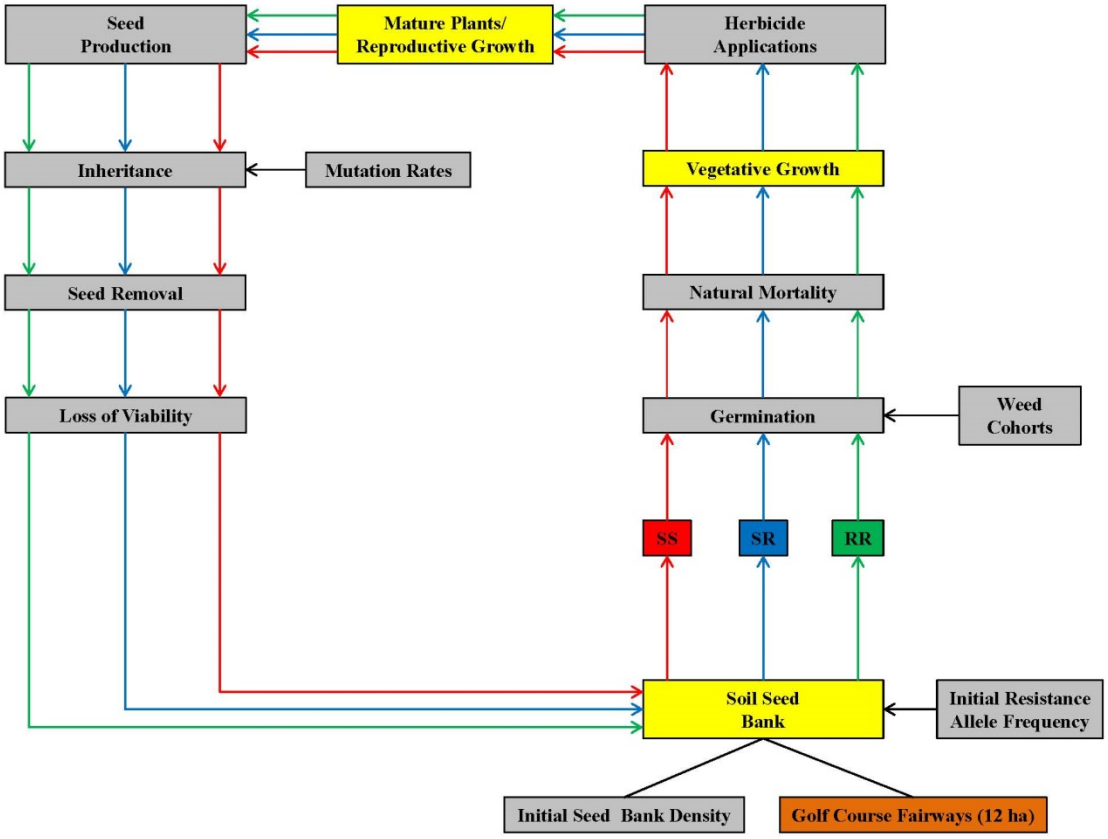


Figure 4.2. A simplified flow chart representing the annual bluegrass herbicide resistance simulation model. Boxes shaded in yellow represent the three life stages of annual bluegrass. Gray boxes are modeled parameters which add to variation in resistance evolution. Red, blue, and green boxes and lines represent the three genotypes, SS, SR, and RR, respectively, which were considered for glyphosate and sulfonylureas.

Table 4.3. Potential management options, timings, and expected levels of annual bluegrass control in golf course fairways. Annual bluegrass control is dependent upon cohort and genotype. Simulations consider different combinations of these management options, results of which are discussed in the text and presented in accompanying figures.

		Annual Bluegrass Control (%)								
		Cohort 1			Cohort 2			Cohort 3		
Management Option	Timing	SS[d,e]	SR	RR	SS	SR	RR	SS	SR	RR
PRE alternate[a]	Early fall	95	95	95	0	0	0	0	0	0
POST alternate[b]	Late fall/early winter	95	95	95	0	0	0	0	0	0
Glyphosate[c]	Late fall/early winter	90	10	5	0	0	0	0	0	0
Sulfonylurea	Late fall/early winter	95	5	5	0	0	0	0	0	0
POST alternate	Late winter	90	90	90	95	95	95	0	0	0
Glyphosate	Late winter	80	10	5	95	25	5	0	0	0
Sulfonylurea	Late winter	90	5	5	95	1	1	0	0	0

[a] Potential PRE herbicides providing 95% control include indaziflam and prodiamine (Brosnan et al. 2012).
[b] Potential POST herbicides providing ≥90% control include simazine, pronamide, glyphosate, flumioxazin, glufosinate, and sulfonylurea herbicides (Flessner et al. 2013; Toler et al. 2007).
[c] The resistance allele is incompletely dominant for glyphosate and completely dominant for sulfonylureas, with sulfonylureas applying greater selection pressure as a result of higher efficacy.
[d] Genotypes pertain only to glyphosate and sulfonylureas as resistance to alternate herbicides is not considered in simulations.
[e] Abbreviations: S, herbicide susceptible allele; R, herbicide resistance allele.

fairways of a golf course. A value for initial resistance allele frequency (F_{Ri}) is generated and used to determine the number of viable seeds of three genotypes (SS, SR, RR) present in the seed bank during the first year of the simulation according to the Hardy-Weinberg equilibrium

$$TSB(F_{Ri}^2) + TSB(2 \times F_{Ri} \times F_{Si}) + TSB(F_{Si}^2) \quad [4.2]$$

where F_{Ri} is the initial resistance allele frequency and F_{Si} is the initial susceptible allele frequency attained by 1-F_{Ri}. A proportion of these viable seeds of each genotype g (V_g) germinate each year according to the germination proportion value (GP_y)

$$G_g = V_g \times GP_y \quad [4.3]$$

where G_g is the total number of germinated seeds of genotype g in year y. Germinated seeds belong to one of three cohorts according to the cohort germination proportion (GP_c)

$$G_{gc} = G_g \times GP_c \quad [4.4]$$

where G_{gc} is the number of germinated seeds of genotype g in cohort c. The survivability to reach reproductive stage is different for each cohort and depends on natural mortality and subjection to herbicide application. For cohorts 1 and 2, this is given by

$$S_{gc} = G_{gc} \times (1 - MP_c) \times E_{hg} \quad [4.5]$$

where S_{gc} is the number of treated plants of genotype g in cohort c reaching reproductive stage, MP_c is the expected proportion of natural mortality of plants of cohort c, and E_{hg} is the herbicide efficacy of herbicide h on genotype g. Cohort 3 seeds germinate after herbicide application and S_{gc} is obtained using equation 4.5 without the E_{hg} term. Seed production per plant for each genotype and cohort is determined using the submodel in equation 4.1 and calculated by

$$TS_{gc} = SP_c \text{ x } S_{gc} \quad [4.6]$$

where TS_{gc} is the total seed produced by genotype g in cohort c and SP_c is the seed production per plant for cohort c. Seed production is assumed to occur in Mendelian fashion and genotypic proportions of new seed are determined considering rates of mutation to produce alleles resistant to herbicide h (MR_h) by

$$SS_{New} = (SS_c \text{ x } p^2) + (SR_c \text{ x } 0.25) + (RR_c \text{ x } q^2) \quad [4.7]$$

$$SR_{New} = (SS_c \text{ x } 2 \text{ x } p \text{ x } q) + (SR_c \text{ x } 0.5) + (RR_c \text{ x } 2 \text{ x } p \text{ x } q) \quad [4.8]$$

$$RR_{New} = (SS_c \text{ x } q^2) + (SR_c \text{ x } 0.25) + (RR_c \text{ x } p^2) \quad [4.9]$$

where SS_{New}, SR_{New}, and RR_{New} are the number of new seeds of genotype SS, SR, and RR, respectively, SS_c, SR_c, and RR_c are the number of seeds of SS, SR, and RR genotypes, respectively, from cohort c, p is $1 - MR_h$, and q is MR_h. The number of viable seeds of each genotype incorporated into the seed bank depends upon the proportion of seed removed prior to incorporation in year y (PSR_y) and the proportion of newly produced seed which is viable (0.9)

$$T_{Ng} = N_g \text{ x } PSR_y \text{ x } 0.9 \quad [4.10]$$

where T_{Ng} is the total new seed of genotype g incorporated into the seed bank and N_g is the new seed produced of genotype g. A proportion of the remaining seeds lying dormant in the seed bank over the simulated year lose viability and is used to calculate the remaining viable seed bank by

$$N_{Vg} = I_{Vg} \text{ x } (1 - LV_y) \quad [4.11]$$

where I_{Vg} is the initial viable seed bank of genotype g. The new viable seed bank of each genotype thus becomes the sum of T_{Ng} and N_{Vg}. This value is used in the following

simulated year. Resistance was considered to have developed at a location when the resistant individuals (SR or RR) comprised greater than 20% of the seed bank. At this point, enough resistant individuals are present in the seed bank such that a turf manager would come to the realization of failure of a herbicide for annual bluegrass control. Further, most previous simulation models for herbicide resistance consider this value as evolution of resistance (e.g., Neve et al., 2010). To prevent over-prediction of resistance, an integer is drawn from a Poisson distribution with mean equal to the predicted value where partial plants are predicted. For example, if 50 plants are exposed to a herbicide application providing 95% control, 2.5 plants are predicted to survive. The one-half plant would be included in further calculations and would produce seed influencing the evolution of resistance. If this number were rounded to the nearest whole number, 3 plants would produce seed. In contrast, there is a greater probability of 2 plants surviving application than 3 plants according to the Poisson distribution (Figure 4.3).

Upon determination of parameter values and ranges, numerous herbicide use strategies for annual bluegrass control were simulated over a period of 50 years and are discussed further below. Each simulated strategy contained 10,000 runs representing a different location (i.e., golf course). The probability of resistance development for each strategy was determined by the proportion of simulation runs (out of 10,000 total) which developed resistance after a period of time of implementing that particular strategy.

Upon evaluating resistance evolution for each herbicide use strategy, biological parameters which were considered to influence resistance development were subjected to sensitivity analysis by fixing the parameter of interest at a range of values while other

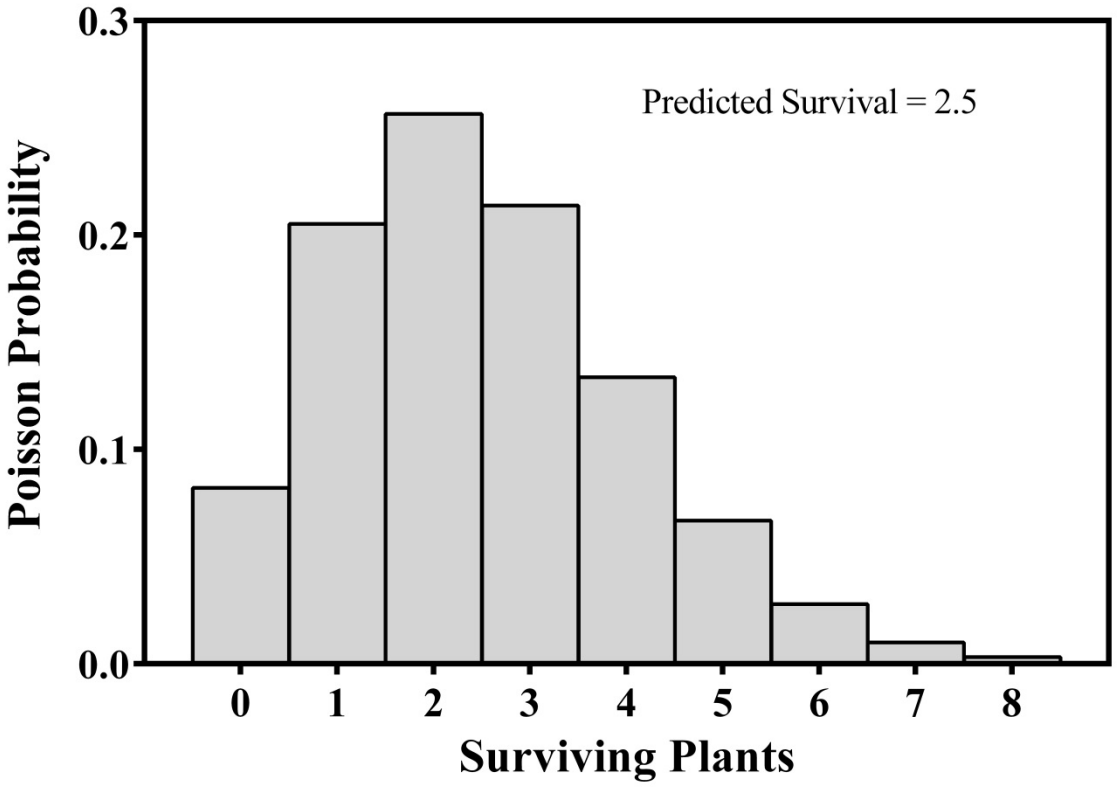


Figure 4.3. Example illustrating the integration of the Poisson distribution into simulation models. Fifty plants exposed to a herbicide application providing 95% control would yield an expected survival of 2.5 plants with the associated Poisson probabilities.

parameters were employed as described above. Effect of the parameter on predicted resistance risk was evaluated by the proportion of model runs (out of 10,000 total) evolving resistance after 15 or 10 yr of consecutive annual applications of glyphosate or ALS-inhibitors, respectively.

Results and Discussion

Single Annual Applications. Many turfgrass managers apply the same mechanism of action at the same general timing to control annual bluegrass each year. This is a result of economic issues, past success with one product or program, more limited options for non-dormant warm-season turfgrass (e.g., use of sulfonylureas in coastal areas), or ease of use with other maintenance practices (e.g., tank-mixing postemergence herbicides during spring preemergence applications). Repeated herbicide applications of the same mechanism creates a shift in the genetic composition of weed populations as intense selection removes susceptible individuals and increases the frequency of resistant individuals over time (Jasieniuk et al., 1996). This has been observed in the past 5 to 10 years on golf courses in the southeastern United States, where, after 7 to 10 years of continuous application of the same herbicide has resulted in resistant annual bluegrass populations (McCarty, 2011).

A worst-case scenario of single yearly applications of both glyphosate and sulfonylureas were considered in these simulations and two application timings of each were compared. For late winter applications (i.e., after cohort 2), glyphosate resistance was first observed in yr 9, but the proportion of resistant populations was below 0.01 until yr 12 (Figure 4.4). Resistance risks increased to 55% by yr 15 and 99% by yr 19. Similar simulations for sulfonylureas resulted in higher risks over a shorter period of time. Resistance was observed in yr 5 with a 99% risk by yr 10 (Figure 4.4).

The timing of application influenced resistance risks for both herbicides, with reduced risks observed when herbicides were applied in late fall (i.e., after cohort 1).

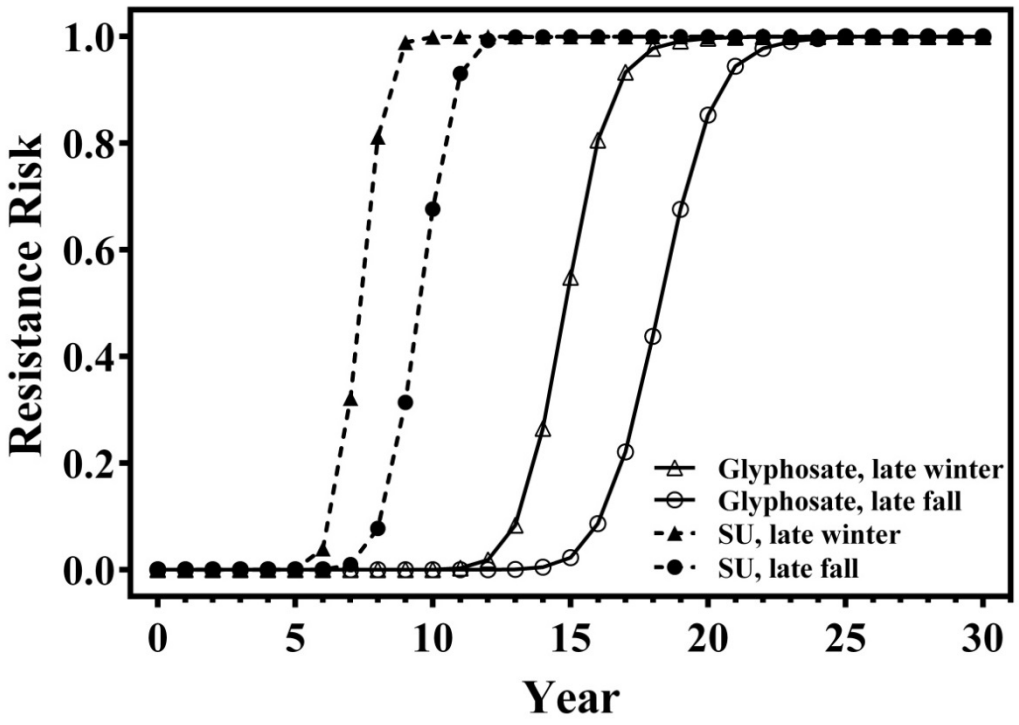


Figure 4.4. Predicted probability of annual bluegrass resistance to glyphosate (solid lines, open symbols) or sulfonylureas (dashed lines, closed symbols) on golf course fairways. Herbicides were applied in either late fall (circles) or late winter (triangles). Resistance risk is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) at the end of a given year.

