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Herbicide Resistance Mechanism(s) in Italian Ryegrass (Lolium perenne ssp. multiflorum) Populations in the Southern United States

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ABSTRACT

Italian ryegrass is a principal weed problem in wheat production fields in the Southern US. Resistance to herbicides diclofop, mesosulfuron, and pinoxaden among ryegrass populations has been reported. Glyphosate-resistant Italian ryegrass populations were identified in Desha County, Arkansas. This research aimed to 1) determine resistance patterns to ACCase (diclofop and pinoxaden) and ALS (imazamox, mesosulfuron, and pyroxsulam) herbicides among Italian ryegrass populations from the southern US; 2) determine if cytochrome P450-mediated enhanced herbicide metabolism contributed to resistance; and 3) elucidate the resistance mechanism to glyphosate in four Arkansas populations (Des03, Des05, Des14, and D8). For objective 1, 30 accessions from problematic fields in the southern US between 2008 and 2010 were subjected to dose-response bioassays. Among the 30 accessions, 27 were resistant to both diclofop and mesosulfuron, 25 of which were also resistant to pyroxsulam. Ten Arkansas accessions collected in 2008 were resistant to diclofop, mesosulfuron, pyroxsulam, and imazamox. One accession from Georgia and three accessions from North Carolina were resistant to diclofop, mesosulfuron, pyroxsulam, and pinoxaden. For objective 2, six ryegrass populations with different resistance patterns to glyphosate, ALS- and ACCase herbicides, were treated with P450 inhibitors malathion (1000 g ai ha^{-1}) and 1-aminobenzotriazole (100 µM ABT) before herbicide application. Malathion improved herbicide activity in some populations; but did not completely overcome resistance to any herbicide. This indicates that P450-mediated metabolism is only partially responsible for resistance in these populations. For objective 3, plants from Des03 population were analyzed for resistance level, *EPSPS* genetic mutation(s), EPSPS enzyme activity, and *EPSPS* gene copy number. The absorption and translocation of ^{14}C-glyphosate were similar in R and S plants. The *EPSPS* gene in the R plants did not contain any point mutation(s)

associated with glyphosate resistance. Resistance to glyphosate in Des03 is due to increased basal EPSPS enzyme activity resulting from amplification of the *EPSPS* gene. Follow-up experiments conducted on other glyphosate-R populations Des05, Des14, and D8 showed 11-fold to 516-fold more copies of the *EPSPS* gene in resistant plants than their susceptible counterparts indicating that *EPSPS* gene amplification also confers resistance to glyphosate in these populations.

This thesis is approved for recommendation
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CHAPTER I

INTRODUCTION

Italian ryegrass [*Lolium perenne* spp. *multiflorum* (Lam) Husnot] is a troublesome weed that infests wheat (*Triticum aestivum* L. ssp. *Aestivum)* production fields, which also carries over to cotton (*Gossypium spp*.) and soybean (*Glycine max* L.*).* It can be an annual or biennial grass that ranges from 30-100 cm tall, either as tufted, heavily tillered plant or with a solitary stem. Italian ryegrass plants are wind-pollinated, are primarily an outcrossing species with vegetative abilities, are capable of adapting rapidly to their environment, produce large amount of seeds, and are easily dispersed (Appleby and Brewster 1992; Terrell 1968). Ryegrass is very competitive because it tillers extensively resulting in significant wheat yield loss, grain quality reduction, and lodging (Carson et al. 1999; Hashem et al. 1998). Heavy ryegrass infestation can reduce wheat yield up to 92% (Hashem et al. 1998).

Wheat is the second most-produced cereal crop after maize in the United States, contributing 8.6 billion dollars to the US economy in 2010 (FAO 2011). The United States has the 3rd largest land area devoted to wheat production next to China and India (FAO 2011). Wheat is grown in 42 states in the United States, with Kansas and North Dakota as the top two wheat-producing states (NASS 2012). Wheat ranks third among the US field crops in both planted acreage and gross farm receipts, behind corn and soybean (USDA ERS 2012). In 2011 and 2012, US farmers grew nearly 2.0 billion bushels of wheat on 22 million hectares of land (USDA ERS 2012). Wheat has increasing demand especially for wheat flour production; however, its yield is significantly reduced by unfavorable environmental conditions, diseases, and pests. Weeds, in particular, are a primary factor in reducing yield by competing with the crop moisture, light, space, and nutrients. Weeds in wheat production field are controlled by cultural practices such as crop rotation, burning, and moldboard plowing, and by the use of herbicides.

The use of herbicide is the most economic and efficient means of weed control in wheat (Kuk et al. 2008). Inhibitors of long-chain fatty acid, photosystem II, microtubule, protoporphyrinogen oxidase, glycine, acetyl-CoA carboxylase (ACCase), and acetolactate synthase (ALS), as well as growth regulators, are some of the herbicide modes of action used in wheat (Scott et al. 2012). Acetyl-CoA carboxylase and ALS herbicides, which inhibit the biosynthesis of fatty acids and branched-chain amino acids, respectively, are frequently used in controlling Italian ryegrass in wheat production fields. Diclofop, an ACCase inhibitor, is the traditional postemergence herbicide used in controlling ryegrass in wheat field since its commercialization in 1980. Acetolactate synthase herbicides have been also used since their introduction in the early 1980s. Glyphosate, which is a nonselective, systemic herbicide, is heavily used in burn-down treatments after wheat harvest to prepare the field for the next cropping season. Since glyphosate commercialization in 1974, its usage significantly increased in the last two decades due to the adoption of no tillage practices and introduction of genetically modified glyphosate-resistant crops (Woodburn 2000). After over three decades of glyphosate usage, weed populations have evolved resistance to glyphosate (Powles and Yu 2010).

Herbicide resistance is the inherited ability of the plant to survive and reproduce following exposure to a dose of herbicide that would normally be lethal to the wild type (WSSA, 1998). Resistance is essentially a natural phenomenon which occurs spontaneously in weed populations, but is only noticed when a selection pressure is applied to the weeds through the application of a herbicide (Nevill et al. 1998). Resistance to ACCase- and ALS-inhibiting herbicides in weeds usually involved either altered target site or enhanced herbicide metabolism. Cytochrome P450 enzymes are implicated in metabolism-based resistance to multiple herbicides in grass weeds such as blackgrass (*Alopecurus myosuroides*), late watergrass (*Echinochloa*

phyllopogon), and rigid ryegrass *(Lolium rigidum*) (Hall et al. 1997; Fischer et al. 2000; Yu et al. 2009; Preston et al. 1996). Yu et al. (2009) reported that resistance to ACCase- and ALS herbicides in a rigid ryegrass population in Australia is due to enhanced herbicide metabolism involving cytochrome P450 enzymes. Glyphosate-resistant weeds usually exhibit either target-site mutation that alters the structure of the EPSPS enzyme or reduced translocation of glyphosate into the meristematic tissues of the plant (Preston et al. 2009). More recently, *EPSPS* gene amplification was reported to confer resistance to glyphosate in *Amaranthus* species (Bell et al. 2009; Gaines et al. 2010). Italian ryegrass populations that are evolving resistance to glyphosate or to ACCase- and ALS herbicides are becoming a problem in wheat productions fields as these increase wheat production cost and reduce wheat yield.

Appropriate weed management strategy should be developed upon confirmation of resistance to a herbicide. The use of alternative herbicides is usually the immediate course of action. Evaluating the resistance pattern profile of a weed species is very helpful in determining potential herbicides that could control the resistant weed species. In addition, it can also give clues on the likelihood of resistance to other herbicides. Determining herbicide resistance patterns in Italian ryegrass is necessary to determine alternative ryegrass management programs.

Italian ryegrass control is becoming more difficult due to its adaptability, high seed production and resistance to many herbicides used for its management. Cross- and multiple-herbicide resistance in weed populations severely limits herbicide options. Determination of the herbicide resistance mechanism in weeds can help in developing effective weed management approaches. For example, metabolic-based mechanism is usually associated with low to moderate level of resistance, thus can be managed with higher herbicide rates. In addition, metabolism-based resistance level is dependent on the health of the plant. Poor ryegrass

condition can weaken the plant's ability to detoxify the herbicide. Conversely, a healthy ryegrass can metabolize the herbicide very efficiently and it would require a higher rate to overcome this resistance level; or, it may not be overcome within the allowable commercial rate. However, resistance due to altered target site implies that higher herbicide dosage will successfully select for resistant populations if the mutation provides virtual immunity (Sammons et al. 2007). With the evolution of Italian ryegrass populations that are resistant to herbicides of different modes of action, new approaches should be implemented to control and decrease the frequency of herbicide-resistant weeds. Understanding the molecular mechanisms endowing herbicide resistance will contribute to wiser use of herbicide resources and enable innovations that, together with integrated control strategies, will help minimize and manage herbicide-resistance evolution (Powles and Yu 2010).

The objectives of these experiments were to determine the resistance patterns to ACCase- and ALS-inhibiting herbicides in Italian ryegrass populations from the southern United States; to determine if cytochrome P450-mediated enhanced herbicide metabolism is the basis of resistance to glyphosate and to ACCase- and ALS-inhibiting herbicides in selected Italian ryegrass populations; and to elucidate the resistance mechanism to glyphosate in four Italian ryegrass populations (Des03, Des05, Des14, and D8) from Arkansas.

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CHAPTER II

REVIEW OF LITERATURE

Italian Ryegrass

Italian ryegrass [*Lolium perenne* ssp. *multiflorum* (Lam.) Husnot] is a cool-season bunchgrass native to southern Europe, but is widely distributed worldwide, including North and South America, New Zealand and Australia. Ryegrass was introduced in the United States in the early colonial days and quickly became an important forage grass. Ryegrass germinates from autumn to early spring (October-March) and flowers in late May to August. It is best adapted to cool, moist climates and grows best at temperatures between 20 and 25 °C and at soil pH levels of 6.0 and 7.0 (Romani et al. 2002). Mature ryegrass can grow to more than 1 m in height, produces many seeds, and can adapt quickly to environmental fluctuations (Smith 2003). Ryegrass plants consume much water and perform poorly during drought or extended periods of extreme temperatures. Italian ryegrass has also shown allelopathy, particularly against clovers and medics (Chung and Miller 1995). Aqueous extracts of Italian ryegrass foliage inhibit the germination and seedling growth of alfalfa (Smith and Martin 1994).

Like other grasses, ryegrass is identified by its vegetative and floral parts. The ligule, which is the outgrowth at the inner junction of the leaf sheath and blade, is membranous (Hannaway et al. 1999). Where the leaf meets the stem, claw-like tissues called auricles wrap around the stem. The clasping auricles are narrow and hairless (Bryson and DeFelice 2009). The leaf blades are green and hairless with a smooth and glossy under-surface. The spikelets on the inflorescence are arranged alternately along the length of the seedhead and are awned (Bararpour et al. 2005)

Italian ryegrass is one of the fastest growing forage grasses. It establishes well and can be used for grazing, hay, silage, and soil conservation purposes (Cosgrove et al. 1992). Ryegrass is widely cultivated as a cool-season forage because of its high seedling vigor, rapid regrowth after

cutting, high quality and forage yield, and adaptability to southern US climatic conditions and soil types (Ball et al. 1996). In the northeast and Pacific Northwest, ryegrass is interseeded with corn and other row crops to absorb excess nitrogen, reduce erosion after row crop harvest, and provide winter feed (Hannaway et al. 1999). High palatability and digestibility, as well as high protein content, makes ryegrass a valued livestock feed.

Despite its value as a forage crop, it is considered as the number one weed problem in wheat (Smith 2003). The widespread use of ryegrass as forage species has greatly increased the incidence of Italian ryegrass infestations in winter wheat throughout the southern United States (Barnes et al. 2001). The ability of ryegrass to tiller extensively even in poor soil makes it a good forage crop, but a threat to wheat (Smith 2003). Ryegrass is highly competitive for minerals, nutrients, light, space, and most importantly water. Ryegrass competition with wheat can reduce wheat yield by 4.2% for every 10 Italian ryegrass plants/m^2 (Liebel and Worsham 1987). Reduction in crop yield is attributed to its interference during the vegetative stage of wheat, severe lodging, and interference with wheat harvest (Appleby and Brewster 1992). According to a study conducted by Stone et al. (1999), the effect of ryegrass interference on wheat yield can be described by a simple linear regression:

% wheat yield loss = 5.7 + (1.15 X)

where X = % of ryegrass plants in the total population

In addition, ryegrass seeds shatter before the wheat harvest. A single ryegrass plant can produce up to 45, 000 seeds which can persist in the soil for up to 5 years (McDonald et al. 1996), thus, it can be found as volunteer weed in the subsequent cropping seasons following the initial infestation (Anonymous 2006).

Italian ryegrass control is becoming more difficult due to its adaptability, high seed production and resistance to many herbicides used for its management. Weed populations with resistance to multiple herbicides severely limit weed management options. The evolution of resistance to herbicides necessitates that other management strategies need to be developed for the control of this species. Repeated use of herbicides with the same mode of action should be minimized and integrated weed management should be adopted. Management options for ryegrass control include one-year fallow with tillage, delaying fall wheat planting, increasing wheat seeding rate, seeding wheat in narrow rows, rotating crops, preemergence treatment with chlorsulfuron plus metsulfuron (Finesse), early postemergence treatment of chlorsulfuron plus flucarbazone (Finesse Grass and Broadleaf), use of soil-active herbicides like flufenacet plus metribuzin (Axiom) or pendimethalin (Prowl H_2O) followed by foliar herbicides, and desiccation of ryegrass seedlings with glyphosate + clethodim (Aldrich-Markham 1992; Appleby and Brewster 1992; Bond et al. 2005; Brewster et al. 1991, 1997; Christoffoleti et al. 2005; Scott et al. 2011, 2012).

Resistance to ACCase Inhibitors

Acetyl coenzyme-A carboxylase inhibitors, known as Group A or Group I herbicides, are selective graminicides that are applied postemergence. They inhibit the acetyl coenzyme A carboxylase (ACCase) enzyme. Acetyl coenzyme-A carboxylase (EC 6.4.1.2) is a biotinylated enzyme that catalyzes the ATP-dependent carboxylation of acetyl coenzyme A into malonyl coenzyme A, which is the first committed step in the de novo fatty acid and lipid biosynthesis (Stryer 1995). Plants contain two isoforms of ACCase found in the cytosol and chloroplast, respectively (Konishi et al. 1996; Sasaki et al. 1995). The chloroplastic isoform is the target of the ACCase herbicides. Both isoforms have three catalytic domains, namely the biotin carboxyl-

carrier (BCC), the biotin carboxylase (BC), and the carboxyltransferase (CT) domains (Nikolau et al. 2003). The ATP-dependent BC activates CO_2 by attaching it to the biotin ring which is covalently linked to the lysine (Lys) residue in the BCC domain. The CT transfers the activated CO_2 from the biotin to the acetyl-CoA.

Acetyl coenzyme-A carboxylase inhibitors are categorized into three chemical families: aryloxypropanoates (AOPPs), also known as "fops", cyclohexanediones (CHD), also known as "dims", and the recently added phenylpyrazolin. Aryloxypropanoates and cyclohexanediones compete with the substrate acetyl coenzyme A in binding to the CT domain of ACCase. Their binding sites are believed to overlap, but not necessarily the same, since they have different chemical structures (Burton et al. 1991). Lipids are involved in the biogenesis and functions of various membranes, cellular signal transduction, and other physiological functions. Because fatty acids are important components of the cell membrane, ACCase inhibition alter the integrity of the cell membrane causing metabolic leakage resulting in plant death (Devine and Shimabukuro 1994). Growth of the meristems is inhibited shortly after contact with ACCase herbicides and chlorosis of emerged leaves is observed 3 to 4 days after herbicide application (Shimabukuro 1990).

Acetyl coenzyme-A carboxylase-inhibiting herbicides have been widely used in controlling a number of grass weed species since their introduction in the late 1970s. Diclofop was the primary herbicide in controlling ryegrass. Good crop tolerance to ACCase herbicides coupled with their excellent efficiency led to the widespread and repeated use of these herbicides (Devine and Shimabukuro 1994). However, the intensive use of ACCase herbicides selected eventually selected for resistant individuals. The first case of resistance to ACCase herbicides was first reported in blackgrass (*Alopecurus myosuroides*) in the UK in 1982 (Heap 2012). The

first case of herbicide-resistant Italian ryegrass was detected in Oregon, USA in 1987 (Stanger and Appleby 1989). As of 2012, resistance to ACCase herbicides has now been reported in at least 42 species (Heap 2012). Diclofop is losing its utility due to the widespread occurrence of diclofop-resistant ryegrass populations.

Alteration of the target site is the primary mechanism of resistance to ACCase herbicides. Generally, whole-plant resistance correlates highly with reduced ACCase sensitivity (Yu et al. 2007a). Resistant biotypes of green foxtail (*Setaria viridis*), wild oat (*Avena sterilis*), johnsongrass (*Sorghum halepense*) and *Lolium* species have altered forms of ACCase (Powles and Holtum 1994). A highly resistant biotype of rigid ryegrass (*Lolium rigidum*) in Spain contained an altered isoform of ACCase while a biotype with moderate level of resistance had an increased rate of oxidation of the aryl ring of diclofop (de Prado et al. 2005). A study conducted by Delye and his colleagues (2003) revealed that an $Ile_{1781}Asn$ substitution within the CT domain of ACCase is a major determinant of sensitivity to AOPP inhibitors in rigid ryegrass. Six distinct amino acid substitutions in the CT domain of the plastidic *ACCase* gene have been previously identified to endow resistance to ACCase herbicides in blackgrass and other weed species (Delye et al. 2005). The $Trp_{2027}Cys$, $Ile_{2041}Asn$, $Gly_{2096}Ala$, and $Trp_{1999}Cys$ mutations confer resistance to AOPP herbicides. In addition, a mutation in $Asp_{2078}Gly$ was identified to endow resistance to many AOPP and CHD (Liu et al. 2007).

Plants can metabolize certain herbicides via the activity of a large group of enzymes belonging to the cytochrome P450 family. Cytochrome P450s are mixed function oxidases which catalyze various reactions such as oxygenation, isomerization, dehydration, and reduction (Durst et al. 1997). Cytochrome P450 enzymes are implicated in metabolism-based resistance to multiple herbicides in blackgrass, late watergrass (*Echinochloa phyllopogon*), and rigid ryegrass

(Fischer et al. 2000; Hall et al. 1997; Preston et al. 1996; Yu et al. 2009). Evolved resistance to ACCase herbicides in a rigid ryegrass population in Spain is due to increase in the rate of diclofop metabolism, which is likely catalyzed by a cytochrome P450 enzyme (de Prado et al. 2005).

Resistance to ALS Inhibitors

Acetolactate synthase, also known as acetohydroxyacid synthase or AHAS (E.C. 4.1.3.18), is the first enzyme in the biosynthesis of the branched chain amino acids Ile, Leu, and Val. Inhibition of ALS leads to depletion of these amino acids disrupting protein synthesis, thereby causing plant death (Shaner 1991). There are five chemical families of ALS herbicides, namely: sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine sulfonanilides (TP), pyrimidinylthiobenzoates (PTB), and sulfonylaminocarbonyltriazolinone. Sulfonylurea and imidazolinone herbicides block the ALS channel preventing the binding of the substrate pyruvate (McCourt et al. 2006). The use of SU and IMI has increased tremendously due to its relatively low use rate, sound environmental properties, low mammalian toxicity, wide crop selectivity, residual activity, and high efficacy (Tranel and Wright 2002).

Selection of ALS-resistant weed populations became evident in 1987, only 5 yr after the introduction of the first SU, with the discovery of chlorsulfuron-resistant prickly lettuce (*Lactuca serriola* L.) and kochia (*Kochia scoparia* L. Shrad) (Mallory-Smith et al. 1990; Primiani et al. 1990). Incidence of ALS resistance steadily increased both in number of sites and species. As of 2012, there are now at least 120 weed species resistant to ALS herbicides (Heap 2012). It is the most resistance-prone herbicidal compound. The high efficacy of ALS herbicides that rapidly selects for resistant phenotypes is ironically the same characteristic that enables these herbicides to be used at very low rates (Powles and Holtum 1994).

The high mutation rate in *ALS* relative to other herbicide target-site genes could theoretically account for the relatively high frequency of resistance to ALS inhibitors (Tranel and Wright 2002). Target site-based ALS resistance is due to point mutations that occur within discrete conserved domains of the *ALS* gene. Six resistance-conferring *ALS* mutations were identified (Pro_{197}Ala, Pro_{197}Arg, Pro_{197}Gln, Pro_{197}Leu, Pro_{197}Ser and Trp-$_{574}$-Leu) in rigid ryegrass population in Australia (Yu et al. 2008). Most resistance mutations occur at the Pro_{197} position which confers a high level of resistance to sulfonylurea but low or no resistance to imidazolinones (Yu at al. 2008). Substitution of Trp_{591} to Leu provides high levels of resistance to all ALS inhibitors (Bernasconi et al. 1995; Yu et al. 2008), whereas the Ser_{670} to Asp and Ala_{122} to Thr mutations confer a high level of resistance to imidazolinones but little change in sensitivity to sulfonylurea and triazolopyrimidine herbicides (Bernasconi et al. 1995; Sathasivan et al. 1990, 1991). Eight different amino acid substitutions for Pro_{197} have been reported in herbicide resistant populations. The relatively large flexibility in the herbicide-binding site in the ALS enzyme can tolerate substitutions at each of the several conserved amino acids with apparently minimal consequences to the normal catalytic activity of the enzyme (Tranel and Wright 2002).

An important mechanism of naturally occurring (as opposed to evolved) resistance to ALS inhibitors is detoxification of the active herbicide in the plant. Inherent selectivity of a particular ALS inhibitor in a given crop is based on the crops' ability to metabolize the herbicide to nonphytotoxic compounds rapidly enough to prevent lethal herbicide levels from reaching the target enzyme ALS (Saari et al. 1994). Among the more common detoxification reactions involved in crop tolerance to sulfonylureas are hydroxylation, O-dealkylation, and deesterification (Saari et al. 1994). Maize is tolerant to nicosulfuron, a sulfonylurea herbicide,

because nicosulfuron is rapidly metabolized to 5-hydroxypyrimidinyl nicosulfuron, a herbicidally inactive derivative which is then conjugated o glucose (Brown et al. 1991). Similarly, flumetsulam, a triazolopyrimidine that is selective in cereals, maize, and soybeans, also owes its selectivity to metabolic detoxification. Tolerant plants oxidize flumetsulam to one or more hydroxylated metabolites, and soybean produces an open pyrimidine ring metabolite (Swisher et al. 1991). This tolerance mechanism in crops also appears to be the same mechanism responsible for poor control of some weeds by certain ALS herbicides (Saari et al. 1994).

Cytochrome P450 monooxygenase enzymes are implicated in metabolism-based resistance to ALS-inhibiting herbicides in grass weeds such as late watergrass and rigid ryegrass (Fischer et al. 2000; Preston et al. 1996; Yu et al. 2009). Malathion is a cytochrome P450 inhibitor that has been used to antagonize cytochrome P450 monooxygensae-mediated chlorsulfuron and pendimethalin resistance in rigid ryegrass (Christopher et al. 1994; Tardif and Powles 1999). Piperonyl butoxide (PBO), also a cytochrome P450 inhibitor, has been used to detect resistance due to metabolism by PBO-sensitive cytochrome P450 enzyme (Kwon and Penner 1995). The addition of these inhibitors were reported to strongly enhance herbicide phytotoxicity toward bispyribac-resistant late watergrass plants, which suggests that metabolic degradation of bispyribac-sodium contributed significantly to the observed resistance (Fischer et al. 2000). Yun et al. (2005) reported that a late watergrass biotype with multiple herbicide resistance to bispyribac-sodium, fenoxaprop-ethyl, and thiobencarb exhibited higher P450 hydroxylation activity toward these herbicides than the susceptible biotype, which suggests the involvement of cytochrome P450 enzymes as a mechanism for resistance. A related study on late watergrass revealed that resistance to penoxsulam is mainly conferred by an enhanced ability to detoxify the herbicide via malathion-sensitive monooxygenases (Yasour et al. 2009). Malathion

reverses chlorsulfuron resistance in rigid ryegrass (Yu et al. 2009). Inhibition of herbicide activity by malathion occurs when atomic sulfur released from the oxygenated organophosphate inhibits the P450 apoprotein (Werck-Reichhart et al. 2000). Enhanced metabolic inactivation is also reported as the basis for cross-resistance to chlorsulfuron in diclofop-resistant *Lolium rigidum* biotype (Cotterman et al. 1992).

Resistance to ACCase- and ALS Herbicides in Italian Ryegrass Populations in the United States

Lolium species have a high propensity to evolve resistance to numerous herbicides (Holtum and Powles 1991). Italian ryegrass is considered as the most troublesome weed in wheat production fields in the United States. Diclofop, an ACCase inhibitor, is the traditional postemergence herbicide used in controlling ryegrass in wheat field since its commercialization in 1980. Acetolactate synthase herbicides have been also used since their introduction in the early 1980s. Evolved resistance to diclofop in Italian ryegrass was confirmed in Oregon in 1987 (Stanger and Appleby 1989). In Arkansas, the occurrence of diclofop-resistant Italian ryegrass was first reported in 1998 (Kuk et al. 2000). Since then, diclofop-resistant ryegrass has been reported in at least nine states in the United States (Heap 2012). Some Italian ryegrass populations were resistant not only to diclofop but also to other herbicides (Eleni et al. 2000; Kuk et al. 2008).

