AN ABSTRACT OF THE DISSERTATION OF

<u>Wilson Vidal Avila Garcia</u> for the degree of <u>Doctor of Philosophy</u> in <u>Crop Science</u> presented on <u>August 18, 2011</u>

Title: <u>Investigation of Inheritance of Glyphosate Resistance and the Mechanisms of Glufosinate Resistance in Italian Ryegrass (*Lolium perenne* L. spp. *multiflorum* (Lam.) Husnot) Populations</u>

Abstract approved:

_____

Carol A. Mallory-Smith

Italian ryegrass populations have been identified with evolved resistance to glyphosate in orchards with a history of glyphosate use. Two of these populations were selected to investigate the inheritance of glyphosate resistance. The mechanisms involved in the herbicide resistance were an altered target site for the population SF and reduced herbicide translocation for the population OR1. Mendelian inheritance studies and dose response experiments were conducted on the two populations. Four F_1 families were formed by reciprocal crosses between each of the glyphosate resistant populations (SF and OR1) and the susceptible population (S) C1. Eight backcross families (BC_1) were formed between the F_1 individuals from each family and the susceptible population C1. Most of the F_1 families resulting from SF and C1 had susceptible:resistant ratios

of approximately 1:1. Similar trends were observed in the backcross families concluding that glyphosate resistance due to target site mutation in the SF Italian ryegrass population is likely conferred by a single, nuclear, partially-dominant gene. For population OR1, there was significant variation in the susceptible:resistant ratios in the F_1 families. Chi-square analysis for backcross families failed to fit the model for a single major gene suggesting that the glyphosate resistance due to reduced herbicide translocation in the Italian ryegrass population OR1 is multigenic.

Italian ryegrass glyphosate resistant populations OR1, OR2, and OR3, the population MG, and three susceptible populations C1, C2, and C3 were selected to conduct dose-response experiments, ammonia accumulation assays, and enzymatic studies to quantify their sensitivity to glufosinate. The glufosinate rates required to reduce the growth by 50% (GR_{50}) were 0.15, 0.18, and 0.21 kg ai ha^{-1} for the susceptible populations C1, C2, and C3, respectively, and for the resistant populations OR1, OR2, OR3, and MG, the GR_{50} values were 0.49, 0.42, 0.40, and 0.45 kg ai ha^{-1} respectively, resulting in an average resistance index of 2.4. The same trend was observed in ammonia accumulation studies between 48 and 96 hours after glufosinate treatment. The susceptible populations accumulated between 1.5 to 2.5 times more ammonia than the resistant populations. The glufosinate concentration required to reduce glutamine synthetase enzyme activity by 50% (I_{50}) was not different for the resistant OR1, OR2, and OR3 and susceptible populations. However, a different response was observed for the population MG. The I_{50} values ranged from 3.1 to 3.6 µM for the resistant

populations OR1, OR2, and OR3, and from 3.7 to 4.3 µM for the susceptible

populations. The population MG had an I_{50} of 10.7 µM resulting in a resistant

ratio 2.6-fold higher than the average of the control populations C1 and C2.

Eighty-three percent of the plastidic GS gene from the resistant population MG

and the susceptible C1 was cloned and sequenced. One amino acid substitution

was found in the population MG that may be responsible for the reduced enzyme

sensitivity. These results are the first reports of target site and non target site

based glufosinate resistance in a weed species.

Investigation of Inheritance of Glyphosate Resistance and the Mechanisms of
Glufosinate Resistance in Italian Ryegrass (*Lolium perenne* L. spp. *multiflorum*
(Lam.) Husnot) Populations

by

Wilson Vidal Avila Garcia

A DISSERTATION

Submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented August 18, 2011

Commencement June 2012

Doctor of Philosophy dissertation of Wilson Vidal Avila Garcia presented on August 18, 2011.

APPROVED:

_____

Major Professor, Representing Crop Science

_____

Head of the Department of Crop and Soil Science

_____

Dean of the Graduate School

I understand that my dissertation will became a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_____

Wilson Vidal Avila Garcia, Author

ACKNOWLEDGMENTS

CONTRIBUTION OF AUTHORS

Dr. Carol Mallory-Smith proposed and advised all the aspects of this research. She was involved in the preparation of all the chapters of the manuscript and provided feedback during all stages of this research. Dr. Andrew G. Hulting was also involved in the improvement of the manuscripts, contributing invaluable suggestions. Elena Sanchez was actively involved in the molecular analysis and DNA sequences that are presented in Chapter 4.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

DEDICATION

This dissertation is especially dedicated to my family and friends, both near and far. Without their encouragement and support none of this would have been possible.

CHAPTER 1: GENERAL INTRODUCTION

Glyphosate

Glyphosate [N-(phosphonomethyl) glycine] has been intensively used in agriculture worldwide for the past 40 years, and currently is the most widely used herbicide in the world (Duke and Powles 2008). Thus, as a result of its continuous and expanding use, 21 weed species have evolved resistance to this herbicide (Heap 2011).

Glyphosate is an amino acid synthesis inhibitor. After glyphosate absorption by the plants, it is readily translocated along with photosynthates, from the point of application on the leaves to distant sinks (Franz et al. 1997). In susceptible plants, the herbicide glyphosate inhibits the activity of the plastidic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). The shikimate pathway can be described by the following reaction:

$$\text{PEP + Shikimate-3-phosphate} \xrightarrow{\text{EPSPS}} \text{EPSP}$$

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvyl-shikimate-3-phosphate (EPSP) (Franz et al. 1997). The shikimate pathway is absent in mammals, but it plays a fundamental role in plants, fungi and bacteria biosynthesis of the aromatic essential amino acids

phenylalanine, tyrosine and tryptophan, and important secondary compounds (Franz et al. 1997).

Herbicide resistance in weeds can be due to an altered binding target site, reduced absorption, reduced translocation, metabolic detoxification, and gene amplification. Altered target site and reduced translocation have been described for glyphosate resistance in Italian ryegrass (*Lolium perenne* L. spp *multiflorum* (Lam.) Husnot) populations (Jaseniuk et al. 2008; Nandula et al. 2008; Perez-Jones et al. 2007) and in several rigid ryegrass (*Lolium rigidum* G.) populations in Australia (Preston and Wakelin 2008).

A new mechanism for herbicide resistance was recently reported for glyphosate resistance in Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Gaines et al. 2010). Gene amplification of five to 160 copies of the EPSPS gene on multiple chromosomes was reported in the resistant population. The increased gene copy number and their location throughout the Palmer amaranth genome suggest that the amplification could have originated via a transposon- or RNA-mediated mechanism, followed by selection through glyphosate applications of highly resistant individuals from the population. Further studies on F_2 populations confirmed that the EPSPS gene amplification was heritable.

A case of multiple mechanisms of resistance was reported by Yu et al. (2007) in a *L. rigidum* biotype from South Africa, with four different mechanisms of herbicide resistance. Even more interesting was that two of those mechanisms (target-site mutation and reduced translocation) were responsible for glyphosate

resistance. Additionally, this biotype was resistant to paraquat due to restricted translocation and resistant to acetyl coenzyme-A carboxylase (ACCase) inhibitors due to an insensitive ACCase enzyme. Similar results were reported by Park et al. (2004) in a *Bromus tectorum* L. population resistant to ALS inhibitors, where target site mutation and metabolism endowed resistance.

Previous studies by Perez-Jones et al. (2003, 2005 and 2007) showed that Italian ryegrass populations from Chile and Oregon (USA) evolved glyphosate-resistance due to two different mechanisms, altered target site and reduced glyphosate translocation, respectively.

Inheritance of Glyphosate Resistance

Italian ryegrass is a widely used forage grass in temperate regions of the world and is one of the most competitive weeds in orchards and crops in several regions of the United States (Hoskins *et al.* 2005 and Tucker *et al.* 2006). Italian ryegrass is an obligate self-incompatible outcrossing species. It is a native of Europe and it is thought to be derived from *Festuca* via inflorescence transformation from panicle to spike (McKell *et al.* 1969). At least seven *Lolium* species including Italian ryegrass have been previously identified and characterized as diploids (Beddows, 1973).

There are few studies that have investigated the inheritance of glyphosate-resistance in grass species. Lorraine-Colwill *et al.* (2001) and Wakelin and Preston (2006) reported that glyphosate resistance in *L. rigidum*

populations in Australia was controlled by a single, partially dominant nuclear gene. The same results were obtained by Ng *et al.* (2004) in a glyphosate resistant *Eleusine indica* L. population in Malaysia. However, Simarmata *et al.* (2005) reported that glyphosate resistance in a *L. rigidum* population in California (US) was inherited as a nuclear, incompletely dominant multigenic trait. In the dicot species, *Conyza canadensis* L., glyphosate resistance was inherited as a single, incompletely dominant nuclear trait (Zelaya *et al.* 2004).

Glufosinate

Glufosinate is a potent inhibitor of the enzyme glutamine synthetase (GS) which is considered a cornerstone of plant productivity due to its importance in nitrogen transformation and thus nitrogen nourishment (Matos-Nogueira et al 2005). The enzyme can be inhibited by several compounds that have similar chemical properties. Three of these compounds, methionine sulfoximide (MSO), phosphinothricin (PPT: glufosinate), and tabtoxinine-β-lactam (tabtoxin,) have been studied extensively in bacteria, higher plants, and animal species (Franco et al. 1996; Tachibana et al 1986; Wu 1963).

Glufosinate and bialaphos are the only GS inhibitors that are currently commercialized as herbicides. Glufosinate is labeled for use in vineyards, orchards, grass seed crop, and transgenic glufosinate resistant Liberty-Link® crops such as corn (*Zea mays* L.), soybean (*Glycine max* L.), and cotton (*Gossypium hirsutum* L.)

The inhibition of GS leads to an accumulation of ammonia to phytotoxic levels. GS is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration (Tachibana et al. 1986). The specific regulatory mechanism of the glutamine biosynthesis catalyzed by GS can be described by the following reaction:

$$\text{Glutamate} + NH_3^+ + ATP \text{ --------> } \text{Glutamine} + ADP + P_i + H^+$$
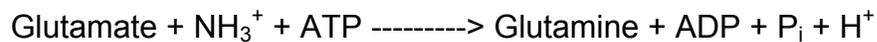
In this reaction, glutamine is synthesized from glutamate. The catalytic reaction of GS proceeds in two steps. The first step is transfer of the terminal phosphoryl group of ATP to the side chain carboxyl moiety of the substrate glutamate, producing the activated intermediate γ-glutamyl phosphate. In the second step, a bound ammonium ion is deprotonated, forming ammonia, which attacks the carbonyl carbon of γ-glutamyl phosphate releasing a free phosphate to yield glutamine (Ray 1989). There are some studies reporting that the inhibition of GS mainly blocks the reassimilation of the ammonia that is released during photorespiration (Manderscheid 1993; Tachibana et al. 1986). The ammonia generated by photorespiration quantitatively is more significant than the ammonia generated by nitrogen assimilation (Masclaux-Daubresse et al. 2006). However, Krieg et al. (1990) reported that the application of up to 40 times of ammonium nitrate in *Medicago sativa* culture media had no effect on callus growth,

suggesting that ammonia accumulation alone may not cause the drastic phytotoxicity observed in plant cells following glufosinate application.

Photosynthesis is also rapidly inhibited by glufosinate under atmospheric levels of O_2, but not under non-photorespiratory O_2 conditions (Sauer et al. 1987; Wild et al. 1987; Lacuesta et al. 1992). Photosynthesis inhibition is not due to ammonia accumulation, but to a decrease in the concentration of the amino acids glutamine, glutamate, aspartate, alanine, glycine and serine. Addition of glutamate and glutamine to glufosinate treated plant tissue increased the content of these amino acids over those treatments with glufosinate alone (Wild and Wendler 1991; Downs et al. 1994).

Differential responses in sensitivity to glufosinate in weeds and non-genetically transformed crops have been attributed to three main mechanisms: altered uptake, reduced translocation, and metabolism (Skora-Neto et al. 2000; Steckel et al. 1997; Pline et al. 1999). Glufosinate efficacy also can be affected by relative humidity and temperature as was demonstrated by Coetzer et al. (2001) in Palmer amaranth, redroot pigweed (*A. retroflexus* L.) and common waterhemp (*A. rudis* L.).

There are two reported cases of glufosinate resistance in weed species. Jalaludin et al. 2010 and Seng et al. 2010 reported that goosegrass (*Eleusine indica*) populations in Malaysia have evolved glufosinate resistance. Greenhouse and field studies confirmed that the goosegrass populations had two and eight-fold resistance compared to the susceptible population. Avila and Mallory-Smith

(2010) reported that three glyphosate resistant Italian ryegrass populations also evolved resistance to glufosinate. Dose response experiments and ammonia accumulation assays showed that the levels of glufosinate resistance of these populations were between two and three-fold compared to three susceptible populations.

Research Objectives

The objectives of this research were: 1) Determine the inheritance of glyphosate resistance due to a target site mutation or due to reduced translocation in Italian ryegrass; 2) Investigate the mechanisms of resistance to the herbicide glufosinate by conducting dose response, ammonia accumulation, and enzyme activity experiments, and cloning and sequencing the plastidic glutamine synthetase (GS) gene in Italian ryegrass populations.

CHAPTER 2: Inheritance of Two Different Mechanisms of Glyphosate Resistance in Italian Ryegrass (*Lolium perenne* L. *ssp. multiflorum* (Lam.) Husnot) Populations

Wilson V. Avila-Garcia, Andrew G. Hulting, and Carol Mallory-Smith

ABSTRACT

The inheritance of glyphosate resistance in two Italian ryegrass populations with different resistance mechanisms was investigated. The mechanisms were a target site mutation for population SF and reduced translocation for population OR1. Mendelian inheritance and dose response experiments were conducted for four F_1 families that were formed by reciprocal crosses between each glyphosate resistant population (SF and OR1) and a susceptible population C1. Additionally, eight backcross families (BC_1) were produced by crossing resistant F_1 individuals from each family and C1. Six of eight F_1 families between SF and C1 had susceptible:resistant ratios of 1:1. Similarly, 50 percent of the sixteen BC_1 families fit the model for 1:1 segregation ratios, while the remaining fifty percent showed variation from 1:1 (2:1 or 1:2). Segregation ratios in F_1 and BC_1 families suggest that glyphosate target site resistance in SF is conferred by a single, nuclear encoded, partially dominant gene. The population OR1 had significant variation for the observed and expected susceptible:resistant ratios in the F_1 and the BC_1 families. Chi-square analyses suggests that the glyphosate resistant trait conferred by reduced herbicide translocation is controlled by multiple genes. The absence of differences in the reciprocal crosses in F_1 and BC_1 families suggest

that the genetic control of glyphosate resistance in both Italian ryegrass

populations is nuclear.

INTRODUCTION

Glyphosate [N-(phosphonomethyl) glycine] is the most widely used herbicide in the world (Woodburn 2000). As a result of its continuous and expanding use, 21 weed species have evolved resistance to this herbicide (Heap, 2011). Glyphosate is a potent and specific inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs). After glyphosate is absorbed by plants, it is readily translocated along with photosynthates, from the point of application leaves to distant sinks (Franz et al. 1997).

The mechanisms of herbicide resistance in plants can be an altered herbicide binding target site, reduced herbicide absorption, reduced herbicide translocation, gene amplification, vacuolar sequestration, or metabolic herbicide detoxification. The most frequent mechanism reported in glyphosate resistant weed species has been limited translocation of glyphosate. This mechanism has been identified in horseweed (*Conyza Canadensis* L.) (Koger and Reddy 2005), hairy fleabane (*C. bonariensis* L.) (Dinelli et al. 2008), rigid ryegrass (*Lolium rigidum* Gaudin) (Preston and Wakelin 2008; Wakelin and Preston 2006), and Italian ryegrass (*Lolium perenne* L.) (Perez et al. 2004; Perez-Jones et al. 2007). Gene amplification was recently reported for glyphosate resistance in Palmer amaranth (*Amaranthus palmeri* L.) (Gaines et al. 2010). Five to 160 copies of the EPSPS gene on multiple chromosomes was reported in the resistant population. The increased gene copy number and their location throughout the Palmer

amaranth genome suggest that the amplification could have originated via a transposon- or RNA-mediated mechanism, followed by selection of highly amplified individuals from the population. Further studies on F_2 families confirmed that the EPSPS gene amplification was heritable. Three mechanisms of resistance have been described for glyphosate resistance in Italian ryegrass populations (Jaseniuk et al. 2008; Nandula et al. 2008; Perez-Jones et al. 2005; Perez-Jones et al. 2007) and in several rigid ryegrass populations from Australia (Preston and Wakelin 2008).

Many inheritance studies of herbicide resistance due to target site mutation have reported that resistance was controlled by a single, nuclear gene with complete or partial dominance. These observations were reported in ALS herbicide resistant prickly lettuce (*Lactuca serriola* L.) (Mallory-Smith et al. 1990), kochia (*Kochia scoparia* (L.) Scrad.) (Saari et al. 1994), annual sowthistle (*Sonchus oleraceus* L.) (Boutsalis and Powles 1995), giant foxtail (*Setaria faberi* Herrm.) (Volenberg et al. 2001), and black nightshade (*Solanum nigrum* L.) (Volenberg and Stoltenberg 2002). Similar results were reported in ACCase herbicide resistant giant foxtail (Volenberg and Stoltenberg 2002), blackgrass (*Alopecurus myosuroides* H.) (Letouze and Gazquez 2001; Moss et al. 2003), and rigid ryegrass (Tal and Rubin 2004), where an altered target site resulted in the resistance.

There are few studies that have investigated the inheritance of glyphosate resistance in weed species. Lorraine-Colwill et al. (2001) and Wakelin and

Preston (2006), reported that glyphosate resistance in rigid ryegrass populations from Australia was inherited as a single incompletely dominant nuclear-encoded gene. The same results were obtained by Ng et al. (2004) in a goosegrasss (*Eleusine indica* L.) population from Malaysia previously reported as glyphosate resistant. However, Simarmata et al. (2005) reported that glyphosate resistance in a rigid ryegrass population from California (US) was nuclear inherited, incompletely dominant and encoded by multiple genes. In the dicot species, horseweed, glyphosate resistance was reported to be inherited as a single, partially dominant nuclear gene (Zelaya et al. 2004).

The objective of this research was to determine the inheritance of the glyphosate resistance trait conferred by either a target site mutation or reduced translocation in Italian ryegrass populations.

MATERIAL AND METHODS

Plant material. The Italian ryegrass populations OR1 and SF were used as the glyphosate resistant parents for the reciprocal crosses. The glyphosate resistance mechanism of OR1 is due to reduced herbicide translocation, while the mechanism of resistance in the SF population is due to the proline by serine amino acid substitution in the EPSPS gene (Perez-Jones et al. 2007). Population C1, the Italian ryegrass cultivar 'Gulf', was used as the susceptible parent. Italian ryegrass is an obligate outcrossing species; therefore, in order to obtain greater homozygous levels in both glyphosate resistant populations, individual plants from each resistant population that survived treatment at the glyphosate rate of 1.0 kg ae ha^{-1} were grown in separate greenhouses and allowed to cross-pollinate. Seeds from each population were harvested at maturity approximately five weeks after flowering. Two selection cycles were performed following the same method.

Seed germination and plant growth. Seeds of SF, OR1 and C1 populations were germinated in 10-cm-diameter petri dishes with blue blotter germination moistened with 10 ml of deionized water. Seeds were kept in germination chambers at 14/10 h day/night at 25/20 ^{o}C, respectively. After four days, the seedlings were transferred to 250 ml plastic pots containing potting mix soil[1]. The

plants were grown in the greenhouse under 25/20 °C day/night temperatures and a 14/10 h day/night photoperiod.

Dose-response bioassay. Dose-response bioassays were conducted to determine the level of glyphosate resistance in the parental resistant populations OR1 and SF after the two selection cycles, and to select individual plants to be used as parents for reciprocal crosses. At the 2-to 3 leaf stage of development, plants were treated with glyphosate[2] at rates of 0, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 kg ae ha^{-1}, using an overhead compressed air cabinet sprayer[3] calibrated to deliver 187 L ha^{-1}. Shoot biomass was harvested two weeks after treatment, dried at 60 C for 72 h and weighed. Resistant/susceptible indices were estimated on the basis of the herbicide rate required to reduce growth by 50% (GR$_{50}$). The experiment was conducted once with three replications per rate. A replication consisted of five individual plants.

Dose-response curves to estimate the glyphosate rate required to reduce biomass growth (GR$_{50}$) by 50% were obtained using nonlinear regression based on the equation described by Streibig et al. (1993):

$$Y = c + \{d - c / 1 + \exp [b(\log x - \log e)]\} \qquad [1]$$

where Y represents shoot dry weight at herbicide rate x, e corresponds to the GR$_{50}$ value. The upper limit is d, the lower limit is c, and b represents the slope of the line at the GR$_{50}$. Data were analyzed using the R software package[4] (Knezevic et al. 2007).

Generation of F_1 families. Four individual plants from OR1 and SF populations

that survived the glyphosate rate of 1.0 kg ae ha^{-1} and eight plants from the

susceptible C1 population that were used as controls in the dose-response

bioassay were retained and allowed to regrow. The plants were repotted into 2-L

plastic pots containing potting mix. The plants were maintained in the

greenhouse under photoperiod and temperature regimes as described

previously. Before flowering, glyphosate resistant plants of OR1 and SF were

paired with individual plants of the C1 population. The criterion to pair the plants

was based on their flowering synchronization. To achieve isolation and to reduce

the risk of cross contamination and restrict external pollen, individual tillers from

the paired plants were bagged with glassine bags[5] just before flowering to

produce the F_1 families. At maturity, seeds were collected separately from each

reciprocal cross.

