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THE STUDY OF GLYPHOSATE RESISTANCE IN RIGID RYEGRASS (*Lolium rigidum* Gaud.) FROM CALIFORNIA

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THE STUDY OF GLYPHOSATE RESISTANCE IN RIGID RYEGRASS (*Lolium rigidum* Gaud.) FROM CALIFORNIA

By

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ABSTRACT

THE STUDY OF GLYPHOSATE RESISTANCE IN RIGID RYEGRASS (*Lolium rigidum* Gaud.) FROM CALIFORNIA

By

Marulak Simarmata

Glyphosate resistance was observed in a rigid ryegrass (*Lolium rigidum* Gaud.) population in an orchard, near Chico, California in 1998. The resistant plants were collected and maintained under greenhouse condition. The objectives of this study were (i) to evaluate the resistance to glyphosate and determine the inheritance of the resistant trait, (ii) to study the potential basis of glyphosate resistance including absorption, translocation, intercellular movement of ^{14}C-glyphosate into the chloroplast, assay and bioassay of shikimic acid accumulation, and (iii) to determine physiological basis including metabolism of glyphosate and an altered target site, 5-enolpyruvylshikimate-3-phosphate synthase, EPSPS (EC 2.5.1.19) for glyphosate resistance in rigid ryegrass from California. The first generation from the collected ryegrass showed diverse sensitivity indicated with 11, 32, 14, 36, and 9 percent of dead plants after treatment with glyphosate 1x, 2x, 4x, 8x, and higher than 8x, respectively; where x = 1.12 kg ai ha^{-1} isopropylamine salt of glyphosate. Based on the magnitude of the glyphosate resistance, the first generation was separated into three groups used as the lines in the further selection. The sensitive (S) group died from (1x), the resistant (R) group survived (1x + 2x + 3x), and the intermediate (I) group responded between R and S groups. These groups respectively were 5, 36, and 59 percent of the 350 plants population. Recurrent selections with the lower and higher rates of glyphosate were continued through several generations of

the S and R lines, respectively. The R biotype that survived 8x of glyphosate was uniform by the 7th generation, whereas the S biotype died from 1/8x of glyphosate and was uniform at the 5th generation. The F_1-hybrids from reciprocal crosses between R and S biotypes were phenotypically homogeneous with respect to glyphosate sensitivity, these survived up to 2x glyphosate. Chi-square analysis of the F_2 population indicated that the inheritance of glyphosate resistance involved more than one gene ($0.75 < P < 0.90$). Foliar absorption and distribution of ^{14}C-glyphosate were not different between the S and R biotypes 1 to 3 days after treatment (DAT). Intercellular movement of ^{14}C-glyphosate into chloroplasts, *invitro* and *invivo*, could not distinguish between S and R biotypes. Compared to the R biotype, the S biotype showed more than a 10-fold increase in shikimic acid 11 DAT with glyphosate. Shikimic acid at 2 to 5 mM did not affect seed germination of either the S or R biotypes, but the length of coleoptiles in the S biotype decreased significantly 5 DAT. Metabolism was not considered as a basis for glyphosate resistance in California rigid ryegrass since the pattern of TLC-images of ^{14}C from extracted plants was not different between the R and S biotypes and the two were similar to ^{14}C-glyphosate. More than 95 percent of extracted ^{14}C was distributed in the Rf region analyzed. Glyphosate sensitivity of EPSPS extracted from the crown region of R and S plants differed significantly. The activity of EPSPS from R plants was not affected, slightly decreased, and decreased to 25 percent, whereas in S biotypes the activity decreased to 60, 50, 40, and remained at only 9 percent with glyphosate at 0.05, 0.5, 5 and 50 mM, respectively. Decreased EPSPS sensitivity in R compared to S biotypes appeared to be a major contributor to glyphosate resistance in rigid ryegrass.

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INTRODUCTION

Weed management is a major factor that contributes to the success of crop production. Controlling weed populations below the economic threshold can optimize the yield of crops. The predominant method of weed management is chemical control by using herbicides. In the past 50 years, the Weed Science Society of America has listed at least 140 herbicides (Vencil, 2002)

Glyphosate, *N-(phosphonomethyl)glycine,* is a systemic, non-selective, and foliar applied herbicide (Baird et al. 1971; Franz, 1985; and Vencil, 2002). Known by various trade names (such as Roundup®, Touchdown®, and Rodeo®, etc.) glyphosate is the most widely used herbicide accounting for about 11 percent of herbicide sales worldwide (Magin, 2003; Powles et al. 1997). The development of glyphosate-resistant crops in the last few years, such as Roundup Ready® corn, soybean, canola, and cotton, has increased the amount of glyphosate used (Duke et al. 1991; Padgette et al. 1996; and Magin, 2003).

When glyphosate is foliar applied to plants, it is rapidly absorbed across the cuticle of the leaves (Sandberg et al. 1980; and Sprankle et al. 1975). Subsequently, glyphosate is translocated in the symplast to the roots, rhizomes, and apical tissues of treated plants. Glyphosate is not metabolized inside the plants when applied at a phytotoxic rate (Malik et al. 1989; and Vencil, 2002), however possible metabolism into

aminomethylphosphonic acid (AMPA) was reported in some crops (Rueppel et al. 1987) and some weed species (Sandberg et al. 1980).

Glyphosate has unique properties and it is the only herbicide reported to inhibit 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in shikimate pathway (Baird et al. 1971; Bradshaw et al. 1977; Rubin et al. 1984; and Steinrucken and Amrhein, 1980). Inhibition of EPSPS will stop the biosynthesis of three aromatic amino acids: phenylalanine, tyrosine, and tryptophan (Steinrucken and Amrhein, 1980).

In the environment, glyphosate is tightly bound to soil particles, thus, this herbicide does not leach into ground water (Vencil, 2002). It also has a low persistence and easily degradable in water and soil (Rueppel et al. 1977; and Wauchope et al. 1992). Microorganisms are able to degrade glyphosate into two general ways, either to glycine or AMPA as intermediate products (Rueppel et al. 1977; and Torstensson, 1985)

Since the introduction of glyphosate 30 years ago, it has often been used for controlling weeds without rotating with other weed control measures (Kishore et al. 1992; and Magin, 2003). In the classic theory, these circumstances will lead to a rapid development of glyphosate resistant weeds (Bradshaw et al. 1997; Dyer, 1994). The appearance of herbicide resistant weeds is not due to a mutation caused by herbicides, rather, it arises from selection of natural mutations or small preexisting populations of resistant plants (Duke et al. 1991; Heap and LeBaron, 2001; Kishore et al. 1992; Powles et al. 1977). Weed resistance had evolved for herbicides in several chemical families including triazines, aryloxyphenoxypropionates, cyclohexanedinones, bipyridiliums, imidazolinones, dinitroanilines, triazoles, nitriles, substitute ureas,

phenoxys, sulfonyl ureas, and glyphosate (Bradshaw et al. 1997; Heap, 2004; Heap and LeBaron, 2001).

Recently, glyphosate resistant weeds have been reported in many parts of the world. Heap (2004) in the web site of weed science has documented six weed species that are resistant to glyphosate including horseweed (*Conyza Canadensis*), hairy fleabane (*Conyza bonariensis*), goosegrass (*Eleusine indica*), rigid ryegrass (*Lolium rigidum*), Italian ryegrass (*Lolium multiflorum*), and buckhorn plantain (*Plantago lanceolata*).

Rigid ryegrass is a widely established grass weed in Australia that has developed resistance to numerous herbicides. The continual use of glyphosate has led to development of resistant biotypes in the rigid ryegrass populations (Powles et al. 1998; Pratley et al. 1996; Pratley et al. 1999; Preston et al. 1999). A similar phenomenon has been reported in rigid ryegrass populations in California. For 15 consecutive years, fruit growers preferred to increase the doses and frequencies of application of glyphosate to get an adequate weed control. These circumstances lead to excessive growth and development of resistant rigid ryegrass populations (J. E. Kaufmann, personal communication).

In 1998, plant samples of resistant rigid ryegrass were collected from orchards near Chico, California. The plants have been grown and seed produced in greenhouse at Michigan State University (Simarmata et al. 2001). Even though the glyphosate resistance in rigid ryegrass has been reported since 1996, the mechanism and the inheritance of glyphosate resistance were not determined.

Research has been conducted to study glyphosate resistance in California rigid ryegrass. This dissertation is divided into three major chapters and the objectives of the

respective chapters are (i) to evaluate the glyphosate resistance in rigid ryegrass from California and to determine the inheritance of the glyphosate resistant trait, (ii) to investigate the potential basis of glyphosate resistance, and (iii) to investigate the physiological basis of glyphosate resistance in rigid ryegrass.

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CHAPTER 1

LITERATURE REVIEW

Review of Glyphosate

Glyphosate was patented by Monsanto as a herbicide (Franz, 1985), and first introduced as a commercial herbicide in 1974 in UK for wheat, in Malaysia for rubber, and in the United States for industrial or non-crop use (Magin, 2003). Since then, glyphosate has been sold around the world with more than 100 trade names such as Roundup@, Touchdown@, Accord®, etc. (Vencil, 2002).

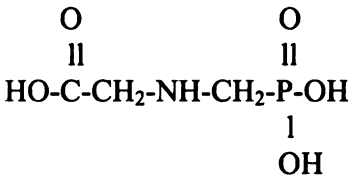

Fig 1. Chemical structure of glyphosate, *N-(phosphonomethyl)glycine* (Vencil. 2003)

Glyphosate is a systemic herbicide and has a broad-spectrum of weed control (Baird et al. 1971). Glyphosate can be used for various purposes such as controlling perennial weeds in orchards, lawn, forestry, nursery, railroads, etc, for land preparation before planting as a conservation technique, and for weed control on Roundup Ready® crops (Vencil, 2002).

Glyphosate is active only when applied post-emergence to the leaves of plants (Vencil, 2002; Baird et al. 1971). An adjuvant or non-ionic surfactant (NIS) and ammonium sulfate (AMS) is required to enhance glyphosate efficacy (O'Sullivan et al. 1981; Sherrick et al. 1986; Thelen et al. 1995). Some formulated products include the surfactant (Vencil, 2002). After reaching the leaf surface, glyphosate is absorbed across the cuticle (Baird et al. 1971). Subsequently, it is translocated in the symplast into meristematic regions, immature leaves, and underground tissue of the plants (Sandberg, et al. 1980). Adjuvants could modify leaf absorption, translocation and herbicidal activity of glyphosate (O'Sullivan et al. 1981; Sherrick et al. 1986) and ammonium sulfate (AMS) can overcome the hard water antagonism in the herbicide solution (Buhler and Burnside. 1983; Thelen et al. 2003).

When glyphosate reaches the site of action, it binds to 5-enolpyruvylshikimate -3-phosphate synthase (EPSPS), a key enzyme in the biosynthesis of EPSP from the condensation reaction of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Rubin et al. 1984 ; Steinrucken and Amrhein, 1980). Inhibition of this enzyme stops biosynthesis of three aromatic amino acids (trypthophan, tyrosine and phenylalanine). Lack of these amino acids within plants causes severe injury and plant death 2-3 weeks after glyphosate application (Vencil, 2002). The accumulation of shikimic acid within plant tissues after glyphosate application can theoretically confirm that glyphosate interferes with EPSPS in the shikimic acid pathway (Singh and Shaner, 1998).

Within plants, glyphosate is not metabolized when applied at phytotoxic rates but remains in its original form (Malik et al. 1989; Vencil, 2002). However, possible metabolism into aminomethyl phosphonic acid (AMPA) was reported in some weed

species, such as field bindweed, Canada thistle, and tall morningglory (Sandberg et al. 1980) and some crop species, such as soybean, maize, and cotton (Rueppel et al. 1987).

When glyphosate reaches the soil, it is tightly adsorbed to soil particles and does not leach in to the ground water (Wauchope et al. 1992). Glyphosate is bound to the charged clay particles an organic matter in the soil and has not demonstrated residual herbicidal activity (Smith and Oehme, 1992). This herbicide has a moderate persistence with typical half-life less than 47 days, and less than 25 days in laboratory experiments (Vencil, 2002). Glyphosate is easily degraded into glycine or AMPA through photo- and chemical-degradation (Wauchope, 1992) and by micro-organisms (Torstensson, 1985; Rueppel et al. 1977).