New herbicides were recently commercialized for grass control in wheat, including pinoxaden (an ACCase inhibitor) and mesosulfuron and pyroxsulam, which are ALS inhibitors. Resistance to mesosulfuron in Italian ryegrass population was first reported in Arkansas in 2003, a year before mesosulfuron was commercialized (Kuk and Burgos 2007). That mesosulfuron-resistant population from Arkansas was cross-resistant to chlorsulfuron, sulfometuron, and

imazamox, but it was not resistant to diclofop or pinoxaden. The next report of mesosulfuron-resistant population was in Texas (Ellis et al. 2008). Resistance to pinoxaden was first reported in Italian ryegrass populations from Arkansas in 2008 (Kuk et al. 2008). Kuk et al. (2008) reported that of 25 diclofop-resistant populations from Arkansas, five were cross-resistant to pinoxaden. A diclofop-resistant Italian ryegrass population from North Carolina was also reported to be resistant to pinoxaden (Ellis et al. 2010). Resistance profiles of diclofop-resistant Italian ryegrass populations to mesosulfuron, imazamox, and pinoxaden were reported in 2008 but included only populations in Arkansas (Kuk et al. 2008). More recently, Italian ryegrass populations with resistance to diclofop, pinoxaden, mesosulfuron, imazamox, and pyroxsulam were reported in North Carolina (Chandi et al. 2011).

Resistance to Glyphosate

Glyphosate [N-(phosphonomethyl)glycine] , a systemic nonselective herbicide, is the world's most widely used herbicide due to its effectiveness in controlling a very broad spectrum of weeds, low mammalian toxicity, and limited residual activity (Woodburn 2000; Baylis 2000). Its lack of soil activity does not contribute to leaching in ground water and poses no risk to crops planted after herbicide application (Baylis 2000; Duke and Powles 2008). It allows simple, cheap, flexible, and effective weed control while possessing excellent environmental properties (Caseley and Copping 2000; Baylis 2000). Glyphosate is a potent inhibitor of the plastidic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), a key enzyme in the shikimate pathway, which catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (Steinrücken and Amrhein 1980). Glyphosate not only mimics the substrate PEP, but also act as an analog of the actual transition state in the enol transfer reaction (Steinrücken and Amrhein 1980). Shikimate

pathway produces the aromatic acids tryptophan, phenylalanine and tyrosine which are precursors to proteins, alkaloids, plastoquinones, flavonoids, lignins, indole acetic acids, phenolics among many others (Herrmann 1995; Stryer 2000). Inhibition of EPSPS leads to the starvation of these metabolites which ultimately results to plant death (Duke and Powles 2008).

Glyphosate is a potent herbicide because of its ability to translocate in the plant to the apical meristems, root meristems, and underground organs (Shaner 2009). The *EPSPS* genes are mostly expressed in the meristems and flowers of plants, followed by the stem, and then by mature leaves and cotyledon (Weaver and Hermann 1997). Glyphosate needs to enter the cell and then translocate to the active meristems to reach the target site in the chloroplast (Schultz et al. 1990). Upon traversing the leaf cuticle, glyphosate moves via the phloem. Glyphosate translocation follows photoassimilate translocation from source to sink (Gougler and Geiger 1984; McAllister and Haderlie 1985). This is important as glyphosate translocation throughout the plant is necessary for its toxicity.

Since the introduction of genetically modified glyphosate-resistant crops in the 1990s, the use of glyphosate significantly increased. Glyphosate-resistant crops are massively adopted in the United States as well as in Latin America (USDA ERS 2011; Cerdeira et al. 2011). About 60% of the 148 million ha of transgenic crops grown are glyphosate-resistant crops (James 2010). Glyphosate-resistant soybean, maize, canola, cotton, and sugarbeet varieties were rapidly adopted because of the economic advantage of the technology, as well as the simple and superior weed control that glyphosate offers (Duke and Powles 2009). Despite the global dominance of glyphosate in the herbicide market, resistance to glyphosate was not identified until relatively recently. Glyphosate resistance was first confirmed in rigid ryegrass in Australia (Powles et al. 1998) in 1996. Since then, the number of cases has increased steadily. Today, glyphosate

resistance occurs in at least 22 different weed species in 19 countries (Heap 2012). Glyphosate-resistant Italian ryegrass population was first reported in 2001 in Chile (Perez and Kogan 2003). Confirmed cases of glyphosate-resistant ryegrass were also detected in Australia, Argentina, Brazil, Spain, France, Italy, South Africa, California, Oregon, Mississippi, and Arkansas (Dickson et al. 2011; Colwill et al. 2003; Heap 2012; Lorraine- Jasieniuk et al. 2008; Nandula et al. 2008; Perez and Kogan 2003; Powles et al. 2009; Yu et al. 2007). In Arkansas, resistance to glyphosate in Italian ryegrass was confirmed in Desha County in 2007 (Dickson et al. 2011).

Weed resistance to glyphosate has been shown to result from different mechanisms. Insensitive altered EPSPS and reduced glyphosate cellular transport to physiologically active meristematic tissues are the common resistance mechanisms in glyphosate-resistance weeds. Recently, *EPSPS* gene amplification was reported in glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) where *EPSPS* genes were reported to be present on every chromosome (Gaines et al. 2010).

Reduced glyphosate translocation was reported in glyphosate-resistant *Lolium* species, johnsongrass (*Sorghum halepense*), and horseweed (*Conyza canadensis*) populations (Lorraine-Colwill et al. 2003; Nandula et al. 2008; Perez-Jones et al. 2005; Riar et al. 2011; Vila-Aiub et al. 2011). Impaired glyphosate translocation mechanism generally confers high resistance levels in horseweed and *Lolium* species, 8- to 12-fold, compared to sensitive populations (Dinelli et al. 2006; Feng et al. 2004; Koger and Reddy 2005; Lorraine-Colwill et al. 2003; Michitte et al. 2007; Preston and Wakelin 2008; Wakelin et al. 2004). Experiments demonstrated that glyphosate resistance is directly correlated with increased transport of the herbicide to the leaf tip (Lorraine-Colwill et al. 2003). In glyphosate –resistant rigid ryegrass, glyphosate largely remains in the treated leaf and less herbicide is translocated to the other organs of the plant (Lorraine-

Colwill et al. 2003). Glyphosate-resistant Italian ryegrass in Mississippi exhibit reduced herbicide absorption and translocation (Nandula et al. 2008). A similar pattern of glyphosate translocation was also found in rigid ryegrass in California (Simarmata and Penner 2008) and horseweed in Delaware (Feng et al. 2004). Ryegrass resistance to glyphosate in Chile resulted from reduced foliar uptake from the abaxial leaf surface and altered translocation pattern (Michitte et al. 2007). Using nuclear magnetic resonance, Ge et al. (2010) discovered that minimal translocation of glyphosate in resistant horseweed is due to rapid sequestration of glyphosate into the vacuole. The extent of glyphosate sequestration in the vacuole also correlated with the level of glyphosate resistance in *Lolium* species (Ge et al. 2012).

Altered target-site based mechanism usually confers low resistance levels to glyphosate (2- to 4- fold) than reduced glyphosate translocation (Dinelli et al. 2006; Kaundun et al. 2008; Sammons et al. 2007). On the contrary, mutation(s) in other herbicide targets (i.e. ALS and ACCase) genrally confers high levels of resistance (Cruz-Hipolito et al. 2011; Kaundun 2010; Warwick et al. 2008). Target site-based resistance is due to a mutation or mutations in the target enzyme such that the affinity of the herbicide to the enzyme catalytic site is reduced; thus, the herbicide no longer effectively inhibits enzyme activity. The crystal structure of *E. coli* EPSPS and molecular modeling show that glyphosate inhibits EPSPS by occupying the PEP binding site (Eschenburg et al. 2002; Healy-Fried et al. 2007; Schönburnn et al. 2001). Alterations of the *EPSPS* gene conferring weed resistance to glyphosate result from point mutation in the substrate-binding region of the target gene. Glyphosate-resistant goosegrass (*Eleusine indica*) populations in Malaysia and the Philippines, Italian ryegrass in Chile and California, and rigid ryegrass in California harbor an amino acid mutation at position 106 in the *EPSPS* gene (Pro_{106} Ser) (Baerson et al. 2002; Kaundun et al. 2008; Jasieniuk et al. 2008; Perez-Jones et al. 2007;

Simarmata and Penner 2008). Transversion at this same site, cytosine$_{875}$ to adenine, encodes a Thr$_{106}$ EPSPS isoform in goosegrass and rigid ryegrass that is less sensitive to glyphosate (Ng et al. 2003; Wakelin and Preston 2006). Nucleotide polymorphism in the *EPSPS* gene resulting in a Pro$_{106}$ Ala substitution was reported in glyphosate-resistant Italian ryegrass in California and rigid ryegrass in South Africa (Jasieniuk et al. 2008; Yu et al. 2007b). More recently, a Pro$_{106}$Leu mutation was demonstrated to partially confer resistance to glyphosate in rigid ryegrass population from South Africa (Kaundun et al. 2011).

Incisive work on *E. coli* Pro$_{106}$ substitutions and the crystal structure of EPSPS-S3P-glyphosate reveals that Pro$_{106}$ substitutions to either Gly/Ser/Ser/Leu cause a structural change in the glyphosate-binding site, which endows some glyphosate resistance but preserves EPSPS functionality (Healy-Fried et al. 2007). In contrast, substitutions at Gly$_{101}$ or Thr$_{102}$ confer high-level glyphosate resistance but reduce the volume of the glyphosate/PEP binding site, thereby significantly reducing affinity for PEP (Eschenburg et al. 2002; Funke et al. 2009). Because the active site of the EPSPS protein is highly conserved, any mutation at this site tend to be deleterious and is likely to cause significant fitness penalty (Mizyed et al. 2003). Single-site mutation at Thr$_{97}$ to Ile or Pro$_{101}$ to Ser (Funke et al. 2009) or Gly$_{96}$ to Ala (Eschenburg et al. 2002) in *E. coli* impairs the binding of glyphosate but at the same time reduces affinity for the susbstrate PEP. Studies comparing glyphosate-resistant goosegrass with Pro$_{106}$Ser mutation versus susceptible population show some differences, but it is not yet evident whether any fitness cost is associated with this target site EPSPS–based resistance mechanism (Ismail et al. 2002; Lee 1999).

Soil microorganisms are able to degrade glyphosate to AMPA, glyoxylate, and sarcosine, however, metabolism of glyphosate is rare in plants (Schuette 1998). A few studies demonstrated

metabolism of glyphosate field bindweed (*Convolvulus arvensis* L.), Canada thistle [*Cirsium arvense* (L.) Scop.] tall morning glory (*lpomoea purpurea* L.), but the metabolites did not reduce phytotoxicity (Sandberg et al. 1980; Simarmata et al. 2003). Glyphosate metabolism does not contribute resistance in rigid ryegrass (Australia), Italian ryegrass (Mississippi, USA) goosegrass (Malaysia) and in horseweed across the United States (Feng et al. 1999; Feng et al. 2004; Lorraine-Colwill et al. 2003; Nandula et al. 2008; Tran et al. 1999). However, it was reported recently that metabolic detoxification plays a role in the resistance of sourgrass [*Digitaria insularis* (L.) Mez] to glyphosate, although three other resistance mechanisms are also involved namely *EPSPS* gene mutations and reduced glyphosate absorption and translocation (de Carvalho et al. 2012).

A rigid ryegrass population in South Africa, which exhibits 14-fold resistant to glyphosate, possessed two resistance mechanisms: (1) *EPSPS* mutation, Pro_{106}Ala and (2) reduced glyphosate translocation to young leaves (Yu et al. 2007b). The two resistance mechanisms occurring in one plant resulted in an additive effect with respect to herbicide resistance. Similar result was obtained in rigid ryegrass from South Africa in which a Pro_{106}Leu mutation and an unknown mechanism(s) act in concert to confer resistance to glyphosate (Kaundun et al. 2011). These studies demonstrated that as glyphosate selection intensifies, so does the potential for multiple resistance mechanisms to act additively particularly in a species with diverse genetic background.

Amplification of the *EPSPS* gene in glyphosate-resistant Palmer amaranth and tall waterhemp (*Amaranthus tuberculatus*) was recently documented (Bell et al. 2009; Gaines et al. 2010). Genomes of the glyphosate-resistant Palmer amaranth contained from 5-fold to more than 160-fold more copies of the *EPSPS* gene resulting in 40-fold *EPSPS* overexpression than the

susceptible plants (Gaines et al. 2010). High copy number of a certain gene can increase the production of the protein it encodes. Increased *EPSPS* copy number in Palmer amaranth is correlated with *EPSPS* mRNA transcript, EPSPS protein level, and EPSPS enzyme activity (Gaines et al. 2011). Furthermore, this *EPSPS* gene amplification is heritable and correlates with the expression level and glyphosate resistance segregating in F2 plants (Gaines et al. 2010). This clear evidence of field-evolved glyphosate resistance endowed by *EPSPS* gene amplification is supported by laboratory selected glyphosate-resistant cell lines of several plant species that have increased EPSPS enzyme activity resulting from *EPSPS* gene amplification (Pline-Srnic 2006). A glyphosate-tolerant carrot cell line obtained by stepwise selection with glyphosate exhibited a 12-fold increase in enzyme activity due to 4- to 25-fold increase in *EPSPS* gene copy number (Nafziger et a. 1984). Similar to the wild carrot cell line, a petunia cell line which exhibited a 20-fold increase in EPSPS activity possessed 20-fold increase in *EPSPS* gene copies relative to the control (Steinrucken et al. 1986). Gene duplication is usually triggered by environmental stresses (Zou et al. 2009). For example, multiple gene duplication in the *CspA* gene family in *E. coli* allows the bacteria to respond to different environmental stresses such as nutritional deprivation and cold-shock stress (Yamanaka et al. 1998). Gene duplication is known to occur repeatedly during evolution of eukaryotes (Soltis and Soltis 1999). Selection pressure imposed by environmental stress, in this case intense glyphosate usage, could potentially favor survival of plants with multiple copies of the glyphosate target gene.

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CHAPTER III

RESISTANCE TO ACCASE- AND ALS INHIBITORS IN ITALIAN RYEGRASS POPULATIONS IN THE SOUTHERN UNITED STATES

Abstract

Italian ryegrass is a major weed problem in wheat production. Ryegrass is an obligate outcrossing species and has a high propensity to evolve resistance as shown by its extensive resistance to numerous herbicides. This study was conducted to determine the resistance patterns of ryegrass populations to ACCase- (diclofop and pinoxaden) and ALS (imazamox, mesosulfuron, and pyroxsulam) herbicides. Thirty accessions from the southern United States collected from problematic fields between 2008 and 2010 were subjected to dose-response bioassays. All accessions were resistant to the commercial dose of diclofop. Among the 12 accessions collected in 2008, 10 were resistant to diclofop, mesosulfuron, and imazamox. Seedling bioassays on 18 accessions from Georgia, Kansas, Mississippi, North Carolina, South Carolina, and Virginia showed 17 accessions resistant to diclofop and mesosulfuron, 15 of which were resistant to pyroxsulam. Four accessions (09-NC-03, 09-NC-05, 10-GA-01, and 10-NC-01) were resistant to the four herbicides tested. Twenty-seven diclofop-resistant accessions are also resistant to at least one ALS inhibitor. Twenty-two percent of the diclofop-resistant accessions are cross-resistant to pinoxaden. This indicates that there are different patterns of cross-resistance to ALS inhibitors, and there are cases of multiple resistance to ALS- and ACCase-inhibiting herbicides. Most diclofop-resistant ryegrass accessions can be controlled by pinoxaden; however, growers should consider that pinoxaden cannot control all diclofop-resistant ryegrass. Because ryegrass populations are already pre-selected for resistance to ACCase inhibitors with diclofop, widespread resistance to pinoxaden can evolve in a short time. A program approach to weed management in wheat has to be planned prior to the growing season.

Introduction

Italian ryegrass [*Lolium perenne* ssp. *multiflorum* (Lam) Husnot] is a major weed problem in wheat production areas in the United States. A heavy infestation of ryegrass can reduce wheat yield up to 92% (Hashem et al. 1998). With the introduction of diclofop in 1980, Italian ryegrass could be chemically controlled in wheat fields (Stanger and Appleby 1989). Diclofop is an aryloxyphenoxypropanoate (AOPP) herbicide that inhibits acetyl coenzymeA carboxylase (ACCase), an enzyme necessary for fatty acid biosynthesis (Burton et al. 1989; Delye 2005). Although diclofop has controlled ryegrass historically, its repeated use has selected for resistant Italian ryegrass populations. The first case of diclofop-resistant Italian ryegrass was reported in Oregon in 1987 (Stanger and Appleby 1989). In Arkansas, diclofop-resistant Italian ryegrass was first documented in 1998 (Kuk et al. 2000). Since then, diclofop-resistant Italian ryegrass has been reported in 10 states in the Unites States and in six other countries (Heap 2012).

Several other ACCase-inhibitor and non-ACCase inhibitor herbicides have been introduced for Italian ryegrass control since the initial discovery of diclofop-resistant Italian ryegrass populations. Relatively new herbicides were commercialized for weed control in wheat, including mesosulfuron, imazamox, pyroxsulam, and pinoxaden (Dickson et al. 2011). Mesosulfuron, imazamox, and pyroxsulam are acetolactate synthase (ALS) inhibitors belonging to sulfonylurea, imidazolinone, and triazolopyrimidine sulfonamide families, respectively (DeBoer et al. 2011; Hand et al. 2002; Kuk et al. 2008). Acetolactate synthase (EC4.1.3.18) is the first enzyme in the biosynthesis pathway of the branched-chain amino acids isoleucine, valine, and leucine (Umbarger 1978). Mesosulfuron controls diclofop-resistant Italian ryegrass populations (Bailey et al. 2003). Sequential postemergence applications of imazamox in

imidazolinone-tolerant (Clearfield®) wheat optimize Italian ryegrass control and wheat yield (Bond et al. 2005). Pyroxsulam is a new triazolopyrimidine sulfonamide herbicide that provides selective postemergence grass and broadleaf weed control in wheat (DeBoer et al. 2011). Pinoxaden, an ACCase-inhibiting herbicide belonging to the phenylpyrazoline family (Porter et al. 2005), has the same mode of action as the other AOPP herbicides but with a novel chemical structure that makes it effective in controlling the majority of ACCase (diclofop)-resistant populations (Boeger et al. 2006). Although alternative herbicides to diclofop are available, populations of Italian ryegrass may evolve resistance to multiple ACCase- and ALS-inhibiting herbicides. The first case of an ALS-resistant population was observed in prickly lettuce (*Lactuca serriola* L.) in 1987, only 5 years after the commercial introduction of chlorsulfuron (Mallory-Smith et al. 1990). Soon thereafter, resistant kochia [*Kochia scoparia* (L.) Schard] was identified in 1990 (Primiani et al. 1990). Now, there are at least 120 weed species with resistance to ALS-inhibiting herbicides including Italian ryegrass (Heap 2012).

Mesosulfuron and pinoxaden were registered in 2004 and 2005, respectively, to manage diclofop-resistant Italian ryegrass in wheat (USA EPA 2004; USA EPA 2005). However, resistance to mesosulfuron was reported in Arkansas, one year before its introduction (Kuk and Burgos 2007), and shortly after, also in Texas (Ellis et al. 2008). The first confirmed mesosulfuron-resistant Italian ryegrass population from Arkansas was also resistant to other ALS inhibitors, chlorsulfuron, imazamox, and sulfometuron, but not to diclofop (Kuk and Burgos 2007). Some diclofop-resistant ryegrass populations are also resistant to other herbicides (Kuk et al. 2008; Eleni et al. 2000; Holtum and Powles 1991). In 2008, Kuk et al. reported that of 25 diclofop-resistant populations from Arkansas, five were cross-resistant to pinoxaden. A diclofop-resistant population from North Carolina was also reported to be resistant to pinoxaden (Ellis et

al. 2010). In the context of this paper, cross-resistance pertains to resistance of a species to herbicides having the same mode of action. These herbicides do not necessarily belong to the same chemical family – for example, the imidazolinones, sulfonylureas and triazolopyrimidines are all ALS inhibitors. Multiple resistance refers to resistance of a species to herbicides having different modes of action, such as the ryegrass populations with resistance both to ACCase- and ALS inhibitors. Resistance of diclofop-resistant Italian ryegrass to mesosulfuron, imazamox, and pinoxaden was reported in 2008, but included only populations from Arkansas (Kuk et al. 2008). So far, resistance to pyroxsulam, the most recent ALS herbicide in wheat, in Italian ryegrass is already confirmed in North Carolina (Chandi et al. 2011). Within the same time frame, bioassays for resistance to pyroxsulam were being conducted on ryegrass populations from Arkansas and other states. How widespread the occurrence of resistance to multiple herbicides is, among ryegrass populations, is not known. Evaluation of resistance patterns in Italian ryegrass is necessary to determine alternative ryegrass management programs. The objective of this research was to determine the resistance patterns of Italian ryegrass populations from southern United States and Kansas to ACCase- and ALS-inhibiting herbicides.

Materials and Methods

Plant Materials. Seeds of 30 Italian ryegrass accessions suspected of resistance to diclofop and mesosulfuron were collected from Arkansas, Georgia, Kansas, Mississippi, North Carolina, South Carolina, and Virginia from 2008 to 2010 (Table 1). Of these, the largest group (12 accessions) was from Arkansas. A commercial Italian ryegrass accession was used as the susceptible standard (SS).

Resistance Patterns to ACCase Herbicides. Seeds of the 30 suspected Italian ryegrass accessions were sown in 11-cm pots with commercial soil mixture (Sunshine Mix®, Sun Gro Horticulture Inc., Bellevue, WA 98008). Susceptible plants were grown for reference. Seedlings were thinned to five plants per pot 1 wk after emergence. Plants were watered daily and fertilized with MiracleGro complete fertilizer (MiracleGro, The Scott's Co., Marysville, OH 43041) every 2 wks. Seedlings were kept in the greenhouse with 12-h days and 24/18 C day/night temperatures. Day length was achieved with natural lighting supplemented by metal halide lamps. At the three- to four-leaf stage, Italian ryegrass seedlings of the 2008 accessions were treated with 0, 560, 1120, 2240, and 4480 g ai ha^{-1} diclofop (Hoelon herbicide, Bayer CropScience, Research Triangle Park, NC 27709) which correspond to 0, 0.5, 1, 2, and 4 times (x) the recommended dose (1120 g ai ha^{-1}). Accessions collected from 2009 and 2010 were treated with 0, 840, and 1680 g ha^{-1} of diclofop and 0, 30, 60, and 121 g ai ha^{-1} of pinoxaden (Axial XL, Syngenta Crop Protection, Inc., Greensboro, North Carolina 27419). The recommended dose of pinoxaden is 60 g ha^{-1}. Diclofop and pinoxaden treatments were applied with 1 and 0.7% non-ionic surfactant (Induce®, Helena Chemical Co. Collierville, TN 38017), respectively. Herbicide treatments were applied using a laboratory sprayer equipped with a flat fan nozzle (TeeJet spray nozzles, Spraying Systems Co., Wheaton, IL 60189) delivering 187 L ha^{-1}. The experiment was conducted in a completely randomized design with four replications. A nontreated check was included for each accession.

Visible injury was evaluated at 4 wk after treatment (WAT) using a 0 to100 % rating scale, with 0 as no control and 100 as complete control. Accessions are categorized based on visible injury at 4 WAT: 0 to 20% control as highly resistant, 21to 60% control as moderately resistant, 61to 80% as slightly resistant, and 81to100% control as susceptible.

Resistance Patterns to ALS Herbicides. The 12 accessions in 2008 were treated with up to 4x the recommended doses of mesosulfuron (Osprey®, Bayer CropScience, Research Triangle Park,NC 27709), pyroxsulam (PowerFlex®, Dow AgroSciences LLC, Indianapolis, IN 46268), and imazamox (Beyond®, BASF Corp., Research Triangle Park, NC 27709). Herbicide doses (g ai ha^{-1}) were 0, 7, 15, 29, and 58 for mesosulfuron; 0, 9, 18, 36, and 72 for pyroxsulam; and 0, 18, 36, 72, and 143 for imazamox. Accessions collected in 2009 and 2010 were treated with up to 2x of the labeled doses of mesosulfuron (0, 7, 15, and 29 g ha^{-1}) and pyroxsulam (0, 9, 18, and 36 g ha^{-1}) except for the SS, which was treated up to the labeled rate only. The recommended doses of mesosulfuron, pyroxsulam, and imazamox are 15, 18, and 36 g ha^{-1}, respectively. A nontreated check was included for each accession. A methylated seed oil (Premium MSO methylated spray oil, Helena Chemical Co., Collierville, TN 38017) at 1.75 L ha^{-1} was added to mesosulfuron. A crop oil concentrate (Agri-Dex crop oil concentrate, Helena Chemical Co., Collierville, TN 38017) at 1.0% (v/v) was used with pyroxsulam. Visual injury was evaluated at 4 WAT. Other procedures and resistance categories were the same as those described in the previous section.

Resistance levels to ACCase and ALS Inhibitors in Selected Italian Ryegrass Accessions with Different Herbicide Resistance Patterns. This experiment included accessions 09-NC-01, 09-NC-04, and 09-NC-05, representing different herbicide resistance patterns. Accession 09-NC-01 is resistant to both diclofop and mesosulfuron; 09-NC-04 is resistant to diclofop, mesosulfuron, and pyroxsulam; 09-NC-05 is resistant to diclofop, mesosulfuron, pyroxsulam, and pinoxaden. A dose-response assay was conducted to evaluate their respective levels of resistance to diclofop, pinoxaden, mesosulfuron, and pyroxsulam. Seedlings were thinned to 10 plants per pot 5 d after emergence. Herbicide doses ranging from 0 to 8x of the recommended

doses of each herbicide were applied to the selected accessions and to the SS at the three- to four-leaf stage. The experiment was conducted in a completely randomized design with four replications. Recommended adjuvants were used in all herbicide treatments. At 4 WAT, the plants were cut at the soil surface, dried for 48 h in a dryer, and weighed. The experiments were repeated once. All other procedures were the same as in the previous section. A nontreated check was included for each accession.

Data Analysis for Dose-Response Experiments. Data were expressed as percentages of the mean of the nontreated control to standardize comparisons among accessions. Regression analysis was conducted using Sigma Plot v.12 (Sigma Plot, Jandel Scientific, Point Richmond, CA 94804). Biomass reduction and visible injury data at 4 WAT with increasing herbicide rates were modeled with either a three-parameter sigmoidal (equation 1) or logistic (equation 2) regression functions.

$$Y = a/[1+ e^{-((x-xo)/b)}] \quad [1]$$

$$Y = a/[(1 + (x/x_o)^b] \quad [2]$$

The amount of herbicide needed to reduce aboveground weight by 50%, or to incur 50% injury (GR_{50}) was calculated from regression equations in Sigma Plot v.12 using the injury ratings for 2008 accessions and biomass reduction data for the 09-NC-01, 09-NC-04, and 09-NC-05 accessions. Herbicide resistance levels (R/S ratios) were estimated from the GR_{50} of the resistant accession relative to the GR_{50} of the SS.

Results and Discussion

Resistance to ACCase-inhibiting Herbicides. All Italian ryegrass accessions tested were resistant to diclofop except for the SS (Tables 2 and 4). The SS was controlled 50% with 458 g ai ha^{-1} diclofop (Table 2). Among the 12 accessions evaluated in 2008, five (08-AR-02, 08-AR-05, 08-AR-09, 09-AR-11, and 08-AR-12) had 2- to 5-fold higher GR_{50} relative to the SS. Seven of the resistant accessions had 9- to >10-fold higher GR_{50} than the SS, six of which could not be controlled by 4480 g ai ha^{-1} diclofop. For the 2008 accessions, GR_{50} ranged from 1085 to >4480 g diclofop ha^{-1}. Based on GR_{50}, the resistant accessions were 2- to >10-fold more resistant to diclofop than the SS.

All 18 accessions evaluated in 2009 and 2010 were poorly controlled by diclofop at 1680 g ai ha^{-1} diclofop (Table 3.3). Italian ryegrass control ranged from 5 to 69%. Of these 18 accessions, seven were highly resistant, seven were moderately resistant and four were slightly resistant to diclofop. None of the accessions tested between 2008 and 2010 were killed with 1680 g ai ha^{-1} diclofop, whereas the recommended dose in wheat is 1120 g ai ha^{-1}. Italian ryegrass accessions with less than 50% control at 1680 g ai ha^{-1} diclofop may harbor more than one resistance mechanisms, most likely target site mutation and enhanced metabolism (Tardif and Powles 1994).

Italian ryegrass has also evolved resistance to diclofop in Brazil, Chile, France, Italy, United Kingdom, and in nine states in the US (Heap 2012). Most of the diclofop-resistant populations in this research are also resistant to other herbicides with the same or different modes of action as was reported by others (Cocker et al. 2001; Eleni et al. 2000; Holtum and Powles 1991; Kuk et al. 2000; Kuk et al. 2008). Anecdotal reports by Extension Agents indicated that resistance to diclofop in Italian ryegrass occurs in all wheat-producing counties in Arkansas

(Kuk et al. 2008) and this is supported by a recent statewide survey (Jim Dickson, Arkansas Cooperative Ext. Service, unpublished data). However, this does not mean that all Italian ryegrass populations in the southern United States are resistant to diclofop. The frequency of occurrence of diclofop-resistant populations in this experiment is higher than the actual distribution of resistant populations across Arkansas or in the southern United States because these samples were collected from fields reporting control failures with diclofop. Because of the increasing number of diclofop-resistant ryegrass populations, diclofop is no longer a viable option for wheat weed control. Pinoxaden, another herbicide in wheat that also targets ACCase, may have a different binding site than diclofop because of differences in their molecular structure (Hofer et al. 2006) and their activity on ryegrass (Kuk et al. 2008). Of the 18 diclofop-resistant accessions evaluated in 2009 and 2010, only four were resistant to pinoxaden (Figure 3.c and 3.d); three were from North Carolina (09-NC-03, 09-NC-04, 10-NC-01) and one from Georgia (10-GA-01) (Table 3). This is the first case of Italian ryegrass with resistance to pinoxaden reported in Georgia. The frequency of cross-resistance to ACCase inhibitors (diclofop and pinoxaden) was 20%, similar to that reported by Kuk et al. (2008). To date, resistance to pinoxaden is confirmed in Italian ryegrass populations from Arkansas, Louisiana, and North Carolina (Kuk et al. 2008; Ellis et al. 2010; Chandi et al. 2011). The three accessions from North Carolina reported in this paper is a warning that pinoxaden-resistant Italian ryegrass in North Carolina may be spreading. Among the four resistant accessions, only 09-NC-05 was moderately controlled (59%); the other three accessions were poorly controlled (20 to 45% control) with the labeled dose (60 g ha^{-1}) of pinoxaden. Even with the 2x dose, these accessions were only controlled by as much as 61% (data not shown). Resistance to pinoxaden was also reported in ryegrass in Chile in 2006 and Israel in 2007 (Heap 2012). Before the commercial release of

pinoxaden, resistance to pinoxaden in blackgrass (*Alopecurus myosuroides* Huds) was already detected in France (Petit et al. 2010). This is because these grass populations have already been preselected with other ACCase inhibitors, including diclofop. Pinoxaden has been commercially used only since 2006, at least 25 yr from the introduction of diclofop (Hofer et al. 2006). Diclofop and pinoxaden inhibit the same enzyme; thus, selection pressure from diclofop could predispose Italian ryegrass accessions to pinoxaden resistance (Kuk et al. 2008). Pinoxaden controls the majority of diclofop-resistant populations; thus, pinoxaden is still an alternative herbicide for controlling Italian ryegrass. However, growers should be cautious in using pinoxaden because some ryegrass populations already exhibit resistance to pinoxaden. Diversified weed control programs should be implemented, and control failures should be monitored. Ryegrass escaping from herbicide treatments should not be allowed to set seeds as this will increase the number of resistant ryegrass in the next growing season. Intensive use of pinoxaden, like any other herbicides, would lead to the evolution of resistant populations.

Resistance Patterns to ALS-inhibiting Herbicides. Traditionally, ALS-inhibiting herbicides such as chlorsulfuron plus metsulfuron and tribenuron are used preemergence and postemergence, respectively, in wheat cropping systems. The occurrence of diclofop-resistant ryegrass has ushered in the postemergence ALS-inhibiting herbicide mesosulfuron. However, before its commercialization, a mesosulfuron-resistant population was confirmed in Arkansas (Kuk and Burgos 2007). The amount of mesosulfuron causing 50% injury to the SS in this recent experiment is only 7.3 g ha^{-1}, which is equivalent to one-half the recommended dose (Table 4). Among the 2008 Arkansas accessions, 10 out of 12 were resistant to mesosulfuron with 5-fold to >eight-fold resistance relative to the SS (Table 3 and Figure 3b). Eight of these

resistant accessions could not be controlled by 58.2 g ha^{-1}, which is more than 4x the recommended dose.

Among the 2009 and 2010 accessions, 17 out of 18 were resistant to mesosulfuron (less than 70% control) (Table 2 and Figure 1c). Of these mesosulfuron-resistant accessions, 15 were poorly controlled at the recommended dose showing less than 50% injury (Table 3). Two accessions in 2010 (10-VA-01 and 10-GA-01) were controlled only 54 to 68% at the recommended dose of mesosulfuron.

Twenty-seven of 30 diclofop-resistant accessions (2008 – 2010) were also resistant to mesosulfuron (Figure 1a). The high frequency of mesosulfuron-resistant accessions (<80% control) is expected since the majority of samples were collected from wheat fields where mesosulfuron applications failed. A similar result was reported by Chandi et al. (2011), with Italian ryegrass populations from North Carolina having resistance to diclofop, pinoxaden, and mesosulfuron. Kuk and Burgos (2007) reported one population in Arkansas resistant to mesosulfuron but not to diclofop. In 2008, a mesosulfuron-resistant population was confirmed in Texas but this population was not resistant to diclofop and pinoxaden (Ellis et al. 2008). Although the level of resistance to mesosulfuron differed among the accessions studied, it appeared that diclofop-resistant Italian ryegrass evolved resistance to mesosulfuron quickly. For example, accession 08-AR-02 in this study was first exposed to mesosulfuron in 2008, but in the same year resistance to mesosulfuron was observed (Salas et al. 2010). Mesosulfuron-resistant populations may have been selected for with other ALS inhibitors such as chlorsulfuron and metsulfuron that were previously used preemergence in wheat. The continued used of mesosulfuron to control diclofop-resistant ryegrass exerted further selection pressure that led to the evolution of mesosulfuron-resistant populations. The high frequency of

mesosulfuron-resistant populations in this study revealed that mesosulfuron is no longer a good alternative in managing these diclofop-resistant ryegrass populations.

Imazamox, an ALS herbicide belonging to the imidazolinone chemistry, is used to manage weeds in Clearfield® wheat in several states in the US including Oklahoma, Colorado, Oregon, Idaho, Washington, Kansas, and Nebraska. Italian ryegrass is naturally susceptible to imazamox. However, some Italian ryegrass populations are already resistant to imazamox, even in locations where imazamox had not been used previously, because of cross-resistance to other ALS inhibitors such as mesosulfuron. The SS was completely controlled by the recommended dose of imazamox (Table 5). Ten of 12 accessions from Arkansas in 2008 were resistant to imazamox, with GR_{50} values ranging from 37 to >143 g ai ha^{-1} (Table 5). The most resistant accession (08-AR-06) requires more than 4x the recommended dose of imazamox to achieve 50% control. These 10 imazamox-resistant accessions were the same accessions resistant to mesosulfuron (Tables 4 and Figure 1b). The first reported mesosulfuron-resistant population in Arkansas was also resistant to imazamox (Kuk et al. in 2007). Related research has shown cross-resistance to sulfonylurea (chlorsulfuron) and imidazolinone (imazethapyr) in prickly lettuce (Mallory-Smith et al. 1990). A sulfometuron-resistant redroot pigweed *(Amaranthus retroflexus* L.) exhibiting cross-resistance to imidazolinone was also reported by Sibony et al. (2001).

A similar resistance pattern was observed with pyroxsulam. In the 2008 Arkansas accessions, ten were resistant to pyroxsulam with GR_{50} values of 13.8 to more than 71.7 g ha^{-1} pyroxsulam (Table 6). The amount of herbicide needed to control the SS by 50% was 6.9 g ha^{-1} pyroxsulam which is one-half the recommended dose (Table 6). Based on GR_{50}, resistant

accessions were 2- to 10-fold more resistant than the SS. These pyroxsulam-resistant accessions were also resistant to mesosulfuron and imazamox (Figure 1b).

The majority of accessions collected in 2009 and 2010 were also poorly controlled by pyroxsulam. Of the 18 accessions, only three (09-NC-01, 09-NC-02, and 10-VA-01) were controlled >80% at the 1x dose of pyroxsulam (Table 3). Of the 15 pyroxsulam-resistant accessions, three were slightly resistant, six were moderately resistant, and six were highly resistant (Table 4). Increasing to 2x the labeled rate did not significantly increase ryegrass control in the resistant accessions (data not shown). The majority of the pyroxsulam-resistant accessions from Arkansas, North Carolina, Mississippi, Georgia, and Kansas were also resistant to mesosulfuron (Table 3 and Figure 1c). Cross-resistance to ALS herbicides within the same or different family is common.

More weed species are resistant to ALS-inhibiting herbicides than any other herbicide group (Heap 2012). The high occurrence of weed populations resistant to ALS-inhibiting herbicides can be attributed to extensive use of these herbicides, the high selection pressure they exert, and the many-resistance conferring mutations in the *ALS* gene (Tranel and Wright 2002). Resistance to ALS herbicides usually results from substitutions in the *ALS* gene. High genetic variability of the *ALS* gene increases the tendency that resistant plants are selected by ALS inhibitors (Tranel and Wright 2002). So far there are eight ALS amino acid substitutions that confer ALS-herbicide resistance in weed species (Tranel et al. 2012). Cross-resistance to ALS herbicides, particularly to sulfonylureas and imidazolinones, had been reported in rigid ryegrass (*Lolium rigidum*), Indian hedgemustard (*Sisymbrium orientale* L.), redroot pigweed, common cocklebur (*Xanthium strumatium* L.), kochia, common ragweed (*Ambrosia artemisiifolia* L.), and giant ragweed (*Ambrosia trifida* L.) (Boutsalis et al. 1999; Foes et al. 1999; Patzoldt et al. 200l;

Patzoldt and Tranel 2002; Sibony et al. 2001; Woodworth et al.1996;Yu et al. 2008). These resistant plants exhibited a mutation in Pro_{197} or Ala_{205} or Trp_{574} in the *ALS* gene. The magnitude of resistance to different ALS herbicides varies widely among *ALS* substitutions (Tranel and Wright 2002). Italian ryegrass populations exhibiting cross-resistance to mesosulfuron, imazamox, and pyroxsulam may exhibit target-site mutation in the *ALS* gene. It is also possible that these populations exhibit enhanced metabolism that can result in rapid detoxification of the herbicide. The mechanism of resistance of the ALS-resistant accessions in this study needs to be investigated.

Resistance Levels to ACCase- and ALS inhibitors in Selected Italian Ryegrass Accessions with Different Herbicide Resistance Patterns. Three resistance patterns were further investigated by selecting a population and evaluating its magnitude of resistance. These resistance patterns were: (1) resistance to diclofop and mesosulfuron represented by 09-NC-01, (2) resistance to diclofop, mesosulfuron, and pyroxsulam represented by 09-NC-04, and (3) resistance to diclofop, mesosulfuron, pyroxsulam, and pinoxaden represented by 09-NC-05. The GR_{50} values for accessions 09-NC-01 and 09-NC-04 for diclofop ranged from 562 and 5432 g ha^{-1}, with R/S values of 2 and 3, respectively (Table 6). Accession 09-NC-05 was shown to be highly resistant to diclofop, with 18-fold higher resistance than the susceptible standard accession (Table 6). Accession 09-NC-05 had a GR_{50} of 28 g ha^{-1} of pinoxaden, which is twice that of the SS (Table 7).

Resistance to mesosulfuron by 09-NC-05, 09-NC-04, and NC-01 was clearly demonstrated in the dose response bioassay in which their GR_{50} values ranged from 20 to 78 g ha^{-1}. Accession 09-NC-04 (71- fold) is more resistant to mesulfuron than the other two accessions; accession 09-NC-04 and 09-NC-01 had 18- and 33-fold higher GR_{50} than the SS

(Table 5). This indicates that 09-NC-04 had a high degree of resistance to ALS-inhibitors whereas 09-NC-05 has a high level of resistance to ACCase inhibitors. These accessions may possess two or more mechanisms that provide resistance to a single herbicide or class of herbicides. An ACCase- and ALS-resistant rigid ryegrass population from Australia (VLR69) harbors multiple resistance mechanisms, including a resistant ACCase, a resistant ALS, and enhanced herbicide metabolism (Preston et al. 1996). Multiple ACCase- and ALS herbicide resistance in two resistant Australian rigid ryegrass populations is due to the presence of enhanced herbicide metabolism mediated by cytochrome P450 monooxygenase (Yu et al. 2009). The mechanisms conferring resistance to diclofop, mesosulfuron, pyroxsulam, and pinoxaden in the accessions tested in this experiment need to be further investigated. Preliminary results on accessions 09-NC-03 and 09-NC-04 suggests that cytochrome P450-mediated enhanced metabolism play a role in their resistance to ACCase- and ALS-inhibiting herbicides and that other mechanisms may also be involved.

Resistance to both ACCase- and ALS inhibitors in Italian ryegrass populations presents a serious problem to wheat growers. In Arkansas, ALS- and diclofop-resistant Italian ryegrass in wheat fields is managed by the application of the commercial mixture of flufenacet plus metribuzin at the one- to two-leaf wheat stage and following it up with pinoxaden and pendimethalin at four-leaf to one-tiller ryegrass (Scott 2011) . However, continued use of pinoxaden should be discouraged because of the tendency of Italian ryegrass to evolve resistance to pinoxaden. Other than the flufenacet plus metribuzin mixture and pendimethalin, all other herbicides currently available for Italian ryegrass control are either ACCase or ALS inhibitors (Scott et al. 2012). Resistance to multiple herbicides and limited herbicide options for Italian ryegrass control in wheat emphasize the need for diversified, integrated weed management to

reduce reliance on herbicides and to delay, if not prevent, the evolution of herbicide-resistant weeds.

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Table 1. List of Italian ryegrass accessions tested for herbicide resistance patterns.

Accession code	Year of collection	County and State
08-AR-01	2008	Phillips, AR
08-AR-02	2008	Lawrence, AR
08-AR-03	2008	Cross, AR
08-AR-04	2008	Cross, AR
08-AR-05	2008	Cross, AR
08-AR-06	2008	Prairie, AR
08-AR-07	2008	Prairie, AR
08-AR-08	2008	Prairie, AR
08-AR-09	2008	Poinsett, AR
08-AR-10	2008	Craighead, AR
08-AR-11	2008	Arkansas, AR
08-AR-12	2008	Arkansas, AR
09-GA-01	2009	GA
09-MS-01	2009	MS
09-MS-03	2009	MS
09-MS-05	2009	MS
09-MS-06	2009	MS
09-MS-07	2009	NC
09-MS-08	2009	NC
09-NC-01	2009	NC
09-NC-02	2009	NC
09-NC-03	2009	KS
09-NC-04	2009	NC
09-NC-05	2009	NC
10-GA-01	2010	GA
10-KS-01	2010	KS
10-NC-01	2010	NC
10-NC-02	2010	NC
10-SC-01	2010	SC
10-VA-01	2010	VA

Table 2. GR_{50}[a] values and resistance levels to diclofop in the 2008 Arkansas Italian ryegrass accessions.

Accession	Regression equation	R^2	GR_{50}	SE[b]	*R/S*[c]
			g ai ha^{-1}		
SS[d]	$Y = 99/[1+ e^{-((x-0.46)/0.059)}]$	0.99	458	1.15	-
08-AR-01	$Y = 73/[1+ e^{-((x-3.69)/1.44)}]$	0.96	>4480	5.17	>10
08-AR-02	$Y = 89/[(1 + (x/1.22)^{-3.28}]$	0.99	1313	3.72	3
08-AR-03	$Y = 72/[1+ e^{-((x-3.77)/1.21)}]$	0.99	>4480	2.97	>10
08-AR-04	$Y = 73/[1+ e^{-((x-3.20)/0.99)}]$	0.99	3955	2.78	9
08-AR-05	$Y = 136/[(1 + (x/3.53)^{-1.08}]$	0.99	2144	1.63	5
08-AR-06	$Y = 14/[(1 + (x/1.03)^{-5.64}]$	0.98	>4480	1.21	>10
08-AR-07	$Y = 29/[(1 + (x/1.85)^{-2.45}]$	0.99	>4480	1.27	>10
08-AR-08	$Y = 5.43/[(1 + (x/2.91)^{-1.84}]$	0.99	>4480	0.23	>10
08-AR-09	$Y = 72/[1+ e^{-((x-1.38)/0.50)}]$	0.99	1785	3.85	4
08-AR-10	$Y = 13.1/[1+ e^{-((x-1.14)/0.039)}]$	0.99	>4480	0.63	>10
08-AR-11	$Y = 82/[1+ e^{-((x-2.05)/0.88)}]$	0.97	2450	7.13	5
08-AR-12	$Y = 63/[1+ e^{-((x-0.66)/0.31)}]$	0.94	1085	9.27	2

[a] GR_{50} is the herbicide concentration that reduced shoot growth by 50%. Data were based on visible injury at 4 WAT.
[b] SE is standard error.
[c] R/S (resistant/susceptible) ratios were calculated based on GR_{50} values of accessions relative to the susceptible standard.
[d] Susceptible standard accession.

Table 3. Control of 2009 and 2010 Italian ryegrass accessions by diclofop, pinoxaden, mesosulfuron, and pyroxsulam.

	Visible injury, 4WAT			
Accession	Diclofop (1680)[a]	Pinoxaden (60)[a]	Mesosulfuron (15)[a]	Pyroxsulam (22)[a]
	----------%----------			
09-GA-01	63	100	6	12
09-MS-01	19	100	25	42
09-MS-03	17	100	43	9
09-MS-05	25	100	11	17
09-MS-06	77	100	14	11
09-MS-07	36	100	23	18
09-MS-08	61	100	14	25
09-NC-01	18	100	19	96
09-NC-02	36	100	97	95
09-NC-03	5	20	15	19
09-NC-04	14	100	3	29
09-NC-05	16	59	20	41
10-GA-01	23	39	68	65
10-KS-01	69	100	40	34
10-NC-01	17	45	30	63
10-NC-02	48	98	30	39
10-SC-01	48	99	33	63
10-VA-01	54	100	54	86
SS[b]	100	100	97	100
$LSD_{0.05}$	19	6	17	14

[a] Herbicide rate, g ai ha^{-1}.

[b] Susceptible standard.

Table 4. GR_{50}[a] values and resistance levels to mesosulfuron among 2008 Italian ryegrass accessions from Arkansas.

Accession	Regression equation	R^2	GR_{50}	SE[b]	*R/S*[c]
			g ai ha^{-1}		
SS[d]	$Y = 100[1+ e^{-((x-0.0073)/0.015)}]$	0.99	7.3	0.90	-
08-AR-01	$Y = 67/[(1 + (x/0.023)^{-2.61}]$	0.98	34.7	4.63	5
08-AR-02	$Y = 133[1+ e^{-((x-0.016)/0.82)}]$	0.99	8.8	2.15	1
08-AR-03	$Y = 40[1+ e^{-((x-0.030)/0.0057)}]$	0.99	>58.2	0.24	>8
08-AR-04	$Y = 102/[(1 + (x/0.023)^{-1.24}]$	0.99	>58.2	0.22	>8
08-AR-05	$Y = 96[1+ e^{-((x-0.0058)/0.0008)}]$	0.99	5.9	3.32	1
08-AR-06	$Y = 8.76/[(1 + (x/0.039)^{-2.23}]$	0.99	>58.2	0.17	>8
08-AR-07	$Y = 21.3/[(1 + (x/0.013)^{-7.82}]$	0.99	>58.2	1.24	>8
08-AR-08	$Y = 12.3/[(1 + (x/0.012)^{-1.36}]$	0.99	>58.2	0.70	>8
08-AR-09	$Y = 37[1+ e^{-((x-0.0027)/0.0084)}]$	0.98	>58.2	3.39	>8
08-AR-10	$Y = 23/[(1 + (x/0.0096)^{-3.45}]$	0.99	>58.2	1.05	>8
08-AR-11	$Y = 30/[(1 + (x/0.016)^{-0.0070}]$	0.98	>58.2	2.51	>8
08-AR-12	$Y = 69[1+ e^{-((x-0.032)/0.017)}]$	0.99	48.3	9.35	7

[a] GR_{50} is the herbicide concentration that reduced shoot growth by 50%. Data were based on visible injury at 4 WAT.
[b] SE is the standard error.
[c] R/S (resistant/susceptible) ratios were calculated based on GR_{50} values of accessions relative to the susceptible standard.
[d] Susceptible standard.

Table 5. GR_{50}[a] values and resistance levels to imazamox in 2008 Arkansas Italian ryegrass accessions.