Dose-response bioassay for the F_1 families. At the 2-to 3 leaf stage of

development, 18 plants (three replications of six plants per each) from the F_1

families and their respective parents were treated with glyphosate at rates of 0,

0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 kg ae ha^{-1}, using an overhead compressed air

sprayer calibrated to deliver 187 L ha^{-1}. Shoot biomass was harvested two weeks

after treatment, dried at 60 C for 72 h and weighed. Resistant/susceptible indices

(RI) were estimated as described previously. The experiment was conducted

once.

Screening test for phenotyping F_1 families. Fifty individuals from each

reciprocal F_1 family and 50 individuals from the parental populations OR1, SF,

and C1 were grown in the greenhouse under 25/20 C day/night temperature and

14/10 h day/night photoperiod. At the 2- to 3 leaf stage, the F_1 families and their

parents were sprayed with the discriminatory glyphosate rate of 0.6 kg ae ha^{-1}

using an overhead compressed air sprayer calibrated to deliver 187 L ha^{-1}. Three

weeks after treatment, plants were phenotyped as dead (susceptible) and alive

(resistant). Preliminary experiments showed that at the glyphosate dose of 0.6 kg

ae ha^{-1}, the susceptible population was 100% controlled, while 90% of the

resistant populations survived at the same dose. Segregation ratios were used to

conduct a goodness of fit test for each F_1 family using chi-square analysis based

on the null hypothesis that the observed ratio and expected ratios were not

different. The null hypothesis was rejected if the test indicated a $P < 0.05$.

Generation of BC families (BC$_1$). The generation of the backcrossed families

(BC$_1$) was performed in the same way as was described for the F_1 families. Two

plants from each of the reciprocal F_1 families that survived the discriminatory rate

of 0.6 kg ae ha^{-1} of glyphosate were retained and transferred to a 2-L plastic pots

containing potting mix soil. Plants from the susceptible population C1 also were

kept until flowering under the same conditions of photoperiod and temperature as

described previously. At maturity, seeds were collected separately from each

reciprocal cross.

Dose-response bioassay for the BC$_1$ families. At the 2-to 3 leaf stage, eight

BC$_1$ families and the original OR1, SF, and C1 populations were treated with

glyphosate at rates of 0, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 kg ae ha^{-1}, using an

overhead compressed air sprayer calibrated to deliver 187 L ha^{-1}. Shoot biomass

was harvested two weeks after treatment, dried at 60 C for 72 h and weighed.

Resistant/susceptible indices were estimated as previously described. The

experiment was conducted once with three replications per rate with each

replication consisting of five individual plants.

Screening test for phenotyping BC$_1$ families. Between 83 and 128 individuals

from each BC$_1$ family and the original parents OR1, SF, and C1 were treated at

the discriminatory glyphosate rate of 0.6 kg ae ha^{-1}. The glyphosate phenotyping

for each BC$_1$ family was determined as previously described. Suceptible:resistant

segregation ratios were used to conduct a goodness of fit test for each family as

previously described.

RESULTS AND DISCUSSION

Dose response bioassay of parents and F1 generations. The glyphosate

dose required to reduce the plant growth by 50% (GR_{50}) was 1.08 kg ae ha^{-1} for

the resistant parent SF and 0.21 kg ae ha^{-1} for the susceptible parent C1 (Table

2.1 and Fig. 2.1).

Table 2.1. Parameter estimates for the nonlinear regression analysis of glyphosate dose response experiments on the basis of aboveground dry weight (% of untreated control) of Italian ryegrass F_1 families formed by the crosses between the glyphosate resistant population SF and the susceptible population C1. Families F_1A corresponds to the cross C1 (female) X SF (male) and families F_1B corresponds to the reciprocal cross SF (female) x C1 (male).

Family	Parameters			GR_{50}[a]	RI[b]
	b (±SE)	c (±SE)	d (±SE)		
C1	1.13 (±0.42)	17.57 (±9.1)	106.8 (±6.21)	0.21 (±0.05)	--
SF	1.78 (±0.68)	41.12 (±11.3)	106.0 (±4.70)	1.08 (±0.32)	5.1
F_1A1	1.53 (±0.37)	14.02 (±12.1)	108.4 (±4.92)	0.79 (±0.22)	3.7
F_1A2	1.65 (±0.48)	21.16 (±7.06)	10.4 (±5.88)	0.46 (±0.10)	2.2
F_1A3	1.27 (±0.49)	23.91 (±8.03)	99.56 (±6.35)	0.27 (±0.07)	1.3
F_1A4	1.86 (±1.14)	28.30 (±9.25)	103.5 (±5.74)	0.25 (±0.07)	1.2
F_1B1	1.54 (±0.48)	10.03 (±10.1)	98.91 (±5.43)	0.73 (±0.15)	3.4
F_1B2	1.27 (±0.35)	16.54 (±8.58)	100.53 (±5.43)	0.41 (±0.11)	1.9
F_1B3	0.72 (±0.27)	13.67 (±9.02)	99.96 (±5.58)	1.0 (±0.16)	4.7
F_1B4	1.83 (±0.60)	25.76 (±5.14)	101.63 (±5.32)	0.23 (±0.04)	1.1

Abbreviations: [a]GR_{50}, rate of glyphosate (kg ae ha^{-1}) required to reduce plant growth by 50%; [b]RI, resistance index on the basis of the ratio between the GR_{50} values from the F_1 families and the GR_{50} value of the population C1.
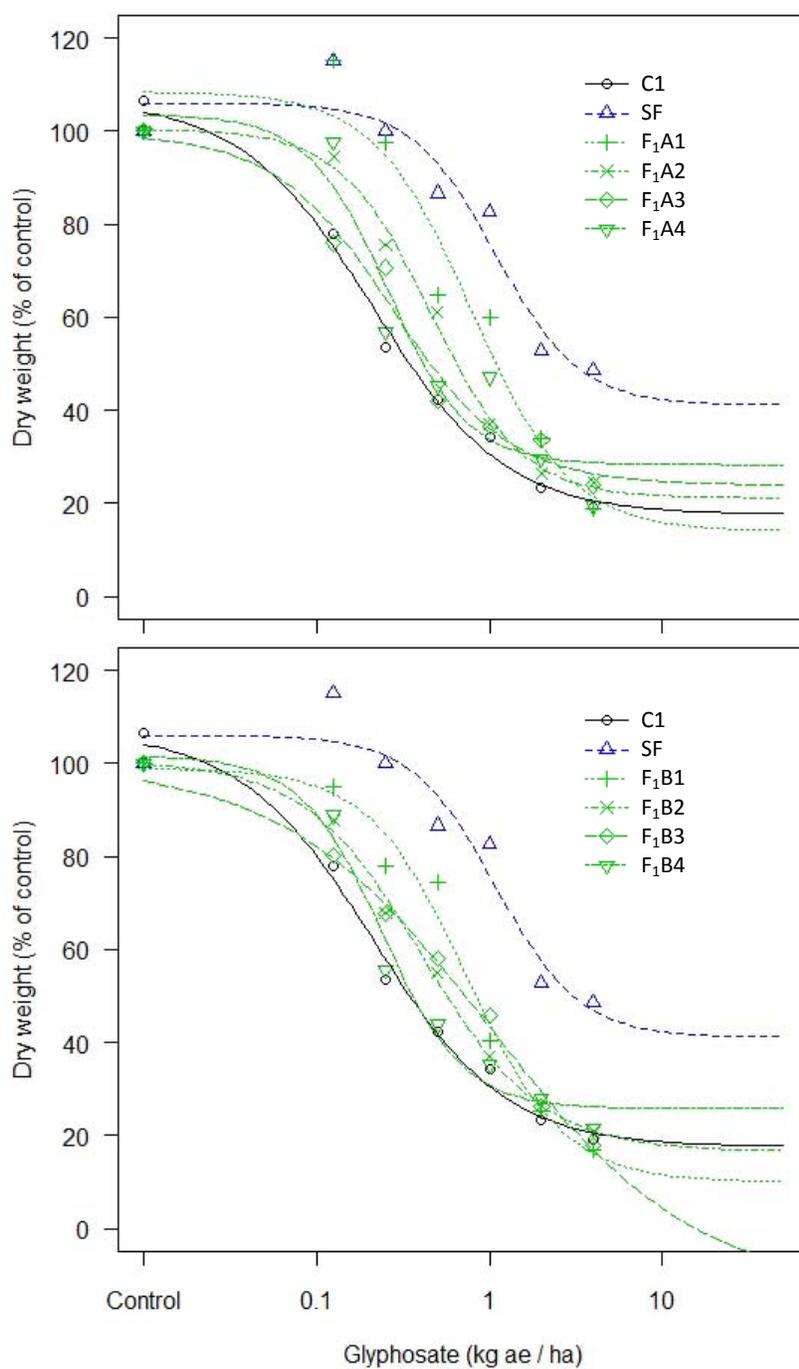
Fig. 2.1. Dry weight (% of untreated control) of glyphosate resistant population SF, susceptible population C1, and the F_1 families of Italian ryegrass. Families F_1A corresponds to the cross C1 (female) x SF (male) and families F_1B corresponds to the reciprocal cross SF (female) x C1 (male).

The resistant/susceptible index (RI) for the resistant parent SF was 5.1. The RI values from the reciprocal crosses between SF and C1 ranged between 1.1 and 4.7. Fifty percent of the eight reciprocal families showed intermediate RI values that ranged between 1.9 and 3.7 (families F_1A1, F_1A2, F_1B1, and F_1B2). Three F_1 families (F_1A3, F_1A4, and F_1B4) had RI values between 1.1 and 1.3, similar to the glyphosate susceptible parent C1, while only one F_1 family (F_1B3) had a RI value of 4.7, closely to the RI value of the glyphosate resistant parent SF. Based on the average response of the reciprocal F_1 families for dose response experiments, it can be concluded that that the resistance in the SF population was nuclear and transferred via pollen.

The results of dose response experiments for the glyphosate resistant population OR1, the susceptible population C1, and the four F_1 families are presented in the Table 2.2 and Figure 2.2. The glyphosate resistant parent OR1 had a RI value of 6.4. The RI values for the F_1 families formed between OR1 and C1 ranged from 1.7 to 5.7. One of the eight reciprocal crosses (family F_1A3) had an RI value of 5.7, similar to the resistant parent OR1. The remainder of the F_1 families had an intermediate response compared with the RI of the resistant and susceptible parents. As was observed in the F_1 families of the SF population, no differences between reciprocal crosses were found, indicating that glyphosate resistance in OR1 is nuclear coded.

Table 2.2. Parameter estimates for the nonlinear regression analysis of glyphosate dose response experiments on the basis of aboveground dry weight (% of untreated control) of Italian ryegrass F_1 families formed by the crosses between the glyphosate resistant population OR1 and the susceptible population C1. Families F_1A corresponds to the cross C1 (female) x OR1 (male) and families F_1B corresponds to the reciprocal cross OR1 (female) x C1 (male).

Family	Parameters			GR_{50}[a]	RI[b]
	b (±SE)	c (±SE)	d (±SE)		
C1	4.04 (±0.83)	15.22 (±2.30)	100.48 (±4.26)	0.17 (±0.01)	--
OR1	1.80 (±0.46)	23.68 (±8.03)	101.46 (±3.42)	1.10 (±0.17)	6.4
F_1A1	1.63 (±0.44)	24.62 (±8.26)	100.97 (±3.62)	0.77 (±0.15)	4.5
F_1A2	1.02 (±0.32)	22.74 (±5.62)	100.85 (±4.06)	0.71 (±0.35)	4.1
F_1A3	1.46 (±0.39)	14.62 (±9.23)	99.06 (±3.83)	0.98 (±0.23)	5.7
F_1A4	1.34 (±0.29)	21.11 (±7.55)	102.67 (±3.90)	0.48 (±0.11)	2.8
F_1B1	1.67 (±0.48)	33.21 (±5.86)	102.50 (±4.68)	0.37 (±0.08)	2.1
F_1B2	1.01 (±0.29)	9.91 (±6.37)	101.05 (±4.93)	0.68 (±0.31)	4.0
F_1B3	1.52 (±0.43)	29.92 (±5.65)	100.94 (±4.95)	0.30 (±0.06)	1.7
F_1B4	1.57 (±0.43)	25.62 (±8.44)	104.10 (±4.93)	0.54 (±0.13)	3.1

Abbreviations: [a]GR_{50}, rate of glyphosate (kg ae ha^{-1}) required to reduce plant growth by 50%; [b]RI, resistance index on the basis of the ratio between the GR_{50} values from the F_1 families and the GR_{50} value of the population C1.

22
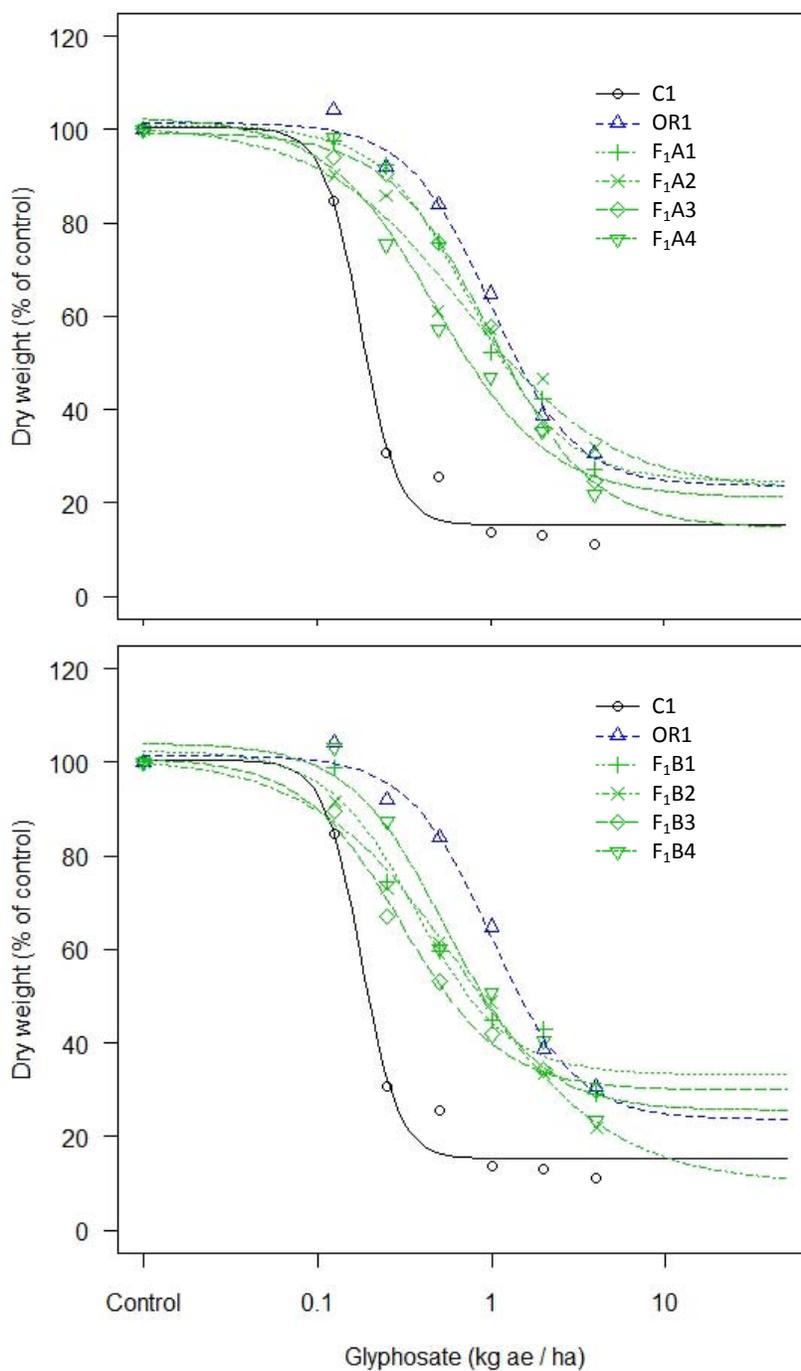
Fig. 2.2. Dry weight (% of untreated control) of the glyphosate resistant population OR1, susceptible population C1, and the F_1 families of Italian ryegrass. Families F_1A corresponds to the cross C1 (female) x OR1 (male) and families F_1B corresponds to the reciprocal cross OR1 (female) x C1 (male).

Phenotyping F$_1$ families. No survivors were recorded in the susceptible

population C1, indicating that the discriminatory rate of 0.6 kg ae ha^{-1} of

glyphosate was effective to obtain 100% control of the susceptible population C1.

However, almost 10% of the individuals from the glyphosate resistant population

SF were susceptible to the same discriminatory rate (Table 2.3).

Table 2.3. Susceptible:resistant segregation ratios and chi-square analysis for glyphosate resistant Italian ryegrass in the F$_1$ family formed by the cross between SF and C1 21 days after treatment with 0.6 kg ae ha^{-1} of glyphosate.

Family	Phenotype		Total	X^2 (1:1)	Probability
	Susceptible	Resistant			
	-------------No. plants-----------				
C1	128	0	128		
SF	11	105	116		
F$_1$A1	54	64	118	0.84	0.36
F$_1$A2	64	52	116	1.24	0.26
F$_1$A3	63	56	119	0.41	0.52
F$_1$A4	79	29	108	23.14	>0.001
F$_1$B1	68	52	120	2.13	0.14
F$_1$B2	39	78	117	13	>0.001
F$_1$B3	50	64	114	1.72	0.19
F$_1$B4	58	44	102	1.92	0.16

Parental cross F$_1$A: C1 (female) x SF (male); Parental cross F$_1$B: SF (female) x C1 (male).

Despite the fact that the rate of glyphosate used during the selection

cycles was 1 kg ae ha^{-1} and only healthy individuals were selected to develop the

next generation, the population was still segregating. The two selection cycles of

the glyphosate resistant population SF were not enough to obtain a homozygous population for the glyphosate resistance trait. This could explain the variation observed in the F_1 families during dose response experiments.

Chi-square analyses of the four reciprocal F_1 families formed between SF and C1 had susceptible:resistant segregation ratios of 1:1 in six of eight reciprocal crosses. Two families (F_1A4 and F_1B2) had frequencies that differed from the expected 1:1 ratio, which may have been influenced by the initial selection of heterozygous resistant individuals as parents to form these families. No maternal effects were found. Similar variations in the frequency of resistant:susceptible ratios among F_1 families were observed by Lorraine et al. (2001) and Wakelin and Preston (2006) in glyphosate resistant rigid ryegrass populations. These variations were mostly attributed to the heterogeneous response of the resistant parent populations in terms of level of resistance. Response to the glyphosate rate of 0.6 kg ae ha^{-1} and chi-square analyses of the F_1 families formed between OR1 and C1 are presented in Table 4. There were no survivors recorded in the susceptible parent C1, however, 128 individuals in the glyphosate resistant parent OR1 survived to the discriminatory rate of 0.6 kg ae ha^{-1}. These results suggest that the selection of homozygous individuals in the population OR1 was more efficient than the selection of homozygous individuals in the population SF. Based on this initial observation, more consistent results for susceptible:resistant ratios of the F_1 families would be expected. However, the segregation ratios in the eight reciprocal F_1 families were

different from the results obtained in dose response experiments. Only three of

eight reciprocal families fit the susceptible:resistant ratio of 1:1, while the rest of

the F_1 families segregated in a variable pattern. Nonetheless, there was a high

proportion of resistant individuals in almost all the reciprocal F_1 families. These

results confirm that the genetic control of glyphosate resistance due to reduced

herbicide translocation OR1 is in the nuclear genome with no evidence of

maternal effects.

Table 2.4. Susceptible:resistant segregation ratios and chi-square analysis for glyphosate resistant Italian ryegrass in the F_1 generation from the cross between OR1 and C1, 21 days after treatment with 0.6 kg ae ha^{-1} of glyphosate.

Family	Phenotype		Total	X^2 (1:1)	Probability
	Susceptible	Resistant			
	------------No. plants-----------				
C1	128	0	128		
OR1	0	128	128		
F_1A1	28	93	121	34.91	>0.001
F_1A2	81	36	117	17.3	>0.001
F_1A3	53	55	108	0.03	0.86
F_1A4	24	85	109	34.13	>0.001
F_1B1	42	76	118	9.79	>0.001
F_1B2	69	53	122	2.09	0.14
F_1B3	63	61	124	0.03	0.86
F_1B4	46	77	123	7.81	>0.001

Parental cross F_1A: C1 (female) x OR1 (male); Parental cross F_1B: OR1 (female) x C1 (male).

Dose response bioassay of BC families. To determine the number of genes involved in both glyphosate resistant populations, eight BC_1 families were formed for each glyphosate resistant population. Dose response results for the backcross populations including the original glyphosate resistant parent SF and the susceptible parent C1 are presented in Table 2.5 and Figure 2.3. As was observed in the F_1 generation, the GR_{50} for the BC_1 families showed variation, with values that ranged between 0.22 and 1.08 kg ae ha^{-1} whereas the original parents SF and C1 had GR_{50} values of 1.35 and 0.19 kg ae ha^{-1}, respectively. Three of the 16 BC_1 reciprocal families had RI values similar to the susceptible parent C1 (1.1 for BC_1B3 and 1.2 for BC_1A2 and BC_1A8). As was observed in the F_1 generation, no maternal effects were observed in the BC families, confirming that the glyphosate resistant trait resides in the nuclear genome and it can be transferred via pollen.