Similar resistance risks for glyphosate were observed about 3 to 4 years later than late winter applications, with the first resistant populations predicted in yr 12 (Figure 4.4). In yr 20, an 85% risk was predicted, and a 99% risk was observed in yr 23. In comparison, resistance to sulfonylureas was first predicted in yr 6 for late fall applications. In yr 10, a 68% risk was observed and 99% of populations were resistant by yr 12.

The differences observed between glyphosate and sulfonylureas are attributed to several characteristics of each herbicide. First, glyphosate resistance was assumed to be inherited in incompletely dominant fashion (Lorraine-Colwill et al., 2001) and resulted in reduced control of the heterozygote (SR). Second, mutation rates to produce herbicide resistant alleles was considered lower for glyphosate and resulted in a lower initial resistance allele frequency. There is evidence mutation to resistance in glyphosate occurs at a lower frequency than ALS-inhibitors (Jander et al., 2003). Third, these simulations considered a more intense selection pressure was applied by sulfonylureas as reflected in relative control levels of both cohorts 1 and 2 (Table 4.3). More intense selection pressure is exerted by herbicides with highly specific mechanisms of action and with longer soil residual activity as would be common with ALS-inhibitors (Jasieniuk et al., 1996; LeBaron and McFarland, 1990). For these reasons, glyphosate resistance was slower to develop when compared to sulfonylureas in these simulations.

With reference to application timing, early season applications were associated with slightly slower predictions of resistance evolution. This is expected as a smaller proportion of the entire population of the year is exposed to herbicide application. Simulations were conducted such that late fall applications were made after all cohort 1 individuals had emerged and late winter applications were made after all cohort 2 individuals had emerged. A mean proportion of 0.2 of annual germination occurred in cohort 2, thus, although there were slight reductions in time to resistance for both herbicides, a vast majority of the population was still exposed to herbicides in late fall

applications. This is true of annual bluegrass since the greatest proportion of germination occurs in early fall as temperatures being to cool (McCarty, 2011).

The intense selection pressure exerted on a weed population resulting from continuous herbicide applications of the same mode of action is presented in Figure 4.5. The relative proportions of each genotype (SS, SR, RR) comprising the seed bank at the end of each year was modeled for continuous glyphosate applications in late winter for 50 years. The majority of the seed bank is comprised of homozygous individuals throughout the simulated period since the majority of annual bluegrass pollination occurs via selfing. A small portion of heterozygous resistant individuals at the beginning of the simulations

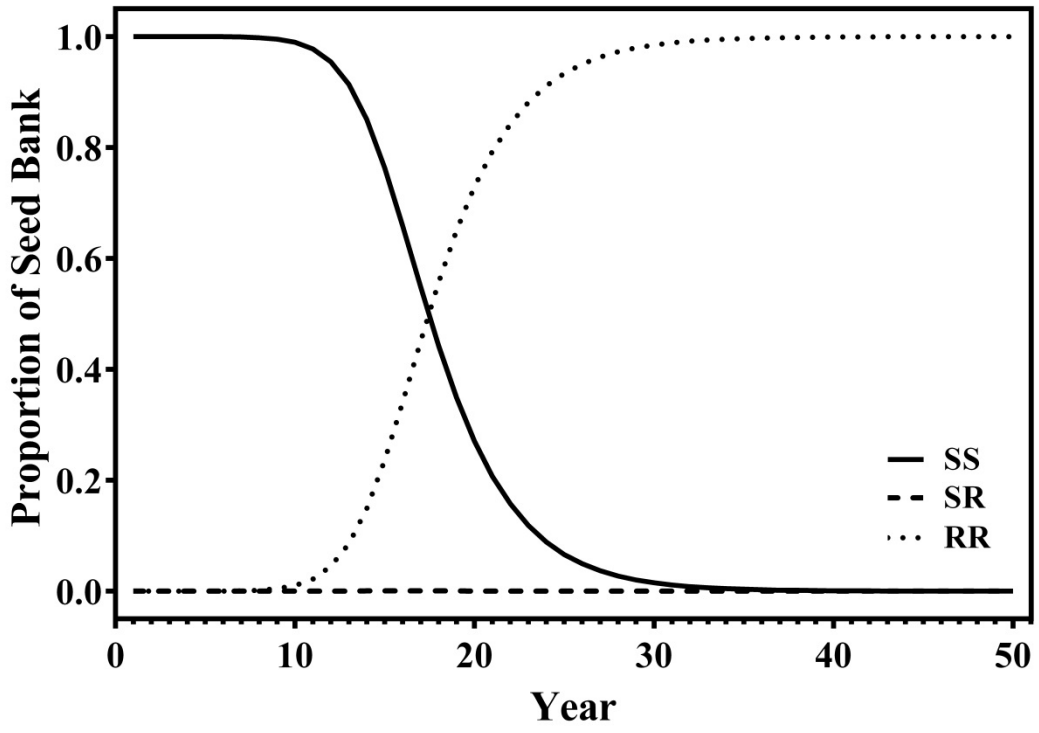


Figure 4.5. Proportion of homozygous susceptible (SS; solid line), heterozygous (SR; dashed line), and homozygous resistant (RR; dotted line) seeds in the seed bank over a 50 year period of annual glyphosate applications in late winter.

in the presence of selection contributes to increasing homozygous resistant individuals, and as selection continuous, the seed bank population shifts to contain mostly homozygous resistant seeds.

Single Annual Applications in Rotation. Using herbicides in rotation is a common strategy for managing herbicide resistance (Beckie, 2006; Beckie and Reboud, 2009; Diggle et al., 2003; Gressel and Segel, 1990). Often, lower budgeted turfgrass facilities rely on single applications of herbicides for weed control. Therefore, simulations were performed to investigate rotating glyphosate or sulfonylureas with other herbicides in single yearly applications as a strategy for managing resistance. Previous studies suggest several mechanisms of action adequately control annual bluegrass pre- or postemergence. Preemergence applications of indaziflam (cellulose biosynthesis inhibition) and prodiamine (mitosis inhibition) controlled annual bluegrass >90% in Tennessee for 18 weeks after treatment (Brosnan et al., 2012b). Postemergence herbicides which are labeled for annual bluegrass control in non-overseeded bermudagrass include simazine (photosystem II inhibition), glyphosate (5-enolpyruvate shikimate 3-phosphate synthase inhibition), glufosinate (glutamine synthetase inhibition), pronamide (mitosis inhibition), flumioxazin (protox inhibition), and several sulfonylureas (ALS inhibition) and have provided >90% control in previous studies (Flessner et al., 2013; Toler et al., 2007).

When alternate herbicides were applied for preemergence or early postemergence (i.e., late fall), 95% control of cohort 1 was assumed (Table 4.3). It is well documented that postemergence herbicides are more effective when applied to immature plants, and the same is true for annual bluegrass (Toler et al., 2007). Thus, when postemergence

herbicides are made in late winter in simulations, decreased control of cohort 1 is assumed (Table 4.3).

The slower time to evolve resistance when herbicides are applied at earlier timings previously established was also observed when glyphosate or sulfonylureas were rotated on a yearly basis (Figure 4.6). However, the importance of rotating herbicide mechanisms of action in order to manage resistance is displayed regardless of timing or herbicide applied. For glyphosate, rotating with an alternate mechanism of action in early fall resulted in a 10 yr delay for the first predicted resistant population (yr 22) compared to annual applications (Figure 4.6a). In yr 30, <10% of populations were resistant, but by yr 40, there was a 97% risk of resistance. A similar resistance risk occurred in yr 23 (98%) when annual applications were made. For late winter applications, a similar delay (10 yr) was observed rotating mechanisms of action, but risks were higher when compared to late fall applications (Figure 4.6b). For example, a 67% risk was predicted for late winter rotations with glyphosate compared to <10% for late fall rotations.

Delays were also predicted when sulfonylureas were applied, but annual rotations were not as effective. Resistance was first predicted 7 yr later (yr 12) than annual sulfonylurea applications when using alternate herbicides in rotation in late fall (Figure 4.6c). However, this delay was only 3 yr (yr 8) when herbicides were applied in late winter (Figure 4.6d). Regardless of timing, a risk >90% was predicted for sulfonylurea herbicides after 22 yr compared to 38 yr for glyphosate.

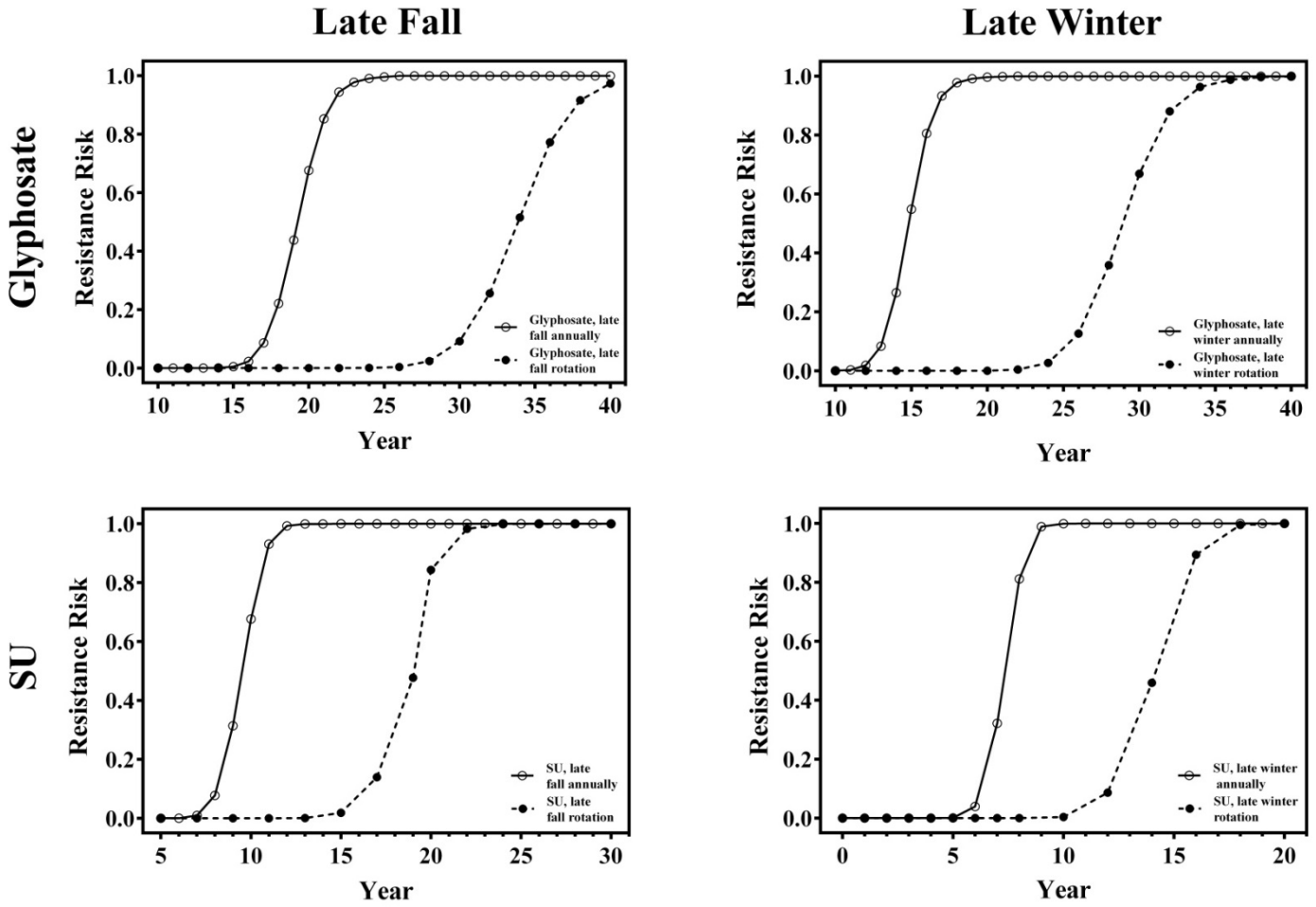


Figure 4.6. Predicted probability of annual bluegrass resistance to glyphosate or sulfonylureas on golf course fairways. Herbicides were applied in either late fall or late winter annually (solid lines, open circles) or in rotation with an alternate herbicide (dashed lines, closed circles). Resistance risk is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) at the end of a given year.

These simulations illustrate the importance of rotating mechanisms of action as often as possible to limit intense selection that occurs after applications of the same herbicide in a short period of time. If herbicides are rotated in greater frequencies (e.g., two years or more), resistance risks remain lower for longer periods of time (data not shown).

Multiple Applications. Considering the previous simulations, the problem remains that acceptable (i.e., season-long) annual bluegrass control is generally not attainable with a single herbicide application each season. Therefore, further simulations were conducted to investigate the effects of multiple herbicide applications in a single year. These included pre- or postemergence applications in early or late fall, respectively, combined with glyphosate or sulfonylureas in late winter. The effect of rotations on these programs were also considered.

When a pre- or postemergence (PRE/POST) herbicide was applied in early/late fall combined with annual glyphosate applications in late winter, risk of resistance increased faster compared to annual glyphosate applications in late winter alone (Figure 4.7). Resistance was still predicted to evolve 9 yr after these programs, but in yr 14, risks were >80% when using PRE/POST herbicides compared to 27% when using glyphosate alone. This occurred because a smaller number of individuals survived to reproductive stage allowing greater seed production. In addition, glyphosate was assumed to control the heterozygote 25% in cohort 2, and homozygous resistant individuals increased at a much faster rate (data not shown). This trend was not observed with sulfonylureas

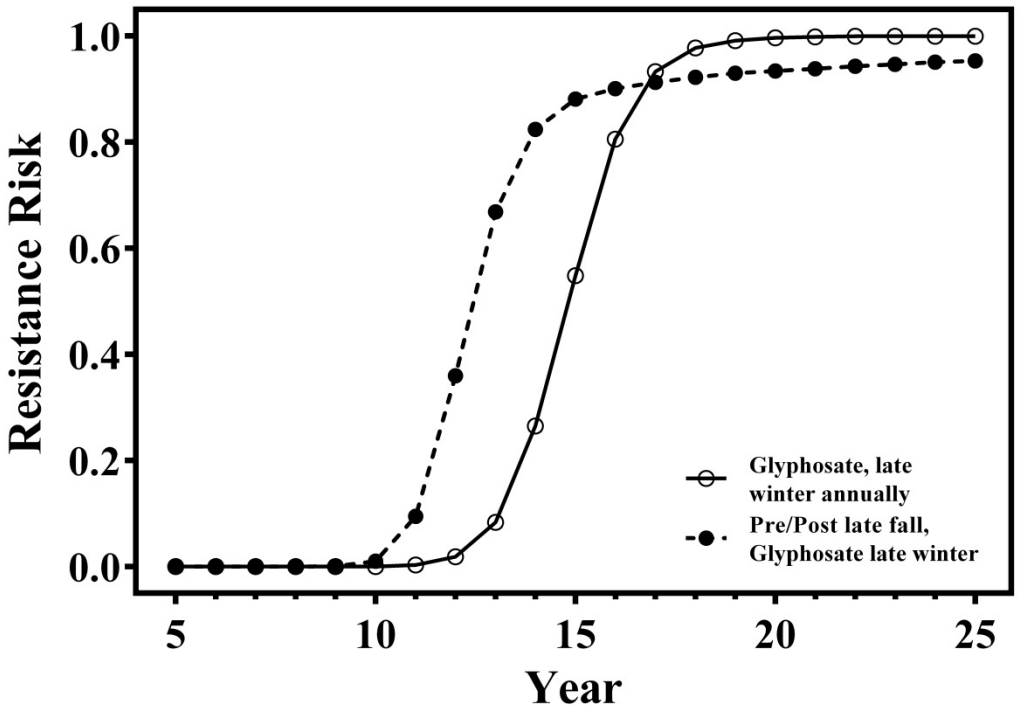


Figure 4.7. Predicted probability of annual bluegrass resistance to glyphosate on golf course fairways. Glyphosate was applied alone in late winter annually (solid lines, open circles) or in a program with a PRE/POST herbicide in late fall (dashed lines, closed circles). Resistance risk is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) at the end of a given year.

(Figure 4.8). A PRE/POST application provided slight delays of resistance, but once resistance was predicted to evolve, risks increased over time at similar rates.