Accession	Regression equation	R^2	GR_{50}	SE[b]	*R/S*[c]
			g ai ha^{-1}		
SS[d]	$Y = 100[1+ e^{-((x-0.017)/0.0031)}]$	0.99	17.3	0.51	-
08-AR-01	$Y = 64[1+ e^{-((x-0.10)/0.028)}]$	0.98	136.6	3.95	8
08-AR-02	$Y = 87[1+ e^{-((x-0.020)/0.0072)}]$	0.99	22.4	5.04	1
08-AR-03	$Y = 98[1+ e^{-((x-0.039)/0.021)}]$	0.94	39.5	13.04	2
08-AR-04	$Y = 68[1+ e^{-((x-0.084)/0.032)}]$	0.99	117.0	3.93	7
08-AR-05	$Y = 98[1+ e^{-((x-0.014)/0.0014)}]$	0.99	13.6	2.60	1
08-AR-06	$Y = 37[1+ e^{-((x-0.049)/0.020)}]$	0.99	>143.4	2.40	>8
08-AR-07	$Y = 68[1+ e^{-((x-0.044)/0.017)}]$	0.99	61.4	3.76	4
08-AR-08	$Y = 62[1+ e^{-((x-0.023)/0.010)}]$	0.98	37.0	5.75	2
08-AR-09	$Y = 86[1+ e^{-((x-0.055)/0.0079)}]$	0.98	57.0	0.59	3
08-AR-10	$Y = 152/[(1 + (x/0.21)^{-0.99}]$	0.99	103.0	2.20	6
08-AR-11	$Y = 331[1+ e^{-((x-0.19)/0.051)}]$	0.98	98.0	7.68	6
08-AR-12	$Y = 61[1+ e^{-((x-0.056)/0.021)}]$	0.98	86.0	5.58	5

[a] GR_{50} is the herbicide concentration that reduced shoot growth by 50%. Data were based by visible injury at 4 WAT.
[b] SE is the standard error.
[c] R/S (resistant/susceptible) ratios were calculated based on GR_{50} values of accessions relative to the susceptible standard.
[d] Susceptible standard.

Table 6. GR_{50}[a] values and resistance levels to pyroxsulam of 2008 Arkansas Italian ryegrass accessions.

Accession	Regression equation	R^2	GR_{50}	SE[b]	*R/S*[c]
			g ai ha^{-1}		
SS[d]	$Y = 99[1+ e^{-((x-0.0069)/0.0007)}]$	0.99	7	1.44	-
08-AR-01	$Y = 71[1+ e^{-((x-0.014)/0.0042)}]$	0.99	18	2.40	3
08-AR-02	$Y = 100/[(1 + (x/0.0082)^{-2.16}]$	0.99	8	1.77	1
08-AR-03	$Y = 86/[(1 + (x/0.018)^{-4.42}]$	0.99	20	5.43	3
08-AR-04	$Y = 24/[(1 + (x/0.012)^{-1.70}]$	0.99	>72	1.44	>10
08-AR-05	$Y = 99/[(1 + (x/0.0069)^{-3.56}]$	0.99	7	0.75	1
08-AR-06	$Y = 61[1+ e^{-((x-0.063)/0.017)}]$	0.99	>72	1.26	>10
08-AR-07	$Y = 57[1+ e^{-((x-0.030)/0.016)}]$	0.94	62	7.19	9
08-AR-08	$Y = 76[1+ e^{-((x-0.013)/0.0020)}]$	0.99	14	0.63	2
08-AR-09	$Y = 52[1+ e^{-((x-0.026)/0.0073)}]$	0.99	52	2.46	7
08-AR-10	$Y = 40[1+ e^{-((x-0.027)/0.014)}]$	0.91	>72	6.23	>10
08-AR-11	$Y = 112/[(1 + (x/0.039)^{-1.33}]$	0.96	33	9.13	5
08-AR-12	$Y = 39[1+ e^{-((x-0.0071)/0.0022)}]$	0.91	>72	7.57	>10

[a] GR_{50} is the herbicide concentration that reduced shoot growth by 50%. Data were based by visible injury at 4 WAT.
[b] SE is the standard error.
[c] R/S (resistant/susceptible) ratios were calculated based on GR_{50} values of accessions relative to the susceptible standard.
[d] Susceptible standard

Table 7. GR_{50}[a] values and resistance levels to diclofop, pinoxaden, mesosulfuron, and pyroxsulam in selected Italian ryegrass accessions.

Herbicide	Accession	Regression equation	R^2	GR_{50}	SE[b]	R/S[c]
				g ai ha^{-1}		
Diclofop	SS[d]	$Y = 110/[(1 + (x/347)^{-1.28}]$	0.93	304	7.87	-
	09-NC-05	$Y = 60/[(1 + (x/1155)^{-1.01}]$	0.98	5432	2.58	18
	09-NC-04[e]	$Y = 94/[(1 + (x/875)^{-4.57}]$	0.99	899	1.15	3
	09-NC-01[f]	$Y = 98/[(1 + (x/532)^{-1.31}]$	0.99	562	1.44	2
Pinoxaden	SS	$Y = 96/[1+ e^{-((x-11.5)/0.0010)}]$	0.99	12	1.14	-
	09-NC-05	$Y = 98/[1+ e^{-((x-28.0)/0.0471)}]$	0.99	28	0.21	2
Mesosulfuron	SS	$Y = 97/[(1 + (x/1.7)^{-2.48}]$	0.99	1	0.68	-
	09-NC-05	$Y = 63/[(1 + (x/10.8)^{-2.16}]$	0.97	20	5.68	18
	09-NC-04	$Y = 105/[1+ e^{-((x-80.8)/0.0301)}]$	0.95	78	8.86	71
	09-NC-01	$Y = 88/[1+ e^{-((x-29.3)/0.0245)}]$	0.82	36	16.29	33
Pyroxsulam	SS	$Y = 97/[1+ e^{-((x-4.3)/0.0006)}]$	0.99	4	3.75	-
	09-NC-05	$Y = 80/[(1 + (x/5.2)^{-0.5559}]$	0.99	13	1.54	3
	09-NC-04	$Y = 196/[(1 + (x/1606.3)^{-0.2636}]$	0.99	28	2.36	6

[a] GR_{50} is the herbicide concentration that reduced shoot growth by 50%. Data were based on biomass reduction at 4WAT.
[b] SE is the standard error.
[c] R/S (resistant/susceptible) ratios were calculated based on GR_{50} values of accessions relative to the susceptible standard.
[d]Susceptible standard accession.
[e]Susceptible to pinoxaden.
[f]Susceptible to pinoxaden and pyroxsulam.

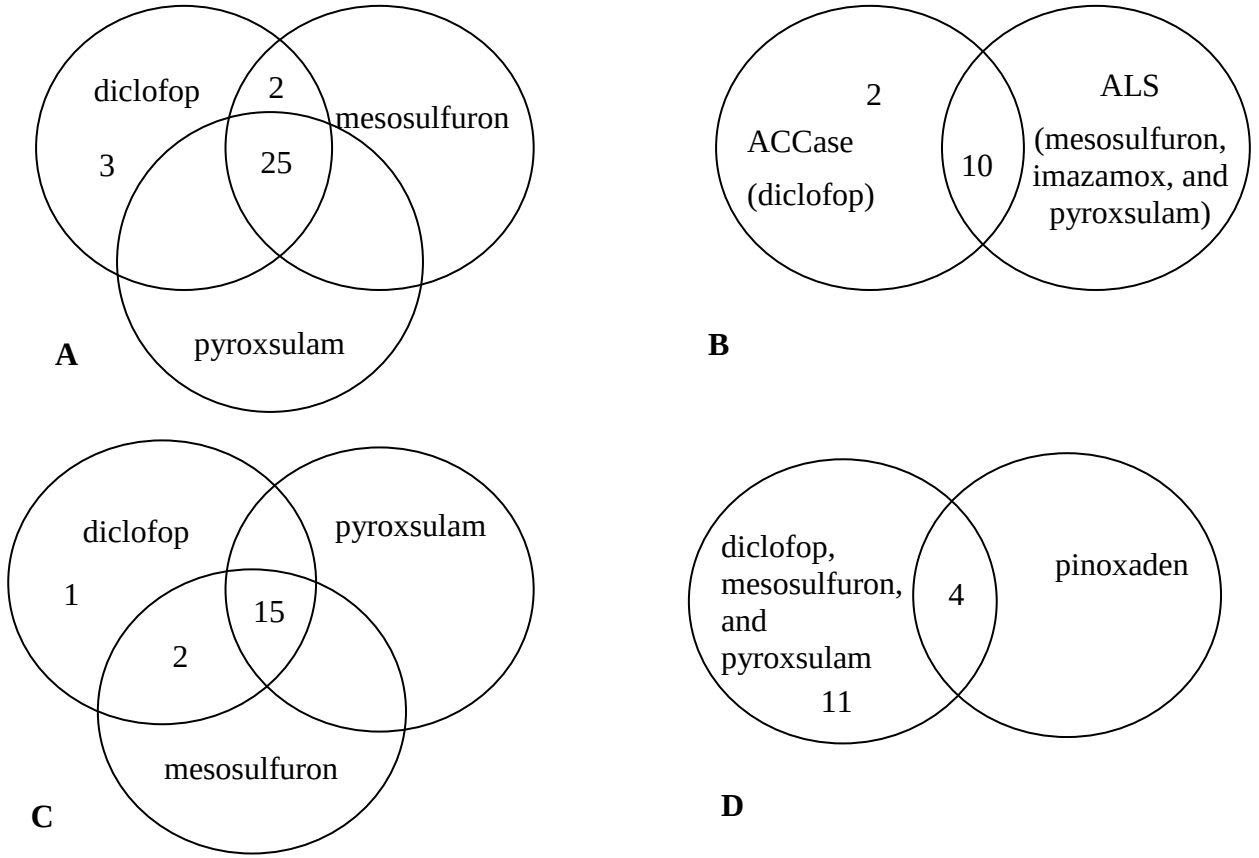

Figure 1. Resistance patterns of 30 Italian ryegrass accessions from the southern US to ACCase- and ALS-inhibiting herbicides. Panel A: total number of accessions resistant to each of the three herbicides (diclofop, mesosulfuron, and pyroxsulam) and their combinations ; Panel B: number of 2008 Arkansas accessions resistant to each of the herbicide groups and their combinations; Panel C: number of accessions collected in 2009 and 2010 that showed resistance to each of the three herbicides and their combinations; Panel D: number of accessions (collected in 2009 and 2010) with multiple resistance to diclofop, mesosulfuron, pyroxsulam, and pinoxaden.

CHAPTER IV

EXPLORATION OF METABOLIC-BASED RESISTANCE IN HERBICIDE-RESISTANT ITALIAN RYEGRASS (*LOLIUM PERENNE* SSP. *MULTIFLORUM*)

Abstract

Plants metabolize certain herbicides via the activity of enzymes belonging to the cytochrome P450 family. The purpose of this experiment is to determine if P450-mediated enhanced metabolism exists in selected herbicide-resistant ryegrass accessions. Six ryegrass populations with different resistance patterns to glyphosate, ALS- and ACCase herbicides were evaluated. P450 inhibitors malathion (1000 g ai ha^{-1}) and 1-aminobenzotriazole (100 µM ABT) were applied 30 min before applying the recommended field rate of either glyphosate, diclofop, pinoxaden, mesosulfuron, and pyroxsulam. Each population was treated with the corresponding herbicides it expresses resistance to. Biomass reduction was evaluated 4 weeks after treatment. Malathion improved the activity of diclofop, mesosulfuron, and pyroxsulam in 09-NC-04 accession to 85%, 54%, and 37%, respectively. The efficacy of pinoxaden and mesosulfuron in 09-NC-03 accession was also enhanced by the addition of P450 inhibitor. Both P450 inhibitors had no effect on the herbicide activity on 08-AR-10, 09-NC-01, 09-GA-01, and Des03 accessions. Overall, malathion elicited the most response in improving herbicide activity. The increased activity, whenever it occurred, did not completely overcome resistance to any herbicide, indicating that P450-mediated metabolism is only partially responsible for resistance in some cases. In many cases, metabolism-based resistance may not be involved at all. Alternatively, herbicide metabolism may still be a factor, but with other monooxygenases or enzyme families. This experiment provides direction for follow-up research on herbicide-resistant ryegrass populations and helps generate more informed decisions on resistance management.

Introduction

Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) is a cool-season annual grass that infests both winter- and spring-planted crops (Rauch et al. 2010). It is widely cultivated as forage because of its high seedling vigor, rapid re-growth after cutting, high quality and forage yield, and adaptability to southern climatic conditions and soil types (Ball et al. 1996). Despite its value as a forage crop, it is considered as the number one problem in wheat (Smith 2003). Italian ryegrass is highly competitive with winter wheat, reducing wheat tillering and interfering with soil nitrogen and phosphorus uptake (Perez-Fernandez and Coble 1998). It also can cause severe lodging which interferes with wheat harvest and contaminates the harvested grain with weed seed (Justice et al. 1994). Liebl and Worsham (1984) reported a 5% grain yield loss for every 10 Italian ryegrass m^{-2}. Heavy ryegrass infestation can reduce wheat yield by as much as 92% (Hashem et al. 1998) and also reported that nine ryegrass plants in 100 winter wheat plants reduced grain yield by 33%.

Diclofop, an ACCase inhibitor belonging to the AOPP family, is the traditional postemergence herbicide used in controlling ryegrass in wheat field since its commercialization in 1980s. However in 1987, diclofop-resistant Italian ryegrass was reported in Oregon. Since then, diclofop-resistant Italian ryegrass has been reported in 10 states in the United States, including five other countries (Stanger and Appleby 1989; Heap 2012). Relatively new herbicides, including pinoxaden, mesosulfuron, and pyroxsulam were introduced to manage herbicide-resistant Italian ryegrass in wheat (Dickson et al. 2011). Pinoxaden, an ACCase herbicide belonging to the phenylpyrazoline family (Porter et al. 2005), has the same mode of action as other AOPP herbicides but with a novel chemical structure that alters its efficacy (Boeger et al. 2006). Mesosulfuron and pyroxsulam are ALS herbicides belonging to the

sulfonylurea and triazolopyrimidine sulfonanilides families, respectively (Hand et al. 2002; deBoer et al. 2011). Glyphosate, a non-selective herbicide, is used pre-harvest of wheat after hard dough stage and at least 7 d prior to harvest) to control perennial and annual weeds and to improve wheat harvest efficiency (Scott et al. 2012). In addition, glyphosate is heavily used in burn-down treatments after crop harvest to prepare the field for the next cropping season.

Repeated use of the same herbicides has led to the evolution of herbicide-resistant weed populations. Ryegrass has evolved resistance to several ACCase- and ALS-inhibiting herbicides, and even to glyphosate (Heap 2012; Yu et al. 2009). *Lolium* species have a high propensity to evolve resistance, with extensive resistance to numerous herbicides (Holtum et al. 1991). Ten states in the US, including six other countries, had reported ACCase- and/or ALS-resistant Italian ryegrass problems (Heap 2012). There are various reports on diclofop-resistant ryegrass populations with resistance also to other ACCase- and ALS inhibitors (Kuk et al. 2008; Elenie et al. 2000; Holtum and Powles 1991). Italian ryegrass populations from Arkansas and North Caroline exhibited resistance to both diclofop and pinoxaden (Kuk et al. 2008; Ellis et al. 2010). More recently, Italian ryegrass populations from North Carolina with cross-resistance to mesosulfuron and pyroxsulam, and multiple-resistance to diclofop and pinoxaden were reported (Salas et al. 2010; Chandi et al. 2011).

Resistance to ACCase and ALS inhibitors usually involved target-site mutation or/ and enhanced herbicide metabolism. Glyphosate-resistant weeds usually exhibit either reduced herbicide translocation or target site mutation; however, sequestration of glyphosate into the vacuole and *EPSPS* gene amplification are recently reported to also make plants insensitive to glyphosate (Powles and Yu et al. 2010; Ge et al. 2012, Gaines et al. 2010). Metabolism of glyphosate is rare in plants (Schuette 1998). Although some plants are able to degrade

glyphosate into aminomethylphosphonic acid (AMPA) to a limited extent, it did not appear to be a common factor in explaining natural resistance levels (Reddy et al. 2008; Sandberg et al. 1980). Plants can metabolize certain herbicides via the activity of a large group of enzymes belonging to the cytochrome P450 family. Cytochrome P450s are mixed function oxidases which catalyze various reactions such as oxygenation, isomerization, dehydration, and reduction (Durst et al. 1997). P450 enzymes are implicated in metabolism-based resistance to multiple herbicides in grass weeds such as blackgrass (*Alopecurus myosuroides*), late watergrass (*Echinochloa phyllopogon*), and rigid ryegrass (*Lolium rigidum*) (Hall et al. 1997; Fischer et al. 2000; Preston et al. 1996; Yu et al. 2009; Yun et al. 2005). Enhanced metabolic inactivation of herbicides is reported as the basis for cross-resistance to chlorsulfuron in diclofop-resistant rigid ryegrass biotype (Cotterman et al. 1992). Evolved ACCase-resistance in a rigid ryegrass population in Spain is due to increased rate of diclofop-methyl metabolism, which is likely catalyzed by a cytochrome P450 enzyme (de Prado et al. 2005). Yu et al. (2009) reported that resistance to ACCase and ALS herbicides in a rigid ryegrass population in Australia is due to enhanced herbicide metabolism involving cytochrome P450 enzymes.

The application of an appropriate P450 inhibitor would increase herbicide activity and potentially overcome the resistance if cytochrome P450-mediated metabolism is involved in herbicide resistance. P450 inhibitors 1-aminobenzotriazole (ABT) and malathion inhibited the metabolism of diclofop and chlorsulfuron, respectively, in herbicide-resistant rigid ryegrass populations in Australia (Preston et al 1996; Yu et al 2009; Bravin et al. 2001). In the present study, we used malathion and 1-aminobenzotriazole to verify if these P450 inhibitors can increase herbicide activity in Italian ryegrass accessions showing different cross- and multiple-resistance profiles to glyphosate, ACCase and ALS herbicides. The objective of this study is to

determine if cytochrome P450-enhanced herbicide metabolism is the basis of resistance to glyphosate, ACCase-inhibitors, and ALS inhibitors in Italian ryegrass accessions from the southern United States.

Materials and Methods

Plant Materials. Six Italian ryegrass accessions (Des03, 09-NC-01, 08-AR-10, 09-NC-04, 09-GA-01, and 09-NC-03) from the southern United States, exhibiting different multiple- and cross-resistance patterns to glyphosate, ACCase and ALS herbicides were used in this study (Table 1). These accessions were confirmed resistant by Salas et al. (2010) and Dickson et al. (2011). A susceptible accession was also included as control.

Greenhouse Bioassay. Italian ryegrass accessions were grown in 11.4-cm pots filled with commercial soil mixture potting medium (Sunshine Mix®, Sun Gro Horticulture Inc., Bellevue, WA 98008). Seedlings were kept in the greenhouse with 12-h days and 24/18 C day/night temperatures. At three- to four-leaf stage, seedlings were sprayed with cytochrome P450 inhibitors malathion (1000 g ai ha^{-1}) and 1-aminobenzotriazole (100 µM). Malathion (1000 g ai ha^{-}) and 1-aminobenzotriazole (100 µM) were applied 30 min before applying the recommended field rate of either glyphosate (Roundup Weathermax, St. Louis, MO 63167), diclofop (Hoelon, Bayer CropScience, Research Triangle Park, NC 27709), pinoxaden (Axial XL, Syngenta Crop Protection,Inc., Greensboro, North Carolina 27419), mesosulfuron (Osprey, Bayer CropScience, Research Triangle Park,NC 27709), and pyroxsulam (PowerFlex, Dow AgroSciences LLC, Indianapolis, IN 46268). The recommended rates of glyphosate, diclofop, pinoxaden, mesosulfuron, and pyroxsulam are 870 g ae ha^{-1}, 1120, 60, 15, and 18 g ha^{-1}, respectively. Each accession was treated with the corresponding herbicides it expresses resistance to. Each herbicide was applied with or without the P450 inhibitor and with adjuvant. Diclofop and

pinoxaden treatments were applied with 1 and 0.7% non-ionic surfactant (Induce non-ionic surfactant, Helena Chemical Co. Collierville, TN 38017), respectively. A methylated seed oil (Premium MSO methylated spray oil, Helena Chemical Co.Collierville, TN 38017) at 1.75 L ha^{-1} was included with mesosulfuron. A crop oil concentrate (Agri-Dex crop oil concentrate, Helena Chemical Co. Collierville, TN 38017) at 1.0% (v/v) was used with pyroxsulam. P450 inhibitors and herbicide treatments were applied using a laboratory sprayer equipped with a flat fan nozzle (TeeJet spray nozzles, Spraying Systems Co., Wheaton, IL 60189) delivering 187 L ha^{-1}. A nontreated check was also provided for each population.

Data Collection and Analysis. The plant material was cut at soil surface 4 weeks after treatment (WAT), placed in brown paper bags, and dried at 70 C for 3 d prior to recording the dry weights. For each accession, results were expressed as the percentage of biomass reduction compared with that of the control treatment without herbicides and P450 inhibitors.

The experiment was set in a factorial treatment design with P450 inhibitor and herbicide as the main factors. Six separate experiments were conducted (by accession). Treatments were replicated three times with five plants per replicate. Data were subjected to analysis of variance in SAS JMP v.10 software. Significant means were separated using Fisher's protected $LSD_{0.05.}$

Results and Discussion

The potential role of herbicide metabolism in glyphosate-, diclofop-, pinoxaden-, mesosulfuron-, and pyroxsulam- resistance in various ryegrass accessions was evaluated using cytochrome P450 inhibitors malathion and 1-aminobenzotriazole. Results showed that in the absence of herbicides, malathion at 1000 g ha^{-1} and 1-aminobenzotriazole (ABT) at 100 μM had

no effect on the growth of the Italian ryegrass accessions when compared with nontreated control. This is similar to results reported by Christopher et al. (1994) and Tardif and Powles (1999). However, when resistant plants in 09-NC-04 accession were treated with both P450 inhibitor malathion and herbicide, plant growth was suppressed (Tables 2 - 7).

The interaction between P450 inhibitor and herbicide in Des03, 09-AR-10, 09-GA-01, 09-NC-01, and 09-NC-03 accessions was not significant (Tables 2 - 6); however, P450 inhibitor and herbicide interaction effect was evident in 09-NC-04 accession (Table 7). Diclofop, mesosulfuron, and pyroxsulam caused greater biomass reduction in 09-NC-04 accession when malathion was applied than when the herbicide is applied alone. In all cases, the main effect of the herbicide within an accession was apparent because the response of the accession to the herbicides they showed resistance varies regardless of the P450 inhibitor. Main effect of P450 inhibitor was significant in 09-NC-03 and 09-NC-04 accessions (Table 6 and 7). Contrast analysis showed that the application of P450 inhibitor in 09-NC-03 accession enhanced the activity of mesosulfuron and pinoxaden (Table 6).

Effect of Cytochrome P450 Inhibitors on the Activity of Glyphosate. The efficacy of glyphosate in resistant Des03 accession was not affected by the addition malathion or ABT (Table 2). This indicated that resistance to glyphosate in Des03 is possibly not contributed by cytochrome-P450 enhanced glyphosate metabolism. This is not suprising as there are no reports on enhanced glyphosate metabolism in glyphosate-resistant weeds although a few studies that showed cell suspensions of soybean, wheat, and maize metabolized glyphosate by degrading the herbicide into aminomethylphosphonate (AMPA) (Komaba et al. 1992). Cleavage of the carboxymethyl carbon-nitrogen bond of glyphosate produces AMPA, which can be further metabolized (Dyer 1994). Reports have shown that resistance to glyphosate in weeds results

from three mechanisms: *EPSPS* gene mutation, reduced absorption and translocation, and *EPSPS* gene amplification (Powles and Yu 2010; Ge et al. 2012). Salas et al. (2012) demonstrated that resistance to glyphosate in Des03 is primarily due to *EPSPS* gene amplification.

Effect of Cytochrome P450 Inhibitors on the Activity of ACCase-inhibiting Herbicides. The response of 08-AR-10, 09-NC-01, 09-GA-01, and 09-NC-03 accessions to diclofop was not improved by the addition of P450 inhibitors (Tables 3- 6). However in 09-NC-04 accession, there was a significant increase in biomass reduction to 85% when plants were pretreated with malathion prior to diclofop application. This result indicated that diclofop in 09-NC-04 accession is possibly detoxified by a cytchrome P450 enzyme, and the addition of malathion antagonized the activity of that enzyme and reduced the metabolism of diclofop. Preston et al. (1996) showed that metabolism of diclofop in rigid ryegrass was inhibited by ABT, but not by malathion. It is possible that in ryegrass, another member of the cytochrome P450 family is responsible for diclofop detoxification. Yu et al. (2009) and Preston and Powles (1998) reported that resistance to diclofop is likely to be metabolism-based, involving cytochrome P450 enzymes, as the addition of a P450 inhibitor amitrole (Yang et al. 1985; Koop 1990) reverses diclofop resistance in a resistant populations. The role of enhanced metabolism in conferring resistance to diclofop was demonstrated in Italian ryegrass population in the UK and in a population of *Avena* spp. (Cocker et al. 2001; Maneechote et al. 1997). Other studies have indicated that similar herbicides like fenoxaprop and quizalofop belonging to the same mode of action and the same family as diclofop are metabolized by resistant grass and dicot species by either cleavage to various phenolic derivatives or aryl oxidation followed by conjugation to polar metabolites (Koeppe et al. 1990; Lefsrud and Hall 1989; Wink et al. 1984).