Glyphosate is not toxic to animals because there is no shikimate pathway within the animals. The oral LD_{50} of isopropylamine salt of glyphosate on rats (lethal doses that kill 50 % of rats) are > 5000 mg/kg (Vencil et al. 2002).

Glyphosate is a most reliable and relatively non-expensive herbicide for weed control (Magin, 2003). Glyphosate was initially used in developed nations and more recently in developing nations. A recent report showed that glyphosate is the 2nd in herbicide sales after the triazine herbicide group and sold in at least 119 countries (Heap, 1999). In developed countries, glyphosate is a major herbicide used for land preparation to minimize cultivation. Recently, it is used as a post-emergence herbicide in glyphosate resistant crops such as Roundup Ready $^{@}$corn, soybean, canola, cotton, etc (Magin, 2003 and Padgette et al. 1996). In developing countries glyphosate is combined with mechanical tillage to make land preparation easier and faster (Heap, 1999).

Glyphosate Resistant Weeds

Herbicide resistant weeds are a relatively new phenomenon compared to the pesticide resistance that has evolved in insects, fungi, and bacteria (Bradshaw et al. 1997). Herbicide resistance is defined as the capacity of weed population to withstand the herbicide and complete its life cycle when the herbicide is used at its normal rate (Heap and LeBaron, 2001). If weed populations survive several herbicides that have the same mechanism of action, this is known as cross-resistance; and if weeds survive many herbicides with different mode of actions, this is defined as a multiple resistance (Heap, 1999).

Herbicide resistant weeds evolve after selection pressure on a susceptible weed population (Heap and LeBaron, 2001). Resistance is not due to a mutation caused by herbicides; rather, it arises from selection of natural mutations or small preexisting populations of resistant plants (Duke et al. 1991). Many factors contribute to the rate of herbicide resistance in a population (Powles et al. 1977). These include the initial frequency of herbicide resistant individuals, the number of individual treated, the mode of inheritance of the gene or gene endowing resistance, and the nature of extent of herbicide use. Most cases of field selected herbicide resistance are due to the action of a single gene with a high degree of dominance. The degree of dominance of resistance genes is particularly important for out crossing weed species because fully recessive genes will tend to be diluted into heterozygous individuals by the larger number of susceptible alleles (Powles et al. 1997).

Some of the herbicides that have been used persistently over large areas of a weed population and lead to the development of resistant weeds are triazines, bipyridiliums,

aryloxyphenoxypropionates, cyclohexanedinones, imidazolinones, dinitroanilines, triazoles, nitriles, substitute ureas, phenoxys, sulfonyl ureas, and glyphosate (Bradshaw et al. 1997; Heap, 1999; LeBaron and Gressel, 1982). A current report from the international survey of resistant weeds indicates that herbicide resistant weeds occurred in most herbicide chemical classes (Heap, 1999). The weed-science website (Heap, 2004) shows 171 different weed species around the world in which herbicide resistant biotypes have been found.

After extensive use of glyphosate for more than 30 years, the weed-science website currently documents (Heap, 2004) at least six weed species that have biotypes resistant to glyphosate. These are horseweed (*Conyza canadensis*), hairy fleabane (*Conyza bonariensis*), goosegrass (*Eleusine indica*), rigid ryegrass (*Lolium rigidum*), Italian ryegrass (*Lolium multiflorum*), and buckhorn plantain (*Plantago lanceolata*).

Glyphosate resistant rigid ryegrass was found in several agricultural sites in Australia following more than 15 years of glyphosate application (Powles et al. 1998; Pratley et al. 1996; Preston et al. 1999). The same phenomenon appeared in a rigid ryegrass population in almond orchards, near Chico, California (Simarmata et al. 2001). This has been documented by Tomaso and Lanini in 1998 and reported on the weed-science website (Heap, 2004).

A glyphosate resistant population of goosegrass was found in Teluk Intan, Perak, Malaysia (Lee et al. 2000; Tran et al. 1999). Fruit growers in Malaysia choose to apply glyphosate 5 to 7 times in a year as this was cheaper than other weed control methods (Lee et al. 2000). A recent report from Malaysia indicates that in less than 4 years after

using glyphosate, fruit growers faced the problem of controlling glyphosate resistant goosegrass (Lee and Ngim, 2000; and Tran et al. 1999).

Horseweed, a dicotylodenous plant, has also developed resistance to glyphosate. The first case was observed in a field planted with glyphosate-resistant soybean for 3 consecutive years in Delaware, USA. Seedlings from the glyphosate resistant plants showed 8- to 13-fold resistance (VanGessel, 2001). Recently, resistant biotypes of this weed were reported in Tennessee, Indiana, Maryland, New Jersey, Ohio, Arkansas, Mississippi, and North Carolina (WSSA, 2004).

Hairy fleabane (*Conyza bonariensis*), and buckhorn plantain (*Plantago lanceolata*) are dicotyledonous weeds reported resistant to glyphosate in South Africa, and Italian ryegrass (*Lolium multiflorum*) has also been reported being glyphosate resistant in Chile (WSSA, 2004)

Biology of Rigid Ryegrass

Rigid ryegrass (*Lolium rigidum*) is an annual herb that was widely used as a pasture crops in northern and southern Australia (Powles et al.1998; Pratley et al. 1996; and Preston et al. 1999). Rigid ryegrass, also known as "wimmera ryegrass", is one of the *Lolium* species in the family Poaceae (Terrel, 1968). Rigid ryegrass is a serious weed in the field of crops in Australia (Preston et al. 1999).

All the *Lolium* species including rigid ryegrass are diploid with 14 chromosome number (Evans, 1926 *In* Thorogood, 2003; and Terrel, 1968). Rigid ryegrass is an allogamus plant and naturally out-crossed because of self-incompatible pollination. It is predicted that self-pollination seeds is less than one percent (Charmet et al. 1997). The

origin of rigid ryegrass was proposed to be from European countries include Corsica, Italy, and Tunisia (Clayton and Revoize, 1986; Thorogood, 2003).

Rigid ryegrass in California, USA, was introduced from elsewhere and naturalized as a forage crop, cover crop, for revegetation of disturbed areas, and wildfire burn rehabilitation (Sawyer et al. 1997; Hickman, 1993; and Skinner and Pavlik, 1994). After natural selection through 13 generations at Pleasanton, California, the wimmera ryegrass was first released in 1962 (PI No-11419) by California AES, Davis, CA and Plant Material Center, SCS, CA. (Hickman, 1993; and Skinner and Pavlik, 1994). Whether or not the glyphosate resistant rigid ryegrass observed in California is a descendant of the original introduction has not been established (Simarmata, 2001). The current distribution of rigid ryegrass in USA includes Oregon, California, Arizona, Texas, Louisiana, and Missouri (WSSA, 2004).

The morphological description of wimmera ryegrass is: awnless, deep bright green, erect, early maturing, uniform in appearance, leafy, and numerous culms annual. This grass tends to lodge the first year in brush-burn seeding habitats (Hubbard, 1984). These characteristics are useful as self-perpetuating cover crop in irrigated orchards and vineyards. Rigid ryegrass has no advantage in areas of high humidity, on fertile soils where rainfall exceeds 300 mm annually, or above 610 m in elevation (Hickman, 1993; Sawyer et al. 1997; and Skinner and Pavlik, 1994)

Basis of Glyphosate Resistance

Although glyphosate resistance in rigid ryegrass was reported in 1996, the basis of the resistance is still not well understood. Theoretical mechanisms of herbicide

resistance in plants are differences in herbicide absorption, translocation or movement of glyphosate to the site of action, plant defense system or metabolism process that may degrade the herbicide to inactive forms inside the plant system, and altered site of action, or interactions of these mechanisms (Bradshaw et al. 1997; Dyer, 1994).

The mechanism of glyphosate resistant in Roundup Ready® crops was well understood because the glyphosate resistant trait was introduced into the crops from the EPSPS gene from bacteria (Duke et al. 1991; and Kishore et al. 1992).

Three genetic transformation mechanisms have been employed to confer resistance (Bradshaw et al. 1997). They are (i) overproduction of EPSPS (target-site amplification mechanism) (Shah et al. 1986); (ii) introduction of EPSPS with decreased affinity for glyphosate (target-site modification mechanism) (Padgette et al. 1996; Padgette et al.1991); and (iii) introduction of a glyphosate degradation gene (metabolic inactivation mechanism (Barry et al. 1992). Over expression of a glyphosate sensitive EPSPS conferred glyphosate resistance, but the level was not sufficient to withstand commercial glyphosate doses (Shah et al. 1986). To obtain the higher levels of resistance, target site modification and or metabolic inactivation mechanism were utilized (Bradshaw et al. 1997).

Studies on the basis for glyphosate resistance in weeds were started when herbicide resistant weeds were found in the plant population. Two of the glyphosate resistant weed species that have been investigated to date are rigid ryegrass and goosegrass. There was similarity in the first step of these studies. All of the published reports documented the accumulation of shikimic acid in the sensitive plants after

treatment with glyphosate (Baerson et al. 2001a & b; Lorraine-Colwill et al, 1999; and Simarmata et al. 2003).

Accumulation of shikimic acid, an intermediate in the shikimate pathway, was observed in the leaf tissue of sensitive (S) plants after the application of glyphosate (Baerson et al 2002a, Lorraine-Colwill et al. 1999, Simarmata, et al. 2003). However, this observation could not explain the basis of glyphosate resistance in rigid ryegrass (Singh and Shaner, 1998). The shikimic acid accumulation in the plant tissues only theoretically confirmed that glyphosate interfered with EPSPS. The shikimic acid assay had been used as a rapid tool to identify glyphosate resistance in plants (Harring et al. 1998, Pline et al. 2002).

Published reports indicated that there is no significant difference in glyphosate absorption, translocation, or metabolism in rigid ryegrass (Feng et al. 999; Lorraine-Colwill et al. 1999; Simarmata et al. 2003).

Lorraine-Colwill et al. (1999) proposed that there might be possible differences in cellular translocation of glyphosate to the chloroplast as the site of glyphosate action. They proposed the possibility that the amount of glyphosate reaching the chloroplast of resistant biotypes was not enough to inhibit the EPSPS and amino acid biosynthesis continued. In a recent publication, Lorraine-Colwill et al. (2003) reported differences in the glyphosate translocation pattern in resistant and sensitive plants. In the resistant biotypes, glyphosate accumulated in the tip of the leaves, whereas in the susceptible biotypes glyphosate accumulated in the roots. This has been considered as one possible mechanism of glyphosate resistance in rigid ryegrass from Australia (Lorraine-Colwill et al., 2003).

However, Simarmata et al. (2003) also recently reported that absorption and translocation, including the amount of glyphosate transported into chloroplast could not distinguish between the resistant and sensitive biotypes of rigid ryegrass collected from California.

The molecular basis for glyphosate resistance in rigid ryegrass had been investigated (Baerson et al. 2002a; Lorraine-Corwill et al. 1999). Alteration in the gene sequence encoding EPSPS in the resistant plants was not observed. Baerson et al. (2002a) proposed that the basis of glyphosate resistance in the Australian population was a non-target mechanism.

A target site mechanism had been elucidated in goosegrass (*Eleucine indica*) from Malaysia (Baerson et al. 2002b). A mutation in the gene encoding EPSP synthase was identified in the resistant biotypes (Baerson et al. 2002b; Ng et al. 2003). A single nucleotide substitution at the sequence number 106 occurred within the R glyphosate binding site that changed an amino acid code from *proline* to *serine* (Baerson et al. 2002b) or to *threonine* (Ng et al. 2003). This mutation is a homologue to the CP4 gene that was introduced into crops (Baerson et al. 2002b; Bradshaw et al. 1997; Padgette et al. 1996; and Padgette et al. 1991).