Previous simulations demonstrated the effectiveness of using herbicides in rotation to delay resistance. Applying a PRE/POST herbicide for early control of annual bluegrass while using alternate mechanisms of action every other year provided a 7 yr

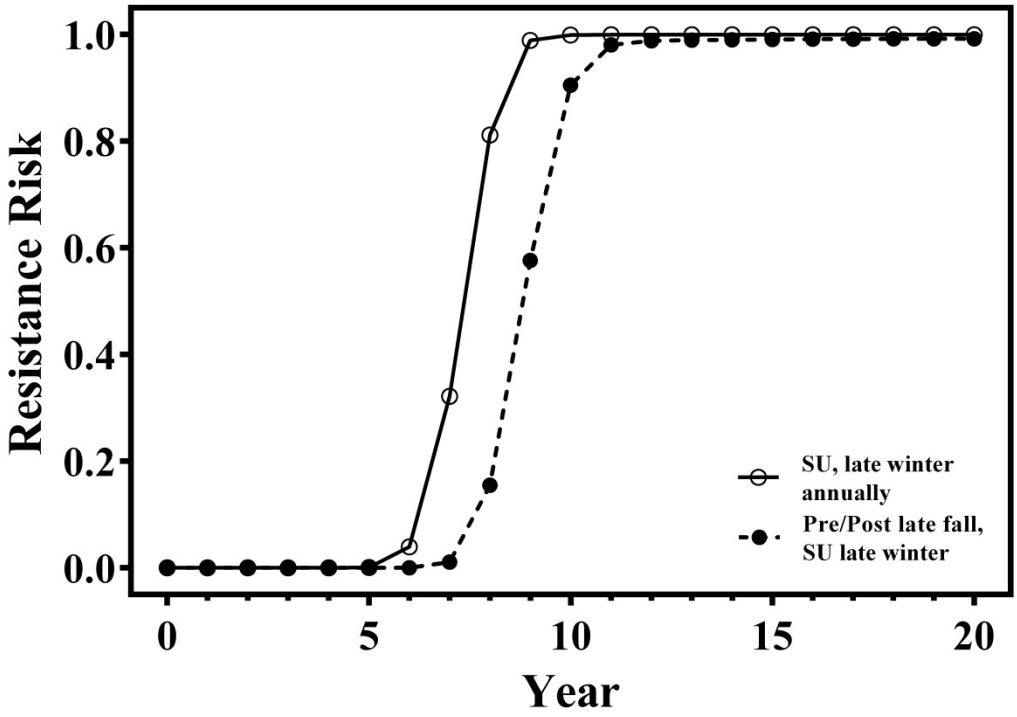


Figure 4.8. Predicted probability of annual bluegrass resistance to sulfonylureas on golf course fairways. Sulfonylureas were applied alone in late winter annually (solid lines, open circles) or in a program with a PRE/POST herbicide in late fall (dashed lines, closed circles). Resistance risk is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) at the end of a given year.

delay in the first predicted glyphosate resistant population compared to annual late winter applications (Figure 4.9). In comparison, alternating herbicides in late winter applications provided a 10 yr delay. Interestingly, after 25 yr of the PRE/POST program with alternate glyphosate applications, approximately 20% of populations had not evolved resistance, and resistance risks were very slow to increase beyond this point. Further investigation revealed populations where low initial seed bank densities were selected resulted in

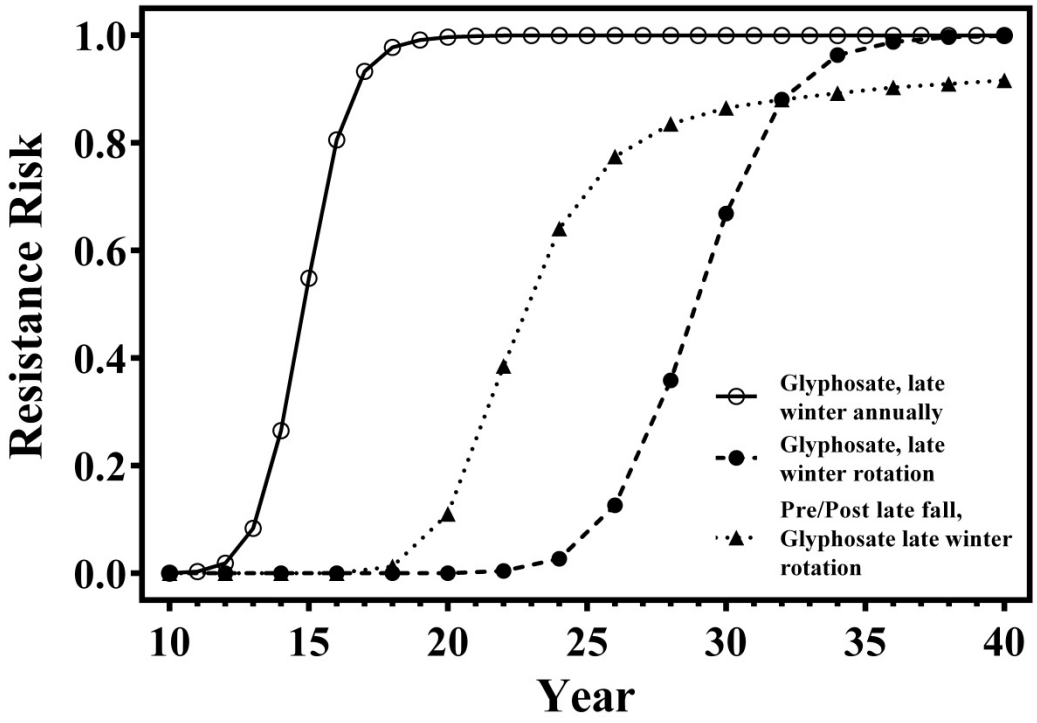


Figure 4.9. Predicted probability of annual bluegrass resistance to glyphosate on golf course fairways. Glyphosate was applied were applied alone in late winter annually (solid lines, open circles), in rotation with an alternate herbicide in late winter (dashed lines, closed circles), or in a program with a PRE/POST herbicide in late fall and in rotation with an alternate herbicide in late winter (dotted lines, triangles). Resistance risk is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) at the end of a given year.

reduced resistance risks using this program (Figure 4.10). Lower initial seed bank densities were not as influential on resistance evolution when glyphosate was alternated in late winter. Therefore, adding a PRE/POST application to a rotation with glyphosate may be a more effective program for golf courses where seed banks are much lower.

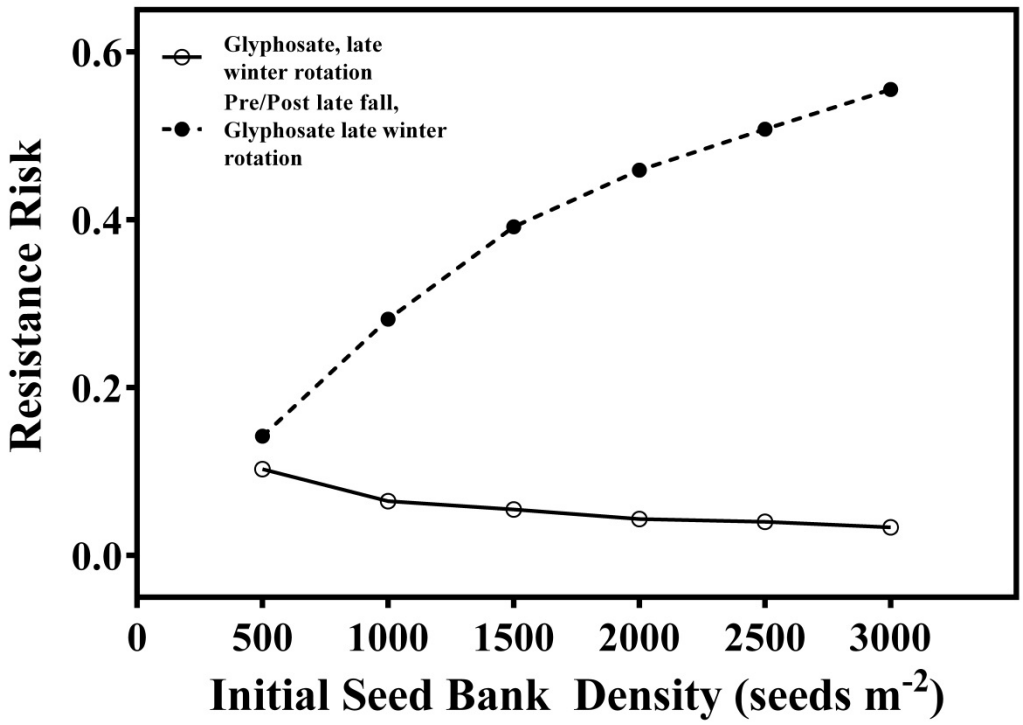


Figure 4.10. Effect of initial seed bank density on risk of annual bluegrass resistance to glyphosate in golf course fairways. Glyphosate was applied in rotation with an alternate herbicide in late winter (solid line, closed cirlces) or in a program with a PRE/POST herbicide in late fall and in rotation with an alternate herbicide in late winter (dashed line, closed circles). Resistance risk is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) at the end 15 years of each program.

Additional herbicide applications would keep seed banks lower for longer periods of time reducing the number of individuals glyphosate is ultimately exposed to.

For sulfonylurea herbicides, the most effective program simulated for delaying resistance was applying a PRE/POST herbicide for early annual bluegrass control, then

alternating a different mechanism of action with sulfonylurea herbicides (Figure 4.11). This program delayed the first prediction of resistance to yr 11 compared to yr 5 with annual applications. Furthermore, a 99% resistance risk was observed in yr 9 with annual applications compared to yr 24 with this program. Regardless of any program modeled, extensive use of sulfonylurea herbicides always resulted in a near 100% risk of resistance

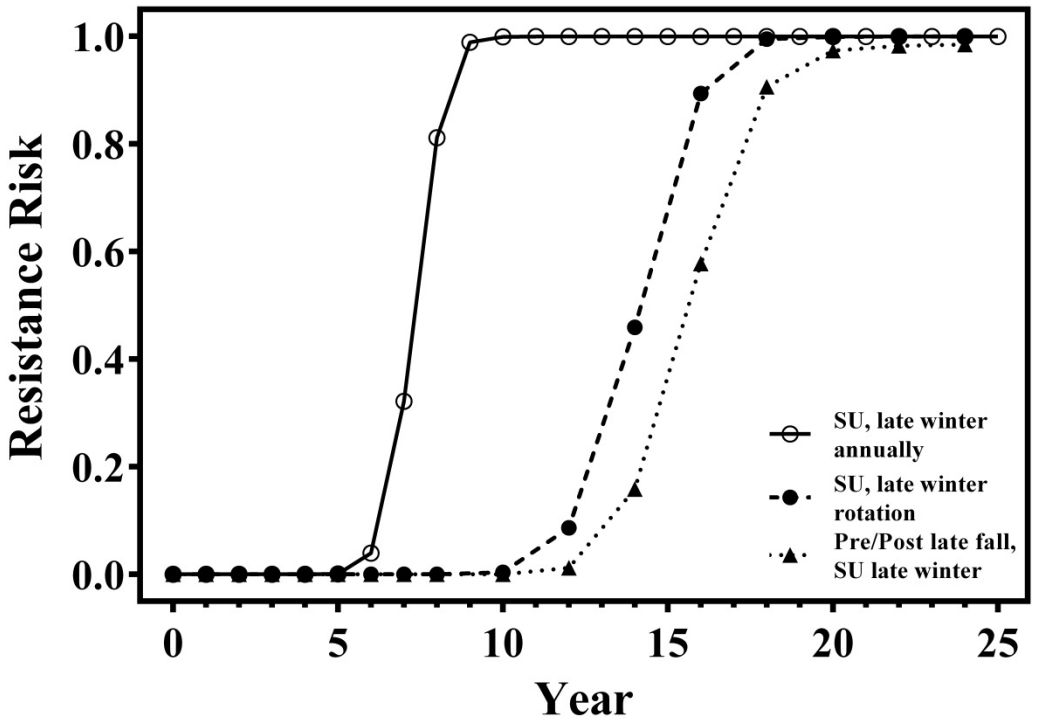


Figure 4.11. Predicted probability of annual bluegrass resistance to sulfonylureas on golf course fairways. Sulfonylureas were applied alone in late winter annually (solid line, open circles), in rotation with an alternate herbicide in late winter (dashed line, closed circles), or in a program with a PRE/POST herbicide in late fall and in rotation with an alternate herbicide in late winter (dotted line, triangles). Resistance risk is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) at the end of a given year.

after approximately 25 yr. This is owed to the resistance allele being completely dominant, higher rates of mutation, and greater selectivity (i.e., greater control of susceptible individuals).

Sensitivity Analysis. In order to evaluate the main effect of modeled parameters on the risk of resistance for both glyphosate and sulfonylureas, a sensitivity analysis was performed. These can provide insight into potential management practices which can accompany an integrated resistance management program. Risks were evaluated by fixing the parameter of interest at a range of values while employing the rest of the parameters stochastically. The risk was determined as the proportion of populations (out of 10,000) which developed resistance after 15 years of annual applications of glyphosate and 7 years after annual applications of sulfonylureas.

Initial seed bank density, annual germination, seed removal, mutation rate, and initial resistance allele frequency influenced resistance evolution for both herbicides (Figure 4.12). Resistance risk increases for both glyphosate and sulfonylureas as initial seed bank densities increase (Figure 4.12a). However, the main effect of this parameter is more sensitive at very low seed bank densities for sulfonylureas which is attributed to the complete dominance of the resistance allele. In comparison, the effect of increasing seed bank density can be observed at higher values for glyphosate. As the proportion of the seed bank which germinates annually increases, resistance also increases (Figure 4.12b). Decreasing the amount of seed incorporated into the seed bank via increasing seed removal reduces resistance (Figure 4.12c). Genetic parameters resistance allele frequency (Figure 4.12d) and mutation rate (Figure 4.12e) are extremely influential to the evolution

of resistance. The true values of these parameters are unknown, but as they increase, risk of resistance also increases. This was observed in all simulations as these values were assumed to be higher for sulfonylureas compared to glyphosate, and is one of the reasons

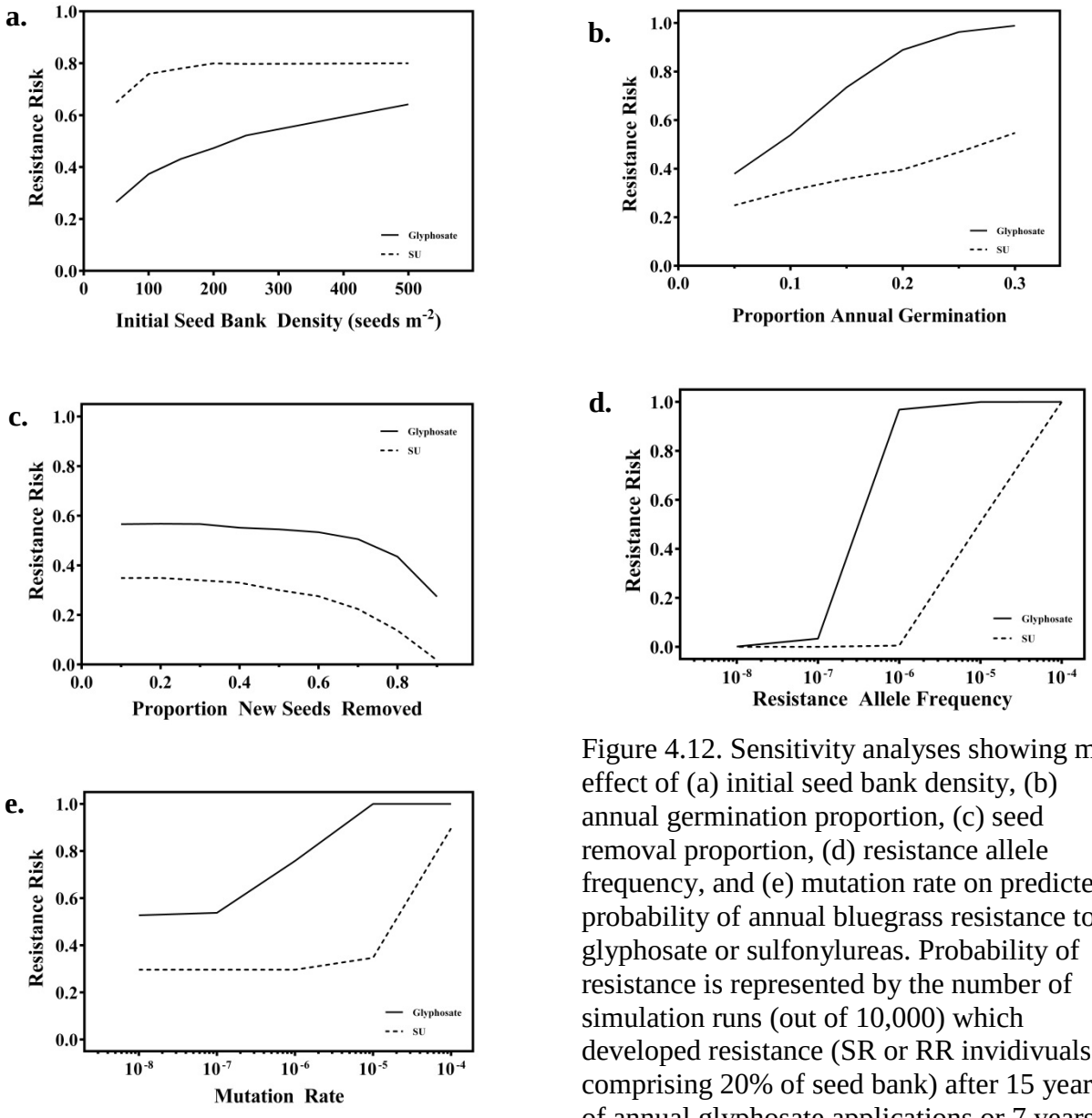


Figure 4.12. Sensitivity analyses showing main effect of (a) initial seed bank density, (b) annual germination proportion, (c) seed removal proportion, (d) resistance allele frequency, and (e) mutation rate on predicted probability of annual bluegrass resistance to glyphosate or sulfonylureas. Probability of resistance is represented by the number of simulation runs (out of 10,000) which developed resistance (SR or RR invidivuals comprising 20% of seed bank) after 15 years of annual glyphosate applications or 7 years of annual sulfonylurea applications in late winter.

why resistance evolved faster than glyphosate. Although parameter interactions are certain to influence predicted resistance evolution, sensitivity analysis of parameter main effects reveal potential cultural strategies for managing annual bluegrass resistance. Practices such as collecting clippings during mowing increases seed removal rates and decreases incorporation of annual bluegrass seed into the soil seed bank. Raising mowing heights and reducing irrigation during peak annual bluegrass germination time can aid in reducing germination rates by limiting sunlight and moisture availability for annual bluegrass seed (McCarty, 2011). Evaluating herbicide efficacy after applications is also important. Scouting for uncontrolled populations could provide initial indications of evolving resistance and allow turf managers to take alternative actions and prevent potentially resistant seed from being incorporated into the seed bank.