The pinoxaden-resistant accession 09-NC-03 showed cross-resistance to diclofop. Pretreatment with P450 inhibitor increased pinoxaden phytotoxicity in 09-NC-03 accession but did not have an effect on diclofop activity (Table 6). Although pinoxaden and diclofop are both ACCase herbicides, they have different structures and chemistries (Scarabel et al. 2011). In the absence of P450 inhibitors, pinoxaden controlled 09-NC-03 accession 39%; however, the efficacy of pinoxaden was improved to 59% to 73% by the addition of P450 inhibitor. This suggests that resistance to pinoxaden in 09-NC-03 accession is conferred in part by increased metabolism, although other mechanisms of resistance, such as target site mutation, cannot be ruled out. It has been shown that resistance to pinoxaden in blackgrass is endowed by non-target-site resistance mechanisms (Petit et al. 2010). However, resistance to pinoxaden in wild oat (*Avena fatua*) and *Lolium* spp. is due to mutation in the ACCase gene (Cruz-Hipolito et al. 2011; Scarabel et al. 2011). Scarabel et al. (2011) reported that the occurrence of pinoxaden-resistant plants not carrying a mutant ACCase allele suggests the presence of non-target-site-based resistance mechanism that can reduce the amount of herbicide molecules from reaching the target site.

Response to ALS-inhibiting Herbicides in Combination with Cytchrome P450 Inhibitors. Among the four pyroxsulam-resistant accessions studied, only 09-NC-04 exhibited increased pyroxsulam phytoxicity when also treated with malathion (Table 7). ABT did not improve the activity of pyroxsulam. Pyroxsulam controlled 09-NC-04 10%; however, its performance was enhanced to 37% with malathion. Resistance to pyroxsulam was not completely overcome by malathion suggesting that resistance to pyroxsulam in 09-NC-04 accession is partially due to enhanced metabolism and that it possibly harbors other resistance mechanisms. Tolerance to a similar herbicide, flumetsulam, belonging to the same family as pyroxsulam, in cereals (maize)

and soybeans is due to metabolic detoxification (Saari et a. 1994). Tolerant plants oxidize flumetsulam to hydroxylated metabolites (Swisher et al. 1990). Cytochrome P450 monooxygenase systems have been implicated in hydroxylation reactions for herbicide metabolic detoxification (Brown 1990). Metabolism of pyroxsulam into hydroxylated metabolites via cytochrome P450 inhibitors is probably a secondary mechanism of resistance in 09-NC-04 accession.

When mesosulfuron, a sulfonylurea herbicide, was applied to 08-AR-10, 09-GA-01, 09-NC-03, and 09-NC-04 accessions, control was 1 to 54% (Tables 2-7). In the absence of malathion and ABT, mesosulfuron controlled 08-AR-10, 09-GA-01, 09-NC-03, and 09-NC-04 accessions 22%, 24%, 29%, and 1% respectively. The addition of P450 inhibitor increased the efficacy of mesosulfuron on 09-NC-03 and 09-NC-04 accessions. The efficacy of mesosulfuron in 09-NC-03 accession was improved to 54% by the addition of P450 inhibitor (Table 6). Malathion increased the performance of mesosulfuron to 54% in 09-NC-04, while ABT did not influence mesosulfuron activity (Table 7). Christopher et al. (1994) reported that malathion, but not ABT, is an excellent synergist for chlorsulfuron in a resistant SLR 31 rigid ryegrass biotype. The synergistic interaction of malathion with sulfonylurea herbicide is likely caused by competitive inhibition of cytochrome P450 degradation enzymes (Tardif et al. 1999). According to Werck-Recichart et al. (2000), the inhibition of herbicide activity by malathion occurs when atomic sulfur released from the oxygenated organophosphate inhibits the P450 apoprotein. The application of malathion with chlorsulfuron slows the degradation rate of the herbicide and lowers the resistance level to chlorsulfuron (Christopher et al. 1994). Malathion has been shown to inhibit the cytochrome P450-dependent detoxification of sulfonylurea herbicides in microsome preparations from maize (Kreutz and Fonne-Pfister 1992). Metabolism of

chlorsulfuron and triasulfuron in wheat involves rapid hydroxylation at the 5 position of the phenyl ring, followed by conjugation to glucose (Saari et al. 1994; Sweetser et al.1982). Our result suggests that malathion synergizes the action of mesosulfuron in 09-NC-04 accession possibly by inhibiting mesosulfuron metabolism. Although malathion did not completely reverse ryegrass resistance to mesosulfuron in 09-NC-03 and 09-NC-04 accessions, enhanced herbicide metabolism contributes resistance to mesosulfuron in these accessions, but does not account for the observed resistance level.

P450 enzymes are involved in secondary metabolism in plants and through different substrate specificities they contribute to herbicide selectivity between crops and weeds, and in some cases confer herbicide resistance to weed biotypes (Durst 1991; Yun et al. 2005). Their role in herbicide conversion is usually hydroxylation or dealkylation (Powles and Yu 2010). Some P450 enzymes metabolize some herbicides to products with reduced phytotoxicity that are further deactivated, often by conjugation with glucose, and transported into the vacuole (Kreutz et al. 1996: Powles and Yu 2010). Naturally occurring ACCase- and ALS-tolerant crops is based on the crop's ability to metabolize the herbicide to nonphytotoxic compounds rapidly enough to prevent lethal herbicide levels from reaching the target site (Saari et al. 1994). This tolerance mechanism in crops also appears to be a mechanism responsible for poor control of some weeds by certain ALS and ACCase herbicides (Cotterman et al. 1992; Cruz-Hipolito et al. 2011; Saari et al. 1994).

Cytochrome P450 monooxygenase-mediated enhanced metabolism most likely endows resistance to diclofop, mesosulfuron, and pyroxsulam in 09-NC-04 accession, and mesosulfuron and pinoxaden in 09-NC-03 accession. Malathion and ABT, although both P450 inhibitors, are not structurally similar and probably inhibit different specific cytochrome P450 monooxygenase

enzymes (Preston et al.1996). Malathion elicited the most response in improving herbicide activity in the accessions studied. The increased activity, whenever it occurred, did not completely overcome the resistance to any herbicide, indicating that P450-mediated metabolism is partially responsible for resistance in some cases. In many cases, metabolism-based resistance may not be involved at all in the accessions studied. Alternatively, herbicide metabolism may still be a factor, but with other monooxygenases or enzyme families. Other resistance mechanism such as target site mutation may be involved. Multiple resistance mechanisms, both target-site and non-target site based, can exist simultaneously in a single plant in *Lolium* spp. (Yu et al. 2009). Wild oat and rigid ryegrass possess multiple resistance mechanisms to diclofop and chlorsulfuron, respectively, exhibiting both altered target site and enhanced herbicide metabolism (Burnet et al. 1994; Christopher et al. 1992; Maneechote et al. 1997). It is likely that the accessions studied harbor multiple resistance mechanisms. Other resistance mechanisms should be investigated. Follow-up research on this study is appropriate to provide additional information and evidence on the metabolism-based resistance of selected ryegrass population. Definitive proof of the direct involvement of cytochrome P450 enzymes in the resistant Italian ryegrass accessions is still required.

Italian ryegrass populations in the southern United States have evolved cross- and multiple resistance to ACCase and ALS herbicides, and even to glyphosate. There is evidence that enhanced herbicide metabolism is partially responsible for resistance to diclofop, pinoxaden, mesosulfuron, and pyroxsulam in some Italian ryegrass accessions. Because Italian ryegrass is an obligate outcrossing species, plants having multiple resistance mechanisms could hybridize, producing progeny plants carrying new combination of resistance genes that may endow new resistant phenotypes (Scarabel et al. 2010). This will complicate ryegrass management in crop

fields. Remaining herbicides options for Italian ryegrass control include pendimethalin and flufenacet plus metribuzin, however over-realiance on these herbicides is discouraged. Chemical weed control should be integrated with biological, mechanical and cultural methods in order to preserve the utility of herbicides.

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Table 1. Resistance profile of six Italian ryegrass accessions used in the study.

Italian ryegrass accession	State	Resistance profile
Des03	Arkansas	Resistant to glyphosate
09-NC-01	North Carolina	Resistant to diclofop
08-AR-10	Arkansas	Resistant to diclofop, mesosulfuron, and pyroxsulam
09-NC-04	North Carolina	Resistant to diclofop, mesosulfuron, and pyroxsulam
09-GA-01	Georgia	Resistant to mesosulfuron and pyroxsulam
09-NC-03	North Carolina	Resistant to diclofop, pinoxaden, mesosulfuron, and pyroxsulam

Table 2. Response of Des03 accession (percent biomass reduction relative to the nontreated control) to glyphosate when pretreated with P450 inhibitor.

	Biomass reduction relative to the nontreated control	
P450 inhibitor	No herbicide	Glyphosate
	----------%----------	
Malathion	2	55
ABT	1	51
No inhibitor	0	7
$LSD_{0.05}$:		
P450 inhibitor effect	----------NS[a]----------	
Herbicide effect	---------- 10----------	
P450 inhibitor X herbicide	----------NS----------	

[a] NS, not significant.

Table 3. Response of 08-AR-10 accession (percent biomass reduction relative to the nontreated control) to diclofop, mesosulfuron and pyroxsulam when pretreated with P450 inhibitor.

P450 inhibitor	Biomass reduction relative to nontreated control			
	No herbicide	Diclofop	Mesosulfuron	Pyroxsulam
	----------%----------			
Malathion	3	25	25	36
ABT	5	0	32	36
No inhibitor	0	3	22	13
$LSD_{0.05}$:				
P450 inhibitor effect	----------NS[a]----------			
Herbicide effect	----------14----------			
P450 inhibitor X herbicide	----------NS----------			

[a] NS, not significant.

Table 4. Response of 09-GA-01 accession (percent biomass reduction relative to the nontreated control) to mesosulfuron and pyroxsulam when pretreated with P450 inhibitor.

P450 inhibitor	Biomass reduction relative to nontreated control		
	No herbicide	Mesosulfuron	Pyroxsulam
	----------%----------		
MalathionABT	1	49	32
ABT	1	29	35
No Inhibitor	0	24	19
$LSD_{0.05}$			
P450 inhibitor effect	----------NS[a]----------		
Herbicide effect	---------- 10----------		
P450 inhibitor X herbicide	----------NS----------		

[a] NS, not significant.

Table 8. Response of 09-NC-01 accession (percent biomass reduction relative to the nontreated control) to diclofop when pretreated with P450 inhibitor.

	Biomass reduction relative to the nontreated control	
P450 inhibitor	No herbicide	Diclofop
	----------%----------	
Malathion	0	68
ABT	0	55
No inhibitor	0	56
$LSD_{0.05}$:		
P450 inhibitor effect	----------NS[a]----------	
Herbicide effect	---------- 21----------	
P450 inhibitor X herbicide	----------NS----------	

[a] NS, not significant.

Table 6. Response of 09-NC-03 accession (percent biomass reduction relative to the nontreated control) to diclofop, pinoxaden, mesosulfuron, and pyroxsulam when pretreated with P450 inhibitor.

	Biomass reduction relative to nontreated control				
P450 inhibitor	No herbicide	Diclofop	Pinoxaden	Mesosulfuron	Pyroxsulam
	----------%----------				
Malathion	5	28	73	54	53
ABT	5	23	59	33	37
No inhibitor	0	17	39	29	37
$LSD_{0.05}$:					
P450 inhibitor effect	----------7----------				
Herbicide effect	----------9----------				
P450 inhibitor X herbicide	----------NS[a]----------				

[a] NS, not significant.

Table 7. Response of 09-NC-04 accession (percent biomass reduction relative to the nontreated control) to diclofop, mesosulfuron and pyroxsulam when pretreated with P450 inhibitor.

	Biomass reduction relative to nontreated control			
P450 inhibitor	No herbicide	Diclofop	Mesosulfuron	Pyroxsulam
	----------%----------			
Malathion	0	85	54	37
ABT	0	33	5	0
No inhibitor	0	47	1	10
$LSD_{0.05}$:				
P450 inhibitor effect	----------8----------			
Herbicide effect	----------10----------			
P450 inhibitor X herbicide	----------17----------			

CHAPTER V

EPSPS GENE AMPLIFICATION IN GLYPHOSATE-RESISTANT ITALIAN RYEGRASS (*LOLIUM PERENNE* SSP *MULTIFLORUM*) FROM ARKANSAS, USA

Abstract

BACKGROUND: Resistance to glyphosate in weed species is a major challenge for the sustainability of glyphosate use in crop and non-crop systems. A glyphosate-resistant Italian ryegrass population has been identified in Arkansas. This research was conducted to elucidate its resistance mechanism.

RESULTS: We investigated resistant and susceptible plants from a population in Desha County, Arkansas (Des03). The amounts of glyphosate that caused 50% overall visual injury were 7 to 13 times greater than those of susceptible plants from the same population. The *EPSPS* gene did not contain any point mutation that has previously been associated with resistance to glyphosate, nor were there any other mutations on the *EPSPS* gene unique to the Des03 resistant plants. The resistant plants had 6-fold higher basal EPSPS enzyme activities than the susceptible plants, but their I_{50} values in response to glyphosate were similar. The resistant plants contained up to 25 more copies of *EPSPS* gene than the susceptible plants. The level of resistance to glyphosate correlated with increases in EPSPS enzyme activity and *EPSPS* copy number.

CONCLUSION: Increased *EPSPS* gene amplification and EPSPS enzyme activity confer resistance to glyphosate in Des03 population. This is the first report of *EPSPS* gene amplification in glyphosate-resistant Italian ryegrass. Other resistance mechanism(s) may also be involved.

1. INTRODUCTION

Glyphosate [*N*-(phosphonomethyl) glycine] is a widely used broad spectrum postemergence herbicide that has low mammalian toxicity and is considered relatively environmentally friendly.[1] Glyphosate inhibits 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) which is an enzyme in the aromatic amino acid biosynthesis pathway. The downstream products of the aromatic amino acids are crucial to plant growth, making glyphosate a potent herbicide.[2] Glyphosate usage has significantly increased in the last two decades due to the adoption of conservation tillage practices and introduction of genetically modified glyphosate-resistant crops.[3] About 60% of the 148 million ha of transgenic crops grown are glyphosate-resistant.[4] Glyphosate-resistant soybean, maize, cotton, canola and sugarbeet varieties were rapidly adopted because of the economic advantage of the technology, as well as the simple and superior weed control that glyphosate offers.[5] Furthermore, glyphosate/glyphosate-resistant crop weed management technology is more environmentally benign than the destructive soil tillage and/or herbicides that it has replaced.[6] Glyphosate-resistant crops accounted for a large majority of canola, soybean, corn and cotton grown in 2011 in the United States.[7,8] The adoption rate of glyphosate-resistant soybean is similar in South America.[9,10,11] Despite the global use of glyphosate, evolved resistance to glyphosate was not identified until 1996.[12,13] Since then, the number of cases has increased steadily. Today, resistance to glyphosate occurs in at least 21 different weed species in 15 countries.[14]

Lolium perenne ssp. *multiflorum* (Lam.) Husnot (Italian ryegrass) is a principal weed problem in *Triticum aestivum* L. ssp. *Aestivum* (wheat), *Gossypium* spp. (cotton) and *Glycine max* L. (soybean) production fields. This obligate outcrosser[15] is particularly prone to evolve resistance to herbicides, with documented cases of resistance to nine different herbicide modes of

action.[14] The evolution of herbicide-resistant ryegrass makes its control in crops difficult due to reduced herbicide options. Resistance to glyphosate was first discovered in *Lolium rigidum* Gaud. (rigid ryegrass) in Australia in 1996,[12] and has now been reported in several populations of *Lolium* species around the world.[14]

Weed resistance to glyphosate results from a number of mechanisms. Reduced glyphosate cellular transport to physiologically active meristematic tissues and insensitive, altered *EPSPS* have been the most common resistance mechanisms in glyphosate-resistant weeds.[16] It has been deduced that minimal translocation of glyphosate in resistant horseweed is due to rapid sequestration of glyphosate in the vacuole.[17] Recently, another glyphosate resistance mechanism (*EPSPS* gene amplification), was reported in glyphosate-resistant *Amaranthus palmeri* S. Wats. (Palmer amaranth) from Georgia.[18] So far, with respect to crop field-evolved glyphosate-resistant weeds, this mechanism has imparted the greatest resistance level to glyphosate (40-fold).[19]

A glyphosate-resistant Italian ryegrass population discovered in Arkansas Desha county exhibited a 23-fold resistance compared with a susceptible population.[20] In this paper, the mechanism of glyphosate resistance in the Desha county population was investigated by biochemical and molecular approaches.

2. EXPERIMENTAL METHODS

2.1 Plant materials

A high degree of genetic diversity is expected among plants within the same population because of the outcrossing requirement of Italian ryegrass. Therefore, three susceptible (S) and five resistant (R) plants of Des03 population were analyzed to determine whether resistance to glyphosate is associated with increased EPSPS activity and *EPSPS* genomic copy number. This

approach enabled us to determine differences between the R and S individuals from the same population without the confounding effects of genotypic or ecological differences.

Seeds from glyphosate-resistant ryegrass population (Des03) in Desha county, Arkansas, USA were collected. Composite seed samples were grown in trays in the greenhouse until the 2-tiller stage. Tillers were separated and transplanted into separate pots to produce two clones of each seedlings. One week after transplanting, shoots were clipped at 5 cm height and allowed to regrow to about 15 cm. Plants were watered daily and fertilized with Miracle-Gro, a water soluble all-purpose plant food containing 15-30-15% NPK, every two weeks. One set of clones was sprayed with 2244 g ae ha^{-1} glyphosate (2.58x of the recommended dose) to identify resistant individuals. Plants that survived at 4 wk after herbicide treatment were considered resistant (R); otherwise, they were classified as susceptible (S). Of the 80 plants from Des-03 population that were sprayed with glyphosate, 73 survived. The nontreated clones corresponding to the confirmed R plants were separated from the S plants and allowed to grow separately for subsequent experiments.

2.2 Whole-plant dose-response bioassay

Four individuals were randomly selected from the S and R groups. Des03-S1, Des03-S2, Des03-S3 and Des03-S4 represented the susceptible group while Des03-R1, Des03-R2, Des03-R3 and Des03-R4 represented the resistant group. These plants were subjected to dose response bioassays to assess their resistance level to glyphosate. Tillers of each plant were separated and planted into 15-cm pots to obtain 24 clones per plant. Susceptible plants were sprayed with 6 doses of glyphosate ranging from 217 g ae ha^{-1} to 1740 g ae ha^{-1} which is equivalent to 0.25x to 2x of the recommended glyphosate dose. Resistant plants were sprayed with 0, 217, 435, 870, 1740, 3480, 6960, and 13920 g ae ha^{-1} glyphosate which is equivalent to up to 16x of the

recommended dose. MON 78623 (potassium salt of glyphosate) was applied with 0.25% vv^{-1} Kinetic HV nonionic surfactant (NIS) (Helena Chemical Co., Memphis, TN, USA). Glyphosate treatments were applied using a laboratory sprayer equipped with a flat fan nozzle delivering 228 L ha^{-1}. The experiment was conducted in a completely randomized design with three replications. Visual injury (%) was evaluated at 28 DAT relative to the nontreated control. Here, injury pertains to the overall visible negative effect of glyphosate on the plant including chlorosis, stunting, or total desiccation (in case of S plants). Visual injury was regressed against glyphosate dose and modeled using a log-logistic equation in the R program.[21] The amount of glyphosate that would cause 50% injury or overall visual growth reduction (GR_{50}) was estimated from the regression equations. Resistance fold (R/S) of the R plants was computed from their respective GR_{50} values divided by the average GR_{50} of the S Des03 plant samples. Des03-S4, which was initially categorized as susceptible, survived the labeled dose of glyphosate (870 g ae ha^{-1}) and, therefore, was reclassified as intermediate and relabeled as Des03-I1. Similarly, Des03-R4 was relabeled as Des03-I2 because of its intermediate level of resistance to glyphosate.

2.3 *EPSP* synthase gene sequencing

Young leaf tissues of 20 confirmed R and S plants from the Des03 population were collected and stored at -80 °C for RNA extraction. Clones of plants used in the dose-response assay (Section 2.2) were among these samples. Leaves from a known S Italian ryegrass population were also collected. Frozen leaf tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using PureLink RNA Mini kit (Ambion). $Oligo(dT)_{20}$ supplied in the Improm-II Reverse Transcription System first-strand cDNA synthesis kit (Promega, Madison, WI, USA) was used to synthesize the first-strand complementary DNA (cDNA). LPM2F (5'-

TSCAGCCCATCARGGAGATCT-3'), designed by Perez-Jones et al. (2005),[22] was used as the forward primer. The reverse primer LPM2R1 (5'- CTAGTTCTTCAC GAAGGTGCTTA-3') was designed based on the *EPSP* synthase gene sequence of *L. multiflorum* (Gene Bank Accession number DQ153168.2). This primer pair amplified a 915 bp fragment of *EPSPS* encompassing codon 106 where the point mutation conferring glyphosate resistance occurred. Mutation that occurred at this locus (substitution of Pro_{106} to either Ser, Ala or Thr), endowed resistance to glyphosate in goosegrass,[23,24] rigid ryegrass[25] and Italian ryegrass.[18,26] The polymerase chain reaction was done in a 25-µL reaction mixture containing 4 µL of cDNA, 0.4 µM of both forward and reverse primers, 12.5 µL of Taq2x master mix (New England Biolabs Inc., Ipswich, MA, USA) and nuclease-free water. Amplification was performed under the following conditions: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s; annealing at 57.5 °C for 30 s; elongation at 72 °C for 90 s, and final extension at 72 °C for 10 min. PCR products were cleaned using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) before sequencing. The resulting DNA sequences were cleaned, aligned using the *EPSPS* sequence of *Lolium multiflorum* as reference, and analyzed for polymorphisms using Sequencher and Bioedit softwares.

2.4 EPSPS enzyme activity assays

Protein extraction and EPSPS assay were conducted generally following the procedures of Sammons *et al.*[27] Twenty grams leaf tissue of the R and S plants (clones of the ones used in the dose-response assay) were ground to fine powder in a chilled mortar. Powdered tissues were transferred to tubes containing 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine) with 1% polyvinylpolypyrrolidone (PVPP) and fresh 70 µL of β-mercaptoethanol. Samples were homogenized for about 5 min with

constant stirring to minimize foaming and then centrifuged for 40 min at 18,000 *g* at 4 °C. The supernatant was decanted through a cheesecloth into a cold beaker. Powdered ammonium sulfate was slowly added to the supernatant to make 45% w v^{-1} concentration, stirred continuously for 30 min and centrifuged at 30,000 *g* for 30 min at 4 °C. Protein extracts were precipitated out of solution by gradual addition of ammonium sulfate to a concentration of 80% (w v^{-1}) with gentle stirring, and then centrifuged at 30,000 *g* for 30 min at 4 °C. Pellets were dissolved in about 3 mL of extraction buffer and dialyzed overnight in 2 L of dialysis buffer using a 30-mm, 10000-MWC dialysis tubing at 4 °C on a stir plate. Protein concentrations were determined using a Bradford assay kit (Bio-Rad protein assay system, Life Science Research, Hercules, CA, USA).

Specific activities of EPSPS from R and S plants were determined in the presence and absence of glyphosate. A continuous assay for inorganic phosphate release[28] was conducted with the EnzCheck phosphate assay kit (Invitrogen, Carlsbad, California, USA) to assay for EPSPS activity. The assay buffer consisted of 100 mM MOPS, 1 mM $MgCl_2$, 10% glycerol, 2 mM sodium molybdate, and 200 mM NaF. The following reagents were added to a cuvette in the following order: 600 µL 2x assay buffer, 300 µL ultrapure water, 0.164 mM of 2-amino-6-mercapto-7-methylpurine riboside (MESG), 1 unit of purine-nucleoside phosphorylase (PNP), 1.02 mM of phosphoenolpyruvate (PEP), 25 µL EPSPS extract and glyphosate. Each sample was assayed in 3 replicates at glyphosate concentrations of 0, 0.1, 1.0, 10, 100, and 1000 µM to obtain the enzyme activity inhibition curve. The solution was allowed to react for 20 min to deplete phosphate contaminants before starting the EPSPS reaction. After obtaining a background phosphate release level, 50 µL of 10 mM (0.41 mM) shikimate-3-phosphate was added. Phosphate release above background level was measured for 10 min at 360 nm in a UV-3101 spectrophotometer (Shimadzu North America, Columbia, MD, USA). The slope was

calculated to determine the amount of phosphate (µmol) released per microgram of total soluble protein (TSP) per min. Enzyme activity (µmol Pi μg^{-1} protein min^{-1}) was regressed against glyphosate dose and modeled using log-logistic in the R program. The glyphosate concentration (µM) that inhibits EPSPS activity by 50% (I_{50}) was estimated from the regression equations.

2.5 Genomic copy number

Quantitative real-time PCR was used to measure the genomic copy number of *EPSPS* relative to cinnamoyl-CoA reductase (*CCR*) in Italian ryegrass. *CCR* is constitutively expressed and is present as a single copy gene in perennial ryegrass.[29] One-hundred milligrams of leaf tissues of clones from the eight S and R Des03 plants were collected and stored at -80 °C. Genomic DNA was extracted using DNeasy plant mini kit from Qiagen (Valencia, CA, USA). Primer pair *EPSPS* F2 (5'- CTGATGGCTGCTCCTTTAGCTC-3') and EPSPS R2 (5'-CCCAGCTATCAGAATGCTCTGC-3') were designed to amplify the *EPSPS* gene of Italian ryegrass. *CCR* primers LpCCR1 F2 (5'-GATGTCGAACCAGAAGCTCCA-3') and LpCCR1 R2 (5'- GCAGCTAGGGTTTCCTTGTCC-3')[29] were used as an internal standard to normalize the samples for differences in the amounts of DNA. The optimal annealing temperature was assessed using gradient PCR. The specificity of the qPCR assay was verified on agarose gel. All primer pairs generated a single band (Figure not shown). A 5-fold serial dilution of genomic DNA samples, ranging from 0.08 ng to 50 ng, was used to construct a standard curve. The slope of the standard curve was used to determine amplification efficiency (E).

Quantitative real-time PCR was performed in a 25-µL reaction containing 10 ng genomic DNA and Bio-Rad iQ SYBR Green Supermix. Real-time PCR detection was performed in a Bio-Rad MiniOpticon System PCR machine under the following conditions: 10 min at 94 °C, 40 cycles of 94 °C for 15 s and 60 °C for 1 min then increasing the temperature by 0.5 °C every 5 s

to access the product melt-curve. Data was analyzed using CFX manager software (version 1.5). Relative quantification of *EPSPS* was calculated as Δ^{Ct} = (Ct, *CCR* – Ct, *EPSPS*) according to the method described by Gaines *et al.* (2010).[18] Increase in *EPSPS* copy number was expressed as $2^{\Delta Ct}$. Each sample was run in three replicates to calculate the mean and standard error of the increase in *EPSPS* copy number. Results were expressed as fold increase in *EPSPS* copy number relative to *CCR*.

2.6 Glyphosate absorption and translocation

Clones of seedlings from Des03-S1, Des03-S3, Des03-I1, and Des03-R1 were allowed to grow to maturity for seed production. Clones of the same plant were kept together and separated from clones of other plants to avoid cross-pollination. Although Italian ryegrass is an obligate outcrosser, we have generated a limited number of fertile seeds from these isolated clones. Seeds from Des03-S1, Des03-S3, Des03-I1, and Des03-R1 plants were planted in 2.5-cm pots in the greenhouse. Representative plants with different sensitivity to glyphosate were chosen for follow-up experiments because reduced absorption and translocation of glyphosate was observed among other glyphosate-resistant populations in the region.[30] Seedlings at one-tiller stage were sprayed with 870 g ae ha^{-1} of formulated glyphosate (MON 78623) containing 0.25% NIS (Kinetic HV, Helena Chemical Company, Memphis, TN, USA) at 187 L ha^{-1} spray volume and then spotted with 4 μL of herbicide solution containing 1.776 kBq ^{14}C-phosphonomethyl-labeled glyphosate (glyphosate-[phosphonomethyl-^{14}C], $HOOCCH_2NH^{14}CH_2PO_3H_2$, 1.85 GBq $mmol^{-1}$ specific activity, American Radiolabeled Chemicals, Inc., 101, ARC Drive, St. Louis, MO) . Plants were harvested at 24 and 48 h after treatment (HAT) and sectioned into four parts: treated leaf (TL), above treated leaf (ATL), below treated leaf (BTL), and roots (R). The treated leaf was rinsed with methanol:water (1:1 v v^{-1}) solution containing 0.25% (v v^{-1}) NIS at each

harvest and the recovered radioactivity was quantified by liquid scintillation spectroscopy. The plant tissues were oven-dried and oxidized and the recovered radioactivity was quantified. The proportion of foliar-absorbed glyphosate was calculated by dividing the total amount of ^{14}C recovered from the oxidized plant parts by the sum of the radioactivity contained in the leaf wash and the total amount recovered from the oxidized plant parts, for each individual plant. The distribution of ^{14}C-glyphosate in plant tissues was expressed as a percentage of absorbed radioactivity.

3. RESULTS

3.1 Whole-plant dose-response

Clonal plants Des03-S1, Des03-S2 and Des03-S3 were sensitive to glyphosate as observed in the preliminary assay. The herbicide doses that caused 50% injury of the S clones ranged from 34 to 264 g ae ha^{-1} while those of the R clones ranged from 945 to 1596 g ae ha^{-1}. Des03-I1 and Des03-I2 had 7- to 9-fold resistance relative to the average GR_{50} values of the S plants while Des03-R1, Des03-R2 and Des03-R3 showed 12- to 13-fold resistance to glyphosate (Table 1). This difference in level of resistance within a population (field) reflects the different degrees of ryegrass injury from glyphosate that has been observed in growers' fields among plants of the same age.

3.2 *EPSPS* gene sequencing

A 915-bp region of the *EPSPS* gene was sequenced from cDNA of the same 8 glyphosate-R and -S clones used in other experiments plus 12 other R and S Des03 plants. The longest *EPSPS* nucleotide sequence of Italian ryegrass in the Genbank is comprised of 1316 bp or 437 amino acids.[31] The fragment we sequenced spanned from amino acid position 77 to 381. Although the full-length *EPSPS* gene of Italian ryegrass was not obtained, the sequenced region included the

domain where point mutations are known to confer resistance to glyphosate, e.g. at Pro_{106}.[18,23-26,31-37] The partial *EPSPS* sequence of the R plants did not reveal any mutation at Pro_{106} (data not shown), which has been associated with resistance to glyphosate in several weed species (Supplemental Table 1). Nucleotide polymorphisms at Cys_{367}, TGC to CGT, were detected in all R plants resulting in a substitution of Cys_{367}Arg; however, some S plants also had this mutation (data not shown) indicating that substitution with arginine at this locus does not confer resistance to glyphosate. The *EPSPS* nucleotide sequence of the R plants did not show any other point mutations that have been associated previously with resistance to glyphosate, nor any mutation that is unique to the R individuals.

3.3 EPSPS enzyme activity

In the absence of glyphosate, the specific activity of EPSPS in the R plants (Des03-I1, Des03-I2, Des03-R1, Des03-R2, and Des03-R3) ranged from 0.075 to 0.186 µmol μg^{-1} protein min^{-1} while that of the S plants (Des03-S1, Des03-S2, and Des03-S3) ranged from 0.00943 to 0.05201 µmol μg^{-1} protein min^{-1} (Fig. 1). The R plants showed 1.4- to 19.8-fold increase in EPSPS enzyme activity relative to the average enzyme activity in S plants. One of the R plants with the highest resistance level (Des03-R1) had 19.4-fold increase in EPSPS enzyme activity relative to the S plant with the lowest GR_{50} (Des03-S2) (Table 1 and Fig. 1). The resistant plants, on average, exhibited six-fold higher basal enzyme activities than their susceptible counterparts.

The EPSPS enzymes of both R and S plants were both inhibited by glyphosate. The amounts of glyphosate needed to reduce the EPSPS activity by 50% (I_{50}), were similar in all samples analyzed, ranging from 4.5 to 6.4 µM glyphosate for the S plants and 3.5 to 6.2 µM glyphosate for the R plants (Fig. 1).

3.4 EPSPS gene-copy number

Agarose gel electrophoresis of the real-time PCR products showed a single band for both *CCR* and *EPSPS* PCR reaction products indicating that the primers used for the target sequences were specific. The reaction efficiency was 102% with an R^2 of 0.992 and a slope of 3.271 (data not shown). The relationship between Ct values and log DNA concentrations was linear, indicating that the Ct values could be used reliably to estimate the relative gene copy number.

Two of three S plants had only one copy of *EPSPS* gene relative to *CCR*; Des03-S3 contained 9 copies (Fig. 2). Des03-S3 had higher enzyme activity compared with the other S plants. Although Des03-S3 was considered as susceptible, its GR_{50} was significantly higher than that of the other S plants, but still lower than those of the R plants (Table 1), which had up to 25 copies of *EPSPS*.

3.5 Glyphosate absorption and translocation

The absorbed glyphosate ranged from 43 to 63% of radioactivity applied, 48 HAT (Table 2). This was within the range of what was reported for the ryegrass populations from Mississippi, USA (43 to 56%).[30] The plant Des03-R1 with the highest resistance index (R/S = 13) and the highest *EPSPS* copy number (25) as well as Des03-S3 absorbed similar fractions of applied ^{14}C-glyphosate at 63 and 56%, respectively. Likewise, Des03-I1 (R/S = 9) and Des03-S1 absorbed practically the same proportions of applied ^{14}C-glyphosate at 43 and 44%, respectively. In all plants, the majority of absorbed glyphosate (52 to 67%) remained in the treated leaf, with negligible amounts translocated to tissues above the treated leaf. Similar small fractions (14 to 19%) were translocated to the roots.

4. DISCUSSION

Anecdotal reports of Italian ryegrass escaping preplant burndown treatments with glyphosate have been increasing in Arkansas since the late 1990s. While these failures were initially attributed to poor timing of application, resistance to glyphosate was eventually confirmed in *Lolium* populations in the southeastern Arkansas Desha County in 2007.[20]

Italian ryegrass is an obligate outcrossing species,[15] thus a high degree of genetic diversity would be expected among plants within the same (Des03) population. Because of this presumed variability, plants from Des03 population were analyzed to determine the differences between R and S plants from the same population, which allowed us to rule out differences in cropping history and localized environmental adaptations. Subjecting the clonal lines derived from glyphosate-S and –R individuals of Des03 population to a wide range of glyphosate doses revealed three categories of plants within this population: resistant (Des03-R1 through Des03-R3), intermediate-resistant (Des03-I1 and Des03-I2) and sensitive (Des03-S1 to Des03-S3) (Table 1). This is expected from a genetically diverse, predominantly outcrossing species at the earlier phase of resistance evolution. Resistance to glyphosate was observed in Arkansas, only about five years ago and it is not yet widespread.

Because of the absence of point mutations in the *EPSPS* that are unique to the ten R plants examined in this population and the absence of other mutations previously associated with resistance to glyphosate (Supplemental Table 1), we determined that target-site alteration is not the resistance mechanism in Des03 population. *EPSPS* is not prone to mutation(s) in the catalytic site in natural plant populations, in contrast to several other herbicide targets. Target-site resistance risk is related to the conservation of the herbicide binding site. Mutation in the *ALS* gene conferring resistance to ALS-inhibiting herbicides usually occurs in sites that are not highly conserved and, therefore, does not come with fitness penalty.[38] Catalytic sites that are

highly conserved indicate that mutations at this site tend to be deleterious. However, an amino acid deletion in the conserved region of one of the *PPO* genes of *Amaranthus tuberculatus* did not seem to affect whole plant fitness,[39] although the deletion significantly altered the enzyme's architecture and affinity for its substrate.[40]

The amino acids at the catalytic site of *EPSPS* are highly conserved, corresponding to less opportunity for mutation. Mutation in the conserved site of *EPSPS* is likely to incur a significant fitness cost. Substitution at Gly_{96} in glyphosate-resistant *E. coli* significantly reduced PEP affinity.[33] So far, there are no published studies on the effect of binding site mutation of EPSPS on the fitness of R plants. The absence of target-site mutation in the conserved region of *EPSPS* in this glyphosate-resistant ryegrass population is not unusual. Consequently, the lack of mutations conferring glyphosate resistance to EPSPS is reflected in the fact that the EPSPS from the various S and R plants had the same sensitivity to glyphosate *in vitro* (Fig. 1).

On the other hand, resistance to glyphosate was generally associated with increased EPSPS activity and gene copy number. Resistant plants (Des03-I1 and Des03-I2, Des03-R1, Des03-R2, and Des03-R3) had increased EPSPS enzyme activity compared with S plants (Des03-S1, Des03-S2 and Des03-S3) (Fig. 1). Likewise, with the exception of Des03-S3, R plants had higher *EPSPS* gene copy number than the S plants (Fig. 2). The strong positive relationship between enzyme activities and GR_{50} values (R^2=89%, $P<0.05$) (Fig. 3) as well as EPSPS activity and *EPSPS* gene amplification (R^2=78%, $P<0.05$) (Fig. 4), further suggests that increased copy number resulted in increased EPSPS activity, which in turn resulted in resistance to glyphosate. Indeed, glyphosate-sensitive clonal plants Des03-S1 and Des03-S2 had the lowest enzyme activity and gene copy number of all plants tested. Conversely, the R plants Des03-R1, Des03-R2, and Des03-R3 had the highest level of enzyme activity and the greatest number of *EPSPS*

gene copy number. The 6-fold increase in the EPSPS enzyme activity and 3-to 25-fold increase in *EPSPS* genomic copy number contributed to the observed 7- to 13-fold resistance on the whole plant level of individual R plants. *EPSPS* gene amplification appears to be the primary mechanism of resistance to glyphosate in the Des03 population.

The same relationship was observed also recently in glyphosate-resistant Palmer amaranth[18] and *Amaranthus tuberculatus* (tall waterhemp)[41] although increased EPSPS activity in glyphosate-tolerant plants had been reported earlier in a wild type population of *Convolvulus arvensis* L. (field bindweed)[42] and a progressively selected population of *Lotus corniculatus* L. (birdsfoot trefoil).[43] Resistance to glyphosate from progressive selection in plant cell cultures is usually attributed to increased EPSPS activity, particularly due to gene amplification.[44] A glyphosate-tolerant *Daucus carota* L. (carrot) cell line obtained by stepwise selection with glyphosate exhibited a 12-fold increase in enzyme activity[45] due to 4- to 25-fold increase in *EPSPS* gene copy number.[46] Similar to the wild carrot cell line, a *Petunia hybrida* (petunia) cell line which exhibited a 20-fold increase in EPSPS activity possessed 20-fold increase in *EPSPS* gene copies relative to the control.[47]

Gaines *et al.*[19] demonstrated that the effect of additional copies of *EPSPS* is additive, and additional copies confer higher levels of resistance.[48] However, we observed that ryegrass plants with similar gene copy number may not necessarily show the same level of resistance to glyphosate. For example, Des03-S3 had significantly higher EPSPS activity than the other S plants and, whereas the intermediate Des03-I1 had a lower gene copy number than Des03-S3, it had higher enzyme activity than Des03-S3. More work remains to be done to better understand this, but a resistant population of rigid ryegrass from Australia has a similar pattern as our

Des03-I1, with increased EPSPS activity but no evidence of *EPSPS* gene amplification.[49] How this happens needs to be investigated.

Overproduction of EPSPS effectively increases the number of target sites that must be inhibited by glyphosate in order to block carbon flow through the shikimate pathway.[50] The increased number of EPSPS per unit of protein or fresh weight dilutes the effect of the herbicide which is no longer able to inhibit enough of the EPSPS protein to sufficiently block the shikimate pathway for herbicide effects to occur.[48]

Gene multiplication might be attributed to genome duplication. The ploidy level of the R and S Des03 plants needs to be investigated to determine whether genome duplication causes *EPSPS* amplification in the R plants. Bunnell *et al.* [51] reported that a higher number of chromosomes (tetraploid) resulted in tolerance to metsulfuron in bahiagrass (*Paspalum notatum)* whereas diploid individuals were susceptible. However, in Palmer amaranth, there was no difference in ploidy level between glyphosate-resistant and –susceptible biotypes.[52] Gene duplication is usually triggered by environmental stresses.[53] Selection pressure with herbicides is akin to a recurring environmental stress that, in the case of glyphosate, favors survival of individuals with multiple copies of the glyphosate target gene. Alternatively, this mechanism could be a manifestation of mutation(s) in the promoter region, which elevates gene transcription.[54] Other than conferring resistance to glyphosate, no physiological advantage has been documented thus far as a consequence of EPSPS overexpression.

Of the representative R, I, and S plants further studied from Des03 population, reduced absorption and translocation did not contribute to the high level of resistance exhibited by De03-R1 nor to the reduced sensitivity to glyphosate in the Des03-I1 plant. Differential translocation was not observed among the R and S ryegrass plants studied here, although reduced glyphosate

translocation is a mechanism common to several glyphosate-resistant species including ryegrass and horseweed.[55]

Increased basal EPSPS enzyme activity and *EPSPS* copy number is the primary mechanism of resistance to glyphosate, but we are not yet certain if these are the only mechanisms involved in this population. Although the correlation between the level of resistance to glyphosate and increased EPSPS enzyme activity or gene copy number is strong, the extent to which the six-fold difference in the EPSPS enzyme activity and 3 to 25 genomic copies of *EPSPS* could contribute to the observed 23-fold[20] increase in glyphosate resistance at the population level is not yet clear. A glyphosate-resistant rigid ryegrass population from South Africa that had a 14-fold resistance level exhibited two mechanisms of resistance to glyphosate.[35]

Differences in glyphosate resistance mechanisms have been reported among ryegrass populations[30,31] and in many other species, but differences in resistance mechanisms among plants within a population are rarely investigated. Whole population studies may reveal principal mechanism(s) but other resistance mechanisms at low frequency may be obscured.[56] The lower *EPSPS* copy number, but higher enzyme activity of Des03-I1 than Des03-S3, suggests that another factor is contributing to the increased enzyme activity in these plants. Des03-I1 could have a more efficient EPSPS, or more efficient aromatic amino acid synthesis pathway (for reasons yet unknown). Other resistance mechanisms that remained to be investigated include vacuolar glyphosate sequestration, glyphosate metabolism, or a concerted action of a network of non-target genes[57]. The contribution of each mechanism to the resistance level of each plant also remains to be investigated. Studies on additional populations and more plants per population are warranted.

We conclude that resistance to glyphosate in Des03 Italian ryegrass population from Arkansas is primarily due to increased *EPSPS* enzyme activity associated with amplification of the *EPSPS* gene copy number. It remains to be seen whether there is cosegregation of *EPSPS* expression and monogenic inheritance of resistance trait in this population. It is not yet known whether increased *EPSPS* gene copy number is stably transmitted to the next generation of plants. The evolution and role of *EPSPS* gene amplification in glyphosate-resistant Italian ryegrass populations is not yet fully understood.

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Table 1. Resistance levels of selected intermediate (I) and resistant (R) plants relative to selected susceptible (S) plants from the Des03 population of Italian ryegrass, Arkansas, USA.

Des03 plants	GR_{50} (g ae ha^{-1})	R/S[a]
Des03-S1	84	-
Des03-S2	34	-
Des03-S3	264	-
Des03-I1	1104	9
Des03-I2	945	7
Des03-R1	1596	13
Des03-R2	1538	12
Des03-R3	1596	13

[a]Resistance levels (R/S) is based on the average GR_{50} of the three susceptible Des03 plants. The recommended dose of glyphosate is 840 g ae ha^{-1}.

Table 2. ^{14}C glyphosate absorption and distribution in various plant tissues of Italian ryegrass (*Lolium perenne* ssp. *multiflorum* L.) from Arkansas, USA.

Des03 plants[a]	^{14}C-glyphosate absorption[b]		^{14}C-glyphosate distribution							
			Treated leaf		Above treated leaf		Below treated leaf		Roots	
	24 HAT	48 HAT[c]	24 HAT	48 HAT	24 HAT	48 HAT	24 HAT	48 HAT	24 HAT	48 HAT
	% of applied		---------- % of absorbed ----------							
Des03-R1	44	63	47	52	2	2	25	27	21	19
Des03-I1	35	43	78	66	1	3	11	15	10	16
Des03-S1	41	44	64	67	1	1	18	19	18	14
Des03-S3	47	56	66	65	1	6	20	12	13	17
$LSD_{0.05}$[d]	14	16	NS	NS	NS	NS	NS	8	NS	NS

[a] R, resistant; I, intermediate; S, susceptible

[b] Values are the average of four plants

[c] HAT, hours after treatment

[d]Least significant difference between means based on Fisher's test at $\alpha = 0.05$

Supplemental Table 1. Amino acid substitutions in EPSPS that confer resistance to glyphosate in different species.

Amino acid substitution	Species	Reference
Pro_{106}-Ser	*Eleusine indica*	Baerson *et al*. (2002)[24]
	Lolium multiflorum	Perez-Jones *et al.* (2007)[48]
	Lolium rigidum	Simarmata and Penner (2008)[25]
Pro_{106}-Thr	*Eleusine indica*	Ng *et al.* (2003)[30]
	Lolium rigidum	Wakelin and Preston (2006)[31]
Pro_{106}-Ala	*Lolium rigidum*	Yu *et al.* (2007)[32]
	Lolium multiflorum	Jasieniuk *et al.* (2008)[26]
Pro_{106}-Leu	*Lolium rigidum*	Kaundun *et al.* (2011)[49]
	Oryza sativa	Zhou et al. (2006)[50]
Gly_{96}-Ala	*Escherichia coli*	Eschenburg *et al.* (2002)[36]

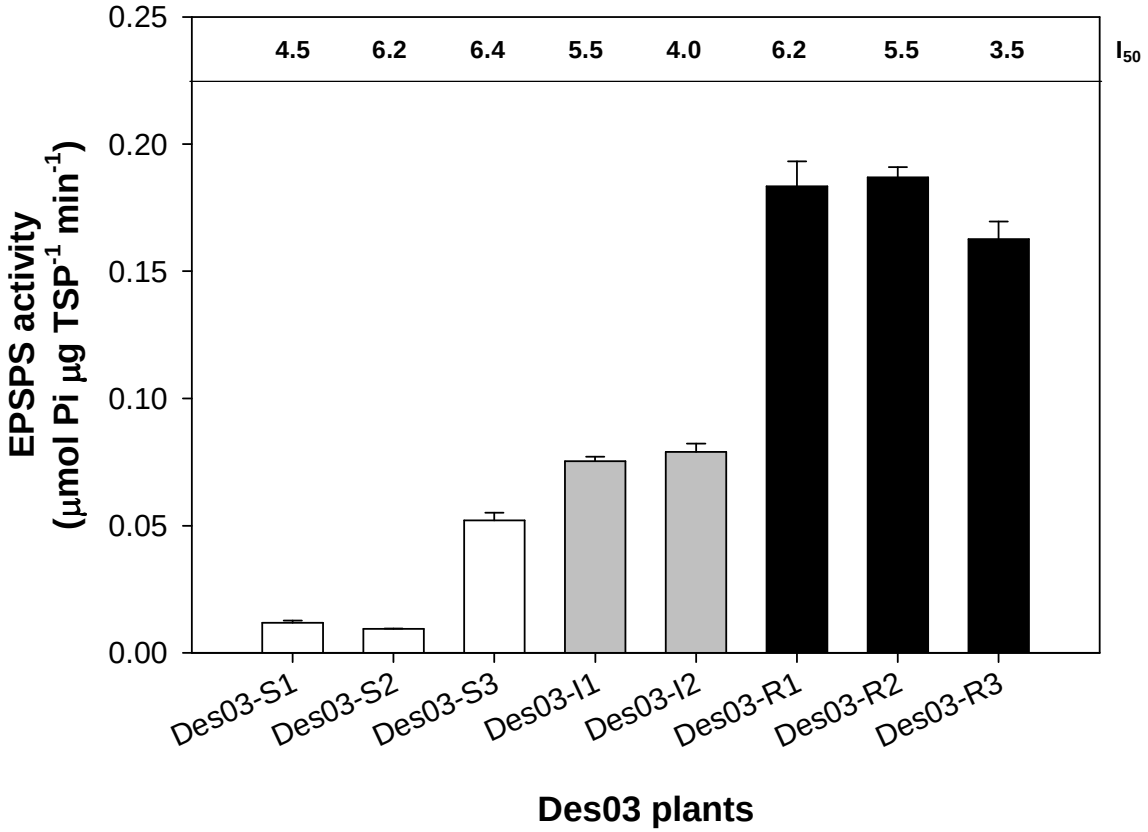

Figure 1. Basal EPSPS activity in intermediate (I) and resistant (R) Italian ryegrass plants relative to their susceptible (S) counterparts. I_{50} values are shown on top. Error bars represent standard deviation. White bars = S plants; Gray bars = I plants; Black bars = R plants.

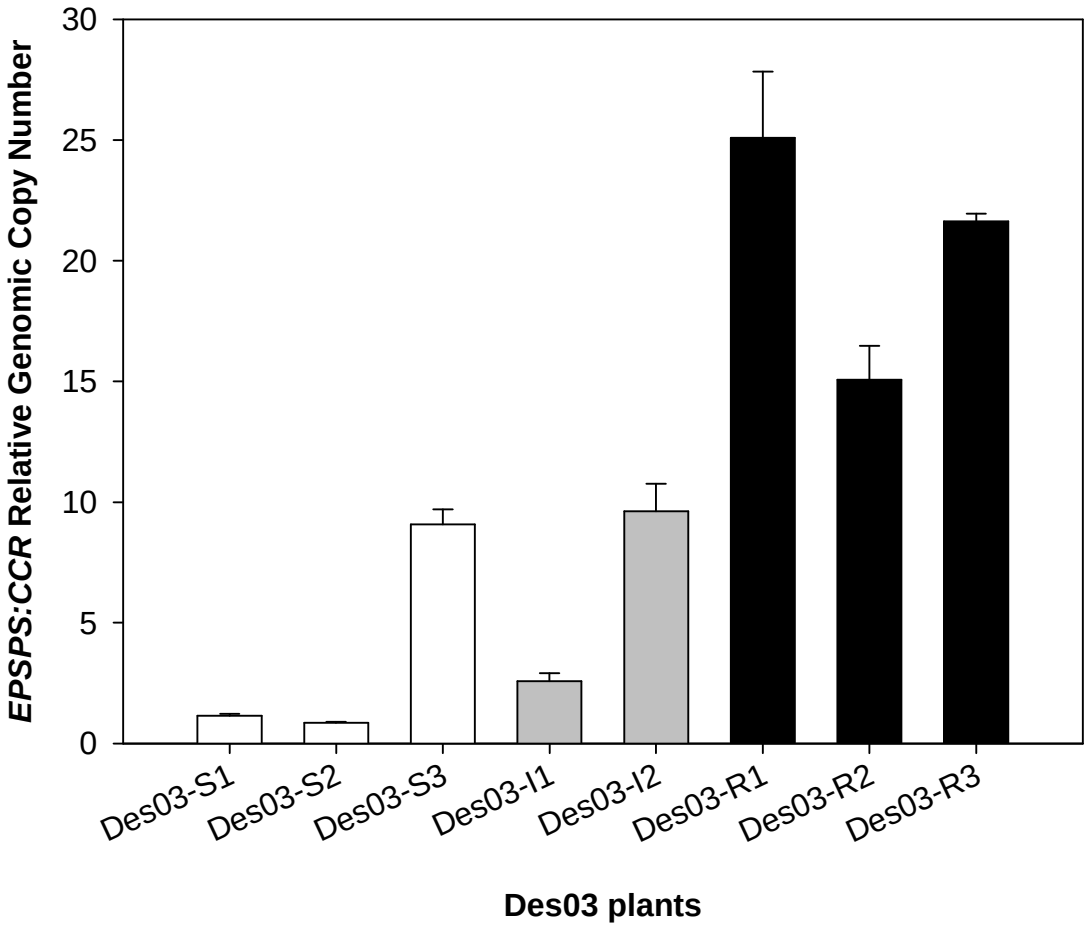

Figure 2. Relative genomic copy number of Italian ryegrass *EPSPS* in susceptible (S), intermediate (I) and resistant (R) plants. Error bars represent standard deviation of the mean. White bars = S plants; Gray bars = I plants; Black bars = R plants.

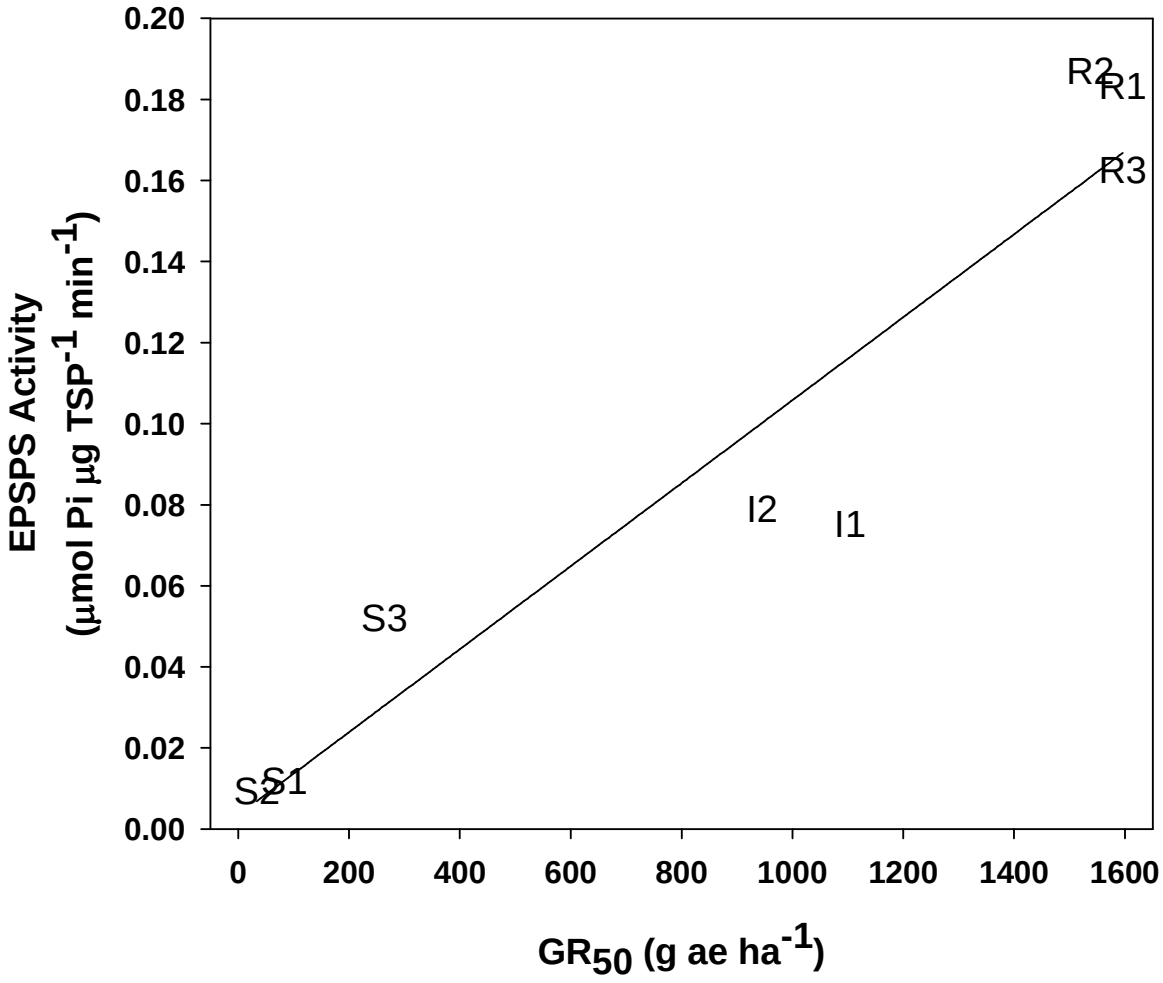

Figure 3. Relationship between Italian ryegrass EPSPS activity and the amount of glyphosate needed to control the plants 50% (GR_{50}); R^2 = 0.89 at α=0.05.

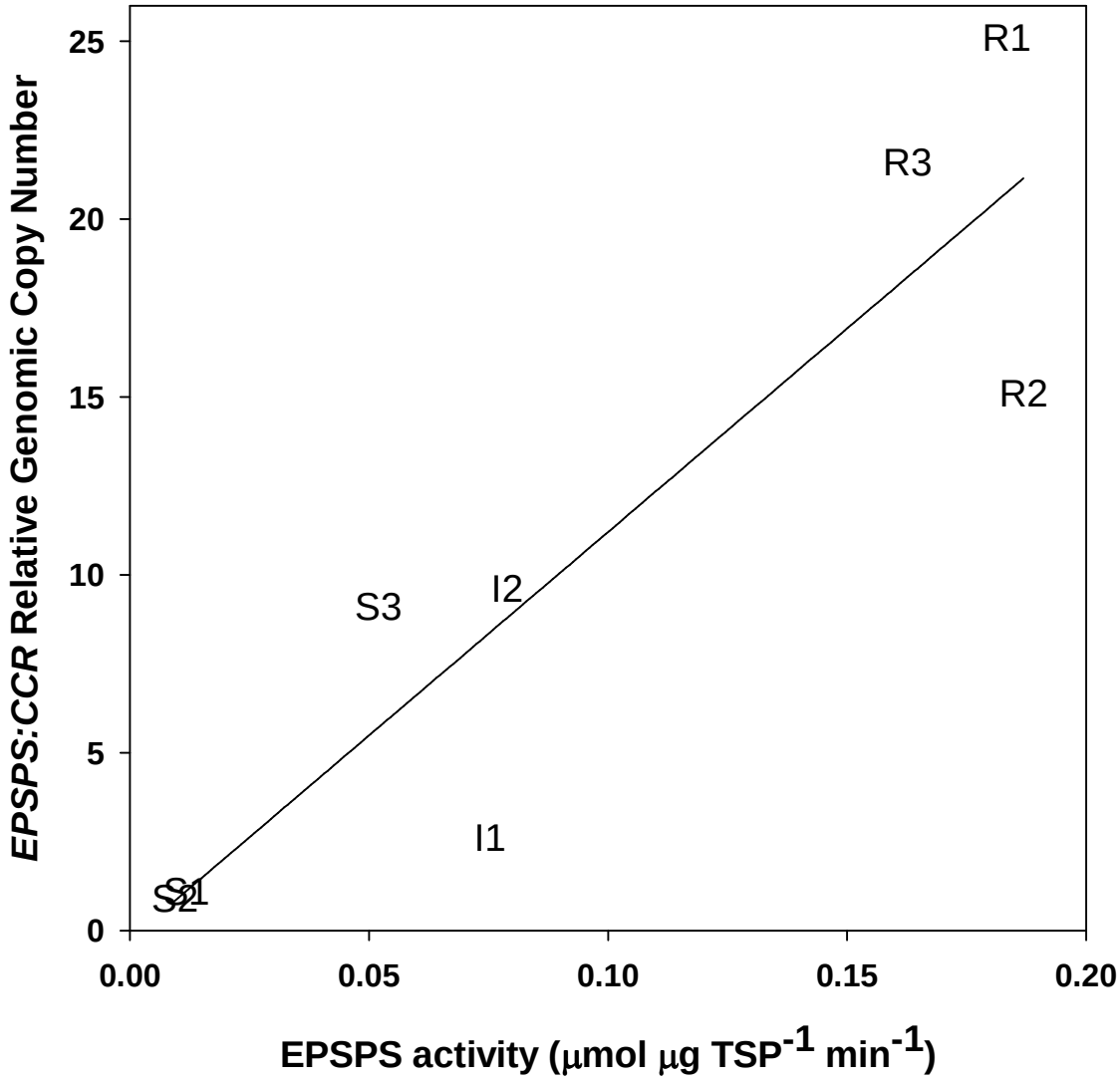

Figure 4. EPSPS activity in glyphosate-susceptible and -resistant Italian ryegrass from Des03 population increases with relative *EPSPS* genomic copy number (R^2 = 0.78).

CHAPTER VI

EPSPS GENE AMPLIFICATION IN GLYPHOSATE-RESISTANT *LOLIUM PERENNE* SSP *MULTIFLORUM* POPULATIONS FROM ARKANSAS, USA

ABSTRACT

Glyphosate-resistant *Lolium perenne* ssp. *multiflorum* population in Arkansas was first detected in Desha County in 2007. Now there are 45 glyphosate-resistant *L. perenne* ssp. *multiflorum* populations confirmed in 8 counties in Arkansas. This research was conducted to determine the level of resistance in Des05, Des14, D4, D8, and D13 populations and the resistance mechanism to glyphosate in selected *L. perenne* ssp. *multiflorum* populations. The resistance level was determined by dose-response bioassay. The absorption and mobility of glyphosate was evaluated using radiolabeled glyphosate. The *EPSPS* gene sequence was analyzed and gene amplification was determined by quantitative real-time PCR. The dose of glyphosate causing 50% growth reduction (GR_{50}) for the resistant populations was 7 to 19 times greater than that of the susceptible population. The *EPSPS* gene did not contain any mutation that has been previously associated with resistance to glyphosate. The uptake and translocation of ^{14}C-glyphosate was similar in resistant and susceptible populations. Resistant plants contained from 11-fold to 516-fold more copies of the *EPSPS* gene than the susceptible plants indicating that *EPSPS* gene amplification confers resistance to glyphosate in Des05, Des14, and D8 populations. Why *EPSPS* gene amplification occurs in these populations, but not in glyphosate-resistant populations in other regions is not yet understood.

INTRODUCTION

Glyphosate is by far the world's most important and widely used herbicide for postemergence control of weeds.[1-3] It is a potent inhibitor of the plastidic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), which catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate.[4] Inhibition of EPSPS by glyphosate results in the accumulation of shikimic acid and depletion of essential aromatic acids, leading to plant death. When commercialized in 1974, glyphosate was mainly used for total vegetation control because it is a nonselective, nonresidual, and environmentally benign herbicide.[5] Glyphosate usage dramatically increased in the past two decades following the introduction of glyphosate-resistant crops in 1996.[6] This expanded the use of glyphosate into millions of crop hectares. Glyphosate-resistant crops accounted for a large majority of canola, soybean, corn and cotton grown in 2011 in the United States.[7] The massive adoptions of transgenic glyphosate-resistant crops caused excessive reliance on glyphosate for weed control across vast areas.[8]

After over three decades of glyphosate use, weed species have evolved resistance to glyphosate. Glyphosate resistance has evolved in populations of several weed species, most often in the genetically diverse and resistance-prone genera *Conyza* and *Lolium*, in situations with persistent, intense glyphosate pressure.[8] The first case of glyphosate resistance was reported in a *Lolium rigidum* population exposed to two to three glyphosate applications per year for 15 years.[9] Today resistance to glyphosate occurs in 22 weed species around the world.[10]

Lolium species, particularly *L. rigidum* (rigid ryegrass), *L. perenne* (perennial ryegrass), and *L. perenne* ssp. *multiflorum* (Italian ryegrass) are self-incompatible and can freely cross-pollinate.[11] They have a high propensity to evolve resistance to herbicides.[11] So far, resistance

has evolved to six and ten different herbicide modes of action in *L. perenne* ssp. *multiflorum* and *L. rigidum*, respectively. [10] Today, *L. rigidum* ranks in the top 10 most important herbicide-resistant species.[10]

Weed resistance to glyphosate results from a number of mechanisms. Reduced herbicide translocation and target site mutation have been the most common resistance mechanisms in glyphosate-resistant weeds.[12] Impaired translocation mechanism has been reported in *Lolium* spp.,[13-16] *Conyza Canadensis,* [17-19] and *Sorghum halepense.*[20,21] This mechanism of resistance appears to provide between 3- and 12-fold resistance to glyphosate.[11] Target site mutation, involving a proline to serine, alanine, threonine or leucine change at position 106 of the EPSPS in *Eleusine indica* [22-26] and *Lolium* species [27-30] have been reported to partially confer resistance to glyphosate. Substitutions of Pro_{182} to Thr and Tyr_{310} to Cys in the *EPSPS* gene were recently reported in glyphosate-resistant *Digitaria insularis*.[31] The level of resistance due to target site mutation is relatively low, ranging from 2- to 4-fold.[32]

Two glyphosate resistance mechanisms have been reported more recently. *Conyza Canadensis* [33] and *Lolium* species [34] reduce the amount of glyphosate that reaches the target site by rapidly sequestering glyphosate into the vacuole. High level of glyphosate resistance in *Amaranthus palmeri* results from *EPSPS* gene amplification on multiple chromosomes.[35] This *EPSPS* gene amplification is heritable and correlates with glyphosate resistance in the F2 population.[35]

Several *L. perenne* ssp. *multiflorum* populations escaping from spring burn-down treatments were observed in Arkansas. The objectives of this study were to determine the level of resistance to glyphosate in these populations and investigate the mechanisms by which selected populations survive what used to be a lethal dose of glyphosate.

MATERIALS AND METHODS

Plant Materials. Mature panicles from suspected glyphosate-resistant *L. perenne* ssp. *multiflorum* plants were collected in Desha County, Arkansas in 2009 and 2010. Des05 and D8 populations were collected from cotton fields; D13 was from a fallow field; and Des14 population was from a soybean field. Seeds were grown in the greenhouse maintained at 24/18 C day/night temperatures with a 12-h photoperiod. Seedlings at three-leaf stage were sprayed with a discriminating rate of 870 g ae ha^{-1} glyphosate. The surviving plants were grown to maturity for seed increase, and seeds from all plants in the same population were bulked at harvest. Populations grown for seed increase were separated in space to avoid cross-pollination between populations, but plants within one population were allowed to cross-pollinate. Seeds generated were used for the subsequent experiments. A susceptible population (98-3) that was never exposed to glyphosate selection was used as reference material in all experiments.

Dose-Response Bioassay. Seeds were planted into flats (25 x 25 x 5 cm) filled with Sunshine Mix LC1soil (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia, Canada). Flats were equally divided in two greenhouses; one maintained at 24/18 C and the other at 30/25 day/night temperatures at 12-h photoperiod. Following emergence, plants were thinned into 15 seedlings per flat. Seedlings (98-3, Des05, Des14, D4, D8 and D13) at three- to four-leaf stage were treated with 8 doses of glyphosate from 0 to 13920 g ae ha^{-1}, which corresponds to 0 to 62 times the commercial rate of 870 g ae ha^{-1}. Treatments of the 98-3 population included a nontreated check and 11 rates of glyphosate from 13 to 3480 g ae ha^{-1} corresponding to 1/64 to 4 times the commercial rate of glyphosate. MON 78623 (58% v/w potassium salt of N-(phosphonomethyl)glycine; Monsanto Co., St. Louis, MO) was applied with 0.25% nonionic surfactant (NIS). Glyphosate treatments were applied using a laboratory sprayer equipped with a

flat fan spray nozzle (TeeJet spray nozzles, Spraying Systems Co., Wheaton, IL) delivering 187 L ha^{-1}. The experiment was conducted in a randomized completely block design with two replications. Each replication consisted of one tray (50 x 25 x 5 cm) accommodating two flats.

The number of survivors was recorded at 28 days after treatment (21 DAT). Plants were cut at the soil surface, stored in a dryer for 3 days, and dry weight was measured and recorded. Data were expressed as the percentage of biomass reduction relative to the untreated control. Regression analysis was conducted using SAS JMP v. 10. The % biomass reduction and % mortality with increasing rate of glyphosate was modeled with a sigmoid, three-parameter, logistic function (Equation 1).

$$Y = a/[(1 + (x/x_o)^b] \quad [1]$$

The rate needed to kill 50% (LD_{50}) or provide 50% biomass reduction (GR_{50}) was calculated from the above equation.

***EPSPS* Gene Sequencing**. Populations with lower level of resistance to glyphosate were chosen for the *EPSPS* gene sequencing. Seeds from Des05 and Des14 populations were planted in 4.5-cm pots filled with Sunshine Mix LC potting soil. Tillers of 12 Des05 and 13 Des14 plants were divided into two pots to produce clones of seedlings. One set of clones was cut to 8 cm and allowed to regrow to 12 cm before being sprayed with glyphosate at 870 g ae ha^{-1}. Plants that survived at 28 DAT were considered resistant (R); otherwise they were classified as susceptible (S). All 13 plants from Des14 population survived while 7 plants from Des05 population stayed alive at 28 DAT. The corresponding nontreated clones were used for the analysis of the *EPSPS* gene.

Young leaf tissues of 7 and 13 confirmed R plants from Des05 and Des14 populations, respectively, were harvested and stored at -80°C for RNA extraction. In addition, leaf tissues of 5 and 10 S plants from Des05 and 98-3 populations, respectively, were also collected for RNA extraction. Frozen leaf tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using PureLink RNA Mini kit (Ambion). Oligo$(dT)_{20}$ supplied in the Improm-II Reverse Transcription System first-strand cDNA synthesis kit (Promega, Madison, WI, USA) was used to synthesize the first-strand complementary DNA (cDNA). LPM2F (5'-TSCAGCCCATCARGGAGATCT-3'), designed by Perez-Jones et al.,[36] was used as the forward primer. The reverse primer LPM2R1 (5'- CTAGTTCTTCAC GAAGGTGCTTA-3') was designed based on the *EPSP* synthase gene sequence of *L. multiflorum* (Gene Bank Accession number DQ153168.2). This primer pair amplified a 915 bp fragment of *EPSPS* encompassing codon 106 where the point mutation conferring glyphosate resistance occurred.

The polymerase chain reaction was done in a 25-µL reaction mixture containing 4 µL of cDNA, 0.4 µM of both forward and reverse primers, 12.5 µL of Taq2x master mix (New England Biolabs Inc., Ipswich, MA, USA) and nuclease-free water. Amplification was performed under the following conditions: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s; annealing at 57.5 °C for 30 s; elongation at 72 °C for 90 s, and final extension at 72 °C for 10 min. PCR products were cleaned using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) before sequencing. The resulting DNA sequences were cleaned, aligned using the *EPSPS* sequence of *Lolium multiflorum* as reference, and analyzed for polymorphisms using Sequencher v.5 and Bioedit v.7 softwares.

***EPSPS* Copy Number Determination**. Leaf tissue samples from 10 confirmed R plants of selected population (Des05, Des14, and D8) and 10 plants of 98-3 population were collected and

stored in -80 C. Genomic DNA was extracted using hexadecyltrimethylammonium bromide (CTAB) method[37] following the modification of Sales et al.[38] Approximately 100 mg of leaf tissue from each plant was ground to a fine powder in liquid nitrogen, transferred to a 1.5-mL centrifuge tube, and suspended in 500 ml of CTAB extraction buffer (100 mM Tris-HCl [pH 8.0], 20 mM ethylenediaminetetra-acetic acid [EDTA] [pH 8.0], 2 M NaCl, 2% CTAB, 2% polyvinylpyrrolidone-40, 1 mM phenanthroline, and 0.3% b-mercaptoethanol). The aqueous extracts were incubated in a water bath at 55 ºC for 40 min, treated with RNAse solution, and extracted with an equal volume of phenol:chloroform:isoamyl alcohol solution (25:24:1). Total nucleic acids were precipitated from the supernatant by addition of an equal volume of isopropanol. The DNA pellet was washed with 500 uL of absolute ethanol, dried in a vacuufuge for 5 min, and resuspended in 30 mL of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Genomic DNA was quantified using a NanoDrop spectrophotometer model ND-1000 (Thermo Scientific, Wilmington DE) and checked for quality by agarose gel electrophoresis.

Quantitative real-time PCR was used to measure the genomic copy number of *EPSPS* relative to cinnamoyl-CoA reductase (*CCR*) in Italian ryegrass. *CCR* is constitutively expressed and is present as a single copy gene in perennial ryegrass. [39] Primer pair *EPSPS* F2 (5'-CTGATGGCTGCTCCTTTAGCTC-3') and EPSPS R2 (5'-CCCAGCTATCAGAATGCTCTGC-3') were designed to amplify the *EPSPS* gene of Italian ryegrass. *CCR* primers LpCCR1 F2 (5'-GATGTCGAACCAGAAGCTCCA-3') and LpCCR1 R2 (5'- GCAGCTAGGGTTTCCTTGTCC-3')[39] were used as an internal standard to normalize the different samples for differences in the amounts of DNA. The optimal annealing temperature was assessed using gradient PCR. The specificity of the qPCR assay was verified on agarose gel. All primer pairs generated a single band (Figure not shown). A 5-fold serial dilution of genomic

DNA samples, ranging from 0.08 ng to 50 ng, was used to construct a standard curve. The slope of the standard curve was used to determine amplification efficiency (E).

Quantitative real-time PCR was performed in a 25-µL reaction containing 10 ng genomic DNA and Bio-Rad iQ SYBR Green Supermix. Real-time PCR detection was performed in a Bio-Rad MiniOpticon System PCR machine under the following conditions: 10 min at 94 °C, 40 cycles of 94 °C for 15 s and 60 °C for 1 min then increasing the temperature by 0.5 °C every 5 s to access the product melt-curve. Negative control consisting of primers with no templates was included. No amplification products were observed in any control lacking a template. Data was analyzed using CFX manager software (version 1.5). Relative quantification of *EPSPS* was calculated as Δ^{Ct} = (Ct, *CCR* – Ct, *EPSPS*) according to the method described by Gaines et al.[35] Increase in *EPSPS* copy number was expressed as $2^{\Delta Ct}$. Each sample was run in three replicates to calculate the mean and standard error of the increase in *EPSPS* copy number. Results were expressed as fold increase in *EPSPS* copy number relative to *CCR*.

Absorption and Translocation of Glyphosate. Seeds from Des05, Des14, and 98-3 population were planted in 2.5-cm pots in the greenhouse maintained at 24/18 C day/night temperatures at 12-h photoperiod. Plants were harvested at 24 and 48 h after treatment (HAT) and sectioned into four parts: treated leaf (TL), above treated leaf (ATL), below treated leaf (BTL), and roots (R). To remove nonabsorbed glyphosate, the treated 5-cm portion of the treated leaf was rinsed for 15 s with 1 ml of a methanol: water (1:1 v/v) solution containing NIS at 0.25% v/v. The leaf wash was collected in a 20-ml scintillation vial, mixed with 10 ml of scintillation cocktail (Ultima Gold Cocktail, PerkinElmer, Waltham, MA), and radio assayed by liquid scintillation spectroscopy (LSS) on a Packard Tri-Carb 2100TR Liquid Scintillation. After rinsing of the treated portion of the treated leaf and dissection, all plant parts were dried for 48 h at 50 ºC.

Individual plant parts were combusted in a sample oxidizer (Biological Oxidizer OX500, R.J. Harvey Instrument Corporation, Tappan, NY) and the evolved CO_2 was trapped in 15 ml of carbon trapping scintillation cocktail (R.J. Harvey Instrument Corporation, Tappan, NY) and radio assayed with the use of LSS. Foliar absorption was calculated by dividing the amount of ^{14}C recovered from the oxidized plant parts by the sum of the radioactivity contained in the leaf wash and that recovered from the oxidized plant parts, for each individual plant. Herbicide translocation was expressed as percentage of total absorbed (total radioactivity recovered minus radioactivity in the leaf wash solution). The experiments were arranged in a completely randomized block design with four replicates. In the absorption experiment, a factorial scheme with two factors, (population and harvest time) was tested by ANOVA. The translocation experiment, which had three factors (population, plant section, and harvest time), was also analyzed by ANOVA using JMP v.9.

RESULTS AND DISCUSSIONS

Dose-Response Bioassay. Dose-response bioassay confirmed resistance of Des05, Des14, D5, D8 and D13 *L. perenne* ssp. *multilforum* populations to glyphosate. The glyphosate dose that caused 50% growth reduction (GR_{50}) of the S population (98-3) was 101 g ae ha^{-1} glyphosate while R populations ranged from 726 to 1264 g ae ha^{-1} glyphosate (Table 1 and Figure 1). Resistant populations Des05, Des14, D13, D8, and D4 were 7, 8, 9, 13 and 19 times, respectively, less sensitive to glyphosate than the S population based on the R/S ratio calculated from GR_{50} values. In addition, the herbicide dose that caused 50% mortality (LD_{50}) of the 98-3 population was 184 g ae ha^{-1}, whereas those of the R populations ranged from 1524 to 2719 g ae ha^{-1} (Table 1 and Figure 2). D13, Des05, Des14, D8, and D4 populations had 8, 9, 9, 12, and 15-fold nine-fold resistance level relative to the LD_{50} value of the 98-3 population (Table 1). The

GR_{50} and LD_{50} values of D4 and D8 populations are higher than Des05, Des14, and D13 populations. Results indicate that the full rate of glyphosate at 840 g ae ha^{-1} is no longer sufficient to control 50% of the plants in these five R populations. Des13, Des05, Des14, D8 and D4 populations need 1.8, 2.0, 1.9, 2.7 and 3.2 times the normal field rate of glyphosate to kill 50% of the plants in these R populations. These results were lower than what was reported in glyphosate-resistant Des03 *L. perenne* ssp. *multiflorum* population, showing 23-fold level of resistance relative to the S population.[40]

Partial *EPSPS* Gene Sequencing. A 915-bp PCR fragment of the *EPSPS* gene was amplified from the cDNA of the R and S *L. perenne* ssp. *multiflorum* plants. This fragment encompassed amino acid position 77 to 381 in the 444 amino acid-long, mature EPSPS. Although the full-length *EPSPS* gene of Italian ryegrass was not obtained, the sequenced region included the domain where point mutations are known to confer resistance to glyphosate, e.g. at Pro_{106},[22-30] Gly_{96},[41] Pro_{182},[31] Tyr_{31},[31] and Thr_{97}.[42] Some nucleotide polymorphisms were detected, however none of them showed association with glyphosate resistance (data not shown). A mutation of Gly_{162} Ser was detected in one resistant Des14 plant, but this mutation was also detected in a S plant from Des05. Comparison of the *EPSPS* sequence between glyphosate-R and -S plants revealed polymorphisms in both nucleotides and deduced amino acid sequences, but there were no amino acid changes in the known resistance mutation sites that confer glyphosate resistance (data not shown). Therefore, mutations in the *EPSPS* gene known to endow resistance to glyphosate are not present in Des05 and Des14 *L. perenne* ssp. *multiflorum* populations.

The absence of point mutations in the *EPSPS* gene that are exclusive to the R plants and the absence of other mutation previously associated with resistance to glyphosate indicates that target-site alteration is not the resistance mechanism in Des05 and Des14 populations. Target-

site resistance risk is associated with the conservation of the herbicide-binding site.[32] Glyphosate interacts with seven invariant amino acids in the active site of the EPSPS protein[43] and mimics the transition state in the enol transfer reaction.[4] Because the active site of the EPSPS protein is highly conserved, any mutation at this site tends to be deleterious and is likely to cause significant fitness penalty.[44] Single-site mutation at Thr_{97} to Ile or Pro_{101} to Ser [42] or Gly_{96} to Ala[41] in *E. coli* impairs the binding of glyphosate but at the same time reduces affinity for the susbstrate phosphoenolpyruvate. Mutation in the *psbA* gene which confers resistance to triazine herbicide results in reduced agroecological fitness.[45] On the other hand, some mutations endowing target site–based resistance to ACCase or ALS herbicides have little or no fitness costs.[12] Studies comparing glyphosate-resistant goosegrass with Pro_{106}Ser mutation versus susceptible population show some differences, but it is not yet evident whether or not there are any fitness costs associated with this target site EPSPS–based resistance.[46,47] Sammons et al.[32] reported that glyphosate has a very low risk for target-site resistance, thus, it is expected for some glyphosate-resistant populations to display resistance mechanism other than target-site mutation.

Uptake and Translocation of Glyphosate. Glyphosate is a potent herbicide because of its ability to translocate in the plant to the apical meristems, root, and underground reproductive organs of perennial plants.[48] Therefore, it is possible that changes in the translocation pattern of glyphosate could endow resistance in plants. Considering the absence of target-site mutation endowing glyphosate resistance, potential difference in the uptake and translocation of glyphosate between resistant and susceptible populations was investigated. Glyphosate absorption by plants was almost 60% in both S and R populations (Table 2). This result is similar to resistant and susceptible biotypes of *L. perenne* ssp. *multiflorum* from Mississippi[16] but differs

from those in Chile[49] where susceptible and resistant biotypes absorbed >90% of ^{14}C-glyphosate at 48 HAT. ^{14}C glyphosate uptake increased from 24 to 48 HAT. On average 40% and 56% of the applied glyphosate was absorbed by the plants at 24 and 48 HAT, respectively, and this response was not significantly different between R (Des05 and Des14) and S populations ($P > 0.05$).

Radioactivity in the treated leaf represented glyphosate loaded into the leaf, but not translocated in the plant. The quantity of the ^{14}C glyphosate recovered from the treated leaf at 48 HAT was not different between R (65 to 68% of absorbed) and S (71% of absorbed) (Table 2). Translocation of ^{14}C glyphosate into the roots and below the treated leaf ranged from merely 11 to 19%; the radioactivity accumulated above the treated leaf was even lower (1 to 3% of absorbed). The proportion of ^{14}C-glyphosate recovered above the treated leaf, below the treated leaf, and in the roots increased between 24 and 48 HAT in the three populations; however, no significant difference was detected between R and S populations in any plant sections at each harvest time. These results were similar to *Lolium* populations from Australia,[50] California,[51] and Chile[52] where the distribution patterns of ^{14}C-glyphosate did not differ between resistant and susceptible plants. On the contrary, glyphosate-resistant *L. perenne* ssp. *multiflorum* populations from Mississippi,[16] Oregon,[14] and Chile[49] showed reduced translocation of glyphosate. Among Arkansas populations, however, resistance to glyphosate was not due to differences in uptake and translocation of glyphosate.

***EPSPS* Genomic Copy Number Relative To *CCR*.** Genomic *EPSPS* copy numbers relative to ALS ranged from 1 to 2 (n =10) for S plants, whereas the relative *EPSPS* copy numbers for R plants (n = 30) were much higher, varying from 11 to more than 516 (Figure 3). Resistant plants from Des05, Des14, and D8 population had up to 122, 444, and 516 *EPSPS* copies, with a

median of 44, 49, and 102 copies, respectively (Table 3). The relative copies of *EPSPS* from the R plants within a population and among populations are highly variable. For example, relative *EPSPS* copy number of 10 Des05 plants ranged from 11 to 122 with a standard deviation of 31, and coefficient of variation of 67. The increased *EPSPS* copy number in the resistant populations indicates that amplification of the *EPSPS* gene imparts resistance to glyphosate in Des05, Des14, and D8 populations.

Amplification of the native, glyphosate-sensitive form of EPSPS enzymes had conferred resistance to glyphosate in several plant species.[53] Resistance to glyphosate in alfalfa, soybean, and tobacco from progressive selection in plant cell cultures is attributed to amplification of the *EPSPS* gene within the genome.[54] In addition, a glyphosate-tolerant wild carrot cell line selected by stepwise glyphosate selection contained a 4- to 25-fold increase in *EPSPS*.[55] Similarly, a petunia cell line contained a 20-fold increase in the copies of *EPSPS* gene.[56] Amplification of the *EPSPS* gene in *Amaranthus palmeri* from Georgia was recently reported by Gaines et al.[35] in which genomes of glyphosate-resistant plants contained from 5-fold to more than 160-fold more copies of the *EPSPS* gene resulting to 40-fold EPSPS overexpression. The level of resistance to glyphosate in this *A. palmeri* population was 6- to 8-fold at the population level.[57] Although the EPSPS enzyme activity was not investigated in this study, there are various studies indicating that *EPSPS* gene amplification results in increased EPSPS enzyme activity in glyphosate-resistant plants.[53-55] Gene amplification can produce abundant supply of EPSPS enzymes that are able to counteract the loss of metabolic function of enzyme molecules that are inhibited by glyphosate.[58] This affords the plant continued synthesis of aromatic acids for normal physiological function and development in the presence of glyphosate.

The degree of *EPSPS* gene amplification differed greatly among the resistant plants indicating high intrapopulation genetic variability. *Lolium perenne* ssp. *multiflorum* is an outcrossing species;[59] thus a high degree of genetic diversity would be expected within a population. A broad range of *EPSPS* copy numbers was detected in glyphosate-resistant *A. palmeri* which is also an obligate outcrossing species.[60] The observation that populations with higher resistance levels to glyphosate had higher copies of *EPSPS* suggests that additional *EPSPS* gene copies have additive effects in conferring glyphosate resistance.[35] Because the level of glyphosate resistance was obtained at the population level and *EPSPS* copies vary widely among resistant plants within a population, the relationship between *EPSPS* copies and glyphosate resistance level requires additional research. Evaluation of the resistance level of the same plants analyzed for *EPSPS* copy number is currently in progress.

Given the lethal consequence of mutations in the binding site of the *EPSPS* gene, the selected glyphosate-resistant plants harbor other mechanisms of survival, such as *EPSPS* gene amplification. Gene duplication is usually triggered by environmental stresses.[61] Selection pressure imposed by environmental stress, in this case intense glyphosate usage, could potentially favor survival of plants with multiple copies of the glyphosate target gene. It is also possible that *EPSPS* gene amplification results from mutation(s) in the promoter region which could potentially elevate gene transcription.[62]

Gene amplification of *EPSPS* provides a certain level of glyphosate resistance in plants;[53] however the stability of *EPSPS* gene amplification is not yet clearly understood. *EPSPS* gene amplification in *A. palmeri* is heritable[35] but the manner by which it is inherited is unknown. Studies on plant cell culture revealed that gene amplification is often not genetically stable or heritable.[53] In the absence of glyphosate selection pressure, resistance is often reduced,

suggesting a fitness penalty for cells containing amplified genes.[53] High levels of EPSPS produced by gene amplification could have a fitness cost. Other than endowing resistance to glyphosate, no physiological advantage has been documented thus far as a consequence of *EPSPS* gene amplification.

In conclusion, the resistance to glyphosate in Des05, Des14, and D8 *L. perenne* ssp. *multiflorum* populations is conferred by amplification of the *EPSPS* gene. A broad range of *EPSPS* genomic copy numbers was observed among resistant plants. The mechanism of *EPSPS* gene amplification and the nature of its heritability are not yet known. Information on the mechanism of amplification, stability and genetic inheritance of copy number, and fitness penalty that may be associated with *EPSPS* gene amplification is necessary to fully understand the novel mechanism of glyphosate resistance due to *EPSPS* gene amplification.

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Table 1. GR_{50} and LD_{50} values of glyphosate-resistant and -susceptible *L. perenne* ssp. *multiflorum* populations.

Population	GR_{50}	R/S[a]	LD_{50}	R/S[b]
Des05[c]	726 (629, 823)[d]	7	1702 (1419, 1986)[d]	9
Des14[c]	831 (771, 892)	8	1587 (1385, 1788)	9
D4[c]	1908 (1485, 2332)	19	2719 (2107, 3329)	15
D8[c]	1264 (1031, 1496)	13	2245 (1916, 2575)	12
D13[c]	917 (795, 1039)	9	1524 (1200, 1847)	8
98-3[e]	101 (91, 111)	-	184 (161, 207)	-

[a]Resistance level (R/S) calculated by GR_{50} of the resistant population relative to the susceptible population.
[b]Resistance level (R/S) calculated by LD_{50} of the resistant population relative to the susceptible population.
[c]Glyphosate-resistant population.
[d]Lower 95%, Upper 95%
[e]Glyphosate-susceptible population

Table 2. ^{14}C glyphosate absorption and distribution in various plant tissues of resistant and susceptible *Lolium perenne* ssp. *multiflorum* populations from Arkansas, USA.

Population	^{14}C-glyphosate absorption[a]		^{14}C-glyphosate distribution[a]							
			Treated leaf		Above treated leaf		Below treated leaf		Roots	
	24 HAT[b]	48 HAT	24 HAT	48 HAT	24 HAT	48 HAT	24 HAT	48 HAT	24 HAT	48 HAT
	% of applied		---------- % of absorbed ----------							
Des05[c]	38	51	80	65	1	2	11	17	8	15
Des14[c]	44	59	79	68	1	1	12	19	8	12
98-3[d]	37	57	77	71	1	3	14	16	8	11

[a] Values are the average of four plants.
[b] HAT, hours after treatment.
[c] Resistant population.
[d] Susceptible population.

Table 9. Summary statistics of the relative *EPSPS* copy number in the glyphosate-resistant *L. perenne* ssp. *multiflorum* populations.

Population	*EPSPS:CCR* copy number				
	Median	Standard deviation	Coefficient of variation	Minimum	Maximum
Des05	44	31	67	11	122
Des14	50	127	144	24	444
D8	102	190	96	19	516
98-3	1	0.4	18	1	2

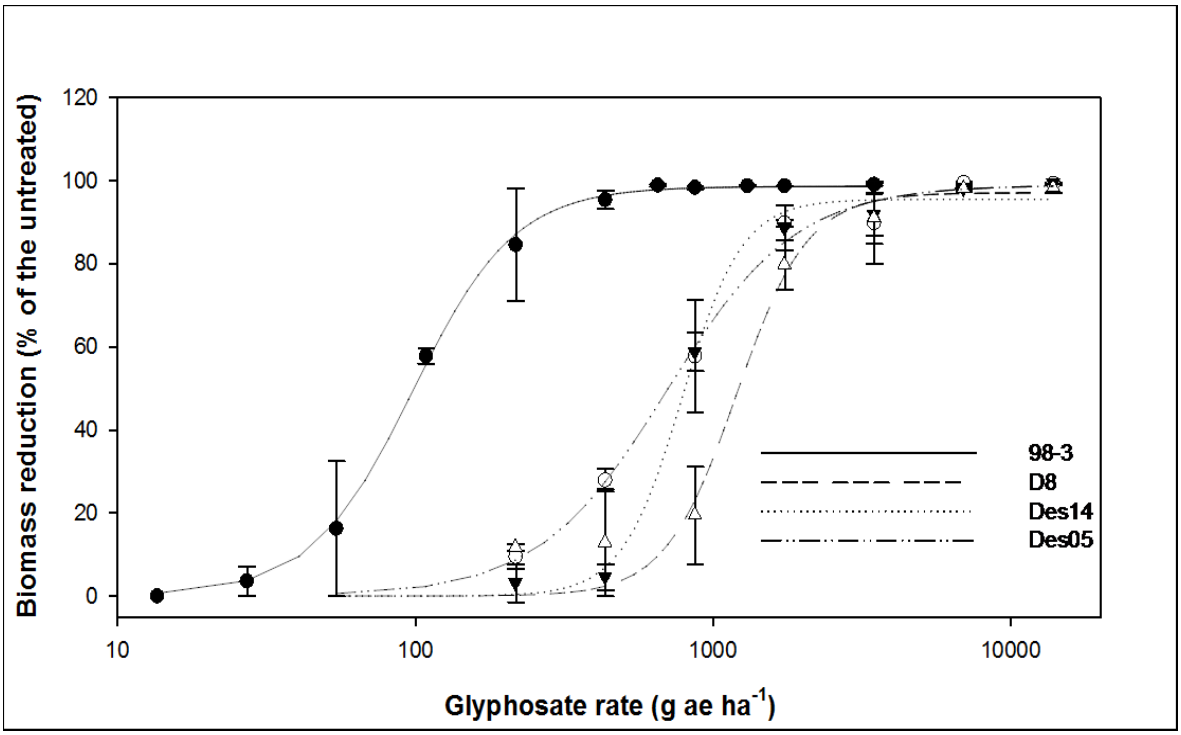

Figure 1. Shoot biomass reduction of selected *L. perenne* ssp. *multiflorum* populations, 28 d after treatment. Error bars are standard error bars of the mean. Des05, Des14, and D8 had an estimated 50% biomass reduction (GR_{50}) value of 726, 831, and 1264 g ae ha^{-1} glyphosate. Susceptible 98-3 population had an estimated GR_{50} of 101 g ae ha^{-1} glyphosate.

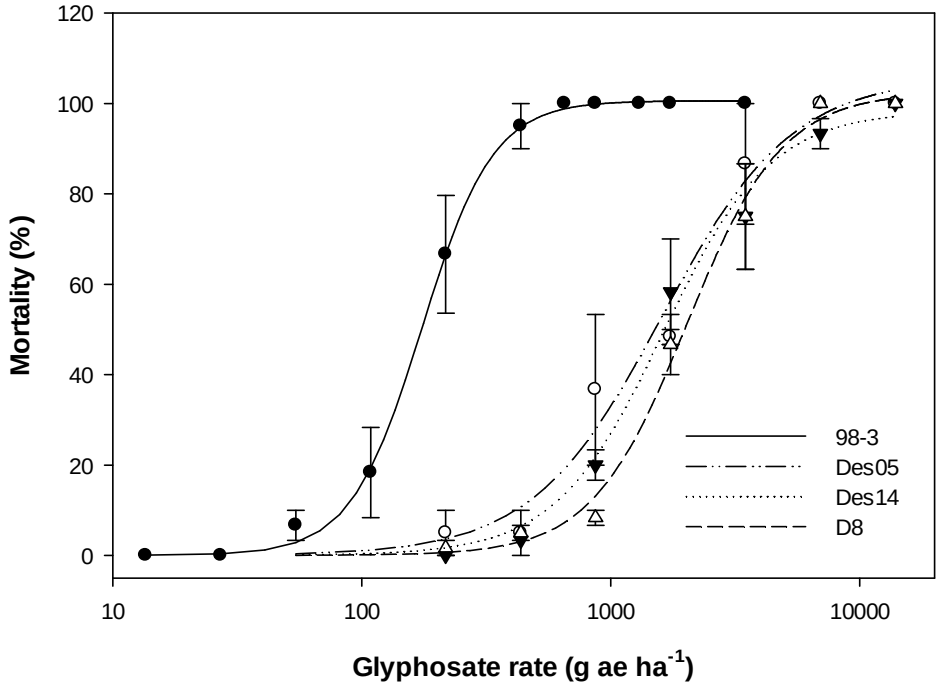

Figure 2. Mortality evaluation of selected glyphosate-resistant and -susceptible *L. perenne* ssp. *multiflorum* populations, 21 d after treatment. Error bars are standard errors of the mean. Des05, Des14, and D8 populations had an estimated 50% mortality of 1702, 1587, and 2245 g ae ha^{-1} glyphosate. The susceptible 98-3 population had an estimated 50% mortality of 184 g ae ha^{-1} glyphosate.

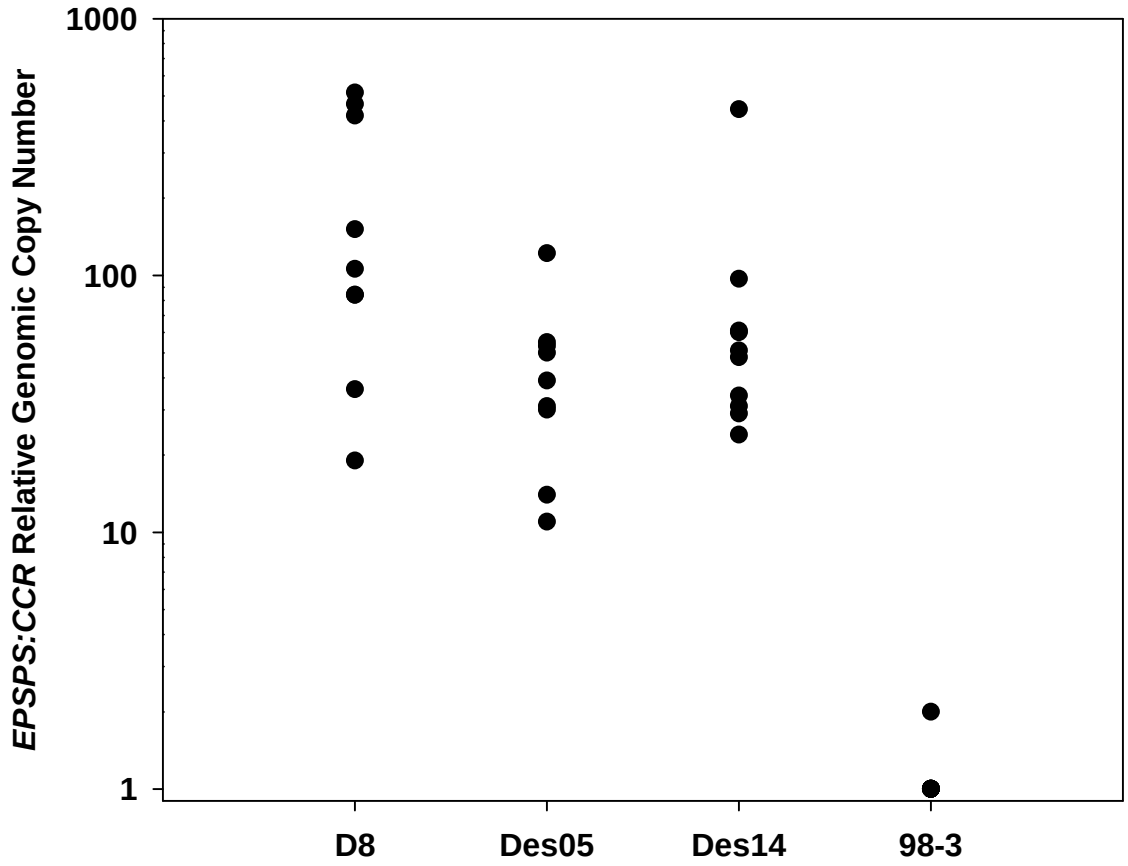

Figure 3. *EPSPS* relative genomic copy number of in glyphosate-resistant and -susceptible *L. perenne* ssp. *multiflorum* plants. Relative copy number of *EPSPS* in resistant populations (D8, Des05, and Des14) ranged from 11 to 516 (n=30), whereas the susceptible population (98-3) contained up to 2 copies (n=10).

CONCLUSIONS

Italian ryegrass populations in the the southern United States have evolved resistance to several ACCase- and ALS-inhibiting herbicides, and even to glyphosate. Different patterns of cross-resistance to ALS-inhibitors and multiple resistance to ALS- and ACCase-inhibiting herbicides were observed. Among the 30 Italian ryegrass accessions collected between 2008 and 2010, 27 were resistant to both diclofop and mesosulfuron, 25 of which displayed resistance to pyroxsulam. Although most diclofop-resistanct accessions can be controlled by pinoxaden; growers should be cautious because some ryegrass populations already exhibit resistance to pinoxaden. There is evidence that P450-mediated enhanced herbicide metabolism is partially responsible for resistance to diclofop, mesosulfuron, and pyroxsulam in 09-NC-04 accession, and to mesosulfuron and pinoxaden in 09-NC-03 accession. Amplification of the *EPSPS* gene confers resistance to glyphosate in Des03, Des05, Des14, and D8 Italian ryegrass populations. Resistance to multiple herbicides and the occurrence of complex herbicide resistance mechanism in Italian ryegrass populations limit herbicide options and complicate ryegrass control in wheat fields. This emphasize the need for diversified, integrated weed management in order to reduce reliance on herbicides and to delay, if not prevent, the evolution of herbicide-resistance weeds.