Dose response results of the backcrossed families involving the population OR1 are presented in Table 6 and Figure 4. As was observed in the F_1 dose response experiments, the GR_{50} values among the BC_1 generations varied. Four BC_1 families had RI values similar to the susceptible parent C1 (BC_1A4, BC_1A5, BC_1A8, BC_1B1, and BC_1B7). The backcross generation BC_1B1 had a RI value lower than the susceptible parent C1 (0.8), whereas BC_1A1 had a RI of 4.0. Ten of the 16 BC families, had intermediate RI values that ranged from 1.7 to 3.7.

Table 2.5. Parameter estimates for the nonlinear regression analysis of glyphosate dose response experiments on the basis of aboveground dry weight (% of untreated control) of Italian ryegrass BC_1 families formed by the crosses between glyphosate resistant F_1 and the susceptible C1 for the SF resistant population. Families BC_1A corresponds to the cross C1 (female) x F_1 (male) and families BC_1B corresponds to the reciprocal cross F_1 (female) x C1 (male).

Family	Parameters			GR_{50}[a]	RI[b]
	b (±SE)	c (±SE)	d (±SE)		
C1	1.06 (±0.39)	15.08 (±7.2)	99.93 (±5.60)	0.19 (±0.03)	--
SF	1.51 (±0.72)	22.30 (±19.7)	99.99 (±5.74)	1.35 (±0.55)	7.1
BC_1A1	1.58 (±0.59)	30.67 (±9.12)	99.80 (±5.55)	0.65 (±0.17)	3.4
BC_1A2	3.95 (±2.9)	36.70 (±3.2)	99.99 (±5.60)	0.23 (±0.03)	1.2
BC_1A3	2.04 (±0.72)	26.75 (±5.7)	99.76 (±5.34)	0.49 (±0.08)	2.5
BC_1A4	2.37 (±0.83)	36.16 (±5.16)	100.0 (±5.36)	0.54 (±0.09)	2.8
BC_1A5	0.67 (±0.43)	28.96 (±4.98)	100.2 (±5.55)	1.08 (±0.60)	5.6
BC_1A6	0.85 (±0.40)	23.96 (±4.89)	99.88 (±5.61)	0.89 (±0.08)	4.6
BC_1A7	1.87 (±0.69)	32.33 (±6.09)	99.92 (±5.59)	0.45 (±0.09)	2.3
BC_1A8	1.27 (±0.64)	19.9 (±8.0)	100.0 (±5.59)	0.23 (±0.08)	1.2
BC_1B1	1.89 (±0.64)	28.06 (±5.19)	99.51 (±5.98)	0.36 (±0.06)	1.9
BC_1B2	1.01 (±0.64)	14.92 (±4.53)	99.76 (±5.91)	0.43 (±0.18)	2.2
BC_1B3	0.79 (±0.62)	17.07 (±9.59)	100.01 (±5.88)	0.22 (±0.11)	1.1
BC_1B4	1.68 (±0.59)	28.97 (±5.93)	99.57 (±5.95)	0.36 (±0.07)	1.9
BC_1B5	3.49 (±1.46)	39.24 (±4.47)	101.44 (±5.57)	0.44 (±0.05)	2.3
BC_1B6	1.64 (±0.68)	29.67 (±9.13)	99.82 (±5.88)	0.59 (±0.15)	3.1
BC_1B7	1.49 (±0.58)	22.11 (±9.12)	100.61 (±5.77)	0.43 (±0.11)	2.2
BC_1B8	1.27 (±0.49)	16.79 (±13.02)	99.60 (±5.94)	0.61 (±0.20)	3.2

Abbreviations: [a]GR_{50}, rate of glyphosate (kg ae ha^{-1}) required to reduce plant growth by 50%; [b]RI, resistance index on the basis of the ratio between the average of the GR_{50} values from the BC_1 families and the GR_{50} value of the population C1.
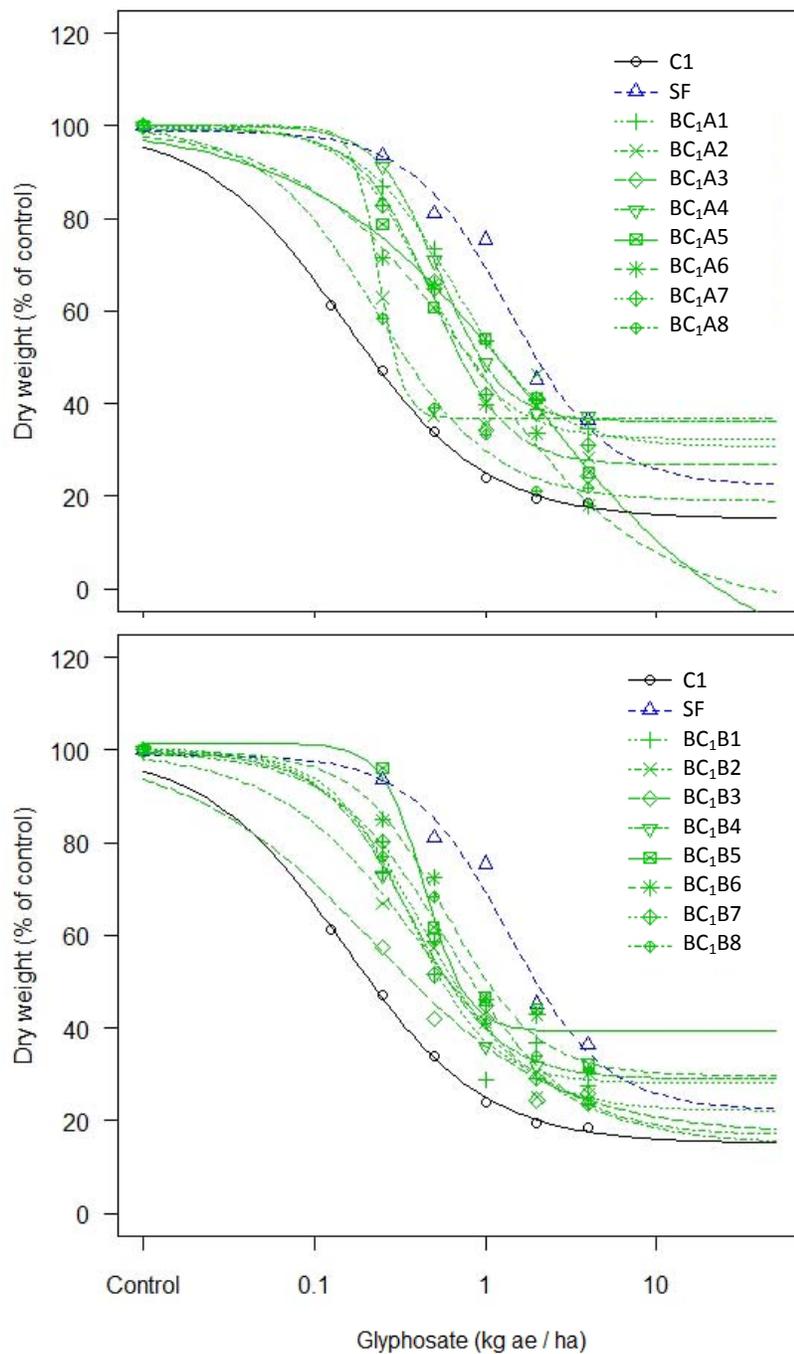
Fig. 2.3. Dry weight (% of untreated control) of the parents SF and C1, and the BC$_1$ families of Italian ryegrass. Families BC$_1$A corresponds to the cross C1 (female) x F$_1$ (male) and families BC$_1$B corresponds to the reciprocal cross F$_1$ (female) x C1 (male).

Table 2.6. Parameter estimates from the nonlinear regression analysis of glyphosate dose response experiments on the basis of aboveground dry weight (% of untreated control) of Italian ryegrass BC_1 families formed by the crosses between glyphosate resistant F_1 and the susceptible C1 for the OR1 resistant population. Families BC_1A corresponds to the cross C1 (female) x F_1 (male) and families BC_1B corresponds to the reciprocal cross F_1 (female) x C1 (male).

Family	Parameters			GR_{50}[a]	RI[b]
	b (±SE)	c (±SE)	d (±SE)		
C1	1.76 (±0.5)	21.96 (±4.1)	99.90 (±5.7)	0.16 (±0.02)	--
OR1	1.85 (±0.4)	17.68 (±8.8)	103.07 (±4.4)	0.89 (±0.15)	5.5
BC_1A1	3.89 (±1.1)	42.21 (±4.8)	110.23 (±3.4)	0.64 (±0.09)	4.0
BC_1A2	16.71 (±5.3)	37.35 (±3.3)	98.04 (±3.3)	0.50 (±0.02)	3.1
BC_1A3	3.86 (±1.6)	25.07 (±4.4)	97.39 (±3.9)	0.60 (±0.06)	3.7
BC_1A4	2.54 (±0.6)	9.1 (±3.5)	100.24 (±5.6)	0.20 (±0.02)	1.2
BC_1A5	4.35 (±1.3)	14.21 (±3.0)	100.75 (±5.6)	0.19 (±0.01)	1.1
BC_1A6	3.96 (±2.8)	33.3 (±3.9)	97.92 (±3.3)	0.49 (±0.20)	3.0
BC_1A7	3.84 (±1.4)	14.26 (±3.3)	98.62 (±4.4)	0.45 (±0.03)	2.8
BC_1A8	3.24 (±0.9)	19.9 (±8.0)	100.88 (±5.6)	0.20 (±0.01)	1.2
BC_1B1	2.20 (±0.6)	7.71 (±3.2)	100.04 (±5.1)	0.13 (±0.01)	0.8
BC_1B2	2.44 (±0.5)	16.7 (±3.6)	100.29 (±4.6)	0.32 (±0.03)	2.0
BC_1B3	1.95 (±0.6)	26.61 (±4.9)	97.65 (±5.2)	0.45 (±0.07)	2.8
BC_1B4	2.07 (±0.4)	15.7 (±3.9)	101.46 (±4.8)	0.28 (±0.03)	1.7
BC_1B5	2.24 (±0.5)	16.62 (±3.9)	101.12 (±4.7)	0.31 (±0.03)	1.9
BC_1B6	1.99 (±0.8)	37.52 (±5.4)	98.93 (±5.1)	0.37 (±0.07)	2.3
BC_1B7	1.41 (±0.3)	13.85 (±5.6)	100.5 (±5.0)	0.22 (±0.04)	1.3
BC_1B8	2.36 (±0.6)	25.53 (±3.9)	98.98 (±4.76)	0.37 (±0.05)	2.3

Abbreviations: [a]GR_{50}, rate of glyphosate (kg ae ha^{-1}) required to reduce plant growth by 50%; [b]RI, resistance index on the basis of the ratio between the GR_{50} values from the BC_1 families and the GR_{50} value of the population C1.
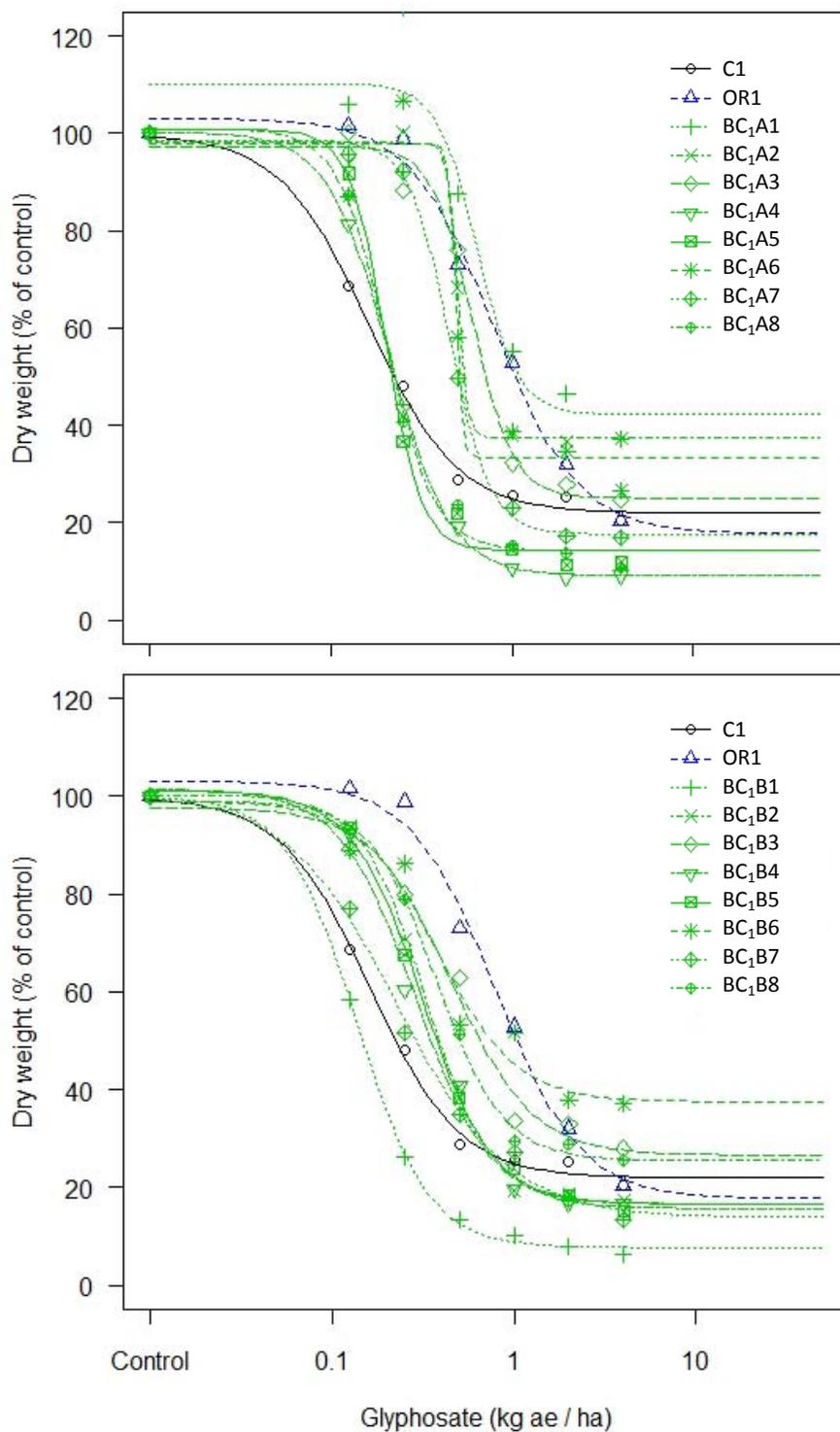
Fig. 2.4. Dry weight (% of untreated control) of the parents OR1 and C1, and the BC$_1$ families of Italian ryegrass. Families BC$_1$A correspond to the cross C1 (female) x F$_1$ (male) and families BC$_1$B correspond to the reciprocal cross F$_1$ (female) x C1 (male).

Phenotyping BC$_1$ families. Susceptible:resistant segregation ratios and chi-

square analyses for the BC$_1$ families involving the glyphosate resistant

population SF are presented in Table 2.7.

Table 2.7. Susceptible:resistant segregation ratios and chi-square analysis for glyphosate resistant Italian ryegrass in the BC$_1$ generation formed by the cross between glyphosate resistant F$_1$ and susceptible C1 (21 days after treatment with 0.6 kg ae ha^{-1} of glyphosate) for the SF glyphosate resistant population.

BC$_1$ Family	Phenotype		Total	X^2 (1:1)	Probability
	Susceptible	Resistant			
	---------------No. plants--------------				
C1	124	0	124		
SF	11	83	94		
BC$_1$A1	55	60	115	0.21	0.64
BC$_1$A2	52	66	118	1.66	0.19
BC$_1$A3	76	41	117	10.47	>0.001
BC$_1$A4	72	40	112	9.14	>0.001
BC$_1$A5	42	76	118	9.79	>0.001
BC$_1$A6	42	48	90	0.40	0.52
BC$_1$A7	60	55	115	0.21	0.64
BC$_1$A8	65	52	117	1.44	0.23
BC$_1$B1	81	39	120	14.7	>0.001
BC$_1$B2	57	55	112	0.03	0.86
BC$_1$B3	80	44	124	10.45	>0.001
BC$_1$B4	78	41	119	11.50	>0.001
BC$_1$B5	52	68	120	2.13	0.14
BC$_1$B6	40	78	118	12.23	>0.001
BC$_1$B7	73	40	113	9.63	>0.001
BC$_1$B8	59	61	120	0.03	0.86

Parental cross BC$_1$A: C1 (female) x F$_1$ (male); Parental cross BC$_1$B: F$_1$ (female) x C1 (male).

The original glyphosate resistant population SF segregated with a high percentage of susceptible individuals (~ 12%). Fifty percent of the 16 reciprocal BC_1 generations fit the susceptible:resistant segregation ratio of 1:1. Even though half of the BC_1 generations deviated from the expected 1:1 ratios, they showed the same trend (2:1 or 1:2). Similar results were reported by Lorraine-Colwill et al. (2001) for a glyphosate resistant rigid ryegrass population from Australia, where inheritance studies revealed susceptible:resistant segregation ratios of 1:1 and 1:2 for the backcross generation F_1 x S at the glyphosate rate of 0.338 kg ae ha^{-1}. These 1:2 ratios were attributed to the degree of dominance of the alleles involved in the resistance. We conclude that the glyphosate resistance due to target site mutation in the Italian ryegrass population SF is likely inherited as a single, partially dominant nuclear gene.

Contrary to what was observed in dose response experiments, susceptible:resistant ratios involving the OR1 population showed a different trend (Table 8). Only one of 16 reciprocal BC_1 families fit the expected segregation ratio of 1:1. The rest of the BC families had susceptible:resistant ratios of 3:1, 4:1; 5:1, and 16:1 without a clear trend between the A and B reciprocal crosses. Similar results were reported by Simarmata et al. (2005) for a glyphosate resistant rigid ryegrass population from California. Dose response results in the F_2 generations revealed a higher proportion of susceptible individuals based on the GR_{50} values, suggesting that the resistance trait was nuclear, partially dominant and multigenic.

Table 2.8. Susceptible:resistant segregation ratios and chi-square analysis for glyphosate resistant Italian ryegrass in the BC_1 generation formed by the cross between glyphosate resistant F_1 and susceptible C1 (21 days after treatment with 0.6 kg ae ha^{-1} of glyphosate) for the OR1 glyphosate resistant population.

BC_1 Family	Phenotype		Total	X^2 (1:1)	Probability
	Susceptible	Resistant			
	-----------------No. plants----------------				
C1	125	0	125		
OR1	0	105	105		
BC_1A1	86	29	115	28.27	>0.00001
BC_1A2	112	4	116	100.55	>0.00001
BC_1A3	87	29	116	29.0	>0.00001
BC_1A4	107	7	114	87.71	>0.00001
BC_1A5	91	13	104	58.5	>0.00001
BC_1A6	81	21	102	35.29	>0.00001
BC_1A7	60	23	83	16.49	>0.00001
BC_1A8	55	50	105	0.23	0.62
BC_1B1	74	33	107	15.71	>0.00001
BC_1B2	107	4	111	95.57	>0.00001
BC_1B3	105	20	125	57.8	>0.00001
BC_1B4	107	20	127	59.59	>0.00001
BC_1B5	91	17	108	50.70	>0.00001
BC_1B6	100	24	124	46.58	>0.00001
BC_1B7	114	14	128	78.125	>0.00001
BC_1B8	100	28	128	40.5	>0.00001

Parental cross BC_1A: C1 (female) x F_1 (male); Parental cross BC_1B: F_1 (female) x C1 (male).

However, Preston and Wakelin (2008) reported that the glyphosate

resistance trait of rigid ryegrass populations with glyphosate resistance due to

reduced herbicide translocation was apparently controlled by a major single dominant or partially dominant nuclear gene.

The differential response observed in both glyphosate resistant populations between dose response and phenotyping studies would be attributed to the genetic diversity that can be found among individuals of the population. Indeed, the accuracy of both approaches seems to be strongly conditioned by the homozygous level of the parental populations and the adequate selection of the discriminatory rate for phenotyping studies. Due to the outcrossing nature of Italian ryegrass, it would be expected that a high number of heterozygous individuals carry the resistant trait. The presence of more than 10% of susceptible individuals in the population SF after two selection cycles is a consistent evidence that the population is still segregating. Segregation would explain the deviation of 2:1 or 1:2 in the BC phenotyping screening for that population. In the case of the OR1 population, the differences between dose response and phenotyping experiments were even more evident. Under this analysis and based on the results presented using both approaches, the authors consider that segregation ratios using chi-square analysis likely to be more consistent than dose response experiments. Consequently, based on the results of susceptible:resistant ratios we conclude that the glyphosate resistant trait in OR1 likely to be multigenic, because only one of the 16 BC families fit model for one single gene.

Glyphosate resistance due to reduced herbicide translocation is a complex mechanism that is not well understood (Shaner 2009). Previous studies on inheritance involving this mechanism of resistance are contradictory (Simarmata et al. 2005; Preston and Wakelin 2008).