While mutation rates and initial resistance allele frequencies are difficult to estimate, the biological characteristics of annual bluegrass have a significant impact on resistance evolution and provide some explanation of its propensity to develop resistance on golf courses. This was demonstrated by Jasieniuk et al. (1996) where higher weed densities increase the probability of finding a resistant individual. With a weed density of 500 m^{-2} and mutation rate of 1×10^{-8}, the probability of a resistant individual occurring in a 30 ha field is 81% for a species that is 95% selfing. The average U.S. golf course has 40 ha of maintained turfgrass and annual bluegrass is a predominantly selfing species (Darmency and Gasquez, 1983; GCSAA, 2007). With viable soil seed banks estimated as high as 185,000 seeds m^{-2} (Watschke et al., 1979) and up to thousands of annual bluegrass plants able to colonize one m^2 (Law, 1981), the probability of a resistant annual

bluegrass plant occurring on a typical golf course is very high, even if mutation rates are low. These dynamics are common with heavy seed producing species, where a single herbicide resistant individual which is exposed to a herbicide application and survives to produce seed can have profound impacts on resistance development.

Although delays in resistance development were observed as the number of applications of the same mechanism of action decreased, >90% risk of resistance was still observed within a 50 yr period regardless of strategy. Therefore, rotating mechanisms of action for annual bluegrass control provides short-term resistance management by itself, but is not an effective long-term resistance management solution. These simulations, however, only considered resistance development to one herbicide with one annual application. In reality, the situation is much more complex with the possibility of developing multiple resistance to several mechanisms of action when more than one herbicide application occurrs each year for annual bluegrass control. Therefore, at a minimum, rotations of mechanisms of action should be extended as long as possible, especially for herbicides such as ALS-inhibitors, and should integrate multiple mechanisms of action. Further, applying herbicides in mixtures is often more effective than using herbicides in rotation (Beckie and Reboud, 2009; Diggle et al., 2003; Gressel and Segel, 1990). This was not considered in the above simulations but could be a focus of future herbicide resistance research and management for turfgrass weed populations.

Conclusions

These basic simulations serve to expand the understanding of annual bluegrass herbicide resistance evolution on golf courses and demonstrate the variables which influence its development in the field. Further improvements to these simulations could be made with studies specific to annual bluegrass on competition-based seed production, emergence patterns under different environmental conditions, fitness costs associated with resistance, and investigations into genetics associated with annual bluegrass resistance. Herbicide resistant annual bluegrass populations pose a serious risk to the future of weed management in commercial turfgrass. Currently, annual bluegrass is resistant to nine mechanisms of action worldwide leaving only three labeled mechanisms of action to which resistance has yet to evolve (Heap, 2014; McElroy et al., 2013). Integrated annual bluegrass management programs are essential to prevent further resistance development to these mechanisms of action

CHAPTER FIVE

EVALUATING NEW ALTERNATIVES FOR ANNUAL BLUEGRASS CONTROL IN CREEPING BENTGRASS AND PERENNIAL RYEGRASS

Introduction

Creeping Bentgrass Putting Greens

Creeping bentgrass is the most commonly used cool-season turfgrass species for golf course putting greens because of its fine leaf texture and stoloniferous (creeping) growth habit. It is most successfully grown in cool, moist climates, like its native habitat, central Europe. However, it is also adapted to portions of the northern United States, where climates are cool and humid (McCarty, 2011). Since creeping bentgrass is a C_3 plant, loss of green color during the winter does not occur, and, unlike bermudagrass, overseeding is not necessary. Due to its desirable morphological characteristics, it is grown on golf course putting greens as far south as coastal South Carolina. It is highly susceptible to stresses common during hot and humid summer months. During these times, plant carbohydrate reserves are depleted and roots shorten, thus reducing its drought, traffic, and disease tolerance. When creeping bentgrass is grown in these environments, cultural practices such as aggressive aerification and thatch management, irrigation and syringing, fungicide applications, and above and below ground air circulation are required.

Annual bluegrass is capable of growing on highly compacted, poorly aerated soils which is common of creeping bentgrass greens not properly managed. Under these conditions, it outcompetes bentgrass due to its shallow roots, allowing the plant to tolerate the lack of soil oxygen (McCarty, 2011). The high input requirements of

bentgrass greens allow annual bluegrass to survive through the summer months, especially if perennial biotypes are present.

Annual Bluegrass Control in Creeping Bentgrass Putting Greens

A USGA publication from the early 1920s contains one of the earliest references to control of annual bluegrass in bentgrass greens. Alexander (1922) noted annual bluegrass could be controlled by mechanical removal with a knife or cup cutter. This is still used today if populations remain low as annual bluegrass-free plugs replace infested areas with minimal disruption. Chemical control is much more difficult to attain due to physiological similarities between creeping bentgrass and annual bluegrass. Both are cool-season (C_3) species, and thus, many metabolic pathways are the same and cannot be exploited with herbicides. Additionally, golf greens are inherently under constant cultural and environmental stresses, and herbicide applications often increase these stress levels. Tolerance for phytotoxicity or turf quality reductions are minimal on golf greens, and thus, many chemical control options for annual bluegrass are not labeled for use on golf greens.

Limited preemergence options exist for bentgrass greens. Since annual bluegrass is a winter annual, preemergence compounds should be applied in late summer to provide control. Due to summer environmental stresses, creeping bentgrass roots are most shallow during this time, and the plant is highly susceptible to many pesticide applications. Preemergence control, therefore, is rarely attempted on creeping bentgrass greens due to the concern of sacrificing the health of the plant. In the past, bensulide has

been effective as a preemergence herbicide in bentgrass greens on the annual biotype. However, control from preemergence bensulide applications is often unacceptable on the perennial biotype commonly associated with bentgrass greens (Callahan and McDonald, 1992).

Like preemergence options, postemergence herbicide options for annual bluegrass in creeping bentgrass putting greens are few. Ethofumesate was investigated as a possibility for postemergence annual bluegrass control in creeping bentgrass putting greens. Lewis and Dipaola (1989) reported 90 to 96% annual bluegrass control and acceptable creeping bentgrass quality with five applications of 0.56 kg ai ha^{-1} or three applications of 0.84 kg ai ha^{-1}. A study in Georgia reported creeping bentgrass was tolerant to sequential ethofumesate treatments of 1.1 kg ai ha^{-1} when applied in September and October, but intolerant at the same rate when applied in October and November (Johnson et al., 1989). Ethofumesate is root absorbed, and applications made in late summer or early fall can increase injury to shallow-rooted bentgrass. Currently, ethofumesate is only labeled for use on creeping bentgrass fairways because of injury concerns to putting greens. Bispyribac-sodium is an ALS-inhibiting herbicide used for annual bluegrass control in overseeded bermudagrass fairways. On creeping bentgrass greens, Park et al. (2002) reported three applications of bispyribac-sodium at 0.075 kg ai ha^{-1} reduced annual bluegrass density to less than 1% with minimal creeping bentgrass injury. However, another study reported bispyribac-sodium rates from 0.018 to 0.075 kg ai ha^{-1} caused unacceptable phytotoxicity and reduced growth of creeping bentgrass in a

greenhouse (Fagerness and Penner, 1998). Like ethofumesate, bispyribac-sodium is not currently labeled for use on creeping bentgrass putting greens.

Two new compounds have recently emerged as possibilities for postemergence annual bluegrass control in creeping bentgrass putting greens. Amicarbazone is a triazolinone herbicide which inhibits photosynthesis at photosystem II (Dayan et al., 2009). McCullough et al. (2010) reported spring applications of amicarbazone were safer on creeping bentgrass, but annual bluegrass control was greater with fall applications. Spring applications did not produce greater than 76% annual bluegrass control at two locations with sequential applications as high as 0.5 kg ai ha^{-1}. This study also reported control of annual bluegrass with amicarbazone was greatly temperature dependent. Perry et al. (2011) reported 93% annual bluegrass control under greenhouse conditions three weeks after a foliar application of 0.53 kg ai ha^{-1}.

Methiozolin is a new isoxazoline compound whose proposed mode of action is disruption of cell wall biosynthesis (Lee et al., 2007) with possible pre and postemergence activity on annual bluegrass and excellent safety on creeping bentgrass putting greens. Askew et al. (2012) reported 93% annual bluegrass control using 6 applications (3 fall, 3 spring) of 0.67 kg ai ha^{-1} each. Venner et al. (2012) reported greater than 85% annual bluegrass control with methiozolin applications of 1 kg ai ha^{-1} or higher regardless of application timing. Neither amicarbazone nor methiozolin are currently labeled in the United States for use on creeping bentgrass putting greens. Further research is required to determine if these compounds hold promise for postemergence annual bluegrass control in creeping bentgrass putting greens.

Plant growth regulators emerged in the 1960s as a possible control strategy for annual bluegrass and are still widely used today (Christians, 1996). Due to the lack of control of the perennial biotype with preemergence herbicides, and the sporadic control and injury to desirable species by postemergence herbicides, PGRs are used to suppress annual bluegrass growth and seedhead formation. Annual bluegrass control with plant growth regulators also provides a gradual transition from annual bluegrass to desirable turf species as opposed to herbicides that may quickly kill annual bluegrass plants leaving voids in the turf stand (Kageyama et al., 1989). Depending upon their classification, PGRs can inhibit cell division or gibberellic acid synthesis. In addition to annual bluegrass control, plant growth regulators can be used to manage clippings and mowing frequency, improve overall turf quality, and reduce bermudagrass growth during overseeding (McCarty, 2011). Mefluidide can be used to prevent seedhead formation of annual bluegrass if timed correctly to seedhead emergence. Timing is highly dependent upon geographic location and climate conditions. However, mefluidide is not recommended for use on bentgrass putting greens (McCarty, 2011). Paclobutrazol is a root-absorbed gibberellin biosynthesis inhibitor. Studies have shown varying paclobutrazol rates and application timings can reduce annual bluegrass density while allowing bentgrass stands to increase. While not directly controlling seedhead production, paclobutrazol can minimize seedhead expression (Kageyama et al., 1989). Johnson and Murphy (1995) reported three spring and three fall applications of paclobutrazol spaced four weeks apart provided 72% annual bluegrass control three weeks after the final treatment. However, the following spring, control was reduced to 57%. Neylan et al.

(1997) reported paclobutrazol alone, or in combination with preemergence herbicides, provided excellent (up to 98%) annual bluegrass control.

Like paclobutrazol, flurprimidol is a root-absorbed gibberellin biosynthesis inhibitor used to manage annual bluegrass populations in creeping bentgrass (McCarty, 2011). A two year study in creeping bentgrass fairways with monthly applications of flurprimidol from May through October with varying rates provided 74 to 78% annual bluegrass control (Bigelow et al., 2007). Although control has been effective in fairways, flurprimidol is not as effective as paclobutrazol on the perennial annual bluegrass biotype typically found in bentgrass greens (Johnson and Murphy, 1995). Trinexapac-ethyl, one of the most widely used PGRs in turfgrass, is also available for annual bluegrass suppression. However, it is commonly used in combination with other PGRs as research shows that control is limited when used alone (McCullough et al., 2005).

Annual bluegrass often invades creeping bentgrass putting greens over time, especially in the transition zone of the United States. Therefore, the purpose of this study was to evaluate various herbicide and plant growth regulator treatment programs for annual bluegrass control and seedhead suppression, including several recently developed herbicides, and to determine their safety for use on creeping bentgrass putting greens.

Overseeding

As temperatures decrease and daylengths shorten during fall months, bermudagrass reduces its growth and eventually goes dormant at temperatures below 10^{o}C for an extended period, resulting in brown turf color. To provide desirable visual

quality (color) and traffic tolerance during winter dormancy, golf course fairways and sports fields are commonly overseeded with a cool-season turf species (typically perennial ryegrass). Although important economically, especially for resort golf courses, consistent overseeding can increase annual bluegrass weed pressure (McCarty, 2011). Overseeding is a major contributing factor to annual bluegrass growth and spread for several reasons. First, bermudagrass must be thinned in fall via scalping, vertical mowing and aerification. Concurrently, annual bluegrass seed is beginning to germinate, and the open bermudagrass canopy allows proficient sunlight into the turf canopy for maximum germination. Youngner (1959) reported light vertical mowing of bermudagrass in the fall had increased annual bluegrass populations. Second, light and frequent irrigation is required to establish perennial ryegrass seed, thus providing sufficient moisture to encourage annual bluegrass germination. Third, overseeding weakens bermudagrass stands during spring transition, providing less competition for annual bluegrass, therefore allowing it to increase over time. Finally, methods of chemical control for annual bluegrass are limited when bermudagrass is overseeded with a C_3 plant. Extreme caution must be used and timing must be exact to prevent preemergence herbicides from interfering with the establishment of the desirable overseeding. Also, due to the similarity of annual bluegrass to overseeding species (especially *Poa trivialis* L., typically used to overseed ultradwarf bermudagrass greens), consistent selective postemergence control is difficult to attain (McCarty, 2011).

Annual Bluegrass Control in Overseeded Golf Course Fairways

When compared to creeping bentgrass putting greens, more preemergence annual bluegrass control options exist for bermudagrass golf course fairways and roughs. However, overseeding bermudagrass can reduce these options and alter timings when applications are made. Fenarimol is a systemic fungicide also with herbicidal properties used for preemergence annual bluegrass control. In addition, its short soil residual makes it an excellent preemergence control option for overseeded golf greens, tees, and fairways (McElroy et al., 2004). However, the cost of this product is considerably higher than other preemergence herbicides, and thus, is typically only used on smaller turf areas, such as greens and tees. In addition, fenarimol will voluntarily become unavailable in 2014. Menn (1986) noted varying rates of subsequent applications of fenarimol provided complete control of annual bluegrass in overseeded golf greens. Trenholm and McCarty (1996) reported annual bluegrass control in overseeded bermudagrass golf greens with two different application sequences, with the sequence closer to overseeding providing greater control; however, this sequence also diminished overseeding density. Oxadiazon also can provide preemergence annual bluegrass control. A 2001 study reported annual bluegrass control of greater than 90% with a single application of oxadiazon applied eight weeks before overseeding (Yelverton and McCarty, 2001). Ryegrass overseeding establishment is extremely sensitive to rates and timings of oxadiazon, and caution must be used in application to avoid the negative effects of herbicide ovarlap (Kopec and Gilbert, 2001). Mitosis-inhibiting herbicides are popular chemistries for preemergence annual bluegrass control prior to overseeding. Dernoeden and Krouse (1994) reported

that various rates and timings gave very good control (88%) with prodiamine and acceptable control (74-80%) with dithiopyr. However, these herbicides have been used for many years, and resistance issues to these herbicides have been reported in the Carolinas (Lowe et al., 2001; Isgrigg et al., 2001). Recent research concluded acceptable annual bluegrass control could still be obtained when these chemistries are no longer effective (Cross et al., 2012).

Fewer options exist for postemergence annual bluegrass control in desirable cool-season grasses due to the physiological similarities of the species. Ethofumesate has been shown to provide early postemergence control of annual bluegrass in perennial ryegrass using varying rates and timings (Carlson and Branham, 1992). However, timing of application is critical. In overseeded bermudagrass, applications must be made after bermudagrass dormancy to avoid damage to the bermudagrass and to prevent a delay in spring transition (McCarty, 1999). Johnson (1983) reported 100% annual bluegrass control in perennial ryegrass with split ethofumesate applications in October and November at 2.2 kg ai ha^{-1}. Additionally, he reported some temporary discoloration to the perennial ryegrass, but no permanent damage. Recently, bispyribac-sodium has emerged as a postemergence option for annual bluegrass control in overseeded bermudagrass. Greater than 90% annual bluegrass control with varying rates of bispyribac-sodium has been reported (McCullough and Hart, 2009). McCullough and Hart (2009) also noted some perennial ryegrass injury (≤15%) with the use of bispyribac-sodium.

Sulfonylurea herbicide applications prior to overseeding can be effective for early postemergence annual bluegrass control (Waltz and Murphy, 2004; McElroy et al.,

2011). These herbicides have soil half-lives from 4 to 56 days, with an average of 35 days, and must be applied at specific intervals prior to overseeding as suggested by the herbicide label (Waltz and Murphy, 2004; WSSA, 2007). Because these herbicides have shorter half-lives than preemergence herbicides, they can be sprayed closer to overseeding without having a negative effect on seed germination. Cross et al. (2012) reported up to 80% annual bluegrass control with rimsulfuron and foramsulfuron applied seven days prior to overseeding and no subsequent problems with establishment of perennial ryegrass.

Golf courses that overseed their bermudagrass fairways, especially year after year, will inevitably combat annual bluegrass in their turf stands. The purpose of these studies was to evaluate various pre- and postemergence herbicides and plant growth regulators to determine their effectiveness in controlling annual bluegrass and their safety for use in perennial ryegrass.