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CHAPTER 2

INHERITANCE OF GLYPHOSATE RESISTANCE IN RIGID RYEGRASS (*Lolium rigidum* Gaud.) FROM CALIFORNIA

ABSTRACT

Glyphosate resistance was observed in a rigid ryegrass (*Lolium rigidum* Gaud.) population in California in 1998. The resistant plants were collected and maintained for seed production under greenhouse condition. The objectives of this study were to evaluate the resistance and determine the magnitude of glyphosate resistance in California rigid ryegrass, to separate the resistant and sensitive biotypes by recurrent selection, and to determine the inheritance of glyphosate resistance in rigid ryegrass. The first generation from the collected ryegrass showed diverse sensitivity indicated with 11, 32, 14, 36, and 9 percent dead plants after treatment with glyphosate 1x, 2x, 4x, 8x, and higher than 8x, respectively; where x = 1.12 kg ai ha^{-1} isopropylamine salt of glyphosate. Based on the magnitude of the glyphosate resistance, the first generation of collected rigid ryegrass was separated into three groups used as the lines in the selection. The sensitive (S) group died from 1x glyphosate, the resistant (R) group survived 3x to 5x, and the intermediate (I) group responded between R and S groups. These groups respectively were 5, 36, and 59 percent of the 350 plants population. Recurrent selections with the lower and higher rates of glyphosate were continued through several generations of S and R lines, respectively. By the 7^{th} cycle of selection the R line was uniform survived 8x glyphosate, whereas in the 5^{th} generation the S line was uniform sensitive to 0.125x. These R and S lines were successfully crossed in greenhouse. The

F_1 population was phenotypically homogeneous in response to glyphosate and survived up to the 2x rate of glyphosate. Chi-square analysis of the F_2 population indicated that the inheritance of glyphosate resistance involved more than one gene.

Nomenclature: Glyphoste; rigid ryegrass, *Lolium rigidum* Gaud. LOLRI

Key words: Glyphosate resistance, inheritance

INTRODUCTION

After glyphosate use for more than 30 years, resistant weeds appeared in some countries (Heap and LeBaron, 2001). In 1998, glyphosate resistant rigid ryegrass was observed and collected in California (Simarmata et al. 2001). Powles et al. (1998) and Pratley et al. (1996) reported glyphosate resistance in rigid ryegrass in Australia. Other reported glyphosate resistant weeds are goosegrass (*Eleusine indica*) in Malaysia (Lee and Ngim, 2000; Tran et al. 1999); horseweed (*Conyza Canadensis*) in Delaware, USA (VanGessel, 2001); Italian ryegrass (*Lolium multiflorum*) in Chile (Perez and Kogan, 2003); hairy fleabane (*Conyza bonariensis*) and buckhorn plantain (*Plantago lanceolata*) in South Africa (Heap, 2004).

Rigid ryegrass found in California was most likely introduced from the Wimmera-Mallee area of Victoria, Australia, where this grass was commonly naturalized (Anderson and Sharp, 1995; Sawyer and Keeler-Wolf, 1997; and Skinner and Pavlik, 1994). Rigid ryegrass was released as a Wimmera 62 cultivar (accession number P-11419) after selection for 13 generations by the Plant Material Center, SCS, Pleasanton, CA (Anderson et al.1995; Hickman, 1993). It was used for grazing, turf, hay, and other uses such as soil preservation (Anderson et al. 1995; Charmet et al. 1996). The current distribution of rigid ryegrass in the USA includes Oregon, California, Arizona, Texas, Louisiana, and Missouri (USDA, 2000).

The genus *Lolium* includes five species worldwide. They can be separated into the allogamus (self-incompatible) group including *Lolium perenne* L. (perennial ryegrass), *L. multiflorum* Lam. (annual ryegrass), and *L. rigidum* Gaud. (rigid ryegrass)

and the autogamus (self-compatible) group including *L. tumelentum* L. and *L. remotum* Schrank (Essad, 1954 *In* Thorogood, 2003). Rigid ryegrass is a diploid species and it has 14 chromosomes (2n = 14) (Evans, 1926 *In* Thorogood, 2003). The origin of ryegrass species has been proposed to be from temperate region of European countries such as Corsica, Italy and France (Clayton and Revoiza, 1986; and Terrel, 1968).

Breeding methods for the first released rigid ryegrass in the USA were by natural selection and roughing (Anderson et al. 1995). These methods identified and disposed of abnormal plants, while those with desirable characteristics were used as parents to generate the following cycles. The breeding method described by Corkill (1956) is ideal for a self-incompatible and allogamus plants. The procedures are most likely similar to a polycross breeding cycle used in the Institute of Grassland and Environment Research (IGER) breeding program (Thorogood, 2003). Individual plants which have phenotypic characteristics were selected from the population and used for parents of the new generation. Although Corkill (1956) found that six plants were the optimum number to obtain and retain in subsequence generation, this number would be dependent on the heterozygosity and heterogeneity for any character.

Currently, rigid ryegrass is intensively used as a pasture crops in Australia (Gill, 1995). However, rigid ryegrass became one of the most widely established grass weeds within the Australian cropping system and caused extreme yield reduction (Powles et al. 1997). Rigid ryegrass has developed resistance to herbicides with various modes of action, such as chlorsulfuron, diclofop-methyl, trifluralin, glyphosate, etc (WSSA, 2004). Prudent management for controlling this putative glyphosate resistant weed requires characterization of the resistance.

The objectives of this study were to evaluate the glyphosate resistance and to determine the magnitude of resistance in California rigid ryegrass, to separate the resistant and sensitive biotypes by modified recurrent selection through multiple generations, and to determine the inheritance of glyphosate resistance in rigid ryegrass.

MATERIALS AND METHODS

Plant Materials

Glyphosate resistant plants were collected from a rigid ryegrass population near Chico, California in 1998. The collection site was an almond orchard intensively treated with glyphosate since it was established in 1984. The field rates of glyphosate had increased from 1x to 2x from 1984 to1997 with 4 to 5 application per year. It is unknown whether the collected ryegrass plants were related to the plants that had been released in 1962.

Rigid ryegrass plants were grown and maintained under greenhouse condition at Michigan State University. Common cultural procedures: seedlings were grown and transplanted into individual plants in 950-ml pots using professional planting mix media[1]. Greenhouse maintenance included supplemental sodium vapor light to provide 1,000 $\mu mol\ m^{-2}\ s^{-1}$ at midday to give a 16-h day, 8-h night. Plants were watered daily and fertilized weekly with water soluble fertilizer 20-20-20 NPK, the percentages of nitrogen (NO_3), phosphate (P_2O_5) and potassium (K_2O), respectively.

Spraying Methods and Evaluation

The herbicide spray solution was prepared from the commercial formulation of the isopropyl amine salt of glyphosate[2] plus 1% (v/v) ammonium sulfate (AMS). Glyphosate spray solutions were applied at 187 L ha^{-1}, 172.5 kPa pressure with a flat-fan nozzle[3]. Plants were recorded as dead or survivors 2 weeks after treatment (WAT). Plant

injury was evaluated visually on a scale from 0 to 100, where 0 indicated no injury and 100 indicated plants were dead.

Evaluation of Glyphosate Resistance in Rigid Ryegrass

Seeds from collected rigid ryegrass designated as the first generation or base population were grown and maintained under greenhouse condition. At the full maturity of vegetative growth, plants were sprayed with glyphosate at 1x, 2x, 4x, and 8x. Untreated plants were included as control. Dead plants were counted 2 WAT. Data presented were the percentages of dead plants in each treatment after subtracted from the percentages of the dead plants at the lower rates of glyphosate. A 180 plant population from duplicate experiment was used in each treatment.

Glyphosate efficacy was evaluated in the 7^{th} and 5^{th} generations of R and S lines. The rates of glyphosate were 0.125, 0.25, 0.5, 1, 2, 4, 6, and 8x. Plant injury was evaluated 2 WAT. Data presented are means of two experiments with four replications in each. The experiment was designed in completely randomized design (CRD), and means were separated by LSD at the 5 percent level of significance.

Inheritance Study

The first generation from collected California rigid ryegrass was used as a base population of selection. At the tillering stage, an individual plant was divided into two clones and re-transplanted into the new pots. One clone was used for evaluation of the magnitude of glyphosate resistance and the other was cultured for seed production.

Methods of selection were modified from recurrent selection based on the phenotypic response of the plant population to glyphosate treatment.

At the fully vegetative mature stage or before seed head formation the plants were sprayed with glyphosate at 1x. The dead plants were recorded 2 WAT and the survivors were re-acclimated. After recovery from glyphosate injury plants were re-sprayed with 2x of glyphosate. Similar procedures were repeated with 3x, 4x, and 5x glyphosate.

The other clones were cultured and grouped based on the sensitivity to glyphosate rates. Because of limitation in the flowering time, the cultured clones could only be divided into three groups: the sensitive group (S) that died from 1x, the intermediate group (I) that survived 1x and 2x but died from 3x, and the resistant group (R) which survived 3x glyphosate. These three groups were isolated as three lines of selection in separated greenhouses. Plants within each group were intercrossed for seed production. Seeds were harvested and designated as the 2^{nd} generation.

Further selection was continued through the generations of the R line with glyphosate applied at 3x, 4x, and 8x until the highest percentages of the very resistant plants were obtained; whereas the S line was selected through several generations with glyphosate applied at 0.5x, 0.25x, and 0.125x until the highest percentages of sensitive plants were obtained.

To determine the inheritance of the glyphosate resistant trait, plants from the 7^{th} generations of R (survived 8x glyphosate) and the 5^{th} generation of S (dead from 0.125x) were cross-pollinated in the greenhouse. Because the *Lolium* species are naturally outcrossed, the pollination was initiated by adjacent placement of two plants, which had similar periods of flowering maturity stage. To prevent pollen contamination from un-

wanted ryegrass, plants were isolated in a separate greenhouse. Plants of the F_1 hybrid were phenotypically evaluated for glyphosate sensitivity at 0.125, 0.5, 0.25, 1, 2, 4, 6, and 8x. Visual injury to glyphosate was recorded from 10 sample plants in each treatment 2 WAT.

Plants of the F_1 hybrid were allowed to intercross to generate the F_2. Glyphosate sensitivity was further evaluated in the F_2 population. At the fully mature vegetative growth the plant population was sprayed with 0.125x glyphosate. The number of sensitive plants (severely injured or dead) was recorded 2 WAT and the survivors were re-acclimated and re-sprayed with 8x glyphosate. The survivors to 8x rate were recorded at 2 WAT. The remainder of the population that showed a response between sensitive and resistance were determined to be intermediate in glyphosate sensitivity. Expected ratios between sensitive, intermediate, and the resistance responses were hypothesized with one, two or more than two genes involved in the inheritance of glyphosate resistance based on Mendelian segregation ratio in the F_2 population. Chi-square analyses were used to determine the most acceptable ratio for F_2 segregation.

RESULT AND DISCUSSIONS

Evaluation of Glyphosate Resistance in Rigid Ryegrass

Glyphosate resistance was observed in the rigid ryegrass population from California. The resistant plants that survived 2x glyphosate were identified, grown and seed produced under greenhouse conditions. The first generation showed diverse sensitivity to glyphosate as indicated by 11, 31, 14, and 36 percent death of the population following glyphosate application at 1x , 2x, 4x and 8x, respectively. Nine percent of the population that survived 8x glyphosate were not further evaluated but assumed to die from > 8x glyphosate (Figure 1).

The distribution pattern of the glyphosate sensitivity indicated a segregation of glyphosate resistance traits. The pattern was neither linier nor a normal distribution rather it showed two peaks, indicating that possibly multiple gene were involved in the glyphosate resistant traits (Figure 1). The collected plants appeared to be a natural hybrid between rigid ryegrass and another ryegrass species. This was considered possible as rigid ryegrass is in the allogamus group in the genus *Lolium.* The allogamus plants are self-incompatible and naturally outcross among other species within the same genus (Terrel, 1968; and Thorogood, 2003).

Efficacy of glyphosate was evaluated the S and R rigid ryegrass after selected for five and seven generation, respectively. The S plants were very sensitive and totally dead from 0.125x glyphosate, whereas the R plants were not affected by glyphosate up to 2x and slightly injuries was observed from 2x to 8x (Figure 2). The ratio of glyphosate sensitivity rates between R and S biotypes were 8x to 1/8x, respectively. This ratio (64-

fold) indicated a very high glyphosate resistance in California rigid ryegrass after recurrent selection through seven and five generations of R and S, respectively, compared to rigid ryegrass from Australia that showed 3-4 fold resistance (Lorraine-Colwill et al. 2001; Pratley et al. 1996), and compared to other weed species that demonstrated resistance to glyphosate (WSSA, 2004)

Inheritance Study

Because of non-homogeneity of glyphosate resistance was observed in the first generation, plants were designated as R, I, and S lines for further evaluation. The magnitude of glyphosate resistance determined by sequential application of glyphosate was used to separate the R, I and S (Table 1, Figures 2). From a population of 350 plants, 95, 70, 36, 16, and 4 % survived glyphosate at 1x, 2x, 3x, 4x, and 5x, respectively (Table 1). Because of the limitation of flowering periods, the clones cultured for seed production were only separated into the three groups for further use as selection lines. The S line died from glyphosate at 1x, the R line survived to glyphosate applied at 3x to 5x, and the intermediate (I) line responded between the S and R lines (Figures 3). Plants of each line were isolated and inter-crossed within each group for seed production. Seeds were harvested and designated as the 2^{nd} generation.

Selection in the R and S lines for the 2^{nd} and subsequent generations was continued to generate the very resistant and susceptible plants. After selection through the 7^{th} cycle, stability of the resistance (uniformly survived 8x glyphosate) was observed in the R line. The S line (dead from 0.125x glyphosate) was uniform after 5 generations of selection. The selection scheme is shown in Figure 4. Phenotype characteristics of

the S biotype at the 5th generation, such as textures, color, direction of the leaves, seed heads were similar to annual ryegrass (*Lolium multiflorum*) (Clayton et al. 1986). On the other hand, characteristics of the 7th generation of the R biotype resembled *Lolium rigidum* as described in the literature (Anderson et al.1995; Clayton et al. 1986).

Reciprocal cross-pollinations were successful between R (7th generation) and S biotype (5th generation). Glyphosate sensitivity in the F_1 hybrid, RS that was harvested from R parents or SR that was harvested from S parents appeared to be homogeneous. This indicated at least partial nuclear control of resistance (Figure 5). At 2 WAT, plants of the F_1 hybrid were slightly (less than 10 percent) and severely (70 to 80 percent) injured by glyphosate 1x and 2x, respectively. The plants were fully recovered by 4 WAT. F1 plants were dead from glyphosate applied at 4x (Figure 5).

Hybrid F_1 (10 plants) were intercrossed to generate an F_2 population. Glyphosate sensitivity evaluation and Chi-square analysis of the F_2 population is shown in Table 2. Plants sensitive to 0.125x and those that survived 8x glyphosate were designated genotypically similar to the S and R parents, respectively. The expected ratio of R, I, and S based on Mendelian segregation ratio in the F_2 population was (1 : 2 : 1) or (1 : 14 : 1) if one or two genes inherited the glyphosate resistance, respectively (Table 2).

The number of plants in F_2 population (total 400 plants) that showed similar sensitivity to the S parent (dead from 0.125x glyphosate), the intermediate (between S and R), and the R parent (survived 8x glyphosate) were 27, 352, and 21, respectively (Table 2). These numbers were tested against the hypothesized expected ratio. Values of the χ^2 ($\chi^2 = 0.8$; $0.75 < P < 0.90$) indicated that at least two genes were involved in the inheritance of glyphosate resistance (Table 2). The involvement of multiple genes for

glyphosate resistance in the rigid ryegrass collected from California is similar to the report on Australian rigid ryegrass by Pratley et al. (1999) and Feng et al. (1999) but different from the inheritance study published by Lorraine-Colwill et al. (2001), who concluded that the inheritance of glyphosate resistance in rigid ryegrass involved a single semi-dominant gene.

In summary, the glyphosate resistant ryegrass collected from an orchard in California appeared to be a natural hybrid between *Lolium* species. Diverse sensitivity to glyphosate (from 1x to 8x) was observed in the first generation (Figure 1). Based on the magnitude of glyphosate resistance, the first generation was separated into R, I and S lines (Table 1, Figure 2). Recurrent selections in the greenhouse successfully separated the R and S biotypes by the 7^{th} and 5^{th} generation, respectively (Fig 3). The ratio of glyphosate sensitivity between R (survived 8x glyphosate) and S (dead at 1/8x glyphosate) was more than 60-fold. This ratio appeared to be the highest reported for glyphosate resistant weeds. Based on the Chi-Square analysis, the inheritance of glyphosate resistance in California rigid ryegrass appeared to be multi-genic (Table 2).

Table 1. Magnitude of glyphosate resistance in California rigid ryegrass (*Lolium rigidum* Gaud.) after sequential treatment with 1x, 2x, 3x, 4x, and 5x glyphosate

Glyphosate rates [1)]	Survival [2)]	Sensitive
	(%)	(%)
1x	95	5
2x (after 1x)	70	25
3x (after 1x and 2x)	36	34
4x (after 1x, 2x, and 3x)	16	20
5x (after 1x, 2x, 3x, and 4x)	4	12

[1)] x = 1.12 kg ai ha^{-1} isopropylamine salt of glyphosate
[2)] Population of 350 plants

Table 2. Chi-square analysis of the F_2 population generated from hybridization between the resistant (survived 8x glyphosate) and the sensitive (dead from 0.125x glyphosate) biotypes of California rigid ryegrass(*Lolium rigidum* Gaud.)

	Sensitive (0.125x)	Intermediate (0.125x – 8x)	Resistance (8x)	Total	P value
F_2 (Observed)	27	352	21	400	
F_2 (Expected)*	100	200	100	400	
χ^2	53	116	62	231 na	
F_2 (Expected)**	25	350	25	400	
χ^2	0.2	0.01	0.6	0.8 aa	$0.75 < P < 0.9$

* *Expected ratio (one gene involved):*

Resistance (RR)	: *1*
Intermediate (Rr)	: *2*
Sensitive (rr)	: *1*

** *Expected ratio (two genes involved):*

Resistance (RRRR)	: *1*
Intermediate (RRRr + RRrr + Rrrr)	: *14*
Sensitive (rrrr)	: *1*

na : hypothesis not accepted; aa: hypothesis accepted

Figure 1. Glyphosate sensitivity evaluation in the first generation of California rigid ryegrass (*Lolium rigidum* Gaud.), data presented from a population of 180 plants in duplicated experiments

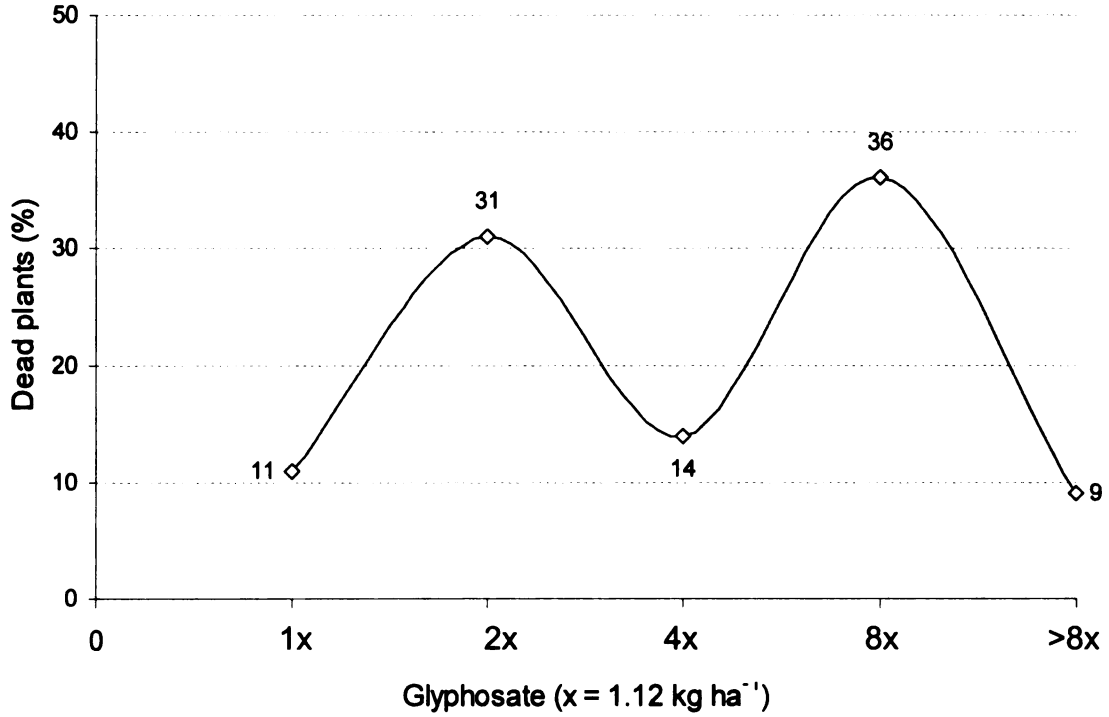


Figure 2. Glyphosate injury of the R and S biotypes of California rigid ryegrass in the 7th and 5th generations, respectively, 2 week after treatment (WAT), data presented are the averages of 10 plants for each treatment

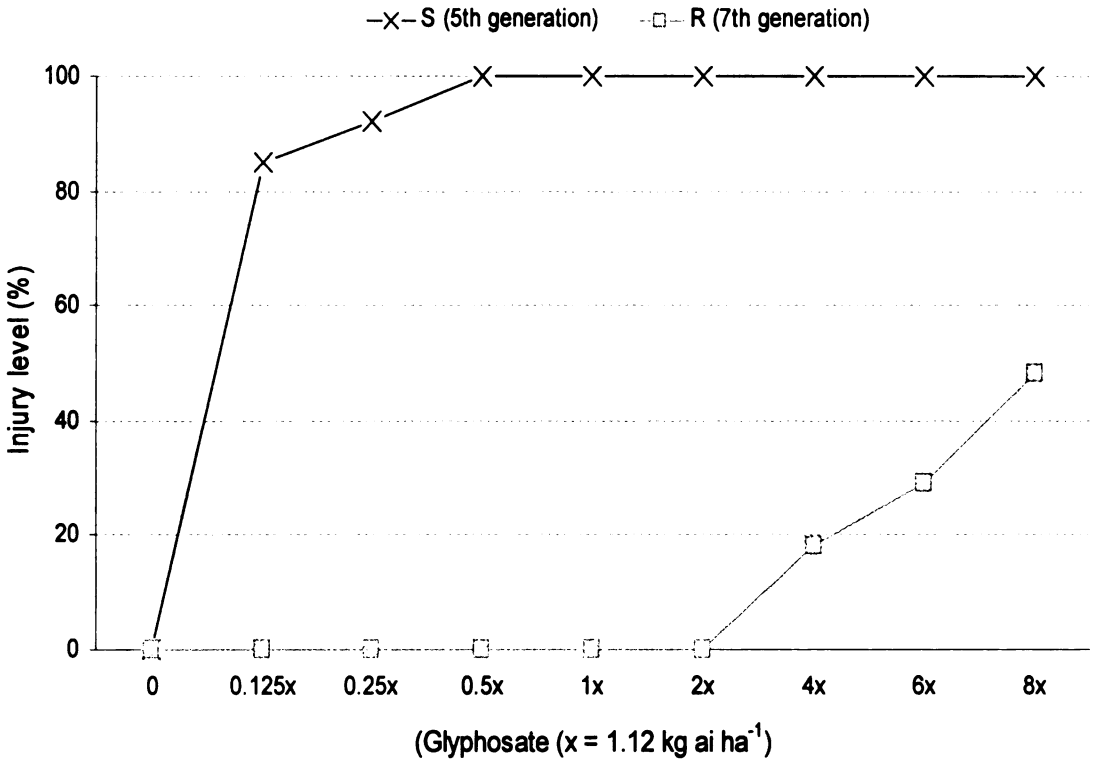


Figure 3. Distribution of the sensitive (S), intermediate (I), and resistant (R) lines of California rigid ryegrass in the first generation based on magnitude of resistance to glyphosate (data presented from a population of 350 plants)

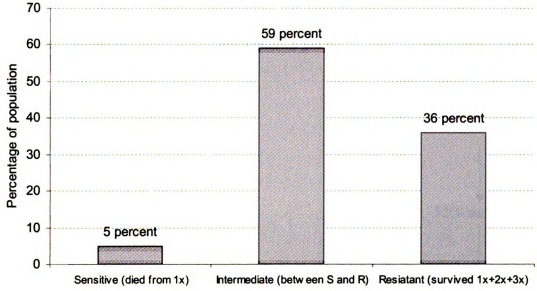


Figure 4. Selection scheme of the glyphosate resistant (R) and sensitive (S) lines of California rigid ryegrass (C_1-C_7 = from the 1st to 7th cycles or generation)

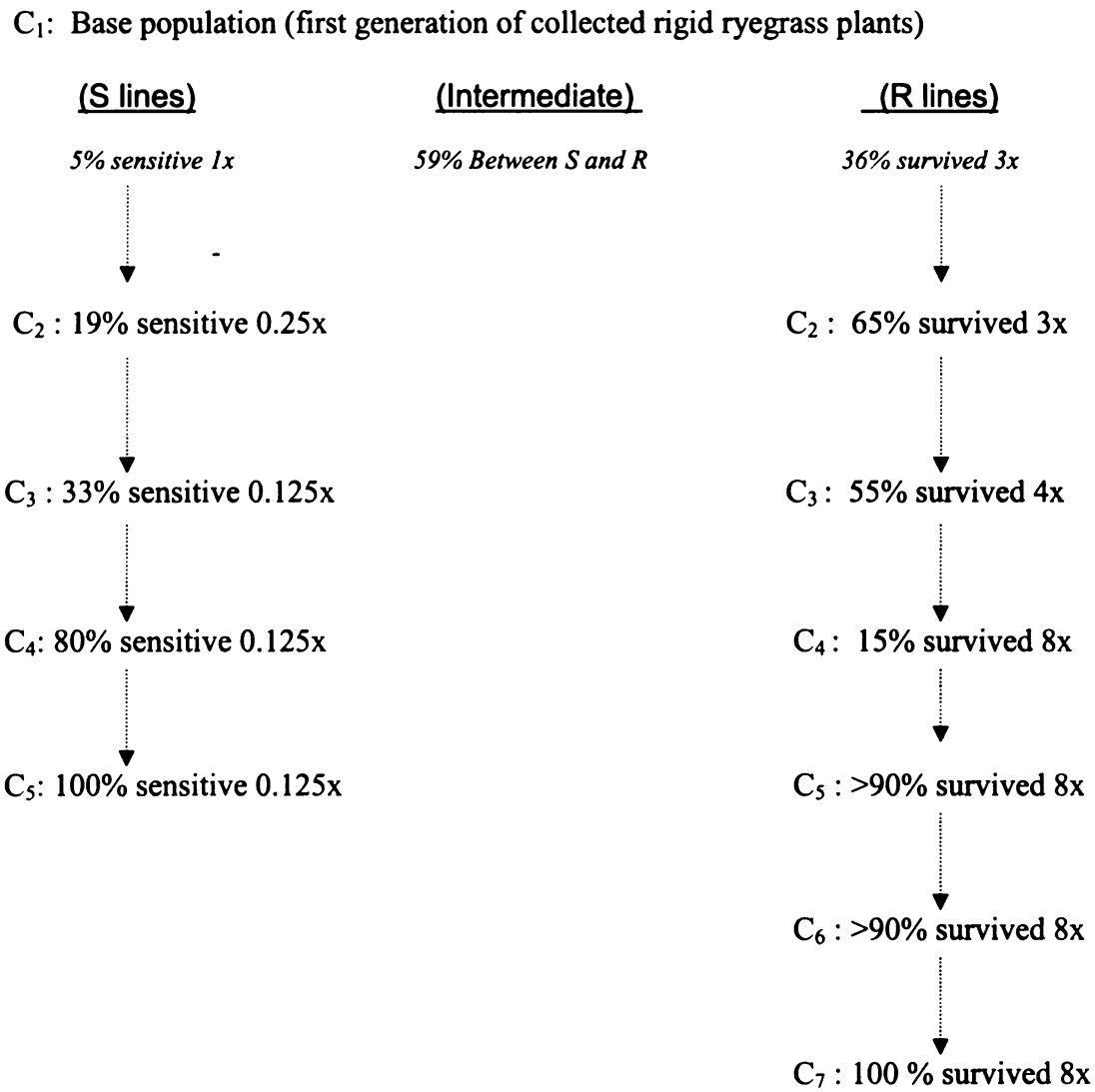
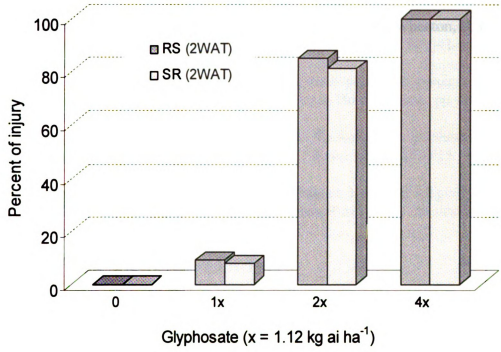


Fig 5. Glyphosate sensitivity of the F_1 hybrid population from cross-pollination between R and S biotypes of California rigid ryegrass 2 weeks after treatment (WAT), data presented are the average of 10 plants for each treatment



Source of materials

[1] BACTO® professional planting mix, Michigan Peat Co., Corporate office P.O. Box 980129, Houston TX 77098

[2] ROUNDUP ULTRA® herbicide, Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198

[3] TeeJet 8001E®, Spraying Systems Co., P. O. Box 7900, Wheaton, IL 60189

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CHAPTER 3.

POTENTIAL BASIS OF GLYPHOSATE RESISTANCE IN RIGID RYEGRASS (*Lolium rigidum* Gaud.) FROM CALIFORNIA

ABSTRACT

Glyphosate resistant rigid ryegrass ryegrass has been identified in California, but research has yet to elucidate the resistance mechanism. The objectives of this study were to examine the differences between the sensitive and resistant rigid ryegrass in foliar absorption and distribution of glyphosate, *in vivo* and *in vitro* absorption by chloroplast, and shikimic acid accumulation after glyphosate treatment. Foliar absorption and distribution of ^{14}C-glyphosate did not differ 1 to 3 d after treatment (DAT) between the susceptible (S) and resistant (R) biotypes. Absorption ^{14}C-glyphosate by isolated chloroplasts also did not differ between the S and R biotypes. After foliar application of ^{14}C-glyphosate, chloroplasts were isolated from treated leaves from both biotypes. Accumulation of ^{14}C-glyphosate in chloroplasts did not differ between the two biotypes. Shikimic acid level increased significantly in the S biotype after treatment with glyphosate at 2.24 kg ai ha^{-1} to levels 10-fold greater than in R biotype 11 DAT. Shikimic acid in the germination media at 2 to 5 mM did not affect seed germination of both S and R biotypes but drastically decreased the length of coleoptiles of both at 5 DAT. Thus, biotype differences in sensitivity or metabolism of shikimic acid do not explain differences in sensitivity to glyphosate.

INTRODUCTION

Glyphosate, *N-(phosphonomethyl) glycine*, is the world's most widely used herbicide. It is a foliar non-selective herbicide and has no activity in the soil (Ahrens, 1994; Baird et al. 1971). It can be used pre-plant to control emerged weeds in a no-tillage planting system or postemergence by spot and direct application to control an extensive range of weeds (Ahrens, 1994) as well as to control weeds on glyphosate resistant crops (Padgette et al., 1996).

Glyphosate in susceptible plant species inhibits biosynthesis of the aromatic amino acids tryptophan, tyrosine, and phenylalanine (Siehl, 1997). In shikimate pathway, glyphosate competes with substrate phosphoenol pyruvate, PEP, for binding site of 5-enolpyruvylshikimate-3-phosphate synthase, EPSPS (E.C. 2.5.1.19). Glyphosate is the only herbicide reported to inhibit EPSPS (Steinrucken and Amrhein, 1980).

Metabolism of glyphosate in higher plants is very limited and not well understood. Glyphosate is not readily metabolized if applied at phytotoxic rates (Sandberg et al., 1980). Coupland (1985) reported that glyphosate metabolism to aminomethyl phosphonic acid (AMPA) is slow.

Since being introduced around 30 years ago, glyphosate has been intensively used for controlling weeds, at times without rotating with other methods. This condition may lead to the selection of the resistant weed species (Bradshaw et al., 1997; Dyer, 1994; Kishore et al., 1992). Glyphosate resistance has been reported in rigid ryegrass in Australia (Powles et al., 1997, 1998; Pratley et al., 1999) and California (Simarmata et al., 2001), in goosegrass (*Eleusine indica*) in Malaysia (Lee and Ngim, 2000; Tran et al., 1999), and in horseweed [Conyza canadensis (L.) Cronq.] (VanGessel, 2000).

Tran et al. (1999) reported that mechanism of glyphosate resistance of Malaysian goosegrass populations is target site based. They showed that EPSPS was not inhibited by glyphosate in the R biotype. However, the basis of glyphosate resistance in rigid ryegrass from Australia is not clearly understood. Uptake, translocation and metabolism were not different between the resistant (R) and susceptible (S) biotypes (Feng et al. 1999; Lorraine-Colwill et al. 1999). The sensitivity of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, DAHPS (E.C. 4.1.2.15) and EPSPS within the plastid cell was also similar for R and S biotypes. Lorraine-Colwill et al. (1999) proposed that possible differences in glyphosate transport into or accumulation of glyphosate in the chloroplast might explain the differential basis of sensitivity.

Lorraine-Colwill et al. (1999) reported that shikimic acid accumulated in leaf tissue of glyphosate susceptible biotype after glyphosate application. Shikimic acid accumulation has been identified in glyphosate-susceptible corn (Singh and Shaner, 1998) as well as glyphosate sensitive cotton (Pline at al., 2002). Singh and Shaner (1998) stated that shikimic acid accumulation may be used as a method to determine whether a plant species is resistant to glyphosate. It may be important as a means of quickly identifying and characterizing glyphosate resistant weed biotypes to avoid their spread and to facilitate their effective management. Harring et al. (1998) stated that this assay was also useful to evaluate the efficacy of different glyphosate formulations.

The objectives of this research were to determine the role of absorption, translocation, and metabolism of ^{14}C-glyphosate in glyphosate resistant rigid ryegrass from California, to study the movement of ^{14}C-glyphosate into the chloroplast, to

investigate shikimic acid accumulation in response to glyphosate and AMPA, and to test for differential phytotoxicity of shikimic acid to rigid ryegrass biotypes.

MATERIALS AND METHODS

Plant Materials

Rigid ryegrass was collected from "Borba Farm", Chico, California in 1998. The site of collection was an almond orchard intensively treated with glyphosate since established in 1984. The rates of glyphosate had increased from 1.12 to 2.24 kg ai ha^{-1} from 1984 to1997 with four to five applications per year. The collected plants were grown and seed produced in the greenhouse. The R, intermediate and S biotypes were selected in the first generation using 1.12, 2.24, and 3.36 kg ai ha^{-1} rates of glyphosate. Further selection rates was continued with high glyphosate rate (8.96 kg ai ha^{-1}) to select for the resistant and with the low rate (0.28 kg ai ha^{-1}) to select for the susceptable individuals. The R and S biotypes used in these studies were generated from the fifth and fourth cycles, respectively.

Absorption and Translocation

S and R biotypes of rigid ryegrasses were grown individually in 950 ml-pots containing commercial soil mixed and maintained in a greenhouse under supplemental sodium vapor light to provide 1,000 µmol m^{-2} s^{-1} at midday to give a 16-h day and 8-h night. At the fully vegetative mature stage before seed head formation, plants were thinned to one tiller in each pot. For the preliminary experiment, two fully mature leaves of each plant were treated with three 2-µl drops of glyphosate[1] solution applied to the upper leaf surface. This corresponded to a field rate of 1.12 kg ai ha^{-1} glyphosate at a volume of 187 L ha^{-1} and pressure 172.5 kPa. One week after treatment, the S biotype was dead but no injury was observed on the R biotype.

The experiment was continued using methyl labeled ^{14}C-glyphosate[2]. Three 2-µl drops ^{14}C-glyphosate solution (specific activity of 370 Bq -µl^{-1}) were placed on each of two fully mature leaves. The solution contained of ^{14}C-glyphosate, isopropylamine, and water plus 1 % (v/v) non-ionic surfactant (NIS)[3]. The treated plants were placed and maintained in the greenhouse at air temperature 25 ± 2 C and 15 ± 2 C for 18- and 6-h periods, day and night, respectively, with supplemental light as described previously.