In summary, segregation of resistance in the F_1 and BC_1 families indicated that the glyphosate resistance trait conferred by a target site mutation in SF is likely controlled by a single, nuclear and partially dominant gene. However, for OR1, a consistent pattern of inheritance could not be elucidated. The results of susceptible:resistant segregation ratios suggests that the trait may be quantitative. Further analyses including molecular tools such as quantitative trait loci (QTL) mapping can be helpful to identify number, contribution, and genomic location of the genes conferring glyphosate resistance due to reduced herbicide translocation in the population OR1.   The absence of differences in the reciprocal crosses in F_1 and BC_1 families suggest that the genetic control of glyphosate resistance involving the two different mechanisms of resistance is in the nuclear genome.

Pollen flow in outcrossing species is important to promote genetic variability but it also can potentially disperse undesirable traits such as herbicide resistance. Busi et al. (2008) reported the introgression of ALS herbicide resistance genes via gene flow in an experiment conducted at landscape level in rigid ryegrass. Mendelian and molecular analyses confirmed that that pollen-mediated gene flow carrying sulfometuron resistance due to target site mutation

occurred even at the maximum distance tested of 3000 m from the source. The partially dominant and nuclear encoded glyphosate resistance trait due to target site mutation in the population SF ensures its spread via pollen. In addition, considering that Italian ryegrass is a wind-pollinated, outcrossing and self-incompatible species, there can be further outcrossing with compatible species such as perennial ryegrass (*L. perenne* L.), rigid ryegrass (*L. rigidum* L.), and fescues (*Festuca* spp.). Therefore, the rate of spread glyphosate resistant trait due to target site mutation may be greater than the glyphosate resistance trait conferred by reduced herbicide translocation in Italian ryegrass. Moreover, based on the results obtained in the backcross progenies, the genes controlling glyphosate resistance due to reduced translocation in the population OR1 may be lost via outcrossing with susceptible relatives. In fact, Ghersa et al. (1994) observed that the inclusion of susceptible individuals into a diclofop resistant Italian ryegrass population decreased the proportion of resistant individuals in the population if there was no selection with diclofop. Similarly, Preston and Wakelin (2008) reported that the frequency of glyphosate resistant individuals in a rigid ryegrass population from Australia declined to 5% after three generations of outcrossing with a susceptible population under natural conditions with no selection pressure from glyphosate.

These results support the importance of knowing not only the mechanism of herbicide resistance but also inheritance. These results may be helpful to

design the most suitable weed management strategy to reduce the impact of

herbicide resistant weeds in the long term.

Source of materials

[1] Sunshine Mix 1 Potting Mix, Sun Gro Horticulture, Inc., 110th Ave. NE, Suite 490, Bellevue, WA 98004.

[2] PowerMax® 660, 540 g ae L^{-1}, Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167.

[3] Generation III Spray Chamber, De Vries Manufacturing, 86956 State HWY 251, Hollandale, MN 56054.

[4] R statistical software, R development core team, http://www.r-project.org/. 63103 USA.

[5] Seedburo Equipment Co. 2293 S. Mt. Prospect Road, Des Plaines, IL 60018.

.

Literature Cited

Beddows, A. 1973. Biological flora of the British Isles, *Lolium multiflorum* Lam. J. of Ecology 61:587-600.

Boutsalis, P. and S. B. Powles. 1995. Inheritance and mechanism of resistance to herbicides inhibiting acetolactate synthase *in Sonchus oleraceus* L. Theor. Appl. Genet. 91:242-247.

Busi, R., Q. Yu, R. Barret-Lennard, and S. Powles. 2008. Long distance pollen-mediated flow of herbicide resistance genes in *Lolium rigidum*. Theor. Appl. Genet. 117:1281-1290.

Chauvel, B. and J. Gasquez. 1994. Relationship between genetic polymorphism and herbicide resistance within *Alopecurus myosuroides* Huds. Heredity 73:336-344.

Davis, V. M., G. R. Krueger, S. G. Hallett, P. J. Tranel, and W. G. Johnson. 2010. Heritability of glyphosate resistance in Indiana horseweed (*Conyza canadensis*) populations. Weed Sci. 58:30-38.

Feng, P., M. Tran, D. Sammons, G. Heack, and C. Cajacob. 2004. Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation and metabolism. Weed Sci.52:498-505.2004.

Franz, J., M. Mao, and J. Sikorski. 1997. Glyphosate a unique global herbicide. *ACS Monograph No. 189*. American Chemical Society, Washington, DC, 653 pp.

Gaines T. A., W. Zhang, D. Wang, B. Bukun, S.T. Chisholm, D. L. Shaner, S. J. Nissen, W. L. Patzoldt, P. J. Tranel, A. S. Culpepper, T. L. Grey, T. M. Webster, W. K. Vencill, R. D. Sammons, J. Jiang, C. Preston, J. E. Leach, and P. Westra. 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. PNAS 107:1029-1034.

Ghersa, C. M., M. A. Martinez-Ghersa, T. G. Brewer, and M. L. Roush. 1994. Use of gene flow to control diclofop-methyl resistance in Italian ryegrass (*Lolium multiflorum*). Weed Tech. 8:139-147.

Heap, I. 2011. International Survey of Herbicide Resistant Weeds. http://www.weedscience.org. Accessed: June 2, 2011.

Hoskins, A., B. Young, R. Krausz, and J. Russin. 2005. Control of Italian ryegrass (*Lolium multiflorum*) in winter wheat. Weed Technol. 19:261-265.

Jaseniuk, M., R. Ahmad, A. Sherwood, J. Firestone, A. Perez-Jones, T. Lanini, C. Mallory-Smith, and Z. Stednick. 2008. Glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in California: distribution, response to glyphosate, and molecular evidence for an altered target enzyme. Weed Sci. 56:496-502.

Knezevic, S., J. Streibig, and C. Ritz. 2007. Utilizing R software for dose-response studies: the concept and data analysis. Weed Technol.21:840-848.

Letouze, A. and J. Gasquez. 2001. Inheritance of fenoxaprop-P-ethyl resistance in a blackgrass (*Alopecurus myosuroides* Huds.) population. Theor. Appl. Genet. 103:288-296.

Lorraine-Colwill, D., S. Powles, T. Hawkes, and C. Preston. 2001. Inheritance of evolved glyphosate resistance in *Lolium rigidum* (Gaud.). Theor. Appl. Genet 102:545-550.

Mallory-Smith, C. A, D. C. Thill, M. J. Dial, and R. S. Zemetra. 1990. Inheritance of sulfonylurea herbicide resistance in *Lactuca* spp. Weed Technol. 4:787-790.

McKell, C., C. Duncan, and C. Muller. 1969. Competitive relationship of annual ryegrass (*Lolium multiflorum* Lam.) Ecology 50:653-657.

Moss, S., C. Cocker, A. Brown, L. Hall, and L. Field. 2003. Characterization of target site resistance to ACCase-inhibiting herbicides in the weed *Alopecurus myosuroides* (black grass). Pest. Manag. Sci., 59:190-201.

Nandula, V., K. Reddy, D. Poston, A. Rimando, and S. Duke. 2008. Glyphosate tolerance mechanism in Italian ryegrass (*Lolium multiflorum*) from Mississippi. Weed Sci. 56:344-349.

Perez, A. and M. Kogan. 2003. Glyphosate-resistant *Lolium multiflorum* in Chilean orchards. Weed Res. 43, 12-19.

Perez-Jones, A., K-W. Park, J. Colquhoun, C. Mallory-Smith, and D. Shaner. 2005. Identification of glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in Oregon. Weed Sci. 53:775-779.

Perez-Jones, A., K-W. Park, N. Polge, J. Colquhoun, and C. Mallory-Smith. 2007. Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. Planta 226:395-404.

Powles, S. and C. Preston. 2006. Evolved glyphosate resistance in plants: Biochemical and genetic basis of resistance. Weed Technol. 20:282-289.

Preston, C. and A. Wakelin. 2008. Mini-review: Resistance to glyphosate from altered herbicide translocation patterns. Pest Manag Sci. 64:372-376.

Saari, L., J. C. Cotterman, and D. C. Thill. 1994. Resistance to acetolactate synthase inhibiting herbicides. Pages 83-140, *in* S. B. Powles and J. A. M. Holtum, eds. Herbicide Resistance in Plants: Biology and Biochemistry. Boca Raton, FL: Lewis Publishers.

Shaner, D. L. 2009. Role of translocation as a mechanism of resistance to glyphosate. Weed Sci. 57:118-123.

Simarmata, M., S. Bughrara, and D. Penner. 2005. Inheritance of glyphosate resistance in rigid ryegrass (*Lolium rigidum*) from California. Weed Sci. 53:615-619.

Streibig, J. C., M. Rudemo, and J. E. Jensen. 1993. Dose–response curves and statistical models. Pages 29–56 in J. C. Streibig and P. Kudsk, eds. Herbicide Bioassays. Boca Raton, FL: CRC.

Tal, A. and B. Rubin. 2004. Molecular characterization and inheritance of resistance to ACCase-inhibiting herbicides in *Lolium rigidum*. Pest. Manag Sci. 60:1013-1018.

Tucker, K., G. Morgan, S. Senseman, T. Miller, and P. Bauman. 2006. Identification, distribution and control of Italian ryegrass (*Lolium multiflorum*) ecotypes with varying levels of sensitivity to triasulfuron in Texas. Weed Technol. 20: 745-750.

Volenberg, D., E. Stoltenberg, and C. Boerboom. 2001. Biochemical mechanism and inheritance of cross-resistance to acetolactate synthase inhibitors in giant foxtail. Weed Sci. 49:635-641

Volenberg, D. and E. Stoltenberg. 2002. Inheritance of resistance in eastern black nightshade (*Solanum nigrum*) to acetolactate synthase inhibitors. Weed Sci. 50:731-736.

Wakelin, A. and C. Preston. 2006. Inheritance of glyphosate resistance in several populations of rigid ryegrass (*Lolium rigidum*) from Australia. Weed Sci.54:212-219.

Woodburn, A. T. 2000. Glyphosate: production, pricing and use worldwide. Pest Manag. Sci. 56:309–312.

Yu, Q., A. Cairns, and S. Powles. 2007. Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a *Lolium rigidum* biotype. Planta 225:499-513.

Zelaya, I., M. Owen, and M. VanGessel. 2007. Transfer of glyphosate resistance: evidence of hybridization in *Conyza* (Asteraceae). Am. J. of Botany 94:660-673.

CHAPTER 3: Glyphosate-Resistant Italian Ryegrass (*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot) Populations also Exhibit Resistance to Glufosinate

Wilson V. Avila-Garcia and Carol Mallory-Smith

Wilson V. Avila-Garcia and Carol Mallory-Smith. Department of Crop and Soil Science, Oregon State University, 107 Crop Science Bldg., Corvallis, OR 97331.

ABSTRACT

Resistance to glufosinate has been confirmed in glyphosate-resistant Italian ryegrass populations collected in hazelnut orchards in Oregon. Dose–response, ammonia accumulation, and enzyme activity studies were conducted to test the sensitivity of three glyphosate resistant and three susceptible Italian ryegrass populations to glufosinate. The glufosinate rates required to reduce the growth by 50% (GR_{50}) were 0.15, 0.18, and 0.21 for the control populations C1, C2, and C3, respectively, whereas for the resistant populations OR1, OR2, and OR3, the GR_{50} values were 0.49, 0.42, and 0.40 kg ai ha^{-1}, respectively, exhibiting an average resistance index of 2.4. The same trend was observed in ammonia accumulation studies between 48 and 96 h after glufosinate treatment where the susceptible populations accumulated on average two times more ammonia than the resistant populations. The glufosinate concentration required to reduce the glutamine synthetase enzyme activity by 50% (I_{50}) was not different for the resistant and susceptible populations. The I_{50}'s ranged from 3.1 to 3.6 μM for the resistant populations and from 3.7 to 4.3 μM for the susceptible populations; therefore, an insensitive target site is not responsible for the glufosinate resistance.

Nomenclature: Glufosinate; glyphosate; Italian ryegrass, *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot LOLMU; hazelnut*, Corylus avellana* L.

Key words: Glutamine synthetase, ammonia accumulation, herbicide resistance.

INTRODUCTION

Glufosinate ammonium is a nonselective broad-spectrum herbicide that is used POST in orchards, vineyards, and glufosinate-resistant (Liberty-Link®) crops such as canola (*Brassica napus* L.), corn (*Zea mays* L.), and soybean (*Glycine max* L. Merr.) (Culpepper et al. 2000; Jones et al. 2001). Glufosinate is a potent inhibitor of the enzyme glutamine synthetase (GS), which plays a major role in the pathway that assimilates inorganic nitrogen into organic compounds and ammonia assimilation derived from nitrate reduction and photorespiration (Ray 1989). Inhibition of GS activity leads to a rapid accumulation of high levels of ammonia due to a lack of nitrogen metabolism, as well as depletion of the amino acid glutamine. As a consequence, excess ammonia in the plant causes reduction in photosynthetic activity, disruption of chloroplastic structure, stroma vesiculation, and glyoxylate accumulation, causing inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase and carbon fixation (Devine et al. 1993; Manderscheid 1993; Tachibana et al. 1986). Ammonia accumulation in plants treated with glufosinate has been used widely as a biochemical marker of GS inhibition (Pornprom et al. 2003; Sankula et al. 1998; Tsai et al. 2006).

Although glufosinate is a nonselective herbicide, there are reports that describe different patterns of sensitivity to glufosinate in weed species (Everman et al. 2009a, 2009b; Skora-Neto et al. 2000). Differential responses in sensitivity to glufosinate have been attributed to three main mechanisms: altered uptake, reduced translocation, and metabolism (Pline et al. 1999; Skora-Neto et al. 2000;

Steckel et al. 1997). Recently field and greenhouse dose–response experiments confirmed a 3.4-fold difference between susceptible and resistant biotypes of goosegrass (*Eleusine indica*) biotype from Malaysia (Jalaludin et al. 2010; Seng et al. 2010).

Glyphosate resistance has been identified in over 21 weed species (Heap 2011), and the most frequently observed mechanism has been limited translocation. Limited translocation has been identified in horseweed (*Conyza canadensis*) (Koger and Reddy 2005), hairy fleabane (*Conyza bonariensis*) (Dinelli et al. 2008), rigid ryegrass (*Lolium rigidum*) (Wakelin et al. 2004; Wakelin and Preston 2006), and Italian ryegrass (*Lolium multiflorum*) (Perez et al. 2004; Perez-Jones et al. 2007).

Italian ryegrass is a widely used forage grass in temperate regions of the world and also is a competitive weed in orchards and crops in the United States (Hoskins et al. 2005; Tucker et al. 2006). The control of Italian ryegrass in orchards is frequently based on the intensive use of glyphosate. As a consequence of the intensive use of glyphosate, seven Italian ryegrass populations have been confirmed to be glyphosate resistant in Oregon. The populations were under glyphosate selection for at least 10 yr with two to three glyphosate applications per year. The glyphosate resistance indices (RI) in these populations ranged from 2.8 to 6.8 (Perez-Jones et al. 2005, 2007).

In 2009, three of the glyphosate-resistant Italian ryegrass populations collected from hazelnut orchards in Oregon were screened using commercial

rates of clethodim, glufosinate, imazamox, paraquat, pinoxaden, quizalofop, and pyroxulam. All the herbicides, except glufosinate, controlled the glyphosate resistant populations. There was no record of the use of glufosinate in the orchards where the populations were collected. Therefore, dose–response, ammonia accumulation and enzyme activity studies were conducted to confirm whether these populations also had evolved resistance to glufosinate.

MATERIAL AND METHODS

Plant material. Three Italian ryegrass glyphosate-resistant populations (OR1, OR2, and OR3) were collected from hazelnut orchards in Oregon. Glyphosate resistance in the OR1 population is due to reduced glyphosate translocation (Perez-Jones et al. 2005). The mechanism of glyphosate resistance in OR2 and OR3 is not an altered target site because no mutations in the 5-enolpyruvylshikimate-3-phosphate synthase gene have been identified. Three known glyphosate and glufosinate susceptible Italian ryegrass populations (C1, C2, and C3) were included as controls. The control populations C1 and C2 were from the Willamette Valley in Oregon, whereas C3 was a standard Italian ryegrass-susceptible population provided by an industry partner.

Greenhouse dose response bioassay. Seeds were germinated in petri dishes containing moistened blotter paper. After 3 d, when the seedling coleoptiles reached on average 1.5 cm, seedlings were transplanted to 267-ml plastic pots containing commercial potting mix[1]. Plants were grown under 25/20 C day/night temperature and natural sunlight in the summer of 2010. At the two- to three-leaf stage, the plants were sprayed with glufosinate[2] at 0.0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 kg ai ha^{-1} using an overhead, compressed-air sprayer and an 8003 even-flat spray nozzle calibrated to deliver 187 L ha^{-1} at 40 psi. The field rate recommended to control Italian ryegrass is between 0.4 and 0.5 kg ai ha^{-1}. Shoot

biomass was harvested 15 d after treatment, dried at 60 C for 72 h, and weighed.

Six plants were used per each of the three replications (18 plants total) per

herbicide concentration. Resistance index ratios were estimated on the basis of

the 50% growth reduction (GR_{50}) values from the susceptible and resistant

populations.

After harvesting, the plants were kept in the greenhouse under the same

conditions as previously described. Fifteen days after harvesting a visual

evaluation of plant re growth was conducted to estimate the percentage of

survivorship per rate and per population. The results are the average percentage

of survivorship from two replications.

Ammonia accumulation. Seeds from resistant and susceptible populations

were germinated and seedlings were transplanted and grown in the greenhouse

as described previously. At the two- to three-leaf stage, the plants were sprayed

with glufosinate at 0.4 kg ai ha^{-1}. Treated and non treated plants from all

populations were assayed for ammonia concentration at 24, 48, 72, and 96 h

after treatment (HAT). The experiment was conducted combining the methods

proposed by D'Hallauin et al. (1992) and Weatherburn (1967). Leaves (250 mg)

were chopped, ground in liquid nitrogen, and homogenized in 1 ml of deionized

water containing 50 mg of polyvinylpyrrolidone. The samples were centrifuged at

16,100 x g for 7 min. An aliquot of 300 µl of the supernatant was added to 700 µl

of deionized water and 100 µl of the diluted extract were reacted with 1.5 ml of

phenol nitroprusside solution,[3] and after mixing, 1.5 ml of an alkaline

hydrochlorite solution (2.5 g of sodium hydroxide, 1.6 ml of sodium hypochloride

5% available chlorine, and 500 ml of distilled water) were added. The samples

were incubated at 37 C for 20 min and the optical density was measured

spectrophotometrically[4] at 625 nm. Ammonia accumulation in $\mu g\ g^{-1}$ of fresh

weight was determined on the basis of a standard calibration curve. The

standard curve was constructed using ammonium chloride with concentrations

ranging from 0.004 to 4.0 mg. Four to five plants were used at each evaluation

time with two replications per time. The experiment was conducted twice.

Enzyme activity. The enzyme activity of the total GS enzyme was measured by

quantifying the L-glutamine synthesized from ammonia and L-glutamate formed

following the protocol proposed by Manderscheid (1993). Studies of enzyme

activity were performed with the three resistant populations and two control

populations (C1 and C3). Seeds were germinated and seedlings were

transplanted and grown in the greenhouse as described previously. At the three-

to four-leaf stage, the plants were assayed for GS activity. Leaves (300 mg) were

chopped, ground in liquid nitrogen, and homogenized in 1.2 ml of an extraction

medium (50 mM Tris[hydroxymethyl] aminomethane, 10 mM of 2-

mercaptoethanol, and 10 mM Mg_2Cl) at 4 C and pH 7–8. Polyvinylpyrrolidone (60

mg) was added to the extraction medium to remove phenolic impurities and to

improve the GS enzyme stability. The homogenate was centrifuged at 16,100 x *g*

for 15 min in a centrifuge pre cooled at 4 C. Supernatant (0.2 ml) was added to 0.8 ml of medium containing 50 mM Tris(hydroxymethyl)aminomethane buffer (pH 7–8), 50 mM MgSO$_4$, 20 mM NH$_2$OH, 3.3 mM L-cysteine, 6 mM adenosine triphosphate, and glufosinate at concentrations ranging from 0.02 to 400 µM. An aliquot of 150 µl of 500 mM of Na-glutamate was added to the medium solution to start the reaction, followed by an incubation of the samples for 40 min at 37 C. The reaction was stopped by the addition of 0.35 ml of a ferric chloride reagent (0.37 M FeCl$_3$ 6H$_2$O, 0.67 M HCl, and 0.20 M trichloroacetic acid). Samples were centrifuged at 1,500 x g for 10 min and 200 µl of the supernatant were taken to measure absorbance at 540 nm. Absorbance levels were transformed to units of GS activity per gram of fresh weight using a standard curve from known concentrations of L-glutamic acid-γ-monohydroxamate. The results are presented as percentage of the control. Three to five plants were used per each of the four replications per herbicide concentration.

Statistical analysis. The experiments were conducted twice and arranged in a completely randomized design with either three or four replications. Levene's ANOVA tests for homogeneity of variances were performed in all the experiments.

Two-way ANOVA analyses was performed for ammonia accumulation data and the differences among the populations and across time were analyzed using the LSD test at P = 0.05 when indicated by ANOVA.