Materials and Methods

Annual Bluegrass Control in Creeping Bentgrass Putting Greens

Treatments and Ratings. A study was conducted on USGA specification creeping bentgrass putting greens from 2010 to 2013 to evaluate multiple herbicides and plant growth regulators for annual bluegrass control. Treatment programs were evaluated at two locations: the Walker Golf Course at Clemson University (WC; Clemson, SC) and Cross Creek Plantation (CC; Seneca, SC). At WC, treatments were evaluated on 'Crenshaw' creeping bentgrass putting greens from fall 2010 to spring 2012. Treatments

at CC occurred on ‘L-93’ creeping bentgrass from fall 2011 to spring 2013. The same plots were treated in the second year of the study at each location for evaluation of treatments over a two year period. All treatments were applied using a CO_2-pressurized backpack sprayer calibrated to deliver 187 L ha^{-1} through 8003 flat-fan nozzles. Treatment programs, active ingredients, rates, and timings are presented in Table 5.1. Plots treated with methiozolin were sprayed three times at one-third the herbicide rate to achieve a recommended spray volume of 561 L ha^{-1} (Moghu Research Center, South Korea). Sites were maintained throughout the studies by the golf course maintenance staffs to normal putting green standards with daily mowing heights of 0.32 cm and regular irrigation cycles to prevent moisture stress. Study sites received approximately 292 kg N ha^{-1} yr^{-1} and were treated with fungicides as needed to prevent disease pressure.

Air temperature, relative humidity, wind speed, cloud cover, soil temperature, and soil moisture conditions were recorded at the time of each application. Subsequent performance ratings were recorded monthly from trial initiation until late spring and included annual bluegrass density and visible seedheads, annual bluegrass and creeping bentgrass phytotoxicity, turfgrass quality, turfgrass density and bermudagrass spring greenup. Annual bluegrass density was visually estimated using a scale of 0 to 100%, where 100% = total annual bluegrass coverage. Density was also quantified using the line intersect method and a 1 x 1 m grid with 36 squares. The number of squares where an annual bluegrass plant appeared was recorded for each plot. At WC, plot sizes were large, thus three repeated measures for each plot were recorded by random placement of the grid in different locations. Control values were calculated using both visual and line-

intersect estimates using equation 2.1 where observed densities were the density/count value at the given rating date and initial densities were the density/count value at the initiation of the study. Annual bluegrass and creeping bentgrass phytotoxicity were visually evaluated using a scale of 0 to 100%, (100% = no green tissue). The maximum acceptable level of phytotoxicity for bentgrass was considered 20%. Seedhead suppression was visually evaluated using a scale of 0 to 9 (9 = bright, vibrant seedheads) and was converted to a percentage of the nontreated control. Turfgrass quality was visually evaluated using a scale of 1 to 9 (9 = dense, dark green, healthy turf) with 7 considered the minimum acceptable turf quality for a golf course putting green. Turfgrass density was visually evaluated using a scale of 0 to 100% (100% = complete turfgrass coverage). Spring bermudagrass greenup was visually evaluated using a scale of 0 to 100% (100% = green, actively growing bermudagrass).

Statistical Design and Analysis. The experimental design for this study was a randomized complete block with four replications. At WC, greens were split horizontally into two equal sections with plots approximately 1.5 x 8 m. Two replications were on the putting green and two replications were on the second green. At CC, plots were 1.5 x 1.5 m. Data for each response variable were subjected to ANOVA to evaluate effects of treatment, location, block, and treatment-by-location interaction. A mixed model was used to adjust for random effect of location and block (replicate). For each response variable, ANOVA assumptions such as equality of variance and normal distribution were evaluated and adjusted if necessary. Where ANOVA indicated significant effects, further comparisons were conducted according to Fisher's protected LSD. Comparisons between

Table 5.1. Treatments, active ingredients, rates, and application timing and notes for annual bluegrass control in 'Crenshaw' and 'L-93' creeping bentgrass putting greens at the Walker Golf Course (Clemson, SC) and Cross Creek Plantation (Seneca, SC), respectively, from 2010 to 2013.

Active Ingredient(s)	Rate	Timing/Application Notes
Nontreated	---	---
Cumyluron 1.5L	1720	Single application in early fall.
Cumyluron	1720	Initial application in early fall followed by a sequential application 9 WAI[a].
Paclobutrazol 2SC	140-280	Initial application in early fall at 140 g ai ha^{-1}. Three WAI, sequential application at 210 g ai ha^{-1}. Applications continued on three wk intervals at 280 g ai ha^{-1}, stopping in late fall and resuming in late winter.
Flurprimidol + Trinexapac-ethyl 1.51L	132	Initial application in early fall with sequential applications made on three wk intervals, stopping in late fall and resuming in late winter.
Flurprimidol 2SC	280	Initial application in early fall with sequential applications made on three wk intervals, stopping in late fall and resuming in late winter.
Bispyribac-sodium 17.6WDG	8.6	Initial application in early fall with sequential applications made on three wk intervals, stopping in late fall and resuming in late winter.
Bispyribac-sodium fb amicarbazone 70WDG[b]	6.2 fb 24.5	Initial application of bispyribac-sodium in early spring. Amicarbazone application WAI bispyribac-sodium, followed by a sequential bispyribac-sodium application and amicarbazone application made one wk apart.
Amicarbazone	49	Initial application made in early spring with three sequential applications following one wk apart.
Cumyluron fb amicarbazone	1720 fb 49	Initial cumyluron application in early fall followed by sequential cumylruon application 9 WAI. Amicarbazone tank mixed at 49 g ai ha^{-1} for the sequential application.
Methiozolin 2EC	560	Three applications in early fall and three in early spring on one wk intervals.
Methiozolin	840	Two applications in early fall and two in early spring on two wk intervals.
Methiozolin	1120	Two applications in early fall and one in early spring. Fall applications four wk apart.
Methiozolin	1120	Three applications in early fall on four wk intervals.
Paclobutrazol fb amicarbazone	140-280 fb 24.5	Paclobutrazol program as described above, with four applications of amicarbazone made on one wk intervals beginning early spring.

[a] Abbreviation: WAI, weeks after initial.

[b] Treatments containing amicarbazone or methiozolin included a nonionic surfactant at 0.25% v v^{-1}. Rates are in g ai ha^{-1}.

locations were conducted using linear contrasts. Significant effects and differences were based on $P < 0.05$. All calculations were performed using JMP Pro version 10 or SAS version 9.3.

Annual Bluegrass Control in Bermudagrass Fairways Overseeded with Perennial Ryegrass

Treatments and Ratings. Five studies were conducted from 2010 to 2014 at various locations to evaluate current and future control options for annual bluegrass in bermudagrass fairways overseeded with perennial ryegrass. Sites included the Preserve at Verdae (PR; Greenville, SC), Boscobel Golf Club (BG; Pendleton, SC), Sage Valley Golf Club (SV; Graniteville, SC), and Clemson University (CU; Clemson, SC). Trials included evaluation of pre- plus postemergence programs (Table 5.2), pre-overseeding control options (Table 5.3), and postemergence control options with amicarbazone and methiozolin (Tables 5.4, 5.4, 5.6). All treatments were applied using a CO_2-pressured backpack sprayer calibrated to deliver 187 L ha^{-1} using 8003 flat-fan nozzles with the exception of granular treatments which were applied evenly in several directions using a shaker jar. Prior to overseeding, typical seedbed preparations were completed, with light vertical mowing, lowered mowing height, and removal of debris. After overseeding, irrigation was applied several times daily to maintain moist conditions until ryegrass seedlings were established. Study sites were overseeded with approximately 336 kg perennial ryegrass seed ha^{-1}. Turf was maintained at approximately 1.9 cm at each site throughout the duration of the studies and received fertility and irrigation according to each site's maintenance program.

Table 5.2. Treatments, rates, and timings for pre- plus postemergence annual bluegrass control programs at the Preserve at Verdae (PR; Greenville, SC) from 2010-2011 and Boscobel Golf Club (BG; Pendleton, SC) from 2011-2012.

Treatment[a]	Rate	Timing
	-----kg ai ha^{-1}-----	
Nontreated	---	---
Prodiamine 4L fb[b]	0.73 fb	8 WBO fb
Trifloxysulfuron 75WG fb	0.018 fb	3 WBO fb
Paclobutrazol 2SC + Prodiamine fb	0.18 + 0.56 fb	10 WAO fb
Paclobutrazol fb	0.18 fb	14 WAO fb
Paclobutrazol	0.18	18 WAO
Foramsulfuron 0.19SC fb	0.015 fb	1 WBO fb
Ethofumesate 1.5EC	1.68	9 WAO
Ethofumesate fb	1.12 fb	9 WAO fb
Ethofumesate	1.12	12 WAO
Prodiamine fb	0.84 fb	8 WBO fb
Foramsulfuron fb	0.015 fb	1 WBO fb
Ethofumesate	1.68	9 WAO
Prodiamine fb	0.84 fb	8 WBO fb
Foramsufluron fb	0.015 fb	1 WBO fb
Ethofumesate fb	1.68 fb	9 WAO fb
Ethofumesate	1.68	12 WAO

[a] Overseeding occurred on 18 Oct. 2010 at PR and 21 Sept. 2011 at BG.
[b] Abbreviations: fb, followed by; WBO, weeks before overseeding; WAO, weeks after overseeding.

Air temperature, relative humidity, wind speed, cloud cover, soil temperature, and soil moisture conditions were recorded at the time of each application. Annual bluegrass density was visually evaluated at numerous dates throughout the study on a scale of 0 to 100% (100% = complete annual bluegrass coverage). Density ratings were converted to percent control using formula 2.1. Perennial ryegrass quality was visually evaluated after

overseeding at numerous rating dates using a scale of 0 to 10 (10 = dark green, dense, uniform ryegrass). Ryegrass injury following treatments was also evaluated at several dates using a visual scale of 0 to 100% (100% = as yellow/brown foliage).

Statistical Design and Analysis. The experimental design for each study was a randomized complete block with three replications and 2- x 3-m plots. Data for each

Table 5.3. Treatments, rates, and timings for pre-overseeding annual bluegrass control options at the Preserve at Verdae (PR; Greenville, SC) from 2010-2011 and Boscobel Golf Club (BG; Pendleton, SC) from 2011-2012.

Treatment[a]	Rate	Timing
	-----kg ai ha^{-1}-----	
Nontreated	---	---
Prodiamine 4L	0.84	8 WBO
Prodiamine	0.56	8 WBO
Prodiamine fb[b]	0.42 fb	8 WBO fb
Prodiamine	0.42	10 WAO
Dithiopyr 2EW	0.56	6 WBO
Dithiopyr fb	0.56 fb	6 WBO fb
Dithiopyr	0.56	10 WAO
Oxadiazon 2G	2.24	8 WBO
Foramsulfuron 0.19SC	0.03	1 WBO
Trifloxysulfuron 75WG[c]	0.018	3 WBO
Rimsulfuron 25DF	0.018	1 WBO
Sulfosulfuron 75WDG	0.092	1 WBO

[a] Overseeding occurred on 18 Oct. 2010 at PR and 21 Sept. 2011 at BG.
[b] Abbreviations: fb, followed by; WBO, weeks before overseeding; WAO, weeks after overseeding.
[c] Trifloxysulfuron and rimsulfuron applications included nonionic surfactant at 0.25% v v^{-1}.

response variable were subjected to ANOVA to evaluate effects of treatment, location, block, and treatment-by-location interactions. A mixed model was used to adjust for random effects of location and block (replicate). For each response variable, ANOVA assumptions such as equality of variance and normal distribution were evaluated and adjusted if necessary. Where ANVOA indicated significant effects, further comparison was conducted according to Fisher's protected LSD. Significant effects and differences were based on $P < 0.05$. All analyses and calculations used either JMP Pro 10 or SAS version 9.3.

Table 5.4. Treatments, rates, and timings for postemergence annual bluegrass control options at the Preserve at Verdae (PR; Greenville, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2010-2011.

Treatment[a]	Rate	Timing
	-----kg ai ha^{-1}-----	
Nontreated	---	---
Ethofumesate 4SC fb[b]	1.12 fb	Late fall fb
Ethofumesate	1.12	21 DAI
Ethofumesate 1.5EC fb	1.12 fb	Late fall fb
Ethofumesate	1.12	21 DAI
Bispyribac-sodium 17.6 WDG fb	0.074 fb	Late winter fb
Bispyribac-sodium	0.074	21 DAI
Amicarbazone 75WG[c] fb	0.1 fb	Late winter fb
Amicarbazone	0.1	21 DAI
Amicarbazone fb	0.2 fb	Late winter fb
Amicarbazone	0.2	21 DAI
Methiozolin 2EC fb	1.12 fb	Late fall fb
Methiozolin	1.12	21 DAI
Methiozolin fb	0.84 fb	Late fall fb
Methiozolin	0.84	14 DAI

[a] Overseeding occurred on 14 Sept. 2010 at SV and 18 Oct. 2010 at PR.
[b] Abbreviations: fb, followed by; DAI, days after initial.
[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

Table 5.5. Treatments, rates, and timings for postemergence annual bluegrass control options at Boscobel Golf Club (BG; Pendleton, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2011-2012.

Treatment[a]	Rate	Timing
	-----kg ai ha^{-1}-----	
Nontreated	---	---
Ethofumesate 4SC fb[b]	1.12 fb	Late fall fb
Ethofumesate	1.12	21 DAI
Bispyribac-sodium 17.6 WDG fb	0.074 fb	Late winter fb
Bispyribac-sodium	0.074	21 DAI
Amicarbazone 75WG[c] fb	0.1 fb	Late winter fb
Amicarbazone	0.1	21 DAI
Amicarbazone fb	0.2 fb	Late winter fb
Amicarbazone	0.2	21 DAI
Methiozolin 2EC fb	1.12 fb	Late fall fb
Methiozolin	1.12	21 DAI
Methiozolin fb	0.56 fb	Late fall fb
Methiozolin	0.56	21 DAI
Methiozolin fb	1.12 fb	Late winter fb
Methiozolin	1.12	21 DAI
Methiozolin fb	0.56 fb	Late winter fb
Methiozolin	0.56	21 DAI
Methiozolin	1.12	Late winter

[a] Overseeding occurred on 15 Sept. 2011 at SV and 21 Sept. 2011 at BG.
[b] Abbreviations: fb, followed by; DAI, days after initial.
[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

Table 5.6. Treatments, rates, and timings for postemergence annual bluegrass control options at Clemson University (CU; Clemson, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2013-2014.

Treatment[a]	Rate	Timing
	-----kg ai ha^{-1}-----	
Nontreated	---	---
Ethofumesate 4SC fb[b]	1.68 fb	9 WAO fb
Ethofumesate	1.68	12 WAO
Bispyribac-sodium 17.6 WDG fb	0.074 fb	Late winter fb
Bispyribac-sodium	0.074	3 WAI
Paclobutrazol 2SC fb	0.21 fb	Early winter fb
Paclobutrazol fb	0.28 fb	4 WAI fb
Paclobutrazol fb	0.35 fb	8 WAI fb
Paclobutrazol fb	0.42 fb	12 WAI fb
Paclobutrazol	0.42	16 WAI
Methiozolin 2EC[c] fb	0.38 fb	Late winter fb
Methiozolin	0.38	3 WAI
Methiozolin fb	0.76 fb	Late winter fb
Methiozolin	0.76	3 WAI
Methiozolin fb	0.38 fb	Late fall fb
Methiozolin fb	0.38 fb	3 WAI
Methiozolin fb	0.38 fb	Late winter fb
Methiozolin	0.38	3 WAI
Paclobutrazol fb	0.21 fb	Early winter fb
Paclobutrazol + Methiozolin fb	0.28 + 0.38 fb	4 WAI fb
Paclobutrazol + Methiozolin fb	0.35 + 0.38 fb	8 WAI fb
Paclobutrazol fb	0.42 fb	12 WAI fb
Paclobutrazol	0.42	16 WAI
Paclobutrazol fb	0.21 fb	Early winter fb
Paclobutrazol + Methiozolin fb	0.28 + 0.76 fb	4 WAI fb
Paclobutrazol + Methiozolin fb	0.35 + 0.76 fb	8 WAI fb
Paclobutrazol fb	0.42 fb	12 WAI fb
Paclobutrazol	0.42	16 WAI
Amicarbazone 70WDG fb	0.15 fb	Late winter fb
Amicarbazone	0.15	3 WAI
Paclobutrazol fb	0.21 fb	Early winter fb
Paclobutrazol + Amicarbazone fb	0.28 + 0.15 fb	4 WAI fb
Paclobutrazol + Amicarbazone fb	0.35 + 0.15 fb	8 WAI fb
Paclobutrazol fb	0.42 fb	12 WAI fb
Paclobutrazol	0.42	16 WAI
Methiozolin fb	0.38 fb	Late fall fb
Methiozolin	0.38	3 WAI
Methiozolin fb	0.76 fb	Late fall fb
Methiozolin	0.76	3 WAI

[a] Overseeding occurred on 15 Sept. 2013 at SV and 1 Oct. 2013 at CU.

[b] Abbreviations: fb, followed by; WAO, weeks after overseeding; WAI, weeks after initial.

[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

Results and Discussion

Annual Bluegrass Control in Creeping Bentgrass Putting Greens

A mixed model was used to analyze data from all response variables to account for random effects of location and block. Analysis of variance revealed significant treatment-by-location interactions for all response variables (Table 5.7). Thus, data are presented separately by location. Annual bluegrass control, seedhead suppression, and turf quality data are presented from a final spring rating date after all treatments had been applied for two years at both sites.