At 1, 2, and 3 d after treatment (DAT), the treated leaves were cut and rinsed vigorously with distilled water plus 1 % (v/v) NIS. Rinse solutions were analyzed for ^{14}C-glyphosate activity by liquid scintillation spectrometry[4]. Absorption was calculated from the total applied minus the activity removed in the rinse solution and the data presented as percentage of applied. Recovery was 85% of applied

Treated leaves, shoot with untreated leaves, and roots were harvested separately, oven dried, and combusted with a biological oxidizer[5]. The $^{14}CO_2$ evolved was trapped in the cocktail solution and radioactivity was quantified by liquid scintillation spectrometry. Data for translocation are presented as percentage of applied.

Glyphosate Movement into Chloroplast *in vivo*

Fully mature plants were sprayed with glyphosate at 1.12 kg ai ha^{-1} before application of ^{14}C-glyphosate. Radioactive solutions were applied with a spot and a dip treatments. Two cm of leaf tips were dipped into 1 ml-micro tubes containing ^{14}C-glyphosate and one 0.5 µl drop of ^{14}C-glyphosate solution (specific activity of 370 Bq µl^{-1}) was applied on each fully mature leaf. The solution applied in the spots contained

^{14}C-glyphosate, isopropylamine, water, and 1% (v/v) NIS, which corresponded to a field rate 1.12 kg ai ha^{-1}.

Two days after treatment, the treated leaves were harvested, macerated, and homogenized in extraction buffer (1 : 2 wt/v). The procedures for chloroplast extraction were modified from Moreland (1986) to maximize integrity in the chloroplast as observed using a confocal microscope. Extraction buffer contained 0.04 M sucrose, 0.0.05 M tricine NaOH (pH 8), 0.01 M NaCl, 0.02 M sodium ascorbate. Homogenized extract was strained through four layers of cheesecloth into cold centrifuge tubes. The extract was centrifuged at 0 C and 500 x g for 1.5 min, and the supernatant was transferred to new tubes and further centrifuged at 1,000 x g for 5 min. The supernatant was discarded and the pellet resuspended with a modified buffer (extraction buffer minus sodium ascorbate) and centrifuged at 1,000 x g for 5 min. The supernatant was discarded and the chloroplast pellets were ovendried, weighed and combusted with the oxidizer, and quantified by liquid scintillation spectrometry. Data are presented as disintegrations per minute (dpm) per milligram dry weight of chloroplast.

Glyphosate Movement to Chloroplast *in vitro*

Chloroplasts were extracted from 50 gram of leaf tissue from mature plants of S and R biotypes as described previously. The chloroplast pellet was diluted with the extraction buffer to 6 ml and divided into 6 microtubes. Ten micro liters of ^{14}C glyphosate solution (specific activity, 7,400 Bq μl^{-1}) was added into each tube. The solution contained ^{14}C glyphosate, isopropylamine, buffer, and 1 % (v/v) NIS as described previously. At 4 and 24 hours after treatment, microtubes were centrifuged at

5,000 x g to concentrate the chloroplast, and the supernatant was carefully discarded from the tubes. Pellets of chloroplasts were oven-dried, weighed and combusted with a biological oxidizer, and ^{14}C was quantified by liquid scintillation spectrometry. Data is presented as dpm per milligram dry weight of chloroplast.

Shikimic Acid Assay

Glyphosate at 2.24 kg ai ha $^{-}$ and AMPA at 4.48 and 11.20 kg ha $^{-1}$ plus 1 % (wt/v) diammonium sulfate were sprayed on mature S and R biotypes of rigid ryegrass. Plant leaves were harvested for shikimic acid assay at 0, 4, 7 and 11 DAT. The procedures for shikimic acid extraction were modified from Singh and Shaner (1998), shikimate content was quantified spectrophotometrically according to the methods of Gaitonde and Gordon (1957).

Leaf tissues were chopped, ground in liquid nitrogen by mortar and pestle, then further ground in 0.25 N HCl (1 : 2 wt/v). The extracts were centrifuged at 25,000 x g for 15 min. The supernatant was collected and used for shikimic acid assay. An aliquot of supernatant was mixed with 1 ml of 1 % solution of periodic acid. After a 3-h oxidation by periodic acid, the sample was mixed with 1 ml of 1 N NaOH and added 0.6 ml of 0.1 M glycine. The solution was mixed thoroughly, and optical density (OD) was measured spectrophotometrically immediately at 380 nm[6]. The amount of shikimic acid was calculated based on standard curve and presented on a fresh weight basis.

Bioassay of Shikimic Acid Accumulation

The effect of shikimic acid on seed germination of S and R biotypes of rigid ryegrass was tested. Ten seeds of each biotype were placed in 90-mm polystyrene Petri dishes on two layers of 80 mm filter papers. The papers were moistened with 10 ml of shikimate solution at 0, 0.25, 0.5, 0.75, 1.0, 2, 3, 4, and 5 mM. The dishes were covered and placed in a seed germinator under controlled conditions at 16 and 8 h (light and dark, respectively) with air temperatures of 22 ± 1 C and 15 ± 1 C, respectively. Seed germination and the length of coleoptiles were measured at 5 DAT.

Statistical Analyses

All experiments in all studies were duplicated and data presented are means of two experiments with three replications in each. Experiments were conducted in a completely randomized design (CRD). After analysis of variance, the means were compared by least significant difference at $P = 0.05$.

RESULTS AND DISCUSSION

Absorption and Translocation of ^{14}C-glyphosate in Rigid Ryegrass

Absorption and translocation of glyphosate tended to increase over time, but there was no significant difference between S and R biotypes (Figure 1). The amounts of ^{14}C-glyphosate absorbed by S and R biotypes at 3 DAT were 62 and 54.6 % of applied, respectively. Most of the ^{14}C-glyphosate remained in the treated leaf, and only small percentage translocated to the shoot and root of the S and R biotypes. The amount of ^{14}C-glyphosate translocated to the roots of the S biotype was slightly greater than for the R biotype, but these differences did not correspond with the level of sensitivity or resistance to glyphosate (Figure 1). The results obtained with glyphosate-resistant rigid ryegrass from California are similar to what was reported for the resistant rigid ryegrass from Australia (Feng et al. 1999; Lorraine-Colwill et al. 1999).

Previous reports found no difference in glyphosate metabolism in S and R biotypes of rigid ryegrass from Australia (Feng et al. 1999; Lorraine-Colwill et al. 1999). On preliminary test, AMPA caused slight to high injury of the S biotype at the rates 6.72 to 11.20 kg ha^{-1}, indicating AMPA penetration to the leaves, but R biotype was not affected by AMPA treatment. The basis for the resistance to AMPA may be similar to the basis for the resistance to glyphosate (Coupland, 1985; Sanberg et al. 1980) but because AMPA showed herbicidal activity, metabolism of glyphosate to AMPA does not appear to be the basis for glyphosate resistance in rigid ryegrass.

In vitro and *in vivo* Movement of ^{14}C-glyphosate into Chloroplasts

Movement of glyphosate into *in-vivo* and *in-vitro* chloroplasts were not significantly different for the S and R biotypes (Table 1). After 4 h of glyphosate exposure, the glyphosate concentration was slightly higher in the R than the S biotypes (1,528.4 and 1,160.3 dpm mg^{-1} of chloroplast), but there was no significant difference after 24 h (1,477.5 and 1,352.2 dpm mg^{-1} of chloroplast).

The same results were also observed in the *in-vivo* chloroplast study. There was no significant difference in the movement of ^{14}C-glyphosate into chloroplasts of S and R biotypes using either dip or spot treatments. But between the two application methods, more glyphosate was absorbed with the spot treatment than with the dip treatment (Table 2).

EPSPS is encoded in the nucleus but is located in the chloroplast (Siehl, 1997). Lorraine-Colwill et al. (1998) proposed that the possible differences in the movement of glyphosate into the chloroplast or the differences in glyphosate absorption by the chloroplast between R and S biotypes of rigid ryegrass formed a potential basis for the resistance. However, data in this study (Tables 1 and 2) do not support that hypothesis because no significant differences in glyphosate translocation into chloroplast of S and R biotypes were observed.

Shikimic Acid Assay and Bioassay on Seed Germination of Rigid Ryegrass

Measurement of the accumulation of shikimic acid is a quick method to identify glyphosate resistance in plants (Singh and Shaner 1998). After glyphosate application shikimic acid increased over time in the S biotype, but no significant increase was

observed in R biotype (Figure 2). A 10-fold increase in shikimic acid was observed in the S biotype compare to the R biotype at 11 DAT with glyphosate at 2.24 kg ai ha^{-1}.

Treatment with AMPA at 4.48 and 11.20 kg ha^{-1} caused slight and severe injury, respectively, to the S biotype of rigid ryegrass (Figure 3). However, shikimic acid levels increased only about two fold in S compare to R biotypes. No differences were observed in shikimic acid levels among AMPA rates within the biotype, and there also was no significant accumulation over time.

It was hypothesized that shikimic acid accumulation after glyphosate application to the plants caused the injury to the S biotype. Seed germination of the S biotype was inhibited by shikimic acid at 2 to 5 mM, but no inhibition of germination was found for the R biotype. Shikimic acid at 2 to 5 mM drastically decreased the length of coleoptiles for both S and R biotypes at 5 DAT (Table 3).

In summary, differences in glyphosate absorption, translocation including movement into the chloroplast, or sensitivity to AMPA or shikimic acid do not explain the basis for resistance of the R biotype of rigid ryegrass from California to glyphosate. The conclusion from the sum of the data presented is that the R biotype has an EPSP synthase insensitive to glyphosate.

Tabel 1. Movement of ^{14}C-glyphosate *in vitro* into chloroplasts of rigid ryegrass (*Lolium rigidum* Gaud.)[a]

Biotype	Reaction time	Absorption
	(h)	(dpm mg^{-1} of chloroplast)
Sensitive	4	1160.6
	24	1352.2
Resistant	4	1528.4
	24	1477.5
$LSD_{0.05}$		323.4

[a] Abbreviations : LSD, least significant difference;
dpm, disintegrations per minute

Tabel 2. Movement of ^{14}C-glyphosate *in vivo* into chloroplasts of rigid ryegrass (*Lolium rigidum* Gaud.)[a]

Biotype	Treatment mode	
	Dipping of leaf tips	Spot on leaf surface
	dpm mg^{-1} dry chloroplast	
Sensitive	1.39	3.63
Resistant	0.93	3.39
$LSD_{0.05}$ = 2.28		

[a] Abbreviations : LSD, least significant difference

Table 3. Effect of shikimic acid accumulation on the seed germination of resistant and sensitive biotypes of rigid ryegrass

Shikimic acid	Seed germinating		Length of coleoptile	
	Susceptible	Resistance	Susceptible	Resistance
mM	---------- % ----------		---------- mm ----------	
0	82	95	27	15
0.25	80	95	26	16
0.50	73	93	26	16
0.75	78	100	26	15
1.00	78	93	20	13
2.00	58	90	22	5
3.00	58	93	9	2
4.00	63	95	7	2
5.00	62	92	5	2
LSD 0.05	12.3		3.9	

[a] Abbreviations : LSD, least significant difference

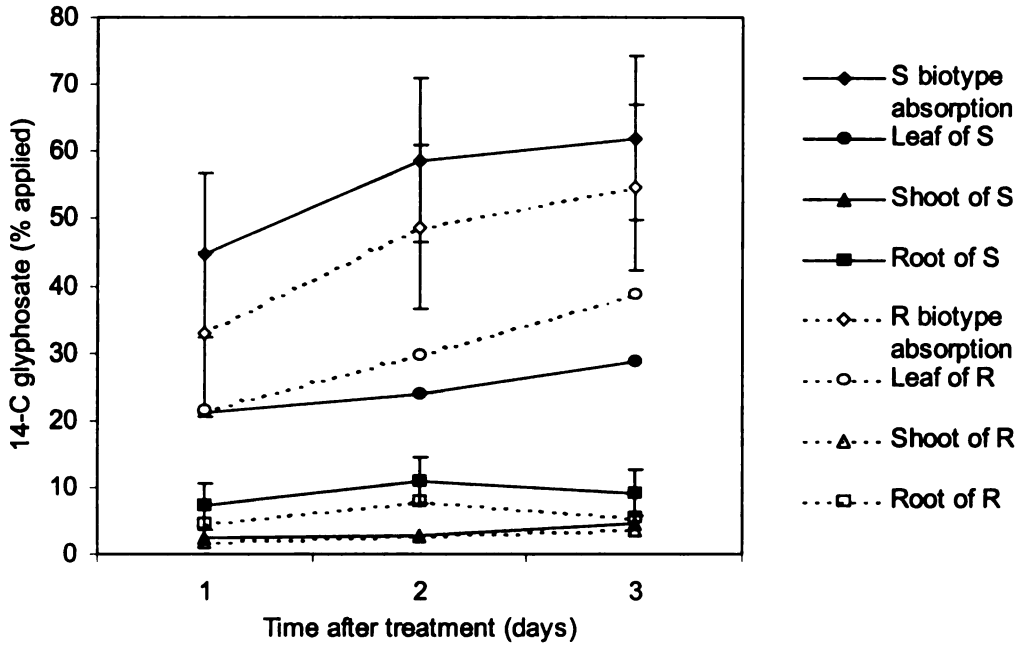

Figure 1. Absorption and translocation of ^{14}C-glyphosate into treated leaves, untreated shoots, and roots of susceptible (S) and resistant (R) biotypes of rigid ryegrass (*Lolium rigidum* Gaud.). Vertical bars represent least significant difference value at P = 0.05

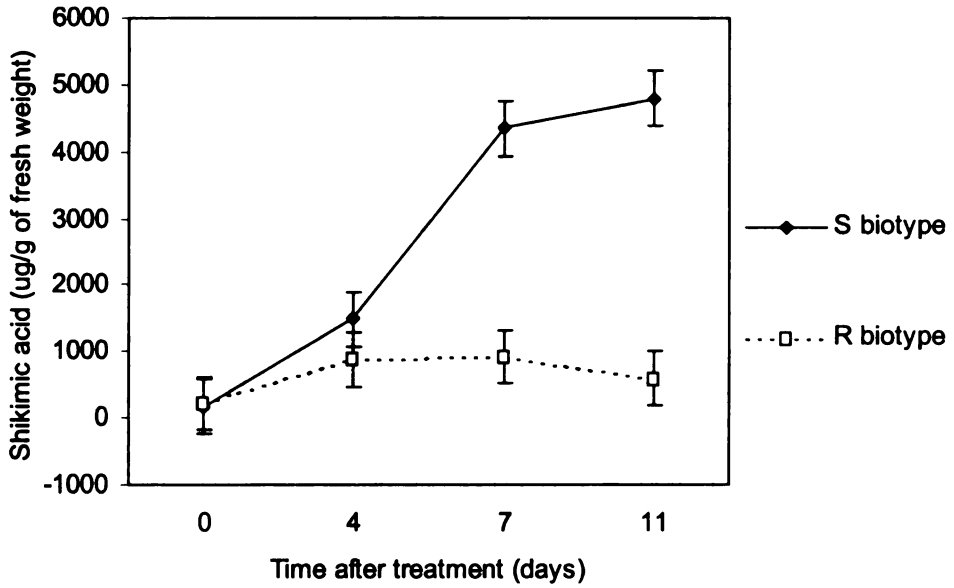

Figure 2. Shikimic acid accumulation in susceptible (S) and resistant (R) biotypes after treatment with glyphosate at 2.24 kg ai ha^{-1}. Vertical bars represent least significant difference value at P = 0.05

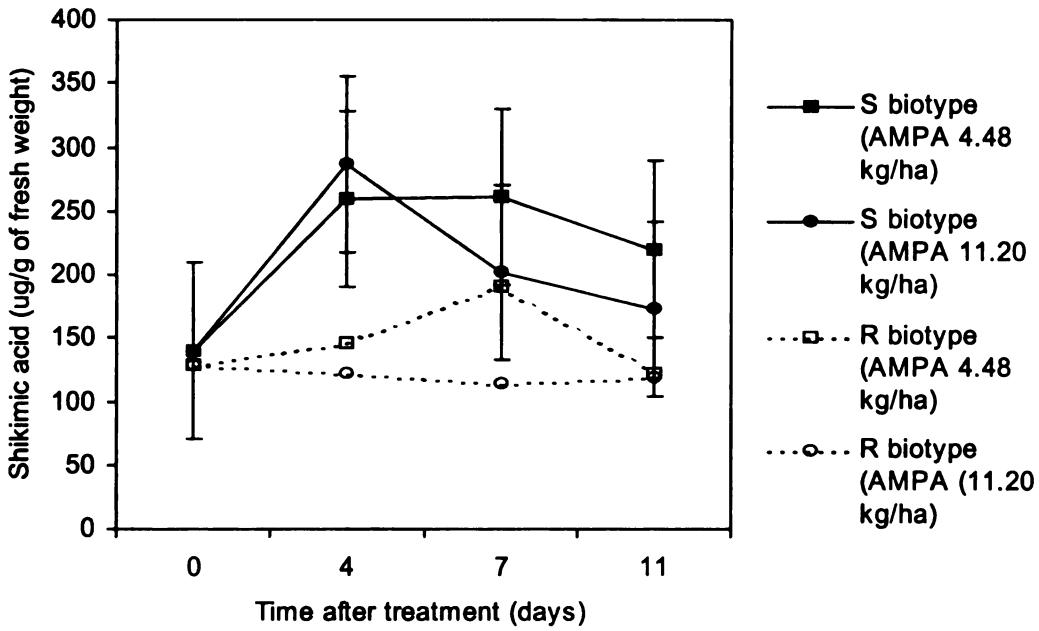

Figure 3. Shikimic acid levels in susceptible (S) and resistant (R) biotypes after treatment with aminomethyl phosphonic acid (AMPA). Vertical bars represent least significant difference value at P = 0.05

Sources of Materials

[1] Glyphosate isopropylamine salt @ Roundup Ultra, dry isopropylamine acid, aminomethyl phosphonic acid (AMPA) from Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198

[2] Radio-labelled glyphosate from Sigma Co.11542 Fort Mims Dr. St Louis, MO 63146-3510.

[3] Nonionic surfactant/AMS blend @Glass Act Surfactant from Agriliance, LCC, P.O. Box 64089, St. Paul, MN 55164-0089

[4] Liquid scintillation counter model LS-6500: Beckman Coulter Inc. 4300 N. Harbor Blvd. Fullerton, CA 92835

[5] Oxidizer model OX300: R.J. Harvey Instrument Corporation, 123 PattersonStreet, Hillsdale, NJ 07642).

[6] Spectrophotometer model Spectronic GENESYS 5, Milton Roy Co. 820 Linden Avenue, Rochester, NY 1425.

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CHAPTER 4

PHYSIOLOGICAL BASIS FOR GLYPHOSATE RESISTANCE IN RIGID RYEGRASS (*Lolium rigidum* Gaud.) FROM CALIFORNIA

ABSTRACT

Rigid ryegrass collected from California has been selected for 7 and 5 generations to separate the resistant (R) and sensitive (S) biotypes. The physiological basis for the resistance of California rigid ryegrass to glyphosate has not been determined. The objectives of this study were to determine the roles of metabolism and altered target site, 5-enolpyruvylshikimate-3-phosphate synthase, EPSPS (EC 2.5.1.19) in California glyphosate resistant rigid ryegrass. No quantitative or qualitative differences were observed in the metabolism of ^{14}C-glyphosate between the R and S biotypes 1 and 3 days after treatment (DAT). Glyphosate sensitivity of EPSPS from R and S plants was significantly different. Activity of EPSPS from R plants was not affected, slightly decreased, and decreased to 25 percent, whereas in S biotypes the activity decreased to 60, 50, 40, and remained only 9 percent of control by glyphosate at 0.05, 0.5, 5 and 50 mM, respectively. Decreased EPSPS sensitivity to glyphosate in the R compared to S biotype appears to be a major contributor in glyphosate resistance in California rigid ryegrass.

INTRODUCTION

Glyphosate resistance in rigid ryegrass was reported in Australia (Pratley et al. 1996; and Powles et al. 1998), and in California, USA (Simarmata et al. 2001). Since then, the efforts to elucidate the basis of glyphosate resistance in rigid ryegrass have been continuing. Possible approaches to investigate glyphosate resistant mechanism include 1) evaluation of non-target basis mechanisms including absorption, translocation, sequestration of absorpted glyphosate inside the plant cell, and metabolism; 2) evaluation of target site for alteration in 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a target enzyme of glyphosate in shikimate pathway (Heap and LeBaron, 2001).

Glyphosate metabolism in plants has been considered insignificant (Malik et al. 1989; Rueppel et al. 1987). Although in some studies metabolism of glyphosate was demonstrated in some plant species, but the transformation products did not significantly reduce phytotoxicity (Rueppel et al. 1987). Plant species that demonstrated varying degrees of metabolism were soybean (*Glycine max* (L.) Merr.), maize (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) (Rueppel et al. 1987), field bindweed (*Convolvulus arvensis* L.), Canada thistle (*Circium arvense* (L.) Scop.), and tall morning glory (*Ipomoea purpurea* (L.) Roth.) (Sandberg et al. 1980)

In the soil environment, glyphosate was easily degraded to aminomethyl phosphonic acid (AMPA) and glyoxylate; or sarcosine and phosphate by micro-biological degradation (Malik et al. 1989; and Rueppel et al. 1977; Tornstensson, 1985). Current reports indicated that a very high dose of AMPA may cause phytotoxicity to plants including ryegrass species (Simarmata et al. 2003). Glyphosate is considered an

environmentally safe herbicide because it has no residual activity from the soil (Smith and Oehme, 1992; and Wauchope et al. 1992).

Current publications showed that metabolism was similar for the resistant (R) and sensitive (S) biotypes of rigid ryegrass from Australia (Baerson et al. 2002a; Feng et al. 1999; Powles et al. 1998). Even though metabolism products were observed in some plant and weed species including rigid ryegrass but the levels were not significantly high to reduce phytotoxicity of glyphosate (Feng et al. 1999),

Published reports on glyphosate resistance in rigid ryegrass concluded that absorption and translocation were not different between resistant and sensitive biotypes (Baerson et al. 2002a; Feng et al. 1999; Lorraine-Colwill et al. 1999; and Simarmata et al. 2003). Intercellular movement of glyphosate into the chloroplast was not different between R and S plants (Simarmata et al. 2003). More recently, Lorraine-Colwill et al. (2003) reported differences in the pattern of glyphosate translocation between the resistant and sensitive rigid ryegrass population from Australia. They concluded that glyphosate accumulated in the roots of sensitive plants, whereas glyphosate accumulated in the leaf tips of resistant plants. They proposed that this difference could account for the difference in glyphosate sensitivity of the two biotypes (Lorraine-Colwill et al. 2003). This is the first report of a non-target basis mechanism for glyphosate resistant weeds.

No significant differences in EPSPS activity were found between resistant and sensitive biotypes of rigid ryegrass from Australia (Lorrraine-Colwill et al. 2003). Baerson et al. (2002a) reported induction of EPSPS 48 h after glyphosate treatment in resistant rigid ryegrass from Australia, but no differences in the sensitivity to glyphosate

of the basal or constitutive EPSPS in sensitive and resistant ryegrass from Australia. Boerboom et al (1990) had related differences in the sensitivity of birdsfoot trefoil (*Lotus carniculatus* L.) selection to differences in the specific activity of EPSPS. Shikimic acid accumulation in the sensitive biotypes was observed in all studies with rigid ryegrass (Baerson et al. 2002a; Lorraine-Colwill et al. 1999; and Simarmata et al. 2003). The latter provides support to the hypothesis that the basis of glyphosate resistant mechanism involved in shikimate pathway.

The objectives of this research were to evaluate the role of metabolism in glyphosate resistance and to determine the role of EPSPS in glyphosate resistance in California rigid ryegrass.