Dose–response curves to estimate the glufosinate GR_{50} rate were obtained using nonlinear regression on the basis of the equation described by Streibig et al. (1993):

$$Y = c + (d - c / 1 + exp [b \{log x - log e\}]) \quad [1]$$

where Y represents shoot dry weight at herbicide rate x and e corresponds to the GR_{50} value. The upper limit is d, the lower limit is c, and b represents the slope of the line at the GR_{50}. Data were analyzed using the R software package[5] (Knezevic et al. 2007).

The concentration of glufosinate required to inhibit 50% of the GS activity (I_{50}) was calculated using the linear regression model:

$$Y = a + bX \quad [2]$$

where Y correspond to the GS enzyme activity (% of control), a is the intercept, b is the slope, and X is the concentration of glufosinate.

RESULTS AND DISCUSSION

Dose-response bioassay. There were no differences on the basis of Levene's ANOVA test for homogeneity of variances between the replications in all experiments; therefore, data were pooled across studies. The GR_{50} rates of glufosinate ranged from 0.15 to 0.49 kg ai ha^{-1}. The GR_{50} values for OR1, OR2, and OR3 populations were 0.49, 0.42, and 0.40 kg ai ha^{-1}, respectively, whereas the GR_{50} values for the control populations C1, C2, and C3 were 0.15, 0.18, and 0.21 kg ai ha^{-1}, respectively (Table 3.1).

Table 3.1. Parameters estimated from the nonlinear regression analysis of glufosinate dose–response experiments based on aboveground dry weight (percentage of untreated control) of Italian ryegrass populations. Values represent pooled data from two experiments.

Population	Parameters			GR_{50}[a]	RI[b]
	b (±SE)	c (±SE)	d (±SE)		
C1	2.97 (±0.59)	10.48 (±2.10)	100.23 (±4.21)	0.15 (±0.01)	–
C2	2.61 (±0.43)	7.60 (±2.16)	100.02 (±4.21)	0.18 (±0.01)	–
C3	1.73 (±0.27)	5.68 (±2.65)	99.89 (±4.21)	0.21 (±0.02)	–
OR1	3.38 (±0.70)	12.28 (±2.63)	105.55 (±2.93)	0.49 (±0.08)	2.7
OR2	2.54 (±0.50)	16.31 (±2.71)	100.81 (±3.54)	0.42 (±0.09)	2.3
OR3	3.35 (±0.94)	11.64 (±2.51)	94.80 (±3.61)	0.40 (±0.09)	2.2

Abbreviations: [a]GR_{50}, rate of glufosinate required to reduce plant growth by 50%; [b]RI, resistance index on the basis of the ratio between the average of the GR_{50} values from the control populations and the GR_{50} value of each resistant population.

RI on the basis of the average of the three control populations were 2.7, 2.3, and

2.2 for OR1, OR2, and OR3, respectively. Although the GR_{50} values represent

the response of the populations, there were recorded on average 23 and 6%

resistant individuals that were able to re grow and survive at rates of 2.0 and 4.0

kg ai ha^{-1}, respectively, in the group of the resistant populations, whereas no

survivors were observed in the three control populations even at 1.0 kg ai ha^{-1}

(Table 3.2).

Table 3.2. Percentage of survivors[a] from dose–response experiments 15 d after harvesting of Italian ryegrass populations. Values represent pooled data from two experiments.

Population	Glufosinate rate (kg ai ha^{-1})			
	0.5	1.0	2.0	4.0
C1	66%	0%	0%	0%
C2	73%	0%	0%	0%
C3	44%	5%	0%	0%
OR1	100%	100%	28%	12%
OR2	100%	100%	28%	5%
OR3	100%	83%	11%	0%

[a]On the basis of the average of 36 plants per rate.

Italian ryegrass is an obligate outcrossing species so the observed survivors at

high rates of glufosinate in the resistant populations indicate that the populations

are still segregating for resistant and susceptible individuals. Therefore, the GR_{50}

values obtained for the resistant populations could be underestimating the real

level of resistance. In the case of the glufosinate resistant goosegrass biotype

reported from Malaysia, the level of resistance was between two- and eight fold

(Jalaludin et al. 2010; Seng et al. 2010).

Ammonia Accumulation. Ammonia accumulation is the biochemical indicator of

the GS inhibition caused by glufosinate toxicity (Pornprom et al. 2000; Tsai et al.

2006). ANOVA indicated differences for accumulation of ammonia among

populations over time and across time. The untreated populations (0 HAT)

showed an ammonia concentration that ranged from 11 to 16 μg of ammonia per

gram of fresh weight. At 24 HAT, all the populations had increased levels of

ammonia; however, the control populations began to accumulate more ammonia

than the OR1, OR2, and OR3 populations (Table 3.3 and Figure 3.1), and

continued this trend at 48, 72, and 96 HAT. Comparing the average ammonia

accumulation between susceptible and resistant populations, the susceptible

populations accumulated 1.6, 1.9, and 2.6 times more ammonia than the

resistant populations at 48, 72, and 96 HAT, respectively, at the rate of 0.4 kg ai

ha^{-1} of glufosinate. It also was observed that the resistant populations reached

the maximum peak of ammonia accumulation at 48 HAT and then the ammonia

concentration decreased at 72 and 96 HAT. In contrast to the pattern observed in

the resistant populations, the three control populations were still accumulating

ammonia until 96 HAT.

Table 3.3. Ammonia accumulation expressed in μg g^{-1} of fresh weight in leaves of Italian ryegrass populations treated with glufosinate (0.4 kg ai ha^{-1}). Values represent pooled data from two experiments. Numbers in parenthesis are the standard errors of the mean of eight samples.

Population	Time after treatment (h)				
	0 (±SE)	24 (±SE)	48 (±SE)	72 (±SE)	96 (±SE)
C1	13.4 (±0.9)a[a]	224.7 (±21.1)a	304.4 (±21.1)a	332.2 (±16.2)a	427.9 (±12.7)a
C2	12.4 (±0.9)ab	251.3 (±6.1)b	336.5 (±8.1)b	341.6 (±10.0)b	385.8 (±25.8)b
C3	16.7 (±2.7)c	243.8 (±6.9)b	320.2 (±5.4)c	353.1 (±9.1)b	384.5 (±18.2)c
OR1	11.0 (±0.8)b	175.5 (±10)ac	196.0 (±5.6)d	173.6 (±12.6)c	148.4 (±7.5)d
OR2	14.7 (±4.0)ad	133.1 (±6.8)d	163.6 (±4.6)e	139.3 (±4.0)c	138.1 (±5.4)e
OR3	15.9 (±5.9)cd	166.3 (±6.0)c	231.3 (±14.4)f	211.7 (±10.2)d	149.4 (±19.6)d

[a]Columns followed by the same letter are not significantly different, according to LSD(<0.05).

The results of ammonia accumulation were strongly correlated with the results obtained in the dose–response experiments, confirming that ammonia accumulation is a valid indicator for glufosinate resistance. The greatest ammonia accumulation recorded in the control populations was similar in magnitude to the results obtained in ammonium accumulation studies reported in other weed species (Petersen and Hurle 2000; Sellers et al. 2004; Tachibana et al. 1986).
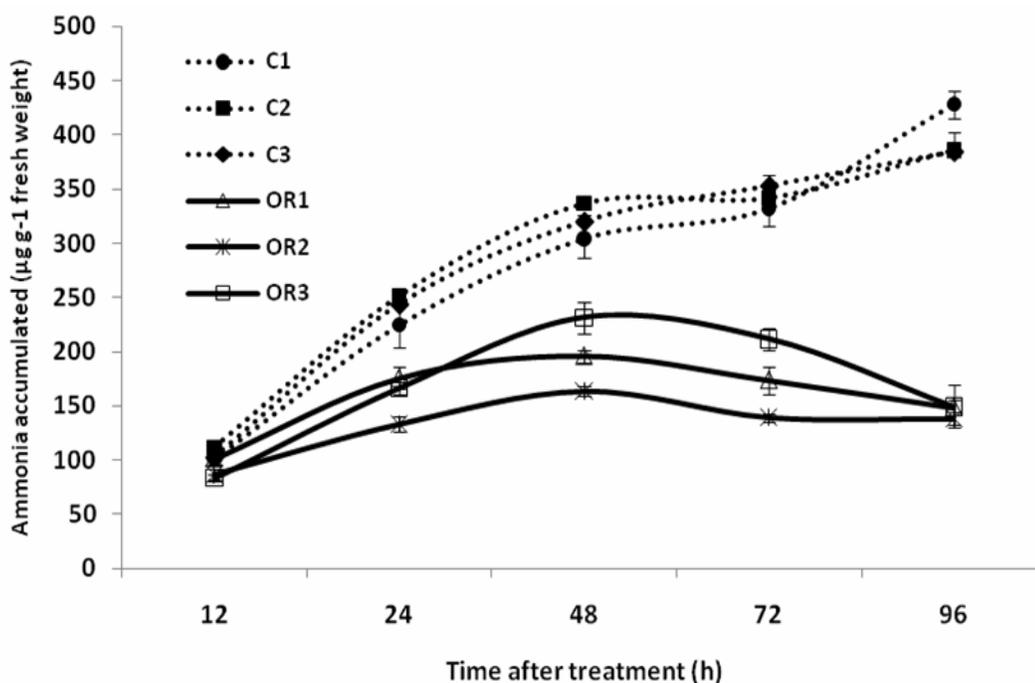
Figure 3.1. Ammonia accumulation in leaves of Italian ryegrass populations treated with glufosinate (0.4 kg ai ha^{-1}). C1, C2, and C3 are susceptible populations, and OR1, OR2, and OR3 are resistant populations. Values represent pooled data from two experiments. Error bars represent the standard errors of the mean from eight samples.

Enzyme Activity. GS enzyme activity was inhibited in all the populations and the inhibition rates were positively correlated with increasing concentrations of glufosinate (Figure 3.2). The I_{50} values for the resistant and susceptible populations were similar, ranging from 3.7 to 4.3 µM for C3 and C1, and from 3.1 to 3.6 µM for the resistant populations.

The similar sensitivity of the GS enzyme between the resistant and the susceptible populations suggests that the glufosinate resistance is not conferred by an insensitive target site. Similar levels of enzyme sensitivity to glufosinate were reported in soybean cells by Pornprom et al. (2009).
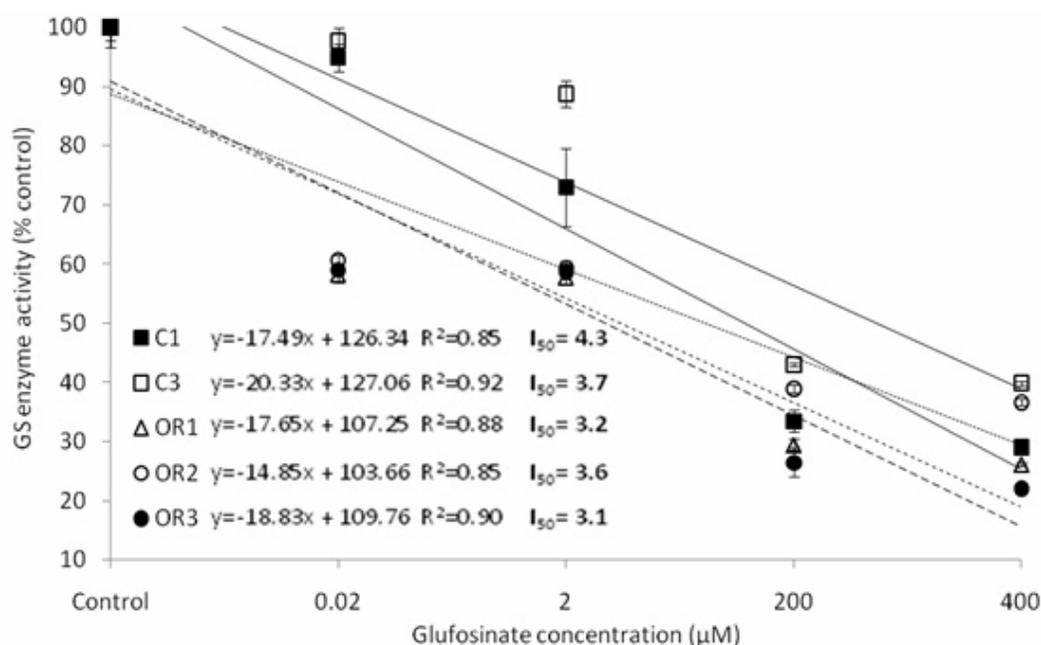
Figure 3.2. Effect of glufosinate concentration on the glutamine synthetase (GS) enzyme activity extracted from leaves of Italian ryegrass populations. C1, and C3 are susceptible populations, and OR1, OR2, and OR3 are resistant populations. Values represent pooled data from two experiments. Error bars represent the standard errors of the mean from eight samples.

We hypothesize that reduced herbicide translocation is responsible for resistance to both glyphosate and glufosinate in these populations. Although the sites of action of these two herbicides are different, this does not preclude the possibility that one mechanism could affect the translocation of both herbicides. Our hypothesis is supported by the fact that there was little or no use of glufosinate in the orchards where the resistant populations were collected, that the resistant populations were not resistant to herbicides with other sites of action, and that there was no difference in GS sensitivity between the resistant and susceptible populations.

Determining if reduced herbicide translocation is the cause of resistance to glufosinate is a key step to understanding the biochemical and physiological basis involved in the evolution of resistance to these two herbicides. In the context of weed management, glufosinate and glyphosate are two of the most important nonselective herbicides used in vineyards and orchards in the United States. Obviously, the evolution of resistance to these two herbicides reduces the chemical options for weed control in these systems. A more alarming weed management issue is the implication for the evolution of weeds with resistance to both herbicides in the systems where both glyphosate and glufosinate resistant crops are grown.

There are no reports of cross-resistance to glufosinate in glyphosate resistant weeds where resistance is due to reduced herbicide translocation. If in the future more cases of cross resistance to these two herbicides are identified, new weed management strategies will be required including herbicides with alternative sites of action or nonchemical methods (or both). The use of additional herbicides in these cropping systems will increase the cost and complexity of weed control and decrease the current benefit of these herbicide-resistant crops.

Sources of Materials

[1]Sunshine Mix 1 Potting Mix, Sun Gro Horticulture, Inc., 110th Ave. NE, Suite 490, Bellevue, WA 98004.

[2]Rely® 200, 182 g ai kg^{-1}, Bayer CropScience, 2 T. W. Alexander Dr., Research Triangle Park, NC 27709.

[3]Phenol nitroprusside solution, Sigma-Aldrich®. 3050 Spruce St., St. Louis, MO 63103.

[4]VERSAmax^{TM} tunable absorbance microplate reader, Molecular Devices Corporation, 1311 Orleans Dr., Sunnyvale, CA 94089.

[5]R statistical software, R development core team, http://www.r-project.org/.

Literature Cited

Culpepper, A. S., A. C. York, R. B. Batts, and K. M. Jennings. 2000. Weed management in glufosinate- and glyphosate-resistant soybean (*Glycine max*). Weed Technol. 14:77-88.

Devine, M., S. O. Duke, and C. Fedtke. 1993. Inhibition of amino acid biosynthesis. Pages 253-262 *in* Physiology of Herbicide Action. Englewood Cliffs, NJ: Prentice-Hall.

D'Hallauin, K., J. DeBlock, J. Janssens, J. Leemans, A. Reynaerts, and J. Botterman. 1992. The *bar* gene as a selectable marker in plant engineering. Methods Enzymol. 216:415-441.

Dinelli, G., I. Marotti, P. Catizone, A. Bonetti, J. M. Urbano, and J. Barnes. 2008. Physiological and molecular basis of glyphosate resistance in *C. bonariensis* (L.) Cronq. biotypes from Spain. Weed Res. 48:257–265.

Everman, W., C. Mayhew, J. Burton, A. York, and J. Wilcut. 2009a. Absorption, translocation, and metabolism of ^{14}C-glufosinate in glufosinate-resistant corn, goosegrass (*Eleusine indica*), large crabgrass (*Digitaria sanguinalis*), and sicklepod (*Senna obtusifolia*). Weed Sci. 57:1-5.

Everman, W., W. Thomas, J. Burton, A. York, and J. Wilcut. 2009b. Absorption, translocation, and metabolism of glufosinate in transgenic and nontransgenic cotton, Palmer amaranth (*Amaranthus palmeri*), and pitted morningglory (*Ipomoea lacunosa*). Weed Sci. 57:357-361.

Heap, I. 2011. The International Survey of Herbicide Resistant Weeds. http://www.weedscience.com Accessed: March 23, 2011.

Hoskins, A., B. Young, R. Krausz, and J. Russin. 2005. Control of Italian ryegrass (*Lolium multiflorum*) in winter wheat. Weed Technol. 19: 261-265.

Jalaludin, A., J. Ngim, B. Bakar, and Z. Alias. 2010. Preliminary findings of potentially resistant goosegrass (*Eleusine indica*) to glufosinate-ammonium in Malaysia. Weed Biology and Management. 10:256-260.

Jones, C., J. Chandler, J. Morrison, S. Senseman, and C. Tingle. 2001. Glufosinate combination and row spacing for weed control in glufosinate-resistant corn (*Zea mays*). Weed Technol. 15:141-147.

Knezevic, S. Z., J. C. Streibig, and C. Ritz. 2007. Utilizing R software package for dose–response studies: the concept and data analysis. Weed Technol. 21:840–848.

Koger, C. H. and K. N. Reddy. 2005. Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53: 84-89.

Manderscheid, R. 1993. Irreversible inhibition of glutamine synthetase from higher plants by the herbicide phosphinothricin. Pages 103-107 *in* Böger, P. and Sandman G., eds. Target Site Assays for Modern Herbicides and Related Phytotoxic Compounds. Boca Raton, Florida: Lewis Publishers.

Perez, A., C. Alister, and M. Kogan. 2004. Absorption, translocation and allocation of glyphosate in resistant and susceptible Chilean biotypes of *Lolium multiflorum*. Weed Biol. Manag. 4:56–58.

Perez-Jones, A., K. W. Park, J. Colquhoun, C. Mallory-Smith, and D. Shaner. 2005. Identification of glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in Oregon. Weed Sci. 53:775-779.

Perez-Jones, A., K. W. Park, N. Polge, J. Colquhoun, and C. Mallory-Smith. 2007. Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. Planta 226: 395-404.

Petersen, J. and K. Hurle. 2000. Influence of climatic conditions and plant physiology on glufosinate-ammonium efficacy. Weed Res. 41:31-39.

Pline, W. A., J. Wu, and K. K. Hatzios. 1999. Absorption, translocation, and metabolism of glufosinate in five weed species as influenced by ammonium sulfate and pelargonic acid. Weed Sci. 47:636-643.

Pornprom, T., S. Surawattananon, and P. Srinives. 2000. Ammonia accumulation as an index of glufosinate-tolerant soybean cell lines. Pestic. Biochem. Physiol. 68:102-106.

Pornprom, T., J. Chompoo, and B. Grace. 2003. Glufosinate tolerance in hybrid corn varieties based on decreasing ammonia accumulation. Weed Biol. Manag. 3:41-45.

Pornprom T., N. Prodmatee, and O. Chatchawankanphanich. 2008. Glutamine synthetase mutation conferring target-site based resistance to glufosinate in soybean cell selection. Pest. Manag. Sci 2009; 65: 216-222.

Ray, T. 1989. Herbicides as inhibitors of amino acid biosynthesis. Pages 106-107 *in* Böger, P. and Sandman G., eds. Target Sites of Herbicide Action. Boca Raton, Florida: CRC Press, Inc.

Sankula, S., M. Braverman, and J. Oard. 1998. Genetic analysis of glufosinate resistance in crosses between transformed rice (*Oryza sativa*) and red rice (*Oryza sativa*). Weed Technol. 12:209-214.

Sellers, B. A., R. J. Smeda, and J. Li. 2004. Glutamine synthetase activity and ammonia accumulation is influenced by time of glufosinate application. Pestic. Biochem. Physiol. 78:9-20.

Seng, C. T., L. V. Lun, C. T. San, and I. B. Sahid. 2010. Initial report of glufosinate and paraquat multiple resistance that evolved in a biotype of goosegrass (*Eleusine indica*) in Malaysia. Weed Biology and Management. 10:229-233.

Skora-Neto, F., H. Coble, and F. Corbin. 2000. Absorption, translocation and metabolism of ^{14}C-glufosinate in *Xantium strumarium*, *Commelina diffusa*, and *Ipomoea purpurea*. Weed Sci. 48:171-175.

Steckel, J., S. Hart, and L. Wax. 1997. Absorption and translocation of glufosinate on four weed species. Weed Sci. 45:378-381.

Streibig, J. C., M. Rudemo, and J. E. Jensen. 1993. Dose–response curves and statistical models. Pages 29–56 in J. C. Streibig and P. Kudsk, eds. Herbicide Bioassays. Boca Raton, FL: CRC.

Tachibana, K., T. Watanabe, Y. Sekizawa, and T. Takematsu. 1986. Accumulation of ammonia in plants treated with bialaphos. J. Pesticide Sci. 11:33-37.

Tsai, C. J., C. S. Wang, and C. Y. Wang. 2006. Physiological characteristics of glufosinate resistance in rice. Weed Sci. 54:634-640.

Tucker, K., G. Morgan, S. Senseman, T. Miller, and P. Baumann. 2006. Identification, distribution and control of Italian ryegrass (*Lolium multiflorum*) ecotypes with varying levels of sensitivity to triasulfuron in Texas. Weed Technol. 20: 745-750.

Wakelin, A. M., D. F. Lorraine-Colwill, and C. Preston. 2004. Glyphosate resistance in four different populations of *Lolium rigidum* as associated with reduced translocation of glyphosate to meristematic zones. Weed Res.44:453–459.

Wakelin, A. and C. Preston. 2006. A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. Weed Res. 46:432-440.

Weatherburn, M. 1967. Phenol-hypochloride reaction for ammonia. Analytical Chemistry 39: 971-974.

CHAPTER 4: Altered Target Site Confers Glufosinate Resistance in Italian Ryegrass (*Lolium perenne* L. *ssp. multiflorum*)

Wilson V. Avila-Garcia, Elena Sanchez, Andrew G. Hulting, and Carol Mallory-Smith

ABSTRACT

Dose-response, ammonia accumulation, glutamine synthetase (GS) activity and DNA sequencing studies were conducted to elucidate the basis for glufosinate resistance in an Italian ryegrass population. Glufosinate rates required to reduce growth by 50% (GR_{50}) were 0.15 and 0.18 kg ai ha^{-1} for the susceptible populations C1 and C2, respectively, and 0.45 kg ai ha^{-1} for the resistant population MG, resulting in a resistance index of 2.8. Ammonia accumulation after glufosinate treatment was on average 1.5 times less for the resistant population MG than for the susceptible populations C1 and C2. The glufosinate concentration required to reduce the GS enzyme activity by 50% (I_{50}) was different between the resistant and susceptible populations. The I_{50} values were 4.3 and 3.7 for C1 and C2, respectively, and 10.7 for the resistant population MG, resulting in a 2.6-fold greater I_{50} than the average of the susceptible populations. Eighty-three percent of the plastidic GS gene was cloned and sequenced. One amino acid substitution that may be involved in the reduced enzyme sensitivity to glufosinate was identified in the MG population. This is the first report of glufosinate resistance involving an altered target site in a weed species.

INTRODUCTION

Glufosinate is a broad-spectrum, nonselective, post-emergence herbicide commonly used to control weeds in vineyards, orchards, and genetically modified crops with the LibertyLink® trait. Glufosinate is a potent inhibitor of the enzyme glutamine synthetase (GS). The GS enzyme plays a major role in the pathway that assimilates inorganic nitrogen into organic compounds and ammonia assimilation derived from nitrate reduction and photorespiration (Ray 1989). Irreversible inhibition of GS activity by glufosinate leads to a rapid accumulation of high levels of ammonia due to a lack of nitrogen metabolism, as well as depletion of the amino acid glutamine. The excess ammonia in the plant causes reduction in photosynthetic activity, disruption of chloroplastic structure, stroma vesiculation and glyoxylate accumulation, causing inhibition of RuBisCo and carbon fixation (Devine et al. 1993; Manderscheid 1993; Tachibana et al. 1986).

GS is a nuclear-coded enzyme with two major isoforms in higher plants: cytosolic (GS1) and plastidic (GS2) (Unno et al. 2006). The GS1 is the predominant isoform present in roots and non-green tissues (McNally et al. 1993). The proportion of GS1 and GS2 in higher plants differs among plant species and plant tissue. McNally et al. (1993) and Miflin and Habash (2002) reported that there are three groups that have different levels of GS isoforms expression in plants. One group has only the GS2 isoform. Tomato (*Solanum licopesicum* L.), tobacco (*Nicotiana tabacum* L.), spinach (*Spinacia oleracea* L.), and common groundsel (*Senecio vulgaris* L.) are part of this group. In a second

group, species such as oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), and peas (*Pisum sativum* L.), the GS2 isoform is predominant and accounts for between 82% and 95% of the total GS. Finally, in a third group of species, mostly C4 plants such as sorghum (*Sorghum bicolor* Pers.), maize (*Zea mays* L.), large crabgrass (*Digitaria sanguinalis* L.), and barnyardgrass (*Echinochloa crus-galli* L.), the isoform GS1 is prevalent over the isoform GS2 in a proportion of two to one. GS1 is encoded by a gene family composed of three to five major genes depending on species (GS1a-GS1e), but GS2 is encoded by a single gene (Bernard et al. 2008). The amino acid sequences of plant glutamine synthetase appear to be highly homologous. Perez-Vicente et al. (1996) reported 95% and 94% homology between the nucleotides of the plastidic GS2 enzyme from Italian ryegrass and wheat, and Italian ryegrass and carrot, respectively.

Differential responses in sensitivity to glufosinate in weed species and non genetically transformed crops susceptible to glufosinate have been attributed to three main mechanisms: altered uptake, reduced translocation, and GS enzyme overproduction (Skora-Neto et al. 2000; Steckel et al. 1997; Pline et al. 1999; Tsai et al. 2006). Several mutations in the GS gene that confer glufosinate resistance have been reported. Pornprom et al. (2008a: 2008b) reported the selection of glufosinate resistant cells of soybean (*Glycine max* L.) and mungbean (*Vigna radiata* L.), respectively. Similarly, Prasertsongskun et al. (2002) and Chompoo and Pornprom (2008) reported the selection of glufosinate resistant cells of vetiver (*Vetiveria zizanioides* Nash) and maize, respectively. In

these studies, an insensitive GS enzyme was involved in the glufosinate resistance. Amplification, cloning and sequencing of the GS gene from the resistant cells revealed amino acid substitutions that were responsible for the insensitive enzyme.

At the whole plant level, Tsai et al. (2006) reported the selection of two rice lines with resistance to glufosinate. Enzymatic studies confirmed that overproduction of the GS conferred resistance to glufosinate in those rice lines.

There are two weed species reported to have glufosinate resistant biotypes: goosegrass (*Eleusine indica* L.) (Jalaludin et al. 2010; Seng et al. 2010), and Italian ryegrass (Avila-Garcia and Mallory-Smith 2011). Greenhouse and field studies confirmed that the goosegrass populations had two to eight-fold level of resistance compared to the susceptible population. Avila-Garcia and Mallory-Smith (2011) reported three glyphosate resistant Italian ryegrass populations with resistance to glufosinate. The levels of glufosinate resistance of these populations were two to three-fold compared to the three susceptible populations. Laboratory studies revealed no differences between the resistant and susceptible populations in GS response to glufosinate which indicated that an altered target site was not the mechanism of resistance in these populations of Italian ryegrass.

An Italian ryegrass population, designated as MG, was controlled with field rates of glyphosate but not controlled with field rates of glufosinate during a greenhouse herbicide screening test. Therefore, physiological and molecular

studies were conducted to determine if the Italian ryegrass population MG was

resistant to glufosinate and to elucidate the mechanism of resistance.

MATERIAL AND METHODS

Greenhouse dose-response bioassay. Seeds of two susceptible populations, C1and C2, and the putative resistant population, MG, were germinated in petri dishes containing moistened blotter paper. The seedlings were transplanted in 267-ml plastic pots containing commercial potting mix (Sun Gro Horticulture, Inc. WA, USA). The seedlings were grown under 25/20 C day/night temperatures with 14 h photoperiod. At the 2- to 3-leaf growth stage, the seedlings were sprayed with glufosinate as Rely® 200 (182 g ai litre^{-1}, Bayer CropScience, NC, USA) at 0.0, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 kg ai ha^{-1}, using an overhead, compressed-air sprayer calibrated to deliver 187 L ha^{-1} at 40 psi. After glufosinate treatment, the seedlings were returned to the greenhouse. Three replications of six plants each (18 seedlings) were treated with each herbicide concentration. Aboveground biomass was harvested 15 d after treatment (DAT), dried at 60 C for 72 h and weighed. The experiment was repeated. Resistance index ratios were estimated based on the average GR_{50} values from the two susceptible populations, C1 and C2, and the GR_{50} value from the resistant population MG.

Ammonia accumulation. Ammonia accumulation assays were conducted following the method proposed by Avila-Garcia and Mallory-Smith (2011). Seeds of C1, C2, and MG were germinated and seedlings were transplanted and grown in the greenhouse as described previously. At the 3-leaf growth stage, the

seedlings were sprayed with 0.4 kg ai ha^{-1} of glufosinate and assayed for

ammonia accumulation at 24, 48, 72, and 96 hours after treatment (HAT). Four to

five plants were used at each evaluation time with three replications assayed per

time (12-15 seedlings). The experiment was conducted twice.

Enzyme activity. Seeds of C1, C2, and MG were germinated and seedlings

were transplanted and grown in the greenhouse as described previously. At the

3- to 4-leaf stage, the seedlings were assayed for GS enzyme activity. GS

activity was measured by quantifying the L-glutamine synthesized from ammonia

and L-glutamate formed following the protocol proposed by Manderscheid

(1993). The results of enzyme activity are expressed in percentage, based on the

enzyme activity of untreated plants of each population and for each glufosinate

concentration. Three to five plants were used per each of the four replications per

herbicide concentration.

GS gene cloning and sequencing

Plant material. Five Italian ryegrass populations were selected to clone and

sequence the plastidic GS2 gene. The glufosinate susceptible populations C1

and C2, the resistant population MG, and the population OR1 previously reported

as glufosinate resistant[20] also was included. Additionally, during the dose

response experiments, susceptible segregant individuals were observed in the

population MG. In order to obtain susceptible segregants from the resistant

population MG, fifty plants were germinated and transplanted as previously described. Approximately four weeks after transplanting when the plants developed two-three tillers a clone (one tiller) was taken from each plant. When the clones reached the 3-leaf stage, they were sprayed with 0.5 kg ai ha^{-1} of glufosinate as previously described. Two weeks after the treatment, five susceptible plants were identified to be used as the susceptible segregants (MGS).

DNA extraction. DNA was extracted from leaf tissue of individual plants using a DNeasy® Plant Mini Kit (QIAGEN Sciences, MD, USA). DNA extraction was performed on a total of fifteen plants consisting of three plants from each population (C1, C2, MG, OR1, and MGS).

Primers design. Eight oligonucleotide primers (Table 1) amplified a region of the GS gene (3,520 bp including exons and introns), which included approximately 83% of the total GS2 protein coding sequence. The eight degenerate primers were designed based on conserved regions from partial or complete GS genes of wheat (*Triticum aestivum* L.) (GenBank accession no. GQ169689), rice (*Oryza sativa* L.) (GenBank accession no. CR855224), barley (GenBank accession no. X16000), and false brome (*Brachypodium distachyon* L) (Bradi 5g24550.2 from http://db.brachypodium.org/).

Table 4.1. Degenerate primers designed for polymerase chain reaction and partial GS gene sequencing of Italian ryegrass

Primer	Orientation	Sequence 5'-3'
1	5'→ 3'	AATGGAA(C/T)TA(C/T)GA(C/T)GGATC
2	3'→ 5'	AGCCCATATGTGATCTCCA
3	5'→ 3'	CCATACTACTGCGC(C/T)GTAGGATC
4	3'→ 5'	GAATAGAGCA(G/T)CCACGGTT

GS2 gene amplification. Fragments of the partial GS2 gene for each population were amplified in separate polymerase chain reactions (PCR) in a C1000[TM] thermal cycler (Bio-Rad Laboratories Inc. CA, USA). The PCR reaction consisted of 30 to 50 ng of template DNA, 0.2 mM of each primer, 0.2 mM of each deoxynucleotide, 3 uL PCR buffer, and 1 unit of Taq DNA polymerase in a total volume of 30 ml. The amplification protocol consisted of a 5-min incubation at 95 C, followed by 35 cycles of a denaturation step at 94 C for 45 s, an annealing step at 55 C for 45 s, an extension step at 74 C for 2 min, and a final extension step at 72 C for 10 min. PCR products were run on a 2% agarose gel. Expected size bands were extracted from the agarose gel using QIAquick® gel extraction kit (QIAGEN Sciences, MD, USA).

GS2 gene cloning. DNA fragments were cloned using a TOPO TA cloning kit and were purified using a QIAquick PCR purification kit (QIAGEN Sciences, MD,

USA). The purified DNA was sequenced using an automatic ABI PRISM® 3770 (Life Technologies Corporation, CA, USA) genetic analyzer and a BigDye® terminator version 3.1 cycle sequencing kit (Life Technologies Corporation, CA, USA). To identify PCR errors and because of the heterozygous nature of the populations, ten clones per PCR product were sequenced in both directions and aligned using the CLC Sequence Viewer version 6.1 (CLC Bio, Weingarten, Germany) allowing the reconstruction of both alleles for each plant.

Statistical analysis. All the experiments except the GS2 DNA sequencing were conducted twice with the treatments arranged in a completely randomized design with either three or four replications. Levene's ANOVA tests for homogeneity of variances were performed for all experiments.

Dose-response curves to estimate the glufosinate rate required to reduce biomass growth (GR_{50}) by 50% were obtained using nonlinear regression based on the equation described by Streibig et al. (1993):

$$Y = c + \{d - c / 1 + \exp [b(\log x - \log e)]\} \qquad [1]$$

where Y represents shoot dry weight at herbicide rate x, and e corresponds to the GR_{50} value. The upper limit is d, the lower limit is c, and b represents the slope of the line at the GR_{50}. Data were analyzed using the R software package (Knezevic et al. 2007).

Two way ANOVA was performed for ammonia accumulation data and the differences among the populations and over time were analyzed using the LSD test at P=0.05.

For the enzymatic studies, the concentration of glufosinate required to inhibit 50% of the GS activity (I_{50}) was calculated using the linear regression model:

$$Y = a + bX \qquad [2]$$

where Y corresponds to the GS enzyme activity (% of control), a is the intercept, b is the slope and X is the concentration of glufosinate.

RESULTS AND DISCUSSION

Dose-response bioassay. Levene's ANOVA test for homogeneity of variances confirmed that there were no significant differences between experiments; therefore, data were pooled across studies. The rates of glufosinate required to reduce plant growth by 50% (GR_{50}) were 0.15 and 0.18 kg ai ha^{-1} for the control populations C1 and C2, respectively, and 0.45 kg ai ha^{-1} for the population MG (Table 4.2 and Fig. 4.1). The resistance index (RI) calculated using mean data of the two control populations was 2.8.

Table 4.2. Parameters for the nonlinear regression analysis of glufosinate dose-response experiments based on aboveground dry weight (% of untreated control) of Italian ryegrass populations. Values represent pooled data from two experiments.

Population	Parameters			GR_{50}[a]	RI[b]
	b (±SE)	c (±SE)	d (±SE)	kg ai ha^{-1}	
C1	2.97 (±0.59)	10.48 (±2.10)	100.23 (±4.21)	0.15 (±0.01)	--
C2	2.61 (±0.43)	7.60 (±2.16)	100.02 (±4.21)	0.18 (±0.01)	--
MG	1.74 (±0.23)	8.83 (±2.68)	99.76 (±3.61)	0.45 (±0.09)	2.8

[a] Rate of glufosinate required to reduce plant growth by 50%
[b] Resistance Index based on the ratio between the average of the GR_{50} values from the susceptible populations C1 and C2, and the GR_{50} value of the resistant population MG.
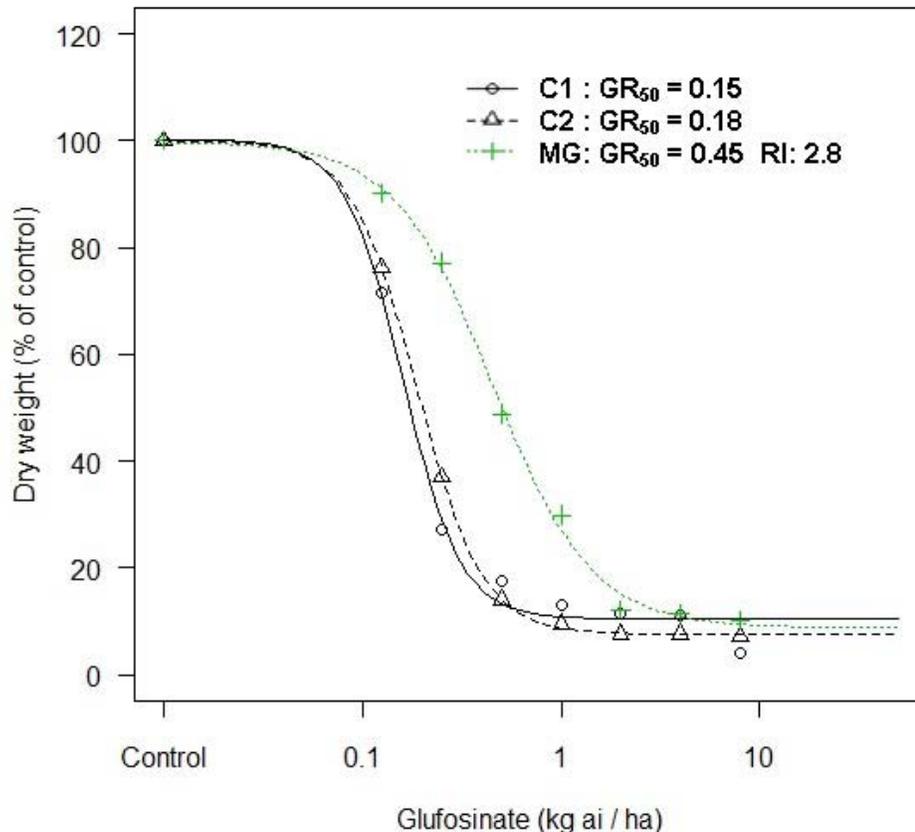
Figure 4.1. Glufosinate dose response curves of Italian ryegrass populations based on aboveground dry weight (percentage of untreated control). C1 and C2 are susceptible populations, and MG is the resistant population. GR_{50} values represent the rate of glufosinate required to reduce plant growth by 50% and correspond to pooled data from two experiments. RI is the resistance index based on the ratio between the GR_{50} value of the population MG and the GR_{50} average value of the control populations C1 and C2.

These results were similar to those reported by Avila-Garcia and Mallory-Smith (2011) for Italian ryegrass populations, where three glufosinate resistant populations had an RI average of 2.4. Similarly, Jalaludin et al. (2010) and Seng et al. (2010) reported a level of resistance between two and eight-fold in goosegrass biotypes from Malaysia.

Ammonia accumulation. Ammonia accumulation is the direct response of the

glutamine synthetase inhibition caused by glufosinate toxicity (Pornprom et al.

2000; Tsai et al. 2006). ANOVA confirmed differences for accumulation of

ammonia among populations and over time. The ammonia concentration of

untreated populations at 0 HAT was 11, 14, and 12 μg of ammonia per g of fresh

weight for the populations C1, C2, and MG, respectively. At 24 HAT, all the

populations accumulated ammonia but in different levels. The control populations

C1 and C2 accumulated more ammonia than the population MG (Figure 4.2).
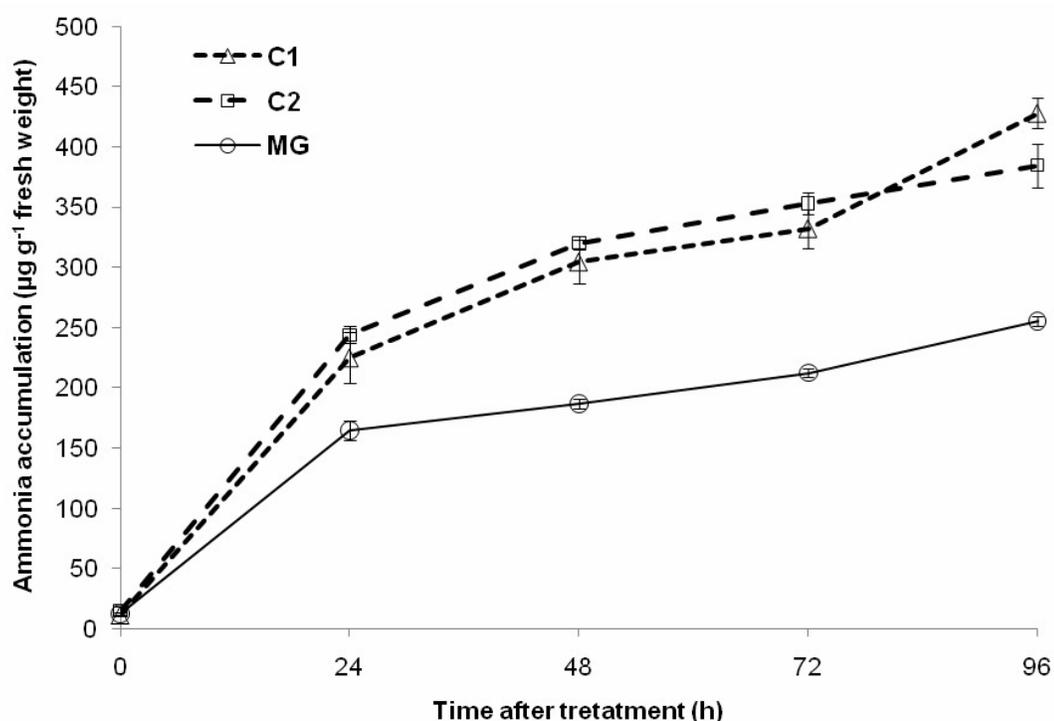
Figure 4.2. Ammonia accumulation in leaves of Italian ryegrass populations
treated with glufosinate (0.4 kg ai ha^{-1}). C1 and C2 are susceptible populations,
and MG is the resistant population. Values represent pooled data from two
experiments. Error bars represent the standard errors of the mean of eight
samples.

The same pattern of ammonia accumulation and the differences between the amount of ammonia accumulation between the two control populations and the population MG continued at 48, 72, and 96 HAT. On average, the susceptible populations C1 and C2 accumulated 1.6 times more ammonia than the resistant population MG. Similar strong relationships between dose response results and ammonia accumulation have been reported in rice (Tsai et al. 2006), soybean cell cultures (Pornprom et al. 2008a), and Italian ryegrass (Avila-Garcia and Mallory-Smith 2011), validating these ammonia accumulation results as a direct plant response of glufosinate toxicity.

Enzyme activity. The concentrations of glufosinate required to inhibit the GS enzyme activity by 50% (I_{50}) were 4.3, 3.7, and 10.2 µM for the populations C1, C2, and MG, respectively (Figure 4.3). The I_{50} value of the resistant population MG was 2.5 greater than the average I_{50} of the susceptible populations C1 and C2. These results suggest that the resistance to glufosinate in the population MG is conferred by an altered GS enzyme. Similar results were reported in soybean and mungbean glufosinate resistant cells by Pornprom et al. (2008a; 2008b). These selected glufosinate resistant cells required between 2.6 and 4.5 times greater glufosinate concentration than the susceptible cells to inhibit the GS enzyme activity by 50% indicating that an altered target site was responsible for the resistance.
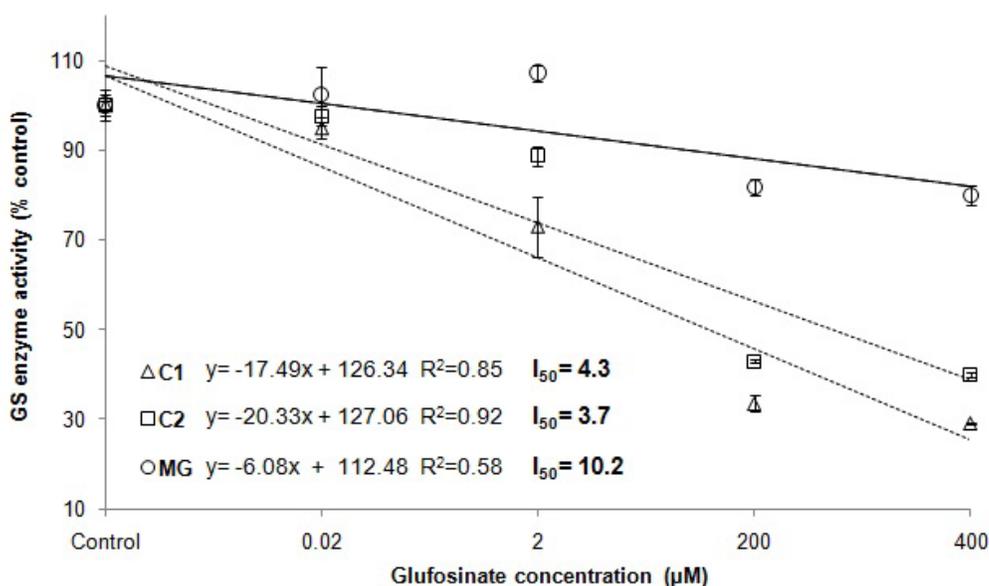
Figure 4.3. Effect of glufosinate concentration on glutamine synthetase (GS) activity extracted from leaves of Italian ryegrass populations. C1 and C2 are susceptible populations, and MG is the resistant population. Values represent pooled data from two experiments. Error bars represent the standard errors of the mean from eight samples. I_{50} value is the concentration of glufosinate required to reduce the enzyme activity by 50%.

GS gene cloning and sequencing. The partial nucleotide sequences of the plastidic GS2 gene from the populations were compared to identify any amino acid substitutions. As Italian ryegrass is an outcrossing self-incompatible species, two alleles were found per plant. The deduced amino acid sequence revealed one substitution that was found only in one of the alleles of the glufosinate resistant population MG. This substitution is due to a nucleotide change from GAC to AAC at the first position of the codon, resulting in the amino acid change of aspartic acid (Asp/D) by asparagine (Asn/N) at the positions 171 (based on the GS2 amino acid sequence from *T. aestivum*) (Figure 4.4).

MG	D T Y T P Q G E P I P T N K R H R A A Q I F S [N] P K V S S Q V P W F G I E Q E Y
OR1	D T Y T P Q G E P I P T N K R H R A A Q I F S [D] P K V S S Q V P W F G I E Q E Y
C1	D T Y T P Q G E P I P T N K R H R A A Q I F S [D] P K V S S Q V P W F G I E Q E Y
C2	D T Y T P Q G E P I P T N K R H R A A Q I F S [D] P K V S S Q V P W F G I E Q E Y
MGS	D T Y T P Q G E P I P T N K R H R A A Q I F S [D] P K V S S Q V P W F G I E Q E Y

Figure 4.4. Alignment of deduced amino acid sequences of the GS2 gene from the glufosinate resistant population MG and OR1, the susceptible populations C1 and C2, and the segregant glufosinate susceptible MGS Italian ryegrass. The shaded box amino acid indicate an asparagine (N) by aspartic acid (D) substitution in the glufosinate resistant MG population. The amino acid position 171 is based on the *Triticum aestivum* sequence of the GS2 gene.

Asparagine is a polar uncharged amino acid, while aspartic acid is a negatively charged amino acid. The physiological and physicochemical stability of the plastidic GS enzyme can be notably changed by a single amino acid substitution (Unno et al. 2006). Therefore, the change of polarity identified in the amino acid substitution Asp by Asn may be playing a major role in the reduced GS sensitivity to glufosinate. Indeed, the amino acid substitution identified in the resistant population MG was not found in the glufosinate resistant population OR1 or in the glufosinate susceptible MG. There are several amino acid substitutions previously reported for the GS gene which are involved in reduced GS sensitivity. Sixteen, eight, and ten amino acid substitutions have been reported in glufosinate resistant cells of soybean (Pornprom et al. 2008), mungbean (Pornprom et al. 2008), and maize (Chompoo and Pornprom 2008), respectively.

However, the amino acid substitution found in Italian ryegrass in this study has not been reported.

CONCLUSIONS

The results presented in this paper provide new insights to understand the biochemical and molecular basis involving glufosinate resistance due to an altered target site. Furthermore, this is the first report of glufosinate resistance conferred by an altered target site in a weed species.

An altered target site is the most common mechanism involved in herbicide resistance. A single amino acid substitution can be enough to prevent or reduce binding between the herbicide and its target site (Fuerst et al. 1986). The level of resistance conferred by a change in the protein expression caused by amino acid substitutions at the herbicide target site differs among weed species and among herbicide sites of action. For instance, a single point of mutation of the *psb*A gene (Gly for Ser at the position 264) can reduce the affinity of atrazine for its substrate Q_B conferring 100-fold atrazine resistance at the whole plant level in smooth pigweed (*Amaranthus hybridus* L.), common lambsquarters (*Chenopodium album* L.), and common groundsel (*Senecio vulgaris* L.) (Fuerst et al. 1986).The single amino acid substitution of Pro by Ser at the position 106 of the EPSPS gene can confer a level of glyphosate resistance between two- and five-fold in Italian ryegrass (Perez-Jones et al. 2007).Further research involving absorption and translocation studies with this population of Italian ryegrass are needed to confirm that an altered target site is

the only mechanism involved in the resistance. Additional research is required to elucidate if a homozygous plant with the amino acid substitution would have an increased level of glufosinate resistance.

Italian ryegrass is a very competitive weed in many field crops and orchards in the Pacific Northwest of the USA. Most of the strategies to control Italian ryegrass in these field crops, vineyards, and orchards are based in the intensive use of herbicides. There are at least 41 biotypes of this weed species that have evolved resistance to five different herbicide groups in eight countries (Heap 2011).

Glufosinate has been proposed as the alternative herbicide to control glyphosate resistant weeds in glyphosate-resistant (Roundup Ready$^®$) cropping systems. Since 1995, the number of weed species that have evolved resistance to glyphosate under those systems has increased dramatically (heap 2011). This first report of glufosinate resistance conferred by an altered target site, coupled with the previous report of glufosinate resistance conferred by reduced herbicide translocation (Avila-Garcia and Mallory-Smith 2011) suggests that in glufosinate-resistant (LibertyLink) cropping systems weeds could evolve glufosinate resistance as quickly as has been documented with glyphosate resistance in the glyphosate-resistant cropping systems. Dependence on a single herbicide to control weeds inevitably will lead to the selection of resistant individuals, limiting the long-term utility of any herbicide technology. To prevent this cyclic scenario it

is imperative to redesign weed management strategies using all available control

options including nonchemical methods.

Literature Cited

Avila-Garcia, W. V. and C. Mallory-Smith. 2011. Glyphosate-resistant Italian ryegrass (*Lolium perenne*) populations also exhibit resistance to glufosinate. Weed Sci. 59:305-309.

Bernard, S. M., A. L. B. Moller, G. Dionisio, T. Kichey, T. P. Jahn, F. Dubois, M. Baudo, M. S. Lopes, T. Terce-Laforgue, C. H. Foyer, M. Parry, B. G. Forde, J. L. Araus, B. Hrel, J. K. Schjoerring, and D. Z. Habash. 2008. Gene expression, cellular localization and function of glutamine synthetase isoenzymes in wheat (*Triticum aestivum* L.). Plant Mol. Biol. 67:89-105.

Chompoo, J. and T. Pornprom. 2008. RT-PCR based detection of resistance conferred by an insensitive GS in glufosinate-resistant maize cell lines. Pest. Biochem. Physiol. 90:189-195.

Culpepper, A. S., A. C. York, R. B. Batts, and K. M. Jennings. 2000. Weed management in glufosinate- and glyphosate-resistant soybean (*Glycine max*). Weed Technol. 14:77-88.

Devine, M., S. O. Duke, and C. Fedtke. 1993. Inhibition of amino acid biosynthesis. Pages 253-262 *in* Physiology of Herbicide Action. Englewood Cliffs, NJ: Prentice-Hall.

D'Hallauin, K., J. DeBlock, J. Janssens, J. Leemans, A. Reynaerts, and J. Botterman. 1992. The *bar* gene as a selectable marker in plant engineering. Methods Enzymol. 216:415-441.

Dinelli, G., I. Marotti, P. Catizone, A. Bonetti, J. M. Urbano, and J. Barnes. 2008. Physiological and molecular basis of glyphosate resistance in *C. bonariensis* (L.) Cronq. biotypes from Spain. Weed Res. 48:257–265.

Everman, W., C. Mayhew, J. Burton, A. York, and J. Wilcut. 2009a. Absorption, translocation, and metabolism of ^{14}C-glufosinate in glufosinate-resistant corn, goosegrass (*Eleusine indica*), large crabgrass (*Digitaria sanguinalis*), and sicklepod (*Senna obtusifolia*). Weed Sci. 57:1-5.

Everman, W., W. Thomas, J. Burton, A. York, and J. Wilcut. 2009b. Absorption, translocation, and metabolism of glufosinate in transgenic and nontransgenic cotton, Palmer amaranth (*Amaranthus palmeri*), and pitted morningglory (*Ipomoea lacunosa*). Weed Sci. 57:357-361.

Fuerst, E. P., C. J. Arntzen, K. Pfister, and D. Penner. 1986. Herbicide cross-resistance in triazine-resistant biotypes of four species. Weed Sci. 34:344-353.

Heap, I. 2011. The International Survey of Herbicide Resistant Weeds. http://www.weedscience.com Accessed: July 22, 2011.

Hoskins, A., B. Young, R. Krausz, and J. Russin. 2005. Control of Italian ryegrass (*Lolium multiflorum*) in winter wheat. Weed Technol. 19: 261-265.

Jalaludin, A., J. Ngim, B. Bakar, and Z. Alias. 2010. Preliminary findings of potentially resistant goosegrass (*Eleusine indica*) to glufosinate-ammonium in Malaysia. Weed Biol. Manage. 10:256-260.

Jones, C., J. Chandler, J. Morrison, S. Senseman, and C. Tingle. 2001. Glufosinate combination and row spacing for weed control in glufosinate-resistant corn (*Zea mays*). Weed Technol. 15:141-147.

Knezevic, S. Z., J. C. Streibig, and C. Ritz. 2007. Utilizing R software package for dose–response studies: the concept and data analysis. Weed Technol. 21:840–848.

Koger, C. H. and K. N. Reddy. 2005. Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53: 84-89.

Manderscheid, R. 1993. Irreversible inhibition of glutamine synthetase from higher plants by the herbicide phosphinothricin. Pages 103-107 *in* Böger, P. and Sandman G., eds. Target Site Assays for Modern Herbicides and Related Phytotoxic Compounds. Boca Raton, Florida: Lewis Publishers.

McNally, S. F., B. Hirel, P. Gadal, A. F. Mann, and G. R. Stewart. 1983. Glutamine synthetases of higher plants. Plant Physiol. 72:22-25.

Miflin, B. J. and D. Z. Habash. 2002. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J. Exp. Bot. 53:979-987.

Perez, A., C. Alister, and M. Kogan. 2004. Absorption, translocation and allocation of glyphosate in resistant and susceptible Chilean biotypes of *Lolium multiflorum*. Weed Biol. Manag. 4:56–58.

Perez-Jones, A., K. W. Park, J. Colquhoun, C. Mallory-Smith, and D. Shaner. 2005. Identification of glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in Oregon. Weed Sci. 53:775-779.

Perez-Jones, A., K. W. Park, N. Polge, J. Colquhoun, and C. Mallory-Smith. 2007. Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. Planta 226: 395-404.

Perez-Vicente, R., G. Dorado, and J. M. Maldonado. 1996. Cross-species amplification of glutamine synthetase cDNA by polymerase chain reaction with degenerate primers. Physiol. Plant. 98:705-713.

Petersen, J. and K. Hurle. 2000. Influence of climatic conditions and plant physiology on glufosinate-ammonium efficacy. Weed Res. 41:31-39.

Pline, W. A., J. Wu, and K. K. Hatzios. 1999. Absorption, translocation, and metabolism of glufosinate in five weed species as influenced by ammonium sulfate and pelargonic acid. Weed Sci. 47:636-643.

Pornprom, T., S. Surawattananon, and P. Srinives. 2000. Ammonia accumulation as an index of glufosinate-tolerant soybean cell lines. Pestic. Biochem. Physiol. 68:102-106.

Pornprom, T., J. Chompoo, and B. Grace. 2003. Glufosinate tolerance in hybrid corn varieties based on decreasing ammonia accumulation. Weed Biol. Manage. 3:41-45.

Pornprom, T., N. Prodmatee, and O. Chatchawankanphanich. 2008a. Glutamine synthetase mutation conferring target-site based resistance to glufosinate in soybean cell selection. Pest. Manag. Sci. 65:216-222.

Pornprom, T., A. Pengnual, N. Udomprasert, and O. Chatchawankanphanich. 2008b. The role of altered glutamine synthetase in conferring resistance to glufosinate in mungbean cell selection. Thai J. Agric. Sci. 41:81-90.

Prasertsongskun, S., N. Sangduen, S. Suwanwong, V. Santisopasri, and H. Matsumoto. 2002. Increased activity and reduced sensitivity of glutamine synthetase in glufosinate-resistant vetiver (*Vetiveria zizanioides* Nash) cells. Weed Biol. Manage. 2:171-176.

Ray, T. 1989. Herbicides as inhibitors of amino acid biosynthesis. Pages 106-107 *in* Böger, P. and Sandman G., eds. Target Sites of Herbicide Action. Boca Raton, Florida: CRC Press, Inc.

Sankula, S., M. Braverman, and J. Oard. 1998. Genetic analysis of glufosinate resistance in crosses between transformed rice (*Oryza sativa*) and red rice (*Oryza sativa*). Weed Technol. 12:209-214.

Sellers, B. A., R. J. Smeda, and J. Li. 2004. Glutamine synthetase activity and ammonia accumulation is influenced by time of glufosinate application. Pestic. Biochem. Physiol. 78:9-20.

Seng, C. T., L. V. Lun, C. T. San, and I. B. Sahid. 2010. Initial report of glufosinate and paraquat multiple resistance that evolved in a biotype of goosegrass (*Eleusine indica*) in Malaysia. Weed Biol. Manage. 10:229-233.

Shaner, D. L. 2009. Role of translocation as a mechanism of resistance to glyphosate. Weed Sci. 57:118-123.

Skora-Neto, F., H. Coble, and F. Corbin. 2000. Absorption, translocation and metabolism of ^{14}C-glufosinate in *Xantium strumarium*, *Commelina diffusa*, and *Ipomoea purpurea*. Weed Sci. 48:171-175.

Steckel, J., S. Hart, and L. Wax. 1997. Absorption and translocation of glufosinate on four weed species. Weed Sci. 45:378-381.

Streibig, J. C., M. Rudemo, and J. E. Jensen. 1993. Dose–response curves and statistical models. Pages 29–56 in J. C. Streibig and P. Kudsk, eds. Herbicide Bioassays. Boca Raton, FL: CRC.

Tachibana, K., T. Watanabe, Y. Sekizawa, and T. Takematsu. 1986. Accumulation of ammonia in plants treated with bialaphos. J. Pesticide Sci. 11:33-37.

Tal, A. and B. Rubin. 2004. Molecular characterization and inheritance of resistance to ACCase-inhibiting herbicides in Lolium rigidum. Pest. Manag. Sci. 60:1013-1018.

Tsai, C. J., C. S. Wang, and C. Y. Wang. 2006. Physiological characteristics of glufosinate resistance in rice. Weed Sci. 54:634-640.

Tucker, K., G. Morgan, S. Senseman, T. Miller, and P. Baumann. 2006. Identification, distribution and control of Italian ryegrass (*Lolium multiflorum*) ecotypes with varying levels of sensitivity to triasulfuron in Texas. Weed Technol. 20: 745-750.

Unno, H., T. Uchida, H. Sugawara, G. Kurisu, T. Sugiyama, T. Yamaya, H. Sakakibara, T. Hase, and M. Kusunoki. 2006. Atomic structure of plant

glutamine synthetase, a key enzyme for plant productivity. Journal of Biological Chemistry. 281:29287-29296.

Wakelin, A. M., D. F. Lorraine-Colwill, and C. Preston. 2004. Glyphosate resistance in four different populations of *Lolium rigidum* as associated with reduced translocation of glyphosate to meristematic zones. Weed Res. 44:453–459.

Wakelin, A. and C. Preston. 2006. A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. Weed Res. 46:432-440.

Weatherburn, M. 1967. Phenol-hypochloride reaction for ammonia. Anal. Chem. 39: 971-974.

CHAPTER 5: GENERAL CONCLUSIONS

Mendelian inheritance studies were conducted to determine the heritability of two mechanisms of glyphosate resistance in Italian ryegrass: target site mutation and reduced herbicide translocation. In addition, experiments were performed to investigate patterns of cross resistance to the herbicide glufosinate conducting dose response experiments, ammonia accumulation and enzyme activity in three glyphosate resistant Italian ryegrass populations.

The Italian ryegrass populations SF and OR1 were selected for the Mendelian inheritance studies. The mechanisms conferring resistance to the herbicide glyphosate were target site mutation for population SF and reduced herbicide translocation for population OR1. Four F_1 families were formed by reciprocal crosses between the glyphosate resistant populations SF and OR1 and a susceptible population C1. Additionally, eight backcross families (BC_1) were produced by crossing resistant F_1 individuals from each family and the susceptible population C1.

Six of eight F_1 families between the population SF and C1 had susceptible:resistant ratios of 1:1 based on chi-square analyses at the discriminatory rate of 0.6 kg ae ha^{-1} of glyphosate. Similar trends were observed in the backcrossed families where 50% of the BC_1 families had the same susceptible:resistant ratio of 1:1. The remaining 50% had ratios of 2:1 or 1:2.

Segregation ratios for F_1 and BC_1 families confirmed that target site mutation glyphosate resistance in the SF population is likely governed by a single, nuclear, partially dominant gene.

The results for the OR1 population were different from those of the SF population. There was a significant variation for the observed and expected susceptible:resistant ratios in F_1 and BC_1 families for 1:1 segregation at the discriminatory rate of 0.6 kg ae ha^{-1} of glyphosate. Chi-square analyses failed to fit the model for a single major gene suggesting that glyphosate resistance due to reduced translocation in the Italian ryegrass population OR1 is multigenic. Furthermore, segregation ratios and the dose response experiments for F_1 and BC_1 families confirmed that both mechanisms of resistance are in the nuclear genome with no evidence of maternal effect.

The partially dominant and nuclear encoded glyphosate resistance trait due to target site mutation in the population SF ensures its spread via pollen. Furthermore, considering the outcrossing nature of Italian ryegrass, there can be gene flow that could transfer the resistance trait to compatible relatives such as perennial ryegrass (*L. perenne* L.), rigid ryegrass (*L. rigidum* G.), and fescues (*Festuca* spp.). A different scenario can be proposed for the glyphosate resistant trait conferred by a reduced herbicide translocation. The results obtained in the F_1 and BC_1 families suggest that the genes responsible for the glyphosate resistance trait of this mechanism could be lost after generations of outcrossing with glyphosate susceptible Italian ryegrass. Both scenarios have been reported

and proven to be feasible. Busi et al. (2008) reported the introgression of an acetolactate synthase (ALS) herbicide resistance gene via gene flow in an experiment conducted at landscape level in rigid ryegrass. Mendelian and molecular analyses confirmed that that pollen-mediated gene flow occurred at 3000 m from the pollen source. For the second scenario, Ghersa et al. (1994) documented that the inclusion of susceptible individuals into a diclofop resistant Italian ryegrass population decreased the proportion of resistant individuals in the population if diclofop was not applied. Similarly, Preston and Wakelin (2008) reported that the frequency of glyphosate resistant individuals in a rigid ryegrass population from Australia declined from 45% in the original population to 5% after three generations of outcrossing with a susceptible population under natural conditions with no selection pressure of glyphosate.

Knowledge of the mechanisms of herbicide resistance and the heritability of the trait can be helpful to reduce spread of resistance genes between and within weed species.

To investigate resistance to the herbicide glufosinate, the populations OR1, OR2, OR3, and MG were selected for dose response, ammonia accumulation, and enzyme activity studies. Three glyphosate and glufosinate susceptible populations (C1, C2, and C3) were included as controls. The glufosinate rates required to reduce plant growth by 50% (GR_{50}) were 0.15, 0.18, and 0.21 kg ai ha^{-1} for the control populations C1, C2, and C3, respectively, whereas the resistant populations OR1, OR2, OR3, and MG had GR_{50} values of

0.49, 0.42, 0.40, and 0.45 kg ai ha^{-1}, respectively. The resistant/susceptible

index (RI) based on the GR_{50} average from both resistant and susceptible groups

was 2.4. Ammonia accumulation studies confirmed the results obtained in dose

response experiments. The susceptible populations C1, C2, and C3 accumulated

on average two times more ammonia than the resistant populations OR1, OR2,

and OR3, 48 and 96 h after glufosinate treatment, and 1.6 times more than the

population MG for the same times. Enzymatic studies revealed that the

glufosinate concentration required to reduce the glutamine synthetase enzyme

activity by 50% (I_{50}) was not different among OR1, OR2, OR3 and the

susceptible populations. However, the population MG had an I_{50} value of 10.2,

resulting in a resistant ratio of 2.6-fold greater than the average of the control

populations. Based on this result, 83% of the GS plastidic gene from the

population MG was cloned and sequenced. One amino acid substitution was

identified that could be involved in the reduced enzyme sensitivity. These results

confirm that glufosinate resistance in the population MG was conferred by an

altered target site. However, the mechanism of glufosinate resistance in the

populations OR1, OR2, and OR3 is not related to an altered target site. Our

hypothesis is that the genes conferring glyphosate resistance due to reduced

herbicide translocation in OR1 and probably OR2 and OR3 also are responsible

for the cross resistance to the herbicide glufosinate. Although the sites of action

of these two herbicides are different, the genes controlling this mechanism could

be affecting the translocation of both herbicides.

In summary, these studies provided evidence that the genetic control of target-site based and reduced herbicide translocation for glyphosate resistance in Italian ryegrass are regulated by a different number of genes. There was also evidence that the Italian ryegrass populations OR1, OR2, and OR3 evolved glufosinate resistance not involving an altered target site. However, for the population MG, enzymatic studies revealed that an insensitive target site conferred glufosinate resistance. Partial sequence of the GS gene from the population MG revealed one amino acid substitution that could be altering the enzyme sensitivity to the herbicide glufosinate.

The approaches used in this research can be used to conduct similar studies in self-incompatible and wind-pollinated weed species. This is the first report of the heritability of glyphosate resistance due to reduced herbicide translocation and target site mutation in Italian ryegrass, as well as the confirmation of glufosinate resistance involving two different mechanisms of resistance in Italian ryegrass.

Bibliography

Avila-Garcia, W. V. and C. Mallory-Smith. 2011. Glyphosate-resistant Italian ryegrass (*Lolium perenne*) populations also exhibit resistance to glufosinate. Weed Sci. 59:305-309.

Bernard, S. M., A. L. B. Moller, G. Dionisio, T. Kichey, T. P. Jahn, F. Dubois, M. Baudo, M. S. Lopes, T. Terce-Laforgue, C. H. Foyer, M. Parry, B. G. Forde, J. L. Araus, B. Hrel, J. K. Schjoerring, and D. Z. Habash. 2008. Gene expression, cellular localization and function of glutamine synthetase isoenzymes in wheat (*Triticum aestivum* L.). Plant Mol. Biol. 67:89-105.

Beddows, A. 1973. Biological flora of the British Isles, *Lolium multiflorum* Lam. J. of Ecology. 61:587-600.

Boutsalis, P. and S. B. Powles. 1995. Inheritance and mechanism of resistance to herbicides inhibiting acetolactate synthase in *Sonchus oleraceus* L. Theor. Appl. Genet. 91:242-247.

Busi, R., Q. Yu, R. Barret-Lennard, and S. Powles. 2008. Long distance pollen-mediated flow of herbicide resistance genes in *Lolium rigidum*. Theor. Appl. Genet. 117:1281-1290.

Chauvel, B. and J. Gasquez. 1994. Relationship between genetic polymorphism and herbicide resistance within *Alopecurus myosuroides* Huds. Heredity 73:336-344.

Chompoo, J. and T. Pornprom. 2008. RT-PCR based detection of resistance conferred by an insensitive GS in glufosinate-resistant maize cell lines. Pest. Biochem. Physiol. 90:189-195.

Culpepper, A. S., A. C. York, R. B. Batts, and K. M. Jennings. 2000. Weed management in glufosinate- and glyphosate-resistant soybean (*Glycine max*). Weed Technol. 14:77–88.

Davis, V. M., G. R. Krueger, S. G. Hallett, P. J. Tranel, and W. G Johnson. 2010. Heritability of Glyphosate Resistance in Indiana Horseweed (*Conyza canadensis*) Populations. Weed Sci. 58:30-38.

Devine, M., S. O. Duke, and C. Fedtke. 1993. Inhibition of amino acid biosynthesis. Pages 253-262 *in* Physiology of Herbicide Action. Englewood Cliffs, NJ: Prentice-Hall.

D'Hallauin, K., J. DeBlock, J. Janssens, J. Leemans, A. Reynaerts, and J. Botterman. 1992. The *bar* gene as a selectable marker in plant engineering. Methods Enzymol. 216:415–441.

Dinelli, G., I. Marotti, P. Catizone, A. Bonetti, J. M. Urbano, and J. Barnes. 2008. Physiological and molecular basis of glyphosate resistance in *C. bonariensis* (L.) Cronq. biotypes from Spain. Weed Res. 48:257–265.

Downs, C.G., M.C. Christey, D. Maddocks, J.F. Seelye, and D.G. Stevenson. 1994. Hairy roots of *Brassica napus*: I. Applied glutamine overcomes the effect of phosphinothricin treatment. Plant Cell Rep. 14:37-40.

Duke S. O. and S. B. Powles. 2008. Mini-review: Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64:319-325.

Everman, W., C. Mayhew, J. Burton, A. York, and J. Wilcut. 2009a. Absorption, translocation, and metabolism of ^{14}C-glufosinate in glufosinate-resistant corn, goosegrass (*Eleusine indica*), large crabgrass (*Digitaria sanguinalis*), and sicklepod (*Senna obtusifolia*). Weed Sci. 57:1–5.

Everman, W., W. Thomas, J. Burton, A. York, and J. Wilcut. 2009b. Absorption, translocation, and metabolism of glufosinate in transgenic and nontransgenic cotton, Palmer amaranth (*Amaranthus palmeri*), and pitted morningglory (*Ipomoea lacunosa*). Weed Sci. 57:357–361.

Feng, P., M. Tran, D. Sammons, G. Heack, and C. Cajacob. 2004. Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation and metabolism. Weed Sci.52:498-505.

Franz, J., M. Mao, and J. Sikorski. 1997. Glyphosate a unique global herbicide. *ACS Monograph No. 189*. American Chemical Society, Washington, DC, 653 pp.

Fuerst, E. P., C. J. Arntzen, K. Pfister, and D. Penner. 1986. Herbicide cross-resistance in triazine-resistant biotypes of four species. Weed Sci. 34:344-353.

Gaines T. A., W. Zhang, D. Wang, B. Bukun, S.T. Chisholm, D. L. Shaner, S. J. Nissen, W. L. Patzoldt, P. J. Tranel, A. S. Culpepper, T. L. Grey, T. M. Webster, W. K. Vencill, R. D. Sammons, J. Jiang, Ch. Preston, J. E. Leach, and P. Westra. 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. PNAS 107:1029-1034.

Ghersa, C. M., M. A. Martinez-Ghersa, T. G. Brewer, and M. L. Roush. 1994. Use of gene flow to control diclofop-methyl resistance in Italian ryegrass (*Lolium multiflorum*). Weed Tech. 8:139-147.

Heap, I. 2011. International Survey of Herbicide Resistant Weeds. http://www.weedscience.org. Accessed: March 23, June 2, and July 22, 2011.

Hoskins, A., B. Young, R. Krausz, and J. Russin. 2005. Control of Italian ryegrass (*Lolium multiflorum*) in winter wheat. Weed Technol. 19:261–265.

Jaseniuk, M., R. Ahmad, A. Sherwood, J. Firestone, A. Perez-Jones, T. Lanini, C. Mallory-Smith, and Z. Stednick. 2008. Glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in California: distribution, response to glyphosate, and molecular evidence for an altered target enzyme. Weed Sci. 2008. 56:496:502.

Jalaludin, A., J. Ngim, B. Bakar, and Z. Alias. 2010. Preliminary findings of potentially resistant goosegrass (*Eleusine indica*) to glufosinate-ammonium in Malaysia. Weed Biol. Manag. 10:256–260.

Jones, C., J. Chandler, J. Morrison, S. Senseman, and C. Tingle. 2001. Glufosinate combination and row spacing for weed control in glufosinate-resistant corn (*Zea mays*). Weed Technol. 15:141–147.

Knezevic, S., J. Streibig, and C. Ritz. 2007. Utilizing R software for dose-response studies: the concept and data analysis. Weed Technol. 21: 840-848.

Koger, C. H. and K. N. Reddy. 2005. Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53:84–89.

Krieg, L. C., M. A. Walker, T. Senaratna, and B. D. McKersie. 1990. Growth, ammonia accumulation and glutamine synthetase activity in alfalfa (*Medicago sativa* L.) shoots and cells treated with phosphinothricin. Plant Cell Rep. 9:80-83.

Lacuesta, M., A. Munoz-Rueda, C. Gonzalez-Murua, and M. N. Sivak. 1992. Effect of phosphinothricin (glufosinate) on photosynthesis and chlorophyll fluorescence emission by barley leaves illuminated under photorespiratory and nonphotorespiratory conditions. J. Exp. Bot. 43:246-251.

Letouze, A. and J. Gasquez. 2001. Inheritance of fenoxaprop-P-ethyl resistance in a blackgrass (*Alopecurus myosuroides* Huds.) population. Theor. Appl. Genet. 103:288-296.

Lorraine-Colwill, D., S. Powles, T. Hawkes, and C. Preston. 2001. Inheritance of evolved glyphosate resistance in *Lolium rigidum* (Gaud.). Theor. Appl. Genet. 102:545-550.

Lorraine-Colwill, D., S. B. Powles, T. R. Hawkes, P. H. Hollinshead, S. A. J. Warner, and C. Preston. 2001. Investigations into the mechanism of glyphosate resistance in *Lolium rigidum.* Pest. Biochem. Physiol. 74:62-72.

Mallory-Smith, C. A, D. C. Thill, M. J. Dial, and R. S. Zemetra. 1990. Inheritance of sulfonylurea herbicide resistance in *Lactuca* spp. Weed Technol. 4:787-790.

Manderscheid, R. 1993. Irreversible inhibition of glutamine synthetase from higher plants by the herbicide phosphinothricin. Pages 103–107 *in* P. Böger, and G. Sandman, eds. Target Site Assays for Modern Herbicides and Related Phytotoxic Compounds. Boca Raton, FL: Lewis Publishers.

McKell, C., C. Duncan, and C. Muller. 1969. Competitive relationship of annual ryegrass (*Lolium multiflorum* Lam.) Ecology, Vol. 50, No. 4. pp 653-657.

McNally, S. F., B. Hirel, P. Gadal, A. F. Mann, and G. R. Stewart. 1983. Glutamine synthetases of higher plants. Plant Physiol. 72:22-25.

Miflin, B. J. and D. Z. Habash. 2002. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J. Exp. Bot. 53:979-987.

Moss, S., C. Cocker, A. Brown, L. Hall, and L. Field. 2003. Characterization of target site resistance to ACCase-inhibiting herbicides in the weed *Alopecurus myosuroides* (Black grass). Pest Manag. Sci. 59:190-201.

Nandula, V., K. Reddy, D. Poston, A. Rimando, and S. Duke. 2008. Glyphosate tolerance mechanism in Italian ryegrass (*Lolium multiflorum*) from Mississippi. Weed Sci. 56:344-349.

Ng, C. H., W. Ratnam, S. Surif, and B. Ismail. 2004. Inheritance of glyphosate resistance in goosegrass (*Eleusine indica*). Weed Sci. 52:564-570.

Perez, A., C. Alister, and M. Kogan. 2004. Absorption, translocation and allocation of glyphosate in resistant and susceptible Chilean biotypes of *Lolium multiflorum*. Weed Biol. Manag. 4:56–58.

Perez, A. and M. Kogan. 2003. Glyphosate-resistant *Lolium multiflorum* in Chilean orchards. Weed Res. 43:12-19.

Perez-Jones, A., K-W. Park, J. Colquhoun,C. Mallory-Smith, and D. Shaner. 2005. Identification of glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in Oregon. Weed Sci. 53:775-779.

Perez-Jones, A., K-W. Park, N. Polge, J. Colquhoun, and C. Mallory-Smith. 2007. Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. Planta 226:395-404.

Perez-Vicente, R., G. Dorado, and J. M. Maldonado. 1996. Cross-species amplification of glutamine synthetase cDNA by polymerase chain reaction with degenerate primers. Physiol. Plant. 98:705-713.

Petersen, J. and K. Hurle. 2000. Influence of climatic conditions and plant physiology on glufosinate-ammonium efficacy. Weed Res. 41:31–39.

Pline, W. A., J. Wu, and K. K. Hatzios. 1999. Absorption, translocation, and metabolism of glufosinate in five weed species as influenced by ammonium sulfate and pelargonic acid. Weed Sci. 47:636–643.

Pornprom, T., J. Chompoo, and B. Grace. 2003. Glufosinate tolerance in hybrid corn varieties based on decreasing ammonia accumulation. Weed Biol. Manag. 3:41–45.

Pornprom, T., N. Prodmatee, and O. Chatchawankanphanich. 2008a. Glutamine synthetase mutation conferring target-site based resistance to glufosinate in soybean cell selection. Pest. Manag. Sci 65:216–222.

Pornprom, T., A. Pengnual, N. Udomprasert, and O. Chatchawankanphanich. 2008b. The role of altered glutamine synthetase in conferring resistance to glufosinate in mungbean cell selection. Thai J. Agric. Sci. 41:81-90.

Pornprom, T., S. Surawattananon, and P. Srinives. 2000. Ammonia accumulation as an index of glufosinate-tolerant soybean cell lines. Pestic. Biochem. Physiol. 68:102–106.

Powles, S. and C. Preston. 2006. Evolved glyphosate resistance in plants: Biochemical and genetic basis of resistance. Weed Tech. 20:282-289.

Prasertsongskun, S., N. Sangduen, S. Suwanwong, V. Santisopasri, and H. Matsumoto. 2002. Increased activity and reduced sensitivity of glutamine synthetase in glufosinate-resistant vetiver (*Vetiveria zizanioides* Nash) cells. Weed Biol. Manage. 2:171-176.

Preston, C. and A. Wakelin. 2008. Mini-review: Resistance to glyphosate from altered herbicide translocation patterns. Pest Manag Sci. 64:372-376.

Ray, T. 1989. Herbicides as inhibitors of amino acid biosynthesis. Pages 106-107 *in* Böger, P. and Sandman G., eds. Target Sites of Herbicide Action. Boca Raton, Florida: CRC Press, Inc.

Saari, L., J. C. Cotterman, and D. C. Thill. 1994. Resistance to acetolactate synthase inhibiting herbicides. Pages 83-140 in S. B. Powles and J. A. M. Holtum, eds. Herbicide resistance *in* plants: Biology and Biochemistry. Boca Raton, FL: Lewis Publishers.

Sankula, S., M. Braverman, and J. Oard. 1998. Genetic analysis of glufosinate resistance in crosses between transformed rice (*Oryza sativa*) and red rice (*Oryza sativa*). Weed Technol. 12:209–214.

Sauer, H., A. Wild, and W. Ruhle. 1987. The effect of phosphinothricin (glufosinate) on photosynthesis: The causes of inhibition of photosynthesis. Z. Naturforsch. 42c:270-278.

Sellers, B. A., R. J. Smeda, and J. Li. 2004. Glutamine synthetase activity and ammonia accumulation is influenced by time of glufosinate application. Pestic. Biochem. Physiol. 78:9–20.

Seng, C. T., L. V. Lun, C. T. San, and I. B. Sahid. 2010. Initial report of glufosinate and paraquat multiple resistance that evolved in a biotype of goosegrass (*Eleusine indica*) in Malaysia. Weed Biol. Manag. 10:229–233.

Shaner, D. L. 2009. Role of translocation as a mechanism of resistance to glyphosate. Weed Sci. 57:118-123.

Simarmata, M., S. Bughrara, and D. Penner. 2005. Inheritance of glyphosate resistance in rigid ryegrass (*Lolium rigidum*) from California. Weed Sci. 53:615-619.

Simarmata, M. and D. Penner. 2008. The basis for glyphosate resistance in rigid ryegrass (*Lolium rigidum*) from California. Weed Sci. 56:181-188.

Skora-Neto, F., H. Coble, and F. Corbin. 2000. Absorption, translocation and metabolism of ^{14}C-glufosinate in *Xantium strumarium*, *Commelina diffusa*, and *Ipomoea purpurea*. Weed Sci. 48:171–175.

Steckel, J., S. Hart, and L. Wax. 1997. Absorption and translocation of glufosinate on four weed species. Weed Sci. 45:378–381.

Streibig, J. C., M. Rudemo, and J. E. Jensen. 1993. Dose–response curves and statistical models. Pages 29–56 in J. C. Streibig and P. Kudsk, eds. Herbicide Bioassays. Boca Raton, FL: CRC.

Tachibana, K., T. Watanabe, Y. Sekizawa, and T. Takematsu. 1986. Accumulation of ammonia in plants treated with bialaphos. J. Pestici. Sci. 11:33–37.

Tal, A. and B. Rubin. 2004. Molecular characterization and inheritance of resistance to ACCase-inhibiting herbicides in Lolium rigidum. Pest. Manag. Sci. 60:1013-1018.

Tsai, C. J., C. S. Wang, and C. Y. Wang. 2006. Physiological characteristics of glufosinate resistance in rice. Weed Sci. 54:634–640.

Tucker, K., G. Morgan, S. Senseman, T. Miller, and P. Baumann. 2006. Identification, distribution and control of Italian ryegrass (*Lolium multiflorum*) ecotypes with varying levels of sensitivity to triasulfuron in Texas. Weed Technol. 20:745–750.

Unno, H., T. Uchida, H. Sugawara, G. Kurisu, T. Sugiyama, T. Yamaya, H. Sakakibara, T. Hase, and M. Kusunoki. 2006. Atomic structure of plant glutamine synthetase, a key enzyme for plant productivity. Journal of Biological Chemistry. 281:29287-29296.

Volenberg, D., E. Stoltenberg, and C. Boerboom. 2001. Biochemical mechanism and inheritance of cross-resistance to acetolactate synthase inhibitors in giant foxtail. Weed Sci. 49:635-641.

Volenberg, D., and E. Stoltenberg. 2002. Inheritance of resistance in eastern black nightshade (*Solanum nigrum*) to acetolactate synthase inhibitors. Weed Sci. 50:731-736.

Wakelin, A. M., D. F. Lorraine-Colwill, and C. Preston. 2004. Glyphosate resistance in four different populations of *Lolium rigidum* is associated with reduced translocation of glyphosate to meristematic zones. Weed Res. 44:453–459.

Wakelin, A., and C. Preston. 2006. Inheritance of glyphosate resistance in several populations of rigid ryegrass (*Lolium rigidum*) from Australia. Weed Sci. 54:212-219.

Weatherburn, M. 1967. Phenol-hypochloride reaction for ammonia. Anal. Chem. 39:971–974.

Wild, A. and C. Wendler. 1991. Effect of glufosinate (phosphinothricin) on amino acid content, photorespiration, and photosynthesis. Pestic. Sci. 30:422-424.

Wild, A., H. Sauer, and W. Ruhle. 1987. The effect of phosphinothricin (glufosinate) on photosynthesis I. Inhibition of photosynthesis and accumulation of ammonia. Z. Naturforsch. 42c: 263-269.

Woodburn, A. T. 2000. Glyphosate: production, pricing and use worldwide. Pest Manag. Sci. 56:309-312.

Yu, Q., A. Cairns, and S. Powles. 2007. Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a *Lolium rigidum* biotype. Planta 225:499-513.

Zelaya, I., M. Owen, and M. VanGessel. 2007. Transfer of glyphosate resistance: evidence of hybridization in *Conyza* (Asteraceae). Am. Journal of Botany 94:660-673.