At WC, treatments containing methiozolin were not different from each other and provided from 76 to 89% control after two years of applications (Table 5.8). The

Table 5.7. ANOVA and correlation between visual and line-intersect control ratings for annual bluegrass control study in creeping bentgrass putting greens at the Walker Golf Course (2010 to 2012) and Cross Creek Plantation (2011 to 2013).

Source	df	Control		Seedhead Suppression	Turf Quality
		Visual	Line-Intersect		
Treatment	14	*	*	*	*
Location	1	NS[a]	*	NS	*
Treatment*Location	14	*	*	*	*
Block	3	NS	NS	NS	NS
		WC	CC		
Pearson Correlation Coefficient[b]		0.9516*	0.9422*		

[a] Abbreviation: WC, Walker Golf Course; CC, Cross Creek; NS, not significant.
[b] Correlation coefficients separated by year due to significant treatment-by-locations.
*Indicates a significant effect at $P < 0.05$.

paclobutrazol program provided less control than methiozolin treatments (53%) but greater than all other treatments. All other treatments provided 0 to 5% control at WC. At CC, all methiozolin treatments again provided greatest control compared to all other treatments (73 to 77%) with the exception of paclobutrazol fb amicarbazone (79%). The paclobutrazol program provided 46% control while all other treatments provided <13% control at CC.

Table 5.8. Annual bluegrass control (± standard error) in creeping bentgrass putting greens as affected by various herbicide/plant growth regulator programs for two years at the Walker Golf Course (WC) and Cross Creek Plantation (CC).

	Annual Bluegrass Control[b]	
Treatment[a]	WC	CC
	----------%----------	
Nontreated	0 ± 0	0 ± 0
Cumyluron 1.5L	0 ± 0	0 ± 0
Cumyluron fb cumyluron	0 ± 0	0 ± 0
Paclobutrazol 2SC	52.5 ± 13.1	46.3 ± 10.1
Flurprimidol + trinexapac-ethyl 1.51L	0 ± 0	0 ± 0
Flurprimidol 2SC	0 ± 0	0 ± 0
Bispyribac-sodium 17.6WDG	0 ± 0	0 ± 0
Bispyribac-sodium fb amicarbazone 70WDG[b]	0 ± 0	0 ± 0
Amicarbazone	0 ± 0	12.5 ± 12.5
Cumyluron fb amicarbazone	0 ± 0	0 ± 0
Methiozolin (6x)	78.3 ± 10.4	75.0 ± 6.8
Methiozolin (4x)	76.0 ± 10.9	72.9 ± 5.3
Methiozolin (3x, fall/spring)	81.9 ± 11.0	76.7 ± 5.6
Methiozolin (3x, fall)	89.2 ± 7.9	74.0 ± 4.8
Paclobutrazol fb amicarbazone	5.0 ± 5.0	79.2 ± 2.4
$LSD_{0.05}$	18.1	14.6

[a] Treatments were applied from 2010-2012 at WC and from 2011-2013 at CC.
[b] Annual bluegrass control was calculated from visual density ratings using the formula [1-(observed/initial)] x 100.

Seedheads were suppressed greatest by treatments containing methiozolin or paclobutrazol regardless of location (Table 5.9). At WC, paclobutrazol alone and in combination with amicarbazone provided 77 to 81% seedhead suppression, while methiozolin treatments suppressed seedheads >82%. Amicarbazone alone provided 52% suppression while all other treatments suppressed seedheads <30%. Similar results were observed at CC where >84% seedhead suppression was observed with all methiozolin treatments and paclobutrazol fb amicarbazone. Paclobutrazol alone suppressed seedheads

Table 5.9. Annual bluegrass seedhead suppression (± standard error) in creeping bentgrass putting greens as affected by various herbicide/plant growth regulator programs for two years at the Walker Golf Course (WC) and Cross Creek Plantation (CC).

	Location	
Treatment[a]	WC	CC
	------Seedhead Suppression (%)------	
Nontreated	0 ± 0	0 ± 0
Cumyluron 1.5L	19.0 ± 7.5	10.4 ± 6.3
Cumyluron fb cumyluron	19.0 ± 11.2	10.4 ± 6.3
Paclobutrazol 2SC	81.0 ± 7.5	67.7 ± 22.8
Flurprimidol + trinexapac-ethyl 1.51L	7.1 ± 4.1	19.8 ± 7.9
Flurprimidol 2SC	22.0 ± 7.0	43.8 ± 9.2
Bispyribac-sodium 17.6WDG	21.4 ± 12.4	11.5 ± 3.9
Bispyribac-sodium fb amicarbazone 70WDG[b]	32.1 ± 12.2	0 ± 0
Amicarbazone	51.8 ± 6.8	6.3 ± 6.3
Cumyluron fb amicarbazone	10.7 ± 10.7	3.1 ± 3.1
Methiozolin (6x)	85.1 ± 5.9	91.7 ± 4.8
Methiozolin (4x)	81.5 ± 6.8	87.5 ± 8.0
Methiozolin (3x fall/spring)	88.7 ± 3.8	87.5 ± 8.0
Methiozolin (3x fall)	88.7 ± 7.0	84.4 ± 6.9
Paclobutrazol fb amicarbazone	77.4 ± 7.6	86.5 ± 5.2
$LSD_{0.05}$	22.9	23.8

[a] Treatments were applied from 2010-2012 at WC and from 2011-2013 at CC.
[b] Annual bluegrass seedhead suppression was calculated from visual seedhead ratings and transformed to a percentage of the nontreated control.

68%, but only methiozolin applied six times per year (92%) provided greater suppression. Less than 44% seedhead suppression was observed with all other treatments at CC.

At WC, greatest turf quality was observed for all methiozolin treatments (8.4 to 8.5), paclobutrazol alone (8.1), paclobutrazol fb amicarbazone (7.9), and flurprimidol (7.9) (Table 5.10). All remaining treatments provided acceptable (≥7.0) turf quality except amicarbazone alone (5.5). In contrast, only one methiozolin treatment (1120 g ai ha^{-1} applied three times in fall) yielded acceptable turf quality (7.3) at CC. Greatest turf

Table 5.10. Creeping bentgrass quality (± standard error) as affected by various herbicide/plant growth regulator programs for two years at the Walker Golf Course (WC) and Cross Creek Plantation (CC).

	Location	
Treatment[a]	WC	CC
	------Seedhead Suppression (%)------	
Nontreated	7.1 ± 0.13	7.0 ± 0
Cumyluron 1.5L	7.3 ± 0.14	7.0 ± 0
Cumyluron fb cumyluron	7.4 ± 0.13	7.0 ± 0
Paclobutrazol 2SC	8.1 ± 0.13	8.0 ± 0
Flurprimidol + trinexapac-ethyl 1.51L	7.3 ± 0.14	7.0 ± 0
Flurprimidol 2SC	7.9 ± 0.13	7.1 ± 0.13
Bispyribac-sodium 17.6WDG	7.0 ± 0	7.0 ± 0
Bispyribac-sodium fb amicarbazone 70WDG[b]	7.1 ± 0.13	7.0 ± 0
Amicarbazone	5.5 ± 0.64	7.0 ± 0
Cumyluron fb amicarbazone	7.3 ± 0.14	7.0 ± 0
Methiozolin (6x)	8.4 ± 0.13	6.9 ± 0.13
Methiozolin (4x)	8.4 ± 0.13	6.8 ± 0.25
Methiozolin (3x fall/spring)	8.5 ± 0	7.3 ± 0.14
Methiozolin (3x fall)	8.4 ± 0.13	6.1 ± 0.13
Paclobutrazol fb amicarbazone	7.9 ± 0.31	7.9 ± 0.31
$LSD_{0.05}$	0.62	0.35

[a] Treatments were applied from 2010-2012 at WC and from 2011-2013 at CC.
[b] Annual bluegrass seedhead suppression was calculated from visual seedhead ratings and transformed to a percentage of the nontreated control.

quality was observed with paclobutrazol alone (8.0) and paclobutrazol fb amicarbazone (7.9) with all other treatments providing turf quality ratings between 7.0 and 7.1.

Currently, there are no postemergence herbicides labeled for creeping bentgrass putting greens which adequately control annual bluegrass. Multiple applications of paclobutrazol is the current standard, but these only provide suppression of annual bluegrass (Kageyama et al., 1989). When applications are not made at frequent intervals, annual bluegrass rebounds quickly and aggressively grows and spreads (Johnson and Murphy, 1995). Regardless of location, <53% annual bluegrass control was observed with this program. However, seedheads were suppressed at both locations (81% at WC, 68% at CC) which reduced the unsightly appearance of annual bluegrass during the spring. Paclobutrazol does not directly control seedhead production but can minimize seedhead expression (Kageyama et al., 1989). Turf quality was >7.9 at both locations for all treatments containing paclobutrazol. Thus, multiple applications of paclobutrazol on two to three week intervals during the fall and spring remain the most effective labeled option for annual bluegrass control in creeping bentgrass.

While paclobutrazol suppresses annual bluegrass, plants remain capable of reproducing and spreading even if applications are consistent. Thus, there is a great need for a selective herbicide to reduce annual bluegrass populations on putting greens. Results from this field study suggest multiple applications of methiozolin may provide a solution to this problem. At both locations, control was >73% regardless of rate or timing, and there were no differences within methiozolin treatments. However, more applications at lower rates (e.g., 6+ applications at ≤560 g ai ha^{-1}) are probably the most

effective option where annual bluegrass infestations are high. In the first year at both locations, high rates of methiozolin (1120 g ai ha^{-1}) removed annual bluegrass too quickly and left voids in creeping bentgrass putting greens producing unacceptable turf quality (data not shown). This was not observed with the lowest rate of methiozolin, where all six applications were required to remove annual bluegrass plants in the spring.

Consistent control and seedhead suppression was observed at both locations with methiozolin programs, but turf quality was unacceptable for three methizolin programs at CC. At both locations, some bentgrass thinning was observed following methiozolin applications. However, thinning was only observed in the first year at WC (2010-2011) while methiozolin plots remained thin into the second year at CC (2012-2013). This may be due to differences in climate as treatments were applied in different years at both locations, but may also indicate a difference in susceptibility of different bentgrass cultivars to this herbicide. Treatments were applied to 'Crenshaw' creeping bentgrass at WC and 'L-93' at CC. Greens are older at CC and contained many visible segregates where differences in susceptibility to treatments were visually observed. Therefore, turf managers should test methiozolin on a nursery or out-of-play area prior to making applications to determine the safety of the herbicide at different locations.

An additional candidate for selective postemergence control in creeping bentgrass is amicarbazone. Four spring amicarbazone applications at 49 g ai ha^{-1} provided <13% control (Table 5.8). This rate significantly injured creeping bentgrass at WC reducing turf quality. However, less injury was observed at CC, and like methiozolin, may suggest some difference in susceptibility to this herbicide across different cultivars. This

herbicide is also temperature dependent and may have been affected by conditions at the time of application. Previous research suggests fall applications of amicarbazone are more efficacious for annual bluegrass control, but greater creeping bentgrass injury occurs at this time (McCullough et al., 2010).

Tank-mixing amicarbazone with other products (e.g., paclobutrazol, bispyribac-sodium) did not improve control. A paclobutrazol program with four applications of amicarbazone in the spring at 24.5 g ai ha^{-1} each provided 79% control, but this was not different from the paclobutrazol program alone (Table 5.8). Control was poor with four applications of 49 g ai ha^{-1}. Thus, higher rates are probably needed to control annual bluegrass, especially when it is actively growing and mature in the spring when this herbicide is usually applied. However, at higher rates, significant injury is likely to occur.

Other treatments evaluated in these trials did not provide effective annual bluegrass control or seedhead suppression. These products may need to be sprayed at higher rates to achieve control or be mixed with other products to enhance activity. Regardless, paclobutrazol remains the most effective control option for creeping bentgrass putting greens. Methiozolin is not currently registered for use in the U.S. but appears as an effective herbicide for selective control of annual bluegrass. Upon registration, superintendents must use this herbicide judiciously as many applications are required to achieve acceptable annual bluegrass control which could quickly select for resistant biotypes. After adequate control of annual bluegrass is achieved after one to two years of methiozolin use, superintendents should revert to the paclobutrazol program for suppression until further methiozolin applications are needed.

Annual Bluegrass Control in Bermudagrass Fairways Overseeded with Perennial Ryegrass

Five studies were conducted to investigate control options for bermudagrass fairways overseeded with perennial ryegrass. The first study investigated current standard pre- plus postemergence programs commonly used for annual bluegrass control in overseeded fairways. For annual bluegrass control, there was no significant treatment-by-location interaction (Table 5.11), thus, data are combined and presented over both locations. All programs provided >82% annual bluegrass control (Table 5.12). A combination of prodiamine fb foramsulfuron fb ethofumesate provided 96% control, while foramsulfuron fb ethofumesate provided 92% control. Only prodiamine fb trifloxysulfuron fb paclobtrazol + prodiamine fb paclobutrazol provided less control (83%) than these two programs. The remaining programs provided 90% control. There was no treatment effect on ryegrass quality (Table 5.11) and treatments were not different from the nontreated control (Table 5.13). These results suggest several programs are currently available combining pre- plus postemergence herbicides and plant

Table 5.11. ANOVA for pre- plus postemergence annual bluegrass control programs at the Preserve at Verdae (Greenville, SC) from 2010-2011 and Boscobel Golf Club (Pendleton, SC) from 2011-2012.

Source	df	Control	Ryegrass Quality
Treatment	5	*	NS
Location	1	NS[a]	*
Treatment*Location	5	NS	NS
Block	2	NS	NS
Error	5		

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

Table 5.12. Annual bluegrass control (± standard error) for pre- plus postemergence annual bluegrass control programs at the Preserve at Verdae (PR; Greenville, SC) from 2010-2011 and Boscobel Golf Club (BG; Pendleton, SC) from 2011-2012.

Treatment[a]	Rate	Annual Bluegrass Control
	-----kg ai ha^{-1}-----	----------%----------
Nontreated	---	0 ± 0
Prodiamine 4L fb[b]	0.73 fb	
Trifloxysulfuron 75WG fb	0.018 fb	
Paclobutrazol 2SC + Prodiamine fb	0.18 + 0.56 fb	82.7 ± 4.9
Paclobutrazol fb	0.18 fb	
Paclobutrazol	0.18	
Foramsulfuron 0.19SC fb	0.015 fb	91.5 ± 2.9
Ethofumesate 1.5EC	1.68	
Ethofumesate fb	1.12 fb	89.6 ± 2.9
Ethofumesate	1.12	
Prodiamine fb	0.84 fb	
Foramsulfuron fb	0.015 fb	89.6 ± 2.9
Ethofumesate	1.68	
Prodiamine fb	0.84 fb	
Foramsufluron fb	0.015 fb	96.4 ± 2.7
Ethofumesate fb	1.68 fb	
Ethofumesate	1.68	
$LSD_{0.05}$	---	7.6

[a] Overseeding occurred on 18 Oct. 2010 at PR and 21 Sept. 2011 at BG.
[b] Abbreviation: fb, followed by.

growth regulators for annual bluegrass control in overseeded fairways. A preemergence herbicide application (e.g., prodiamine) followed by an early postemergence application of a sulfuonylurea (e.g., foramsulfuron, trifloxysulfuron) followed by a postemergence application of ethofumesate provides the best program for overseeded fairways. These results were also observed by McElroy et al. (2011). Paclobutrazol may be an additional option for seedhead suppression, but on large scale areas such as fairways, this would be an expensive option and would require multiple applications to achieve adequate seedhead suppression. These programs are safe on overseeded fairways as long as

Table 5.13. Perennial ryegrass quality (± standard error) for pre- plus postemergence annual bluegrass control programs at the Preserve at Verdae (PR; Greenville, SC) from 2010-2011 and Boscobel Golf Club (BG; Pendleton, SC) from 2011-2012.

Treatment[a]	Rate	Ryegrass Quality
	-----kg ai ha^{-1}-----	----------0-10----------
Nontreated	---	5.8 ± 0.8
Prodiamine 4L fb[b]	0.73 fb	
Trifloxysulfuron 75WG fb	0.018 fb	
Paclobutrazol 2SC + Prodiamine fb	0.18 + 0.56 fb	5.1 ± 0.9
Paclobutrazol fb	0.18 fb	
Paclobutrazol	0.18	
Foramsulfuron 0.19SC fb	0.015 fb	6.1 ± 0.7
Ethofumesate 1.5EC	1.68	
Ethofumesate fb	1.12 fb	5.7 ± 0.7
Ethofumesate	1.12	
Prodiamine fb	0.84 fb	
Foramsulfuron fb	0.015 fb	4.8 ± 2.5
Ethofumesate	1.68	
Prodiamine fb	0.84 fb	
Foramsufluron fb	0.015 fb	5.2 ± 1.0
Ethofumesate fb	1.68 fb	
Ethofumesate	1.68	
$LSD_{0.05}$	---	NS

[a] Overseeding occurred on 18 Oct. 2010 at PR and 21 Sept. 2011 at BG.
[b] Abbreviation: fb, followed by.

treatments are applied according to label instructions (i.e., following seeding interval restrictions, rates, timings).

A second study investigated pre-overseeding control options including preemergence herbicides and early postemergence herbicides applied prior to overseeding. There were no significant model effects for annual bluegrass control (Table 5.14). Treatments provided between 25 and 60% annual bluegrass control (Table 5.15). These data suggest these treatments alone are not sufficient for season-long annual

Table 5.14. ANOVA for pre-overseeding annual bluegrass control options at the Preserve at Verdae (Greenville, SC) from 2010-2011 and Boscobel Golf Club (Pendleton, SC) from 2011-2012.

Source	df	Control	Ryegrass Quality	
			Fall	Spring
Treatment	10	NS[a]	NS	ns
Location	1	NS	*	*
Treatment*Location	10	NS	NS	ns
Block	2	NS	*	ns
Error	10			

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

bluegrass control. Although most germination occurs in fall months, some germination will take place through the spring. Thus, these herbicides should be used as a component of a complete control program. This was indicated in the previous study where a mixture of pre- and postemergence herbicides and plant growth regulators were used and provided >80% control. Ryegrass quality was not acceptable (>7) for any treatment (Table 5.15), but this was probably not related to individual treatment effects on perennial ryegrass and more related to maintenance of study sites. At both PR and BG, a small section of bermudagrass turf was overseeded with perennial ryegrass for these studies while the rest of the turf at these golf courses remained dormant (nonoverseeded). Therefore, additional inputs (e.g., irrigation, fertilizer, mowing) were minimal and contributed to poor ryegrass quality.

Table 5.15. Annual bluegrass control and ryegrass quality (± standard error) for pre-overseeding options at the Preserve at Verdae (PR; Greenville, SC) from 2010-2011 and Boscobel Golf Club (BG; Pendleton, SC) from 2011-2012.

Treatment[a]	Rate	Annual Bluegrass Control	Ryegrass Quality	
			Fall	Spring
	-----kg ai ha^{-1}-----	----------%----------	----------0-10----------	
Nontreated	---	0 ± 0	5.2 ± 0.6	5.7 ± 1.0
Prodiamine 4L	0.84	56.8 ± 7.8	4.0 ± 0.4	4.9 ± 0.3
Prodiamine	0.56	56.8 ± 7.8	4.2 ± 0.7	3.5 ± 1.1
Prodiamine fb[b]	0.42 fb	38.9 ± 4.3	4.8 ± 0.5	5.3 ± 0.7
Prodiamine	0.42			
Dithiopyr 2EW	0.56	26.4 ± 8.8	4.3 ± 0.8	4.3 ± 0.9
Dithiopyr fb	0.56 fb	40.4 ± 10.4	5.5 ± 0.7	5.7 ± 0.7
Dithiopyr	0.56			
Oxadiazon 2G	2.24	58.5 ± 7.2	4.8 ± 0.7	5.3 ± 0.7
Foramsulfuron 0.19SC	0.03	48.6 ± 10.9	4.8 ± 0.7	5.6 ± 0.8
Trifloxysulfuron 75WG[c]	0.018	45.2 ± 10.0	5.3 ± 0.6	6.2 ± 0.5
Rimsulfuron 25DF	0.018	45.7 ± 14.5	4.2 ± 0.7	5.4 ± 1.0
Sulfosulfuron 75WDG	0.092	52.0 ± 6.5	4.5 ± 0.6	5.1 ± 1.1
$LSD_{0.05}$	---	NS	NS	NS

[a] Overseeding occurred on 18 Oct. 2010 at PR and 21 Sept. 2011 at BG.
[b] Abbreviations: fb, followed by.
[c] Trifloxysulfuron and rimsulfuron applications included nonionic surfactant at 0.25% v v^{-1}.

Three studies were conducted to investigate annual bluegrass control in overseeded ryegrass with amicarbazone and methiozolin compared to other postemergence standards. The first study was conducted from 2010 to 2011 at PR and SV. There was a significant effect of treatment but no significant interaction between treatment and location (Table 5.16). Thus, data are combinated and presented across both locations. Greater than 88% control was achieved with sequential applications of ethofumesate 1.5EC at 1.12 kg ai ha^{-1} and amicarbazone at 0.2 kg ai ha^{-1} (Table 5.17). These treatments were not different from sequential applications of methiozolin at 0.84 kg ai ha^{-1} (83%), ethofumesate 4SC at 1.12 kg ai ha^{-1} (75%), or amicarbazone at 0.1 kg ai ha^{-1} (74%). Only two treatments provided <74% control including sequential applications of bispyribac-sodium at 0.074 kg ai ha^{-1} (61%) and methiozolin at 1.12 kg ai ha^{-1} (53%).

There were treatment-by-location interactions for ryegrass quality (Table 5.16). At PR, ryegrass quality was between 5.2 and 7.7 and similar for all treatments not containing methiozolin (Table 5.18). Methiozolin treatments yielded ryegrass quality of 4.0 (1.12 kg ai ha^{-1}) and 0.5 (0.84 kg ai ha^{-1}). The lower rate reduced ryegrass quality

Table 5.16. ANOVA for postemergence annual bluegrass control options in perennial ryegrass at the Preserve at Verdae (PR; Greenville, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2010-2011.

Source	df	Control	Ryegrass Quality
Treatment	7	*	*
Location	1	NS[a]	*
Treatment*Location	7	NS	*
Block	2	NS	NS
Error	7		

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

Table 5.17. Annual bluegrass control (± standard error) for postemergence control options in perennial ryegrass at the Preserve at Verdae (PR; Greenville, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2010-2011.

Treatment[a]	Rate	Annual Bluegrass Control
	-----kg ai ha^{-1}-----	-----%-----
Nontreated	---	0.0 ± 0.0
Ethofumesate 4SC fb[b]	1.12 fb	75.0 ± 0.0
Ethofumesate	1.12	
Ethofumesate 1.5EC fb	1.12 fb	88.9 ± 7.3
Ethofumesate	1.12	
Bispyribac-sodium 17.6 WDG fb	0.074 fb	61.1 ± 30.9
Bispyribac-sodium	0.074	
Amicarbazone 75WG[c] fb	0.1 fb	73.6 ± 8.4
Amicarbazone	0.1	
Amicarbazone fb	0.2 fb	91.7 ± 8.3
Amicarbazone	0.2	
Methiozolin 2EC fb	1.12 fb	52.8 ± 26.5
Methiozolin	1.12	
Methiozolin fb	0.84 fb	83.3 ± 16.7
Methiozolin	0.84	
$LSD_{0.05}$		30.4

[a] Overseeding occurred on 14 Sept. 2010 at SV and 18 Oct. 2010 at PR.
[b] Abbreviations: fb, followed by.
[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

compared to the higher rate probably because the application interval was shorter (14 DAI compared to 21 DAI). In contrast, ryegrass quality was 10.0 for all treatments at SV with the except of the high rate of amicarbazone (7.3). Methiozolin was safer at SV due to a more mature ryegrass stand. However, the high rate of amicarbazone was phytotoxic to ryegrass at SV. This displays the tradeoff with amicarbazone, where higher rates are needed for greater annual bluegrass control, but are more injurious to ryegrass. To further investigate these interactions, a second postemergence study was initiated at BG and SV from 2011 to 2012. Here, lower rates of methiozolin were included and fall and spring

Table 5.18. Spring ryegrass quality (± standard error) for postemergence control options in perennial ryegrass at the Preserve at Verdae (PR; Greenville, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2010-2011.

Treatment[a]	Rate	Ryegrass Quality[b]	
		Preserve	SV[c]
	-----kg ai ha^{-1}-----	----------0-10----------	
Nontreated	---	6.7 ± 0.7	10.0 ± 0.0
Ethofumesate 4SC fb Ethofumesate	1.12 fb 1.12	6.8 ±0.4	10.0 ± 0.0
Ethofumesate 1.5EC fb Ethofumesate	1.12 fb 1.12	6.5 ± 1.0	10.0 ± 0.0
Bispyribac-sodium 17.6 WDG fb Bispyribac-sodium	0.074 fb 0.074	6.3 ± 0.9	10.0 ± 0.0
Amicarbazone 75WG[d] fb Amicarbazone	0.1 fb 0.1	7.7 ± 0.8	10.0 ± 0.0
Amicarbazone fb Amicarbazone	0.2 fb 0.2	5.2 ± 2.2	7.3 ± 0.7
Methiozolin 2EC fb Methiozolin	1.12 fb 1.12	4.0 ± 0.6	10.0 ± 0.0
Methiozolin fb Methiozolin	0.84 fb 0.84	0.5 ± 0.0	10.0 ± 0.0
$LSD_{0.05}$		3.1	0.71

[a] Overseeding occurred on 14 September 2010 at Sage Valley and 18 October 2010 at the Preserve.
[b] Ryegrass quality visually evaluated on a scale of 0 to 10 (10 = dense, dark green turf).
[c] Abbreviations: SV, Sage Valley Golf Club; fb, followed by.
[d] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v/v.

applications were compared. For this study, the location effect was significant for annual bluegrass control which was probably due to differences in annual bluegrass pressure (Table 5.19). The treatment effect was not significant and no interaction between location and treatment was detected. Data are pooled and presented across both locations. In contrast to the 2011-2012 postemergence study, no treatment provided >63% control (Table 5.20). Annual bluegrass pressure was minimal at both locations which contributed to difficulty estimating control.

Table 5.19. ANOVA for postemergence annual bluegrass control options in perennial ryegrass at Boscobel Golf Club (BG; Pendleton, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2011-2012.

Source	df	Control	Ryegrass Quality	
			Fall	Spring
Treatment	9	NS[a]	*	*
Location	1	*	*	*
Treatment*Location	9	NS	*	NS
Block	2	NS	NS	NS
Error	9			

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

Table 5.20. Annual bluegrass control (± standard error) for postemergence annual bluegrass control options in perennial ryegrass at Boscobel Golf Club (BG; Pendleton, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2011-2012.

Treatment[a]	Rate	Annual Bluegrass Control
	-----kg ai ha^{-1}-----	-----%-----
Nontreated	---	0 ± 0
Ethofumesate 4SC fb[b]	1.12 fb	45.8 ± 20.8
Ethofumesate	1.12	
Bispyribac-sodium 17.6 WDG fb	0.074 fb	29.2 ± 18.7
Bispyribac-sodium	0.074	
Amicarbazone 75WG[c] fb	0.1 fb	62.5 ± 20.2
Amicarbazone	0.1	
Amicarbazone fb	0.2 fb	54.2 ± 20.8
Amicarbazone	0.2	
Methiozolin 2EC fb	1.12 fb	50.0 ± 22.4
Methiozolin	1.12	
Methiozolin fb	0.56 fb	54.2 ± 20.8
Methiozolin	0.56	
Methiozolin fb	1.12 fb	29.2 ± 18.7
Methiozolin	1.12	
Methiozolin fb	0.56 fb	29.2 ± 18.7
Methiozolin	0.56	
Methiozolin	1.12	45.8 ± 20.8
$LSD_{0.05}$	---	NS

[a] Overseeding occurred on 15 Sept. 2011 at SV and 21 Sept. 2011 at BG.
[b] Abbreviations: fb, followed by.
[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

Fall methiozolin treatments provided poor spring ryegrass quality in the previous study (Table 5.18), thus, different rates and timings were added to this study for comparison. There were significant treatment by location intercations for fall ryegrass quality (Table 5.19). At BG, only sequential fall applications of methiozolin at 1.12 kg ai ha^{-1} provided lower ryegrass quality than the nontreated (Table 5.21). Sequential spring

Table 5.21. Ryegrass quality (± standard error) for postemergence annual bluegrass control options in perennial ryegrass at Boscobel Golf Club (BG; Pendleton, SC) and Sage Valley Golf Club (SV; Graniteville, SC) from 2011-2012.

Treatment[a]	Rate	Ryegrass Quality[b]		
		Fall		Spring
		BG	SV[c]	
	---kg ai ha^{-1}---	----------0-10----------		
Nontreated	---	6.7 ± 0.3	8.8 ± 0.2	9.2 ± 0.5
Ethofumesate 4SC fb[b] Ethofumesate	1.12 fb 1.12	6.7 ± 0.3	8.7 ± 0.3	9.3 ± 0.4
Bispyribac-sodium 17.6WDG fb Bispyribac-sodium	0.074 fb 0.074	7.0 ± 0	8.7 ± 0.4	8.7 ± 0.5
Amicarbazone 75WG[c] fb Amicarbazone	0.1 fb 0.1	6.7 ± 0.3	9.0 ± 0.3	8.3 ± 0.8
Amicarbazone fb Amicarbazone	0.2 fb 0.2	6.3 ± 0.3	9.3 ± 0.2	8.1 ± 0.8
Methiozolin 2EC fb Methiozolin	1.12 fb 1.12	6.0 ± 0	7.7 ± 0.7	7.5 ± 0.5
Methiozolin fb Methiozolin	0.56 fb 0.56	6.3 ± 0.3	6.3 ± 0.9	7.3 ± 0.6
Methiozolin fb Methiozolin	1.12 fb 1.12	5.7 ± 0.3	9.2 ± 0.2	8.3 ± 0.7
Methiozolin fb Methiozolin	0.56 fb 0.56	6.0 ± 0.6	8.7 ± 0.3	9.3 ± 0.4
Methiozolin	1.12	6.7 ± 0.3	9.2 ± 0.2	9.1 ± 0.5
$LSD_{0.05}$	---	0.98	1.28	1.33

[a] Overseeding occurred on 15 Sept. 2011 at SV and 21 Sept. 2011 at BG.
[b] Abbreviations: fb, followed by.
[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

applications at 0.56 and 1.12 kg ai ha^{-1} also provided lower fall ryegrass quality than the nontreated, but these applications had not yet been made at the time of rating, thus, differences cannot be attributed to treatments. At SV, only sequential fall applications of methiozolin at 0.56 kg ai ha^{-1} reduced ryegrass quality in the fall compared to the nontreated. Ryegrass quality was higher at SV because of higher overseeding rates and maintenance (e.g., fertility, irrigation). There was no treatment by location interaction for spring ryegrass quality, thus, data are pooled across locations. Ryegrass quality was not different from the nontreated for any treatments except fall applications of methiozolin (Table 5.21). Therefore, methiozolin is not conducive for annual bluegrass control in young ryegrass (i.e., fall applications). In the spring, ryegrass is mature and has begun tillering, thus, methiozolin applications are not as injurious. However, these spring applications did not adueqately control annual bluegrass.

To further investigate how control with methiozolin and amicarbazone can be improved, an additional study was initiated in 2013 at CU and SV. Multiple methiozolin and amicarbazone rates and timings with and without the combination of a paclobutrazol program. Ethofumesate and bispyribac-sodium were included as standards. There was a significant main effect of location on annual bluegrass control, but no effect of treatment (Table 5.22). Due to no significant treatment-by-location interaction, data are presented across locations. Treatments provided between 47 and 78% annual bluegrass control, but no conclusions can be drawn with respect to individual treatments (Table 5.23).

Ryegrass quality was influence by treatment, location, block and treatment-by-location interaction (Table 5.22). Data are presented separately by location. Ryegrass

Table 5.22. ANOVA for postemergence annual bluegrass control options at Clemson University (Clemson, SC) and Sage Valley Golf Club (Graniteville, SC) from 2013-2014.

Source	df	Control	Ryegrass Quality
Treatment	12	NS	*
Location	1	*	*
Treatment*Location	12	NS[a]	*
Block	2	NS	*
Error	50		

[a] Abbreviation: NS, not significant.
*Indicates a significant effect at $P < 0.05$.

quality was lower at CU due to lower seeding rates (168 kg perennial ryegrass ha^{-1} at CU vs. 392 kg ha^{-1} at SV). Regardless of treatment, ryegrass quality at CU was ≤5.3 and ≥8.0 at SV (Table 5.24). Only two treatments reduced ryegrass quality compared to the nontreated at both locations including sequential methiozolin applications in late winter at 0.76 kg ai ha^{-1} and sequential amicarbazone applications in late winter at 0.15 kg ai ha^{-1}. There were no indiciations of improved annual bluegrass control or ryegrass quality with or without the combination of four applications of paclobutrazol.

Conclusions

Annual bluegrass is difficult to selectively control in other cool season grasses (e.g., creeping bentgrass and perennial ryegrass) because all are C_3 species. These difficulties were confirmed in field trials involving creeping bentgrass putting greens at two locations and overseeded bermudagrass fairways at four locations. Methiozolin provided the greatest annual bluegrass control (>80%) in creeping bentgrass putting greens, but required two years to achieve this level of control. There was some thinning

Table 5.23. Annual bluegrass control for postemergence annual bluegrass control options at Clemson University (Clemson, SC) and Sage Valley Golf Club (Graniteville, SC) from 2013-2014.

Treatment[a]	Rate	Annual Bluegrass Control
	-----kg ai ha^{-1}-----	-----%-----
Nontreated	---	0 ± 0
Ethofumesate 4SC fb[b]	1.68 fb	55.0 ± 18.2
Ethofumesate	1.68	
Bispyribac-sodium 17.6 WDG fb	0.074 fb	46.7 ± 19.6
Bispyribac-sodium	0.074	
Paclobutrazol 2SC fb	0.21 fb	
Paclobutrazol fb	0.28 fb	
Paclobutrazol fb	0.35 fb	60.0 ± 19.1
Paclobutrazol fb	0.42 fb	
Paclobutrazol	0.42	
Methiozolin 2EC[c] fb	0.38 fb	56.7 ± 18.0
Methiozolin	0.38	
Methiozolin 2EC fb	0.76 fb	78.3 ± 15.8
Methiozolin	0.76	
Methiozolin 2EC fb	0.38 fb	
Methiozolin fb	0.38 fb	60.0 ± 19.0
Methiozolin fb	0.38 fb	
Methiozolin	0.38	
Paclobutrazol fb	0.21 fb	
Paclobutrazol + Methiozolin fb	0.28 + 0.38 fb	
Paclobutrazol + Methiozolin fb	0.35 + 0.38 fb	53.3 ± 18.2
Paclobutrazol fb	0.42 fb	
Paclobutrazol	0.42	
Paclobutrazol fb	0.21 fb	
Paclobutrazol + Methiozolin fb	0.28 + 0.76 fb	
Paclobutrazol + Methiozolin fb	0.35 + 0.76 fb	53.3 ± 15.0
Paclobutrazol fb	0.42 fb	
Paclobutrazol	0.42	
Amicarbazone 70WDG fb	0.15 fb	60.0 ± 16.9
Amicarbazone	0.15	
Paclobutrazol fb	0.21 fb	
Paclobutrazol + Amicarbazone fb	0.28 + 0.15 fb	
Paclobutrazol + Amicarbazone fb	0.35 + 0.15 fb	53.3 ± 15.8
Paclobutrazol fb	0.42 fb	
Paclobutrazol	0.42	
Methiozolin fb	0.38 fb	60.0 ± 19.1
Methiozolin	0.38	
Methiozolin fb	0.76 fb	54.2 ± 17.2
Methiozolin	0.76	
$LSD_{0.05}$	---	NS

[a] Overseeding occurred on 15 Sept. 2013 at Sage Valley and 1 Oct. 2013 at Clemson.
[b] Abbreviations: fb, followed by.
[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

Table 5.24. Spring ryegrass quality for postemergence annual bluegrass control options at Clemson University (Clemson, SC) and Sage Valley Golf Club (Graniteville, SC) from 2013-2014.

Treatment[a]	Rate	Ryegrass Quality	
		CU[b]	SV
	-----kg ai ha^{-1}-----	----------0-10----------	
Nontreated	---	4.3 ± 0.9	9.7 ± 0.2
Ethofumesate 4SC fb	1.68 fb	5.0 ± 1.2	9.3 ± 0.4
Ethofumesate	1.68		
Bispyribac-sodium 17.6 WDG fb	0.074 fb	5.3 ± 0.3	9.2 ± 0.6
Bispyribac-sodium	0.074		
Paclobutrazol 2SC fb	0.21 fb		
Paclobutrazol fb	0.28 fb		
Paclobutrazol fb	0.35 fb	5.3 ± 1.2	8.5 ± 0.8
Paclobutrazol fb	0.42 fb		
Paclobutrazol	0.42		
Methiozolin 2EC[c] fb	0.38 fb	3.3 ± 0.7	9.2 ± 0.2
Methiozolin	0.38		
Methiozolin fb	0.76 fb	2.0 ± 0.6	8.3 ± 0.2
Methiozolin	0.76		
Methiozolin 2EC fb	0.38 fb		
Methiozolin fb	0.38 fb	2.0 ± 0	9.0 ± 0.3
Methiozolin fb	0.38 fb		
Methiozolin	0.38		
Paclobutrazol fb	0.21 fb		
Paclobutrazol + Methiozolin fb	0.28 + 0.38 fb		
Paclobutrazol + Methiozolin fb	0.35 + 0.38 fb	2.7 ± 0.3	9.0 ± 0.5
Paclobutrazol fb	0.42 fb		
Paclobutrazol	0.42		
Paclobutrazol fb	0.21 fb		
Paclobutrazol + Methiozolin fb	0.28 + 0.76 fb		
Paclobutrazol + Methiozolin fb	0.35 + 0.76 fb	3.0 ± 0.6	8.0 ± 0.8
Paclobutrazol fb	0.42 fb		
Paclobutrazol	0.42		
Amicarbazone 70WDG fb	0.15 fb	2.0 ± 0	8.0 ± 0.6
Amicarbazone	0.15		
Paclobutrazol fb	0.21 fb		
Paclobutrazol + Amicarbazone fb	0.28 + 0.15 fb		
Paclobutrazol + Amicarbazone fb	0.35 + 0.15 fb	5.3 ± 1.2	8.0 ± 0.6
Paclobutrazol fb	0.42 fb		
Paclobutrazol	0.42		
Methiozolin fb	0.38 fb	2.3 ± 0.3	9.2 ± 0.3
Methiozolin	0.38		
Methiozolin fb	0.76 fb	3.0 ± 0	8.8 ± 0.4
Methiozolin	0.76		
$LSD_{0.05}$	---	1.88	1.09

[a] Overseeding occurred on 15 Sept. 2013 at Sage Valley and 1 Oct 2013 at Clemson.
[b] Abbreviations: CU, Clemson University; SV, Sage Valley; fb, followed by.
[c] All amicarbazone and methiozolin applications included a nonionic surfactant at 0.25% v v^{-1}.

of creeping bentgrass, but was acceptable if multiple applications (≥6) were made at low rates (<0.5 kg ai ha^{-1}) and occurred over a period of six months (Oct. through March). This allowed sufficient time for creeping bentgrass to fill voids left from controlled annual bluegrass. Other options did not adequately control annual bluegrass or were not safe for creeping bentgrass putting greens. Most of these products require higher rates to control annual bluegrass which would significantly injure creeping bentgrass. Seedheads were suppressed most effectively by paclobutrazol. This plant growth regulator does not remove annual bluegrass but suppresses seedheads to provide enhanced turfgrass putting quality. This is currently the best option for annual bluegrass suppression in creeping bentgrass putting greens as long as applications are consistently made. Due to herbicide resistance issues, methiozolin can be used initially to remove annual bluegrass, but paclobutrazol should be used between methiozolin applications. Seed banks are high in infested putting greens, thus, even after control with methiozolin, it is likely annual bluegrass will reappear after some time.

Control is more difficult in perennial ryegrass. Many different rates and timings of methiozolin either did not adequately control annual bluegrass or severely injured perennial ryegrass. Thus, further investigations are required to determine if this may be a viable option for annual bluegrass control in overseeded fairways in the Southeast U.S. Similar results were observed with amicarbazone treatments. These studies further confirmed single applications of postemergence products are not adequate alone for annual bluegrass control. The best control with currently labeled options will be achieved with a program consisting of a preemergence herbicide, a sulfonylurea prior to

overseeding, and postemergence herbicides after overseeding (e.g., ethofumesate or bispyribac-sodium). Resistance poses a significant threat to golf courses which overseed each year as effective options are not plentiful enough to be used in rotation or combination. These golf courses should consider rotating years of overseeding so additional mechanisms of action can be used to control annual bluegrass.

APPENDICES

APPENDIX A

SAS Code for Herbicide Resistance Simulations

(Glyphosate Applied Annually in Late Winter)

```
data resistance;
do location =1 to 10000;
do year =1 to 50;
seed=47639087;
TTA=120000;
ISB=round(ranuni(seed)*20000,1);
LV=0.25+(rannor(seed)*0.05);
if Year=1 then TSB=ISB*TTA;
PR=0.5+(rannor(seed)*0.05);
GP=0.1+(rannor(seed)*0.025);
if GP<0.01 then GP=0.01;
if GP>0.2 then GP=0.2;
if Year=1 then RF=0.00000001+(0.0000005*rand('lognormal'));
MR=0.000000001+(0.00000005*rand('lognormal'));
SF=1-RF;
if year=1 then SSsb=round(TSB*SF*SF,1); else SSsb=NSSsb;
if year=1 then SRsb=round(2*TSB*RF*SF,1); else SRsb=NSRsb;
if year=1 then RRsb=round(TSB*RF*RF,1); else RRsb=NRRsb;
TGSS=round(SSsb*GP,1);
TGSR=round(SRsb*GP,1);
TGRR=round(RRsb*GP,1);
C1GP=0.75+(rannor(seed)*0.05);
if C1GP<0.6 then C1GP=0.6;
if C1GP>0.9 then C1GP=0.9;
C2GP=0.2+(rannor(seed)*0.025);
if C2GP<0.15 then C2GP=0.15;
if C2GP>0.25 then C2GP=0.25;
if C1GP+C2GP>1 then C2GP=1-C1GP;
C3GP=1-(C1GP+C2GP);
C1SS1=TGSS*C1GP;
C1SR1=TGSR*C1GP;
C1RR1=TGRR*C1GP;
C2SS1=TGSS*C2GP;
C2SR1=TGSR*C2GP;
C2RR1=TGRR*C2GP;
C3SS1=TGSS*C3GP;
C3SR1=TGSR*C3GP;
C3RR1=TGRR*C3GP;
if C1SS1<1000 then C1SS=ranpoi(seed,C1SS1); else C1SS=round(C1SS1,1);
if C1SR1<1000 then C1SR=ranpoi(seed,C1SR1); else C1SR=round(C1SR1,1);
if C1RR1<1000 then C1RR=ranpoi(seed,C1RR1); else C1RR=round(C1RR1,1);
if C2SS1<1000 then C2SS=ranpoi(seed,C2SS1); else C2SS=round(C2SS1,1);
if C2SR1<1000 then C2SR=ranpoi(seed,C2SR1); else C2SR=round(C2SR1,1);
if C2RR1<1000 then C2RR=ranpoi(seed,C2RR1); else C2RR=round(C2RR1,1);
```

```
if C3SS1<1000 then C3SS=ranpoi(seed,C3SS1); else C3SS=round(C3SS1,1);
if C3SR1<1000 then C3SR=ranpoi(seed,C3SR1); else C3SR=round(C3SR1,1);
if C3RR1<1000 then C3RR=ranpoi(seed,C3RR1); else C3RR=round(C3RR1,1);
Cohort1Survival=0.5+(rannor(seed)*0.1);
Cohort2Survival=0.2+(rannor(seed)*0.05);
Cohort3Survival=0.05+(rannor(seed)*0.01);
if cohort1survival < 0.01 then cohort1survival=0.01;
if cohort2survival < 0.01 then cohort2survival=0.01;
if cohort3survival < 0.01 then cohort3survival=0.01;
SC1SS1=round(C1SS*Cohort1Survival,1);
SC1SR1=round(C1SR*Cohort1Survival,1);
SC1RR1=round(C1RR*Cohort1Survival,1);
SC2SS1=round(C2SS*Cohort2Survival,1);
SC2SR1=round(C2SR*Cohort2Survival,1);
SC2RR1=round(C2RR*Cohort2Survival,1);
SC3SS1=round(C3SS*Cohort3Survival,1);
SC3SR1=round(C3SR*Cohort3Survival,1);
SC3RR1=round(C3RR*Cohort3Survival,1);
if SC1SS1<1000 then SC1SS=ranpoi(seed,SC1SS1); else
SC1SS=round(SC1SS1,1);
if SC1SR1<1000 then SC1SR=ranpoi(seed,SC1SR1); else
SC1SR=round(SC1SR1,1);
if SC1RR1<1000 then SC1RR=ranpoi(seed,SC1RR1); else
SC1RR=round(SC1RR1,1);
if SC2SS1<1000 then SC2SS=ranpoi(seed,SC2SS1); else
SC2SS=round(SC2SS1,1);
if SC2SR1<1000 then SC2SR=ranpoi(seed,SC2SR1); else
SC2SR=round(SC2SR1,1);
if SC2RR1<1000 then SC2RR=ranpoi(seed,SC2RR1); else
SC2RR=round(SC2RR1,1);
if SC3SS1<1000 then SC3SS=ranpoi(seed,SC3SS1); else
SC3SS=round(SC3SS1,1);
if SC3SR1<1000 then SC3SR=ranpoi(seed,SC3SR1); else
SC3SR=round(SC3SR1,1);
if SC3RR1<1000 then SC3RR=ranpoi(seed,SC3RR1); else
SC3RR=round(SC3RR1,1);
TrC1SS1=round(SC1SS*0.2,1);
TrC1SR1=round(SC1SR*0.9,1);
TrC1RR1=round(SC1RR*0.95,1);
TrC2SS1=round(SC2SS*0.05,1);
TrC2SR1=round(SC2SR*0.75,1);
TrC2RR1=round(SC2RR*0.95,1);
if TrC1SS1<1000 then TrC1SS=ranpoi(seed,TrC1SS1); else
TrC1SS=round(TrC1SS1,1);
if TrC1SR1<1000 then TrC1SR=ranpoi(seed,TrC1SR1); else
TrC1SR=round(TrC1SR1,1);
if TrC1RR1<1000 then TrC1RR=ranpoi(seed,TrC1RR1); else
TrC1RR=round(TrC1RR1,1);
if TrC2SS1<1000 then TrC2SS=ranpoi(seed,TrC2SS1); else
TrC2SS=round(TrC2SS1,1);
if TrC2SR1<1000 then TrC2SR=ranpoi(seed,TrC2SR1); else
TrC2SR=round(TrC2SR1,1);
if TrC2RR1<1000 then TrC2RR=ranpoi(seed,TrC2RR1); else
TrC2RR=round(TrC2RR1,1);
```

```
GS=TGSS+TGSR+TGRR;
d=(TrC1SS+TrC1SR+TrC1RR+TrC2SS+TrC2SR+TrC2RR+SC3SS+SC3SR+SC3RR)/TTA;
C1g=2000;
C2g=1000;
C3g=250;
C1b=200000;
C2b=100000;
C3b=25000;
C1seed=round((c1g*d)/(1+((c1g*d)/C1b)),1);
C2seed=round((c2g*d)/(1+((c2g*d)/C2b)),1);
C3seed=round((c3g*d)/(1+((c3g*d)/C3b)),1);
C1seeds=round(C1seed/d,1);
C2seeds=round(C2seed/d,1);
C3seeds=round(C3seed/d,1);
TrC1SSSeed=TrC1SS*c1seeds;
TrC1SRSeed=TrC1SR*c1seeds;
TrC1RRSeed=TrC1RR*c1seeds;
TrC2SSSeed=TrC2SS*c2seeds;
TrC2SRSeed=TrC2SR*c2seeds;
TrC2RRSeed=TrC2RR*c2seeds;
SC3SSSeed=SC3SS*c3seeds;
SC3SRSeed=SC3SR*c3seeds;
SC3RRSeed=SC3RR*c3seeds;
TrC1SSseed1=ranpoi(seed,TrC1SSseed);
TrC1SRseed1=ranpoi(seed,TrC1SRseed);
TrC1RRseed1=ranpoi(seed,TrC1RRseed);
TrC2SSseed1=ranpoi(seed,TrC2SSseed);
TrC2SRseed1=ranpoi(seed,TrC2SRseed);
TrC2RRseed1=ranpoi(seed,TrC2RRseed);
SC3SSseed1=ranpoi(seed,SC3SSseed);
SC3SRseed1=ranpoi(seed,SC3SRseed);
SC3RRseed1=ranpoi(seed,SC3RRseed);
y=MR;
z=1-MR;
NewSSseed=(TrC1SSseed1*z*z)+(TrC2SSseed1*z*z)+(SC3SSSeed1*z*z)+(TrC1SRs
eed1*0.25)+(TrC2SRseed1*0.25)+(SC3SRseed1*0.25)+(TrC1RRSeed1*y*y)+(TrC2
RRseed1*y*y)+(SC3RRseed1*y*y);
NewSRseed=(TrC1SSseed1*2*y*z)+(TrC2SSseed1*2*y*z)+(SC3SSSeed1*2*y*z)+(T
rC1SRseed1*0.5)+(TrC2SRseed1*0.5)+(SC3SRseed1*0.5)+(TrC1RRSeed1*2*y*z)+
(TrC2RRseed1*2*y*z)+(SC3RRseed1*2*y*z);
NewRRseed=(TrC1SSseed1*y*y)+(TrC2SSseed1*y*y)+(SC3SSSeed1*y*y)+(TrC1SRs
eed1*0.25)+(TrC2SRseed1*0.25)+(SC3SRseed1*0.25)+(TrC1RRSeed1*z*z)+(TrC2
RRseed1*z*z)+(SC3RRseed1*z*z);
TSS1=NewSSseed*0.9;
TSR1=NewSRseed*0.9;
TRR1=NewRRseed*0.9;
TSS=round(TSS1*(1-PR),1);
TSR=round(TSR1*(1-PR),1);
TRR=round(TRR1*(1-PR),1);
if TSR<1000 then TSR=ranpoi(seed,TSR);
if TRR<1000 then TRR=ranpoi(seed,TRR);
VSSseeds=(SSsb-TGSS)*(1-LV);
VSRseeds=(SRsb-TGSR)*(1-LV);
VRRseeds=(RRsb-TGRR)*(1-LV);
```

```
VSB=SSsb+SRsb+RRsb;
TNS=TSS+TSR+TRR;
NTSB=VSB+TNS-GS;
PSS=NSSsb/NTSB;
PSR=NSRsb/NTSB;
PRR=NRRsb/NTSB;
Check=(NSSsb+NSRsb+NRRsb)/TTA;
output;
NSSsb=VSSseeds+TSS;
NSRsb=VSRseeds+TSR;
NRRsb=VRRseeds+TRR;
Res=(NSRsb+NRRsb)/(NSRsb+NSSsb+NRRsb);
RF=TRA/NTSB;
if Res>0.2 then R=1; else R=0;
end;
end;
proc sort; by year;
proc means noprint; by year;
var R; output out=c sum=sumR;
proc print data=c; var sumR;
run;
quit;
```

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