MATERIALS AND METHODS

Metabolism of Glyphosate

R and S rigid ryegrass plants from California from the 5th and 4th selection cycles of the original collection were grown individually in 950-ml pot with professional planting mix media[1]. Plants were maintained in greenhouse with the supplement light, watered daily, and fertilized weekly with NPK water soluble fertilizer (20-20-20). At full vegetative growth, plants were pre-sprayed with glyphosate[2] at 1.12 kg ai ha^{-1} (1x) plus 1 % ammonium sulfate (AMS) prior to treatment of radio label ^{14}C-glyphosate[3]. Glyphosate was sprayed at 187 L ha^{-1}, 172.5 kPa pressure using a nozzle tip 8001E[4].

Two leaves of each plant were each treated with two 5-µl drops of ^{14}C-glyphosate solution (specific activity is 370 Bq. ul^{-1}). Final concentration of solution was similar to 1x glyphosate containing ^{14}C-glyphosate, isopropylamine[2], plus 1 % non-ionic surfactant (NIS)[5]. Treated plants were placed and maintained in a greenhouse with temperature 25 and 15 ± 2 C and 18 h day and 6 h night.

At 1 and 3 days after treatment (DAT), treated leaves were harvested and rinsed with water. Four leaves from two plants were pooled in one treatment for extraction. Leaves were macerated and homogenized in 10 ml water for 2 min and then centrifuged at 17,300 x g for 10 min. The supernatant was separated and pellet was re-homogenized and centrifuged two more times. Total volume of 30 ml supernatant was concentrated to one ml at 60 C under N_2 .

A 20-µl drop of concentrated supernatant was developed on 50-µm cellulose TLC-plates[6] with the solvent system solution containing 55 % (v/v) ethanol, 35% (v/v)

H_2O, 2.5% (v/v) 15N NH_4OH , 3.5 % (w/v) TCA (trichloroacetic acid), and 2 % (v/v) 17 N acetic acid (Sprankle et al., 1978).

After development for 16 cm, the plates were scanned for 10 h in a Radio-analytical Imaging System[7]. To obtain greater separation, the plates were developed and scanned two more times with the similar procedures. Rf value (ratio of front) was calculated from the ratio between the distance of ^{14}C-extract and solvent development. The amount of ^{14}C inside the Rf were counted automatically by the imaging system and presented in percent per sample.

EPSPS Study

Methods of extraction and assaying EPSP synthase were modified from Boerboom et al. (1990). Crown tissue was harvested from rigid ryegrass plants at full vegetative growth. Approximately 0.5 gram fresh weight per sample was frozen in liquid nitrogen and ground in a cold mortar in the presence of 150 mg polyvinylpolypyrrolidone (PVPP). A fine powder tissue was further ground with 0.5 ml extraction buffer (pH 7.5) containing 10 mM Tris HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 1mg ml^{-1} bovine serum albumin (BSA), 10 mM ascorbate, 1mM benzamidine (BAM), and 5 mM dithiothreitol (DTT). The mixture was centrifuged at 15,000 x g for 10 min, and supernatant was desalted in 1.0-ml Sephadex G-50 column[8] and further centrifuged at 1,000 x g for 3 min. Total protein in the supernatant was quantified by the method of Bradford (1976) using BSA as a standard solution.

Assay of EPSPS activity was modified from Westwood and Weller (1997). Condensation reaction of shikimate 3-phosphate (S3P) and phosphoenol pyruvate (PEP)

was assayed in the buffer mixtures (pH 7.5) in the presence of 10 µl enzyme extract. The final concentration of the reaction mixtures were 50 mM HEPES, 1 mM $(NH_4)_6Mo_7O_{24}$, 5 mM KF, 1mM PEP, 2 mM shikimate 3-phosphate and glyphosate acid at 0, 0.05, 0.5, 5.0, and 50 mM. Reactions were incubated at 25 C for 20 min and stopped with 100 C for 2 min.

The reaction mixtures were further centrifuged at 15,000 x g for 10 min for eliminating denatured protein. PEP used in the reaction mixture was determined from the remaining PEP which was analyzed spectrophotometrically[9] at 340 nm by adding NADH, pyruvate kinase and lactate dehydrogenase (PK/LDH). The control was determined by using denatured plant extract in the condensation reactions. Activity of EPSPS was expressed in enzyme unit (EU) per mg protein, 1 EU equaled 1 mole of PEP used per min in the assayed reaction.

Statistical Analyses

Experiments were duplicated and data presented are the means of two experiments with three replications in each. Analysis of variance was done using a SAS program. Experiments were conducted in a completely randomized design (CRD) and means were separated with Least Significant Difference (LSD) at 5 percent.

RESULTS AND DISCUSSION

Matabolism Study

The ^{14}C containing concentrated extracts from the leaves of R and S plants were successfully developed on the 50-μm cellulose TLC-plates and the images are shown in Fig 1. The images were more readily visualized after three sequential developments in the same solvent system. The image comparisons could not distinguish differences between the extracts from R and S biotypes at 1 and 3 DAT. The patterns from both the R or S extracts were similar to the original ^{14}C-glyphosate. Distribution of the scanned radiolabel in the Rf region was not statistically different between R and S biotypes. The percentages of ^{14}C equivalent to ^{14}C-glyphosate ranged from 95 to 99 percent per sample (Table 1).

The results from this study indicated that glyphosate was not metabolized in R and S biotypes of California rigid ryegrass. In the previous study, we found no differences in absorption, translocation, and sequestration of radiolabel ^{14}C-glyphosate into chloroplasts of the two biotypes (Simarmata et al. 2003). Similar results have been reported for glyphosate resistant goosegrass (Baerson et al. 2002b), and rigid ryegrass (Feng et al. 1999; Lorraine-Colwill et al. 1999). The results are consistent with previous reports indicating lack of glyphosate metabolism in plants (Malik et al. 1989; Rueppel et al. 1975). Phytotoxicity of aminomethyl phosphonic acid (AMPA) in S plants also provided a clue that metabolism might not contribute to the observed glyphosate resistance in California rigid ryegrass (Simarmata et al. 2003).

EPSPS Study

At the enzyme level, the activity of the constitutive EPSPS was inhibited significantly more in S than in R plants by the glyphosate. Activity of EPSPS in S plants decreased to 64, 63, 49 and 9 percent of control, whereas R plants remained at 100, 95, 75 and 25 percent with glyphosate 0.05, 0.5, 5 and 50 mM, respectively (Fig. 2). Decreased sensitivity of EPSPS in R plants appeared to be a major contributor in glyphosate resistance in rigid ryegrass from California. This is in contrast to the results reported with Australian rigid ryegrass by Baerson et al. (2002a)

In glyphosate resistant goosegrass from Malaysia, Baerson et al. (2002b) observed decreased sensitivity of EPSPS to glyphosate. Accumulation of shikimic acid had been identified in almost all studies on glyphosate resistance mechanisms. This evidence is consistent with glyphosate inhibition of EPSPS in the shikimate pathway (Baerson et al. 2002ab; Simarmata et al. 2003).

In summary, metabolism appears not to be the basis for glyphosate resistance in rigid ryegrass collected from California. Decreased sensitivity of EPSP synthase to glyphosate in the R biotype appears to be a major contributor to the observed resistance.

Sources of Materials

[1] BACTO® professional planting mix, Michigan Peat Co., Coorporate office P.O. Box 980129, Houston TX 77098

[2] ROUNDUP ULTRA® herbicide, Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198

[3] Radio-label glyphosate, Sigma Co.11542 Fort Mims Dr. St Louis, MO 63146-3510.

[4] TeeJet 8001E®, Spraying Systems Co., P. O. Box 7900, Wheaton, IL 60189

[5]GLASS ACT® Surfactant, Agriliance, LCC, P.O. Box 64089, St. Paul, MN 55164-0089

[6]UNIPLATE® Thin Layer Chromatography Plate, Analtech Inc. P.O. Box 7558, Blue Hen Drive. Newark, DE 19714

[7]AMBIS® Radioanalytical Imaging System, Ambis System Inc. 3939 Ruffin Rd. San Diego, California 92123

[8]MICRO-SPINS® Shephadex G-50 column, Life Science Products Inc. 5989 Iris Parkway, P. O. Box 1150, Frederick, Colorado 80530

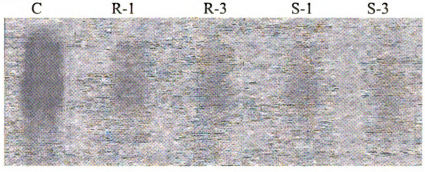
[9]GENESYS 5® Spectrophotometer model Spectronic, Milton Roy Co. 820 Linden Avenue, Rochester, NY 1425.

Table 1. Rf and distribution of ^{14}C from concentrated extract of resistant (R) and sensitive (S) biotypes of California rigid ryegrass at 1 and 3 DAT

Developed	Rf	R biotype:		S biotype:	
		1 DAT	3 DAT	1 DAT	3 DAT
		..... % of ^{14}C			
1st	0.31-0.63	98	96	95	95
2nd	0.59-0.88	98	96	96	98
3rd	0.72-0.94	99	98	99	99

Fig 1. TLC-images of ^{14}C from concentrated extracts of resistant (R) and sensitive (S) biotypes of California rigid ryegrass after development in the solvent system one times (I) and 3 times (II) (C = Control or ^{14}C-glyphosate solution, R-1 and R-3 = extracts from the R plants 1 and 3 DAT, S-1 and S-3 = extracts from the S plants 1 and 3 DAT)

(I):



(II) :

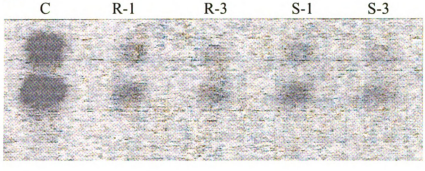
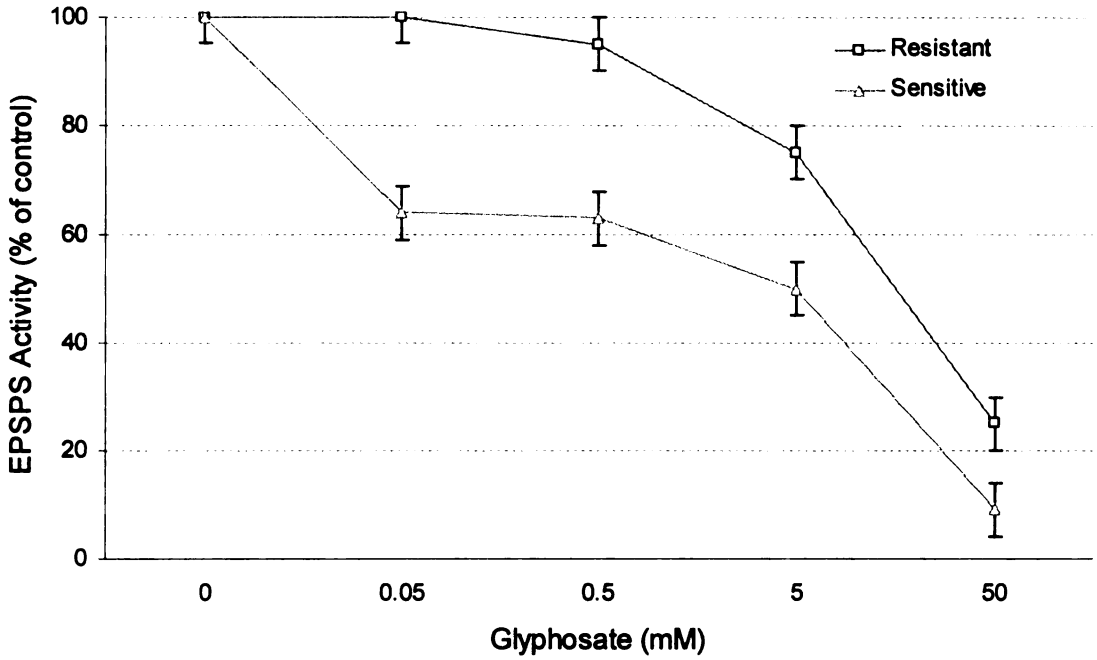


Fig 2. Differences in EPSPS activity between resistant (R) and sensitive (S) biotypes of California rigid ryegrass



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