Glyphosate Resistance in Annual Ryegrass (*Lolium rigidum* Gaud.) with Multiple Resistance Mechanisms

Yazid Bostamam

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ABSTRACT

Glyphosate (*N*-(phosphonomethyl)glycine) is a post-emergent, systemic and non-selective herbicide for the control of annual and perennial weeds. This herbicide has very low toxicity to the mammals. The target enzyme for glyphosate in plants is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Glyphosate inhibits the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan in the plant. The first case of glyphosate resistance was reported in *Lolium rigidum* in Australia after 15 years of persistence use of this herbicide and the number weeds reported resistant to glyphosate has increased around the world. So far, two mechanisms known to be involved in resistance to glyphosate are target-site mutation and reduced herbicide translocation. Recently, two populations of *L. rigidum* from Australia have been discovered with very high levels of resistance to glyphosate. This project aims to determine the levels of glyphosate resistance in these populations, investigate glyphosate resistance mechanisms in the populations and finally assess the mode of inheritance of resistance.

In this project, four resistant (NLR70, SLR77, SLR80 and SLR88) and one susceptible (VLR1) *L. rigidum* populations were evaluated for their response to glyphosate. From the dose response experiments, the susceptible population of VLR1 was completely controlled with the recommended rate of glyphosate (450 g a.e ha^{-1}). In contrast, the resistant populations were not fully controlled by this herbicide rate. There was considerable variation between the populations in their resistance to glyphosate. In comparison to the susceptible population VLR1, SLR77 was 2.2 to 3.5 fold resistant to glyphosate, NLR70 was 3.7 to 8.4 fold resistant to glyphosate, SLR88 was 5.6 to 11.4 fold resistant to glyphosate and SLR80 was 8.2 to 76.7 fold resistant to glyphosate.

The mechanism of glyphosate resistance in the populations was investigated. ^{14}C-glyphosate was used to determine the absorption and translocation of glyphosate among the populations. There was no significant difference on the absorption of ^{14}C-glyphosate 48 hours after treatment in the population. However, the accumulation of ^{14}C-glyphosate in the stem region was higher in the susceptible VLR1 population (25.9%) and in resistant SLR77 (25%) than the other three populations. The resistant populations NLR70, SLR88 and SLR80 had about half the amount of glyphosate

accumulating in the stem region. These three resistant populations appear to be resistant to glyphosate as a result of reduced translocation of glyphosate to the shoot meristem.

Part of the EPSP synthase gene of the susceptible and four resistant populations was amplified and sequenced to identify any changes in the nucleotide sequence. The predicted amino acid sequence from the susceptible population VLR1 was the same as the consensus sequence from other plant species in the conserved region sequenced. However, the resistant populations of NLR70, SLR77, SLR80 and SLR88 showed polymorphisms within the nucleotide sequence in this region. Single nucleotide substitutions of A for C at codon 106 were observed in the resistant populations SLR77 and SLR80. This nucleotide change is predicted to substitute threonine for proline at position 106. In the resistant population SLR88, a nucleotide substitution of T for C was observed at the same codon. This nucleotide substitution is predicted to change the amino acid from proline 106 to serine. Therefore, these three populations appear to be resistant to glyphosate as a result of a target-site mutation.

An inheritance study was conducted by cross pollinating the susceptible VLR1 and resistant SLR88 population. From the dose response, the parent susceptible was completely killed with the recommended rate of glyphosate and higher rates of glyphosate were required to control parental resistant and both F_1 progenies (maternal susceptible and resistant). Both F_1 progenies showed an intermediate response to glyphosate compared with the parental populations. This indicated that the resistance to glyphosate in population SLR88 is inherited by nuclear gene(s) through the transfer of pollen during the cross pollination.

It is suggested that SLR88 and SLR80 population contain both glyphosate resistant mechanisms due to the cross pollination between individuals with different resistant mechanisms. Having two resistant mechanisms results in populations being highly resistant to glyphosate compared to those with one resistance mechanism. The higher level of glyphosate resistance in these multiple glyphosate resistance populations will likely make them harder to manage.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference is made in the text.

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This poem goes to you all……

Pisang emas dibawa belayar,
Masak sebiji di atas peti,
Hutang emas boleh ku bayar,
Hutang budi ku bawa mati.

TABLE OF CONTENTS

CHAPTER ONE

Literature Review

1.1 Introduction

In agriculture, there are many obstacles for growers to produce a quality product with high yields. One of the major problems faced by farmers is competition with the crops from weeds. Weeds can be defined as unwanted plants that grow in undesirable places (Monaco *et al*., 2002). Weeds become a problem when settled agriculture started around 10,000 years ago (Zimdahl, 2007). Weeds create problems because they compete for space, light, moisture and nutrients, and thus affect the ability of the crop to produce higher yield. Farmers have many methods at their disposal to control weeds. The earliest technique was hand weeding. Later, special instruments were invented to help in weeding. This started with the invention of primitive hoes and then followed by animal-powered implements. The implements later were improved by using mechanical power (Heap and LeBaron, 2001).

The first chemical used to kill weeds was accidentally discovered by Bonnet in 1896 when Bordeaux mixture used in controlling downy mildew in grapevines turned the leaves of *Sinapsis arvensis* black (Brian, 1976). The introduction of synthetic chemicals for weed control began in 1932 with 2-methyl-4,6-dinitrophenol (DNOC) (Brian, 1976). This chemical was used in Europe and America. Weed control come to a new era when the first crop selective herbicides: 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxy acetic acid (2,4-D), were introduced in 1945 (Brian, 1976; Heap and LeBaron, 2001). Starting from that year, various types and mode of action herbicide were invented, introduced and used by farmers around the world. An example of the herbicide modes of action used in Australia is given in Table 1.1. Herbicides play a major role on the weed management and result increase on the crop production (Powles *et al*., 1997). Herbicides have allowed intensive agriculture and horticulture systems to be practiced with minimum tillage.

As had happened with fungicides and insecticides, the persistence and continuous use of herbicides was predicted to result in weed populations with resistance to herbicides (Harper, 1956). Herbicide resistance refers to the ability of previously susceptible weed population to survive the application of herbicide at the recommended rate that control majority of the population and pass that triat to its progeny (Heap, 1997; Powles *et al*., 1997; Heap, 2009). The first case of herbicide resistance was identified in a population of *Senecio vulgaris* resistant to triazine herbicides from a nursery in 1958 (Ryan, 1970). Since then, weeds have evolved resistance to most

herbicide modes of action and the number of new cases reported is increasing every year as shown in Figure 1.1 (Holt *et al*., 1993; Heap, 1997; Powles *et al*., 1997; Heap, 2009). The latest report documented 332 cases of weed species resistant to various herbicides worldwide (Heap, 2009). The inhibitors of the enzyme acetolactate synthase (ALS) have the most resistance with 102 cases and this is followed by inhibitors of photosynthesis at photosystem II with 68 cases.

Table 1.1: Group of herbicides and their mode of action for Australia (Anonymous, 2009).

NOTE:
This table is included on page 4
of the print copy of the thesis held in
the University of Adelaide Library.

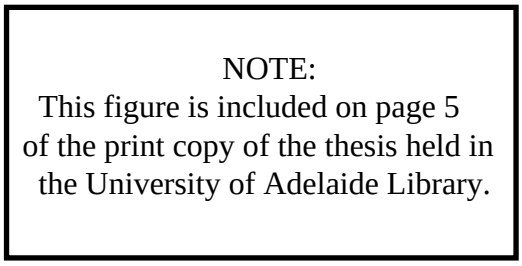

Figure 1.1: The accumulation of resistant weeds species to herbicides in worldwide (Heap, 2009).

In Australia, synthetic herbicides were first released in 1946. After several decades of herbicide use, the first occurrence of resistance was reported in the population of *Lolium rigidum* from Bordertown, South Australia to diclofop-methyl (Heap and Knight, 1982). This was followed by the occurrence of a resistant population of *Hordeum leporinum* ssp. *glaucum* from Willaura, Victoria to paraquat (Warner and Mackie, 1983). According to Heap (2009), there are 34 weeds species in Australia reported resistant to different type of herbicides up to date (Table 1.2).

Table 1.2: The resistant weed species to various types of herbicide reported in Australia (Heap, 2009).

NOTE:
This table is included on pages 6-7 of the print copy of the thesis held in the University of Adelaide Library.

In some cases, weed populations are resistant to more than one herbicide. Therefore, two other common terms used in herbicide resistance are cross resistance and multiple resistance. Cross resistance refers to the evolution of resistance to herbicides to which the weed has never been exposed (Holt *et al*., 1993). There are many studies showing the phenomenon of cross resistance in weeds. One example of this is atrazine resistant *Amaranthus hybridus* that shows cross resistance to cyanazine, metribuzin, linuron and desmedipham, which are from different herbicide chemistries, but have the same mode of action (Fuerst *et al*., 1986). A resistant population of *L. rigidum* to diclofop-methyl was reported cross resistance to three herbicides from the same chemical group as well as two sulfonylurea herbicides (Heap and Knight, 1986). A further study on cross resistance of SLR31 population to chlorsulfuron suggested that the mechanism was due to detoxification (Christopher *et al*., 1991). Other studies on cross resistance of weeds were reported by Moss (1990), Burnet *et al*. (1991), Tardif *et al*. (1993) and Seefeldt *et al*. (1994).

Multiple resistance refers to the evolution of resistance to herbicides with different modes of action through multiple mechanisms (Holt *et al*., 1993). A study by Pölös *et al*. (1988) demonstrated the occurrence of multiple resistance in *Conyza canadensis* resistant to paraquat and atrazine, two herbicides with different modes of action. In Australia, a population of *L. rigidum*, VLR69, exhibited multiple resistance to nine chemical classes of herbicides after application of five different classes of herbicides for the period of 21 years (Burnet *et al*., 1994). This population also exhibited herbicide cross resistance. The increase in herbicide resistance cases has greatly reduced the usefulness of herbicides as the main tool to control weeds. This makes some growers less confident in the effectiveness of herbicides.

1.2 Evolution of Herbicide Resistance

Significant factors contributing to the evolution of herbicide resistance in weed populations are the initial gene frequency, herbicide selection pressure, gene flow and relative fitness of herbicide resistant and susceptible individuals. These factors influence the variability of resistant among weed population.

1.2.1 Initial Gene Frequency

It is generally believed that mutations in genes endowing resistance to herbicides are present in weed populations, but at a very low level, before herbicides are ever used (Moss and Rubin, 1993). The initial frequencies or resistant alleles are unpredictable and likely to be different between weed species, localities and types of resistance. In general, it was assumed that the initial frequency gene of herbicide resistant in the weed population was about 10^{-6} (Maxwell and Mortimer, 1994). When the initial gene frequency is lower, resistance appears later. For example, it was suggested that triazine resistance might have an initial resistance gene frequency of 10^{-10} to 10^{-20}, which would result in resistance after 10 years as compared to sulfonylurea herbicides with an initial frequency gene of 10^{-6}, which only required 3 years for resistance to evolve (Gressel, 1991). However, when investigated the initial gene frequency for sulfonylurea herbicide resistance in *L. rigidum* populations was between 2.2×10^{-5} to 1.2×10^{-4} (Preston and Powles, 2002). The high frequency of this mutation meant resistance evolved rapidly to this herbicide mode of action once it was used.

1.2.2 Selection Pressure

Selection pressure is related to the survival rate of susceptible and resistant weeds after herbicide application (Gressel, 1991). Higher selection pressure creates faster evolution of resistance in weeds to herbicides. There are several factors contributing to the intensity of selection pressure. Among these are: the frequency of herbicide application and the persistence of the herbicide in the environment (Jasieniuk *et al*., 1996). Herbicides that do not control much of the weed population will not apply as much selection pressure as those that control more of the population. Herbicides that persist in the environment will control a greater proportion of the population than those that dissipate quickly. Repeated application of herbicides over several years increases selection pressure by selecting multiple generations of the weed species.

In monoculture farming system, growers normally apply herbicides, as they are most reliable form of weed control (Moss and Rubin, 1993) and can result in 90 to 99% mortality of the susceptible weed population (Diggle and Neve, 2001). This exceptional control of the weed population applies considerable selection pressure for resistance. As a result, there are many cases of weeds resistant to herbicides in cereal crops (Heap and Knight, 1982; Tucker and Powles, 1988; Gill, 1995; VanGessel, 2001), in horticultural

crops (Powles *et al*., 1998; Pérez and Kogan, 2003) and other areas where herbicides are used persistently (Burnet *et al*., 1991).

1.2.3 Gene Flow

Gene flow is a transfer of genes through gametes, dispores or individuals to a different location and establishment of a new population at the new location (Golenberg, 1987). In plants, this typically happens through pollen and seed dispersal (Schaal, 1980; Hamrick, 1982). Gene flow transferred through pollen varies between species with different pollination mechanisms and with environment conditions during flowering (Stallings *et al*., 1995). Generally, wind can disperse pollen further from the source compared with insects (Hamrick, 1982). The further pollen is distributed; the greater the potential for gene flow. However, pollen dispersal curves decline with distance. For example, the percentage of gene flow to susceptible *Kochia scoparia* was less (0.01% to 1.4%) at a distance of 28.9 m from the resistant plant than at 1.5 m (4% to 13%) (Stallings *et al*., 1995). A study by Govindaraju (1988) on the dispersal ability and level of gene flow in plants also demonstrated that pollination mechanisms, such as wind and animals, are important to generate different levels of gene flow among populations. One feature of pollen mediated gene flow is that it allows the accumulation of resistance mechanisms within individuals in populations. If a plant with one resistance mechanism crosses with a plant with a different resistance mechanism, some of the progeny will carry both mechanisms.

Another method of gene flow transfer is by seeds. The dispersal of weed seeds occurs through natural dehiscence mechanisms, wind, water, animals and human activities (Thill and Mallory-Smith, 1997). These factors contribute to the distance of seeds dispersal from the parent plants. A study of the appearance of resistant biotypes of *H. glaucum* at different farms suggested the possibility of movement through stock, hay and machinery between the fields (Tucker and Powles, 1988). Andrews *et al*. (1998) showed the spread of resistant *Avena fatua* in a field was due to farming activities, such as harvesting. Pollen or seed dispersal of resistance genes is an important mechanism for the spread of the herbicide resistance, allowing it to establish to a new site where it was not previously present. Resistant populations of *Solanum nigrum* in Poland, for instance, were dispersed by birds. According to Stankiewicz *et al*. (2001), the migration of birds during spring from France into Poland probably carried the resistant *S. nigrum* seeds in their digestive system.

1.2.4 Fitness

Fitness is the ability of an individual to compete and contribute to the gene pool of the next generation (Gressel and Segel, 1978). Factors affecting the fitness of weeds are: their ability to germinate; their ability to compete for resources with other individuals of the same or other species; and their ability to produce new seeds. Conard and Radosevich (1979) found that susceptible biotypes of *S. vulgaris* and *A. retroflexus* had a higher level of fitness than the resistant biotypes in the absence of herbicide. Susceptible biotypes of *Echinochloa colona* were also more competitive than resistant ones in the absence of herbicide application (Fischer *et al*., 1993). With resistance to photosystem II-inhibiting herbicides, the mutation providing resistance reduces electron transport in the photosynthetic apparatus. This reduces photosynthetic rates in resistant biotypes and contributes a fitness penalty to individuals carrying the resistance trait (Warwick, 1991). For example, *S. vulgaris* resistant biotypes were found less photoefficent compared to the susceptible biotypes (Holt *et al*., 1981). Because of the poor photosynthesis performance, the resistant biotype is less competitive in the absence of herbicides.

The fitness of weeds is important, because it provides a possible strategy for the management of resistant weeds. When the resistant weed biotype has a lower level of fitness than the susceptible biotypes, the susceptible weeds are going to replace the resistant biotype in the population in the absence of herbicide selection (Matthews, 1994). Jordan (1999) in studies on fitness effects of the triazine resistance mutation in *A. hybridus* found that simulation studies of population dynamics of resistant and susceptible *A. hybridus* indicated that interannual variations in fitness penalties can have a large effect on resistance dynamics in cropping systems. Jordan (1999) also showed growing a competitive crop could expose the fitness penalty in resistant biotypes.

1.3 Resistance Mechanisms

Herbicide resistance occurs because of biochemical changes in the plants that exhibit the resistance trait (Moss and Rubin, 1993). There are several ways this can occur. However, the most common are: where the ability of the herbicide to bind at the target site is reduced in resistant weeds; the herbicide is metabolised before it can reach the target site; and the amount of herbicide translocated to the target site is reduced (Preston and Mallory-Smith 2001). Target-site resistance is the most common mechanism observed (Devine and Preston, 2000).

1.3.1 Target-site Resistance

Target-site resistance is a change in the specific plant enzyme that is inhibited by a particular herbicide (Saari *et al*., 1994; Powles and Preston, 2006). According to Devine and Preston (2000), this is usually conferred by a mutation in the target protein that decreases herbicide binding without seriously compromising the function of the protein. An example of this is resistant to photosystem II inhibitors of triazine herbicides like atrazine and simazine. In resistant to photosystem II inhibitors, the herbicide binds to the D1 protein and blocks the transfer of electron donor (Q_A) to the mobile electron carrier of Q_B (Gronwald, 1994). It was determined that most resistant cases involve the mutation at the substitution of glycine for serine at amino acid residue 264 of D1 protein.

Other modes of action where target-site resistance occurs are inhibitors of acetyl-coenzyme A carboxylase, acetolactate synthase, tubulin elongation and 5-enolpyruvylshikimate-3-phosphate synthase (Preston and Mallory-Smith, 2001). The occurrence of resistant weed populations with target-site resistance to inhibitors of acetyl-coenzyme A carboxylase was reported to the resistant of *L. multiflorum* (Gronwald *et al*., 1992), *Eleusine indica* (Leach *et al*., 1995), *L. rigidum* (Tardif *et al*., 1993), *Setaria. viridis* (Marles *et al*., 1993) and *A. fatua* (Shukla *et al*., 1997).

1.3.2 Enhanced Herbicide Metabolism

Enhanced herbicide metabolism was documented as a mechanism in the resistant weeds to photosystem II inhibitors (Gronwald, 1994). In Australia, this mechanism was reported contributing in chlorotoluron and diclofop methyl resistance in a *L. rigidum* population (Preston *et al*., 1996; Preston and Powles, 1998) and a population of *Digitaria sanguinalis* resistant to fluazifop-butyl (Hidayat and Preston, 1997). In Europe, two resistant populations of *Alopecurus myosuroides* to chlorotoluron were also reported to have this mechanism (Hall *et al*., 1995). Enhanced herbicide metabolism refers to the capability of weeds to degrade the herbicide to less toxic compounds (Moss and Rubin, 1993). Many examples of herbicide metabolism are due to the activity of cytochrome P450 manooxygenases (Devine, 1997), but other enzymes can also contribute. A study of *L. rigidum* resistant to simazine found enhanced detoxification of the herbicide by cytochrome P450 monooxygenases (Burnet *et al.,* 1993a). Likewise, in *L. rigidum* resistant to chlorotoluron cytochrome P450 monooxygenases contributed to metabolism of the herbicide (Burnet *et al*., 1993b). However, a study by Gronwald *et*

al., (1989) showed a resistant biotype of *Abutilon theophrasti* was able to detoxify atrazine through more rapid glutathione conjugation.

1.3.3 Reduced Herbicide Translocation

Reduced herbicide translocation is a resistance mechanism where less herbicide is translocated into the site of action from the treated site of the plant. This type of resistance mechanism has been documented only in weed populations resistant to photosystem I disrupting herbicides (Preston, 1994) and glyphosate (Powles and Preston, 2006). For paraquat resistance, reduced herbicide translocation was reported as the mechanism of resistance in resistant populations of *C. bonariensis* (Fuerst *et al*., 1985), *Erigeron philadelphicus*, *E. canadensis* (Tanaka *et al*., 1986), *H. leporinum* (Preston *et al*., 1992) and *Crassophalum crepidioides* (Ismail *et al*., 2001). The same mechanism was also reported in glyphosate resistance weeds of *L. rigidum* (Lorraine-Colwill *et al*., 2003; Wakelin *et al*., 2004), *C. canadensis* (Feng *et al*., 2004; Koger and Reddy, 2005), *L. multiflorum* (Perez-Jones *et al*., 2005; Michitte *et al*., 2007) and *C. bonariensis* (Dinelli *et al*., 2008; Ferreira *et al*., 2008).

1.4 Glyphosate

Glyphosate (*N*-(phosphonomethyl)glycine) was first synthesised and prepared by Monsanto Agricultural Products Company in 1970 (Franz *et al*., 1997). Glyphosate is a post-emergent non-selective herbicide and was originally formulated as mono-isopropylamine salt. Now there are many formulations of glyphosate in the market, such as trimesium, diphenylamine, potassium and mono-ammonium salts. The chemical structure of glyphosate is shown in figure 1.2.

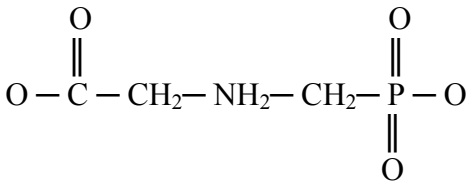

Figure 1.2: Chemical structure of glyphosate

Glyphosate was initially introduced as a plant growth regulator in cane to promote sucrose production, but later in 1974 was marketed as Roundup herbicide (Franz *et al*., 1997). Glyphosate has become the world's most widely used herbicide (Baylis, 2000). Glyphosate has been used in agricultural and non agricultural activities (Powles and Preston, 2006). In agriculture, glyphosate is normally used to control weeds before planting field crops, between rows in row crops and around perennial crops. Its non agricultural uses are to control weeds along roadsides, irrigation channels and around recreation areas. In modern minimum tillage systems, herbicides such as glyphosate are widely used to kill weeds prior to sowing a crop. This reduces damage to the structure of soil and erosion of soil by wind and rain. The introduction of glyphosate resistance crops has also drastically increased the use of glyphosate (Powles and Preston, 2006; Duke and Powles, 2008).

1.4.1 Glyphosate Characteristics

As a non selective herbicide, glyphosate is used for the control of both annual and perennial weeds. It is a systemic herbicide and the herbicide translocates readily from the treated leaves to the meristematic zone and roots of weeds (Franz *et al*., 1997). However, the appearance of visual symptoms is slow. The phytotoxic symptoms of glyphosate can be observed 2 to 3 weeks after application (Aston and Crafts, 1981).

Glyphosate has very low toxicity to the mammals (Franz *et al*., 1997). Glyphosate also has low dermal toxicity in humans (Wester *et al*., 1991). The compound does not leach into the ground water, because it binds tightly to soil particles. Glyphosate is metabolised in soil by microorganisms, initially to aminomethyl-phosphonic acid (AMPA) (Rueppel *et al*., 1977) and then to other compounds that are used as nutrients. The low mammalian toxicity and lack of soil residual effects help make glyphosate the most widely use and important herbicide in the world (Powles and Preston, 2006).

The efficacy of glyphosate to target weeds depends on several environmental factors. McWhorter and Azlin (1978) reported that the temperature, humidity and soil temperature affect the efficacy of glyphosate to *Sorghum halepense*. Besides these factors, rain also was suggested to influence the effectiveness of glyphosate. Rainfall after glyphosate application was found to reduce the degree of weed control (Bryson, 1987; Bariuan *et al*., 1999). In other studies there were differences in the glyphosate absorbed by young and old plants of *S. halepense* (Camacho and Moshier, 1991). In addition, glyphosate efficacy varies with growth stage in *Agropyron repens* (Sprankle *et al*., 1975). However, under field conditions, the effect of glyphosate on different growth stages was inconsistent (Sprankle *et al*., 1975).

To control weeds actively growing through rhizomes, such *S. halepense* and *A. repens*, a systemic herbicide rather than a contact herbicide is more practical. This is because contact herbicides only kill the upper part of the plant system. Therefore, after a certain period of time, the plant growth will recover through their rhizomes. In contrast, glyphosate translocation from the treated leaves to the roots and rhizomes will kill the plant (Camacho and Moshier, 1991).

1.4.2 Mode of Action

The target enzyme for glyphosate in plants is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Steinrucken and Amrhein, 1980). EPSPS is the penultimate enzyme of the shikimate pathway and catalyses a conversion of shikimite-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to yield EPSP and inorganic phosphate (Figure 1.3). Glyphosate occupies the binding site for PEP in the enzyme and is a potent inhibitor of EPSPS. This inhibits the biosynthesis of the plant aromatic amino acids phenylalanine, tyrosine and tryptophan and also increases the concentration

of shikimate acid. The shikimate pathway is also important in the biosynthesis of a number of secondary compounds (Steinrucken and Amrhein, 1980), and thus affects the physicochemical and physiological processes of treated weeds (Cole, 1985). This includes reduction of photosynthesis, inhibition of the transportation of auxin and enhancement of auxin oxidation (Baylis, 2000). Glyphosate inhibits EPSPS in plants, some fungi and some bacteria (Franz *et al*., 1997).

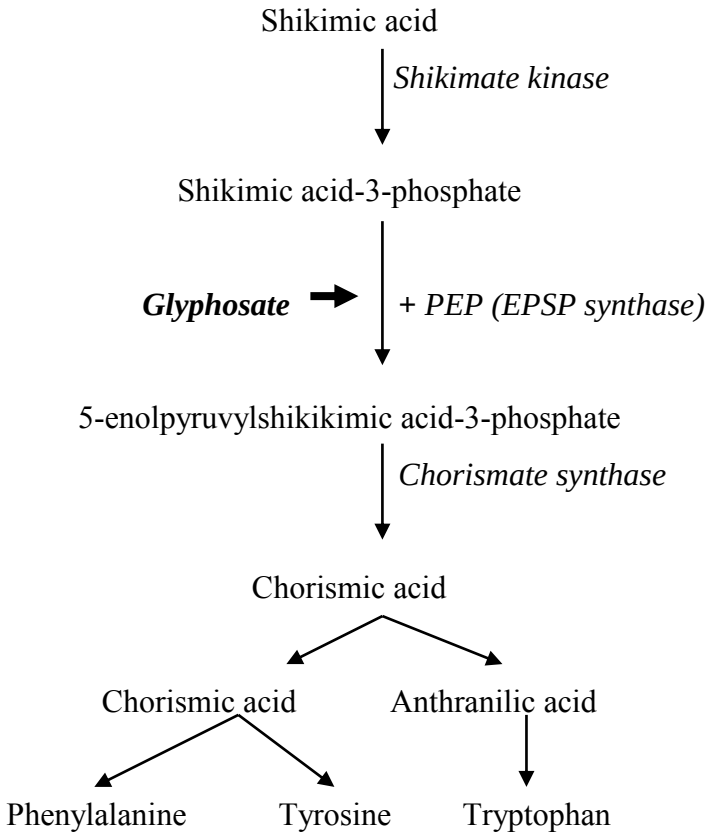

Figure 1.3: Glyphosate mode of action (Dill, 2005)

1.4.3 Weed Resistance to Glyphosate

The first cases of glyphosate resistant were reported from two populations of *L. rigidum* from Australia in 1996 (Powles *et al*., 1998; Pratley *et al*., 1999). The number of weeds reported resistant to glyphosate has increased around the world after that year. In 1999, *E. indica* in Malaysia (Lee and Ngim, 2000) and *L. multiflorum* in Chile (Pérez and Kogan, 2003) were reported resistant to glyphosate. In USA, *C. canadensis* was reported resistant to glyphosate in 2001 (VanGessel, 2001). To date, 16 weed species are confirmed resistant to glyphosate in 14 countries. This is shown in Table 1.3 (Heap, 2009).

The time taken for resistance to glyphosate to evolve is different from one country to another. *L. rigidum* in Australia took about 15 years from the first application to evolve resistance to glyphosate (Powles *et al*., 1998). In Chile, *L. multiflorum* took about 10 years to evolve resistance to glyphosate (Pérez and Kogan, 2003). *E. indica* in Malaysia took a shorter time of about 3 years to evolve resistance (Lee and Ngim, 2000). In USA, *C. canadensis* was reported to be resistant to glyphosate only within 3 years of glyphosate application (VanGessel, 2001).

Table 1.3: List of weed resistant to glyphosate (Heap, 2009).

NOTE:
This table is included on pages 18-19 of the print copy of the thesis held in the University of Adelaide Library.

1.4.4 Mechanisms of Glyphosate Resistance

Two mechanisms of resistance to glyphosate have been identified. These are target-site mutations and reduced herbicide translocation. The level of resistance conferred by the two mechanisms is different. The translocation mechanism produces a higher level of resistance compared to the target-site mutations (Powles and Preston, 2006).

1.4.4.1 Target-site Mutations

Target-site mutations for glyphosate resistance in weed populations were first reported in *E. indica* from Malaysia (Baerson *et al*., 2002). In that study, the resistant *E. indica* population showed 2 to 4 fold resistance to glyphosate compared with susceptible population. The amino acid change was determined at the position of proline 106 to serine in the resistant population compared with susceptible population. In Australia, a resistant population of *L. rigidum* from South Australia also exhibited resistance due to target-site mutation (Wakelin and Preston, 2006a). At the same position of amino acid, proline 106, a change of proline to threonine was identified in the resistant population. In other studies, target-site mutation mechanism also was determined in the resistant population of *L. multiflorum* from Chile (Perez-Jones *et al*., 2007), *L. rigidum* from California (Simarmata and Penner, 2008), *L. multiflorum* from California (Jasieniuk *et al*., 2008), *L. rigidum* from South Africa (Yu *et al*., 2007) and *E. indica* from the Philippines (Kaundun *et al*., 2008). In all these examples, changes at proline 106 were identified.

1.4.4.2 Reduced Herbicide Translocation

The reduced herbicide translocation mechanism of resistance to glyphosate was first reported in *L. rigidum* from Australia (Lorraine-Colwill *et al*., 2003). In that study, most of the absorbed glyphosate was accumulated in the leaf tips of the resistant population well away from the growing shoot meristem, while in susceptible population, glyphosate accumulated more in the stem and roots of the plant. *L. rigidum* resistant populations with reduced herbicide translocation mechanism exhibited 10 fold resistance compared with susceptible population (Wakelin *et al*., 2004). In other weed species, reduced herbicide translocation has been identified in resistant populations of *C. canadensis* (Feng *et al*., 2004; Koger and Reddy, 2005), *L. multiflorum* from Chile (Michitte *et al*., 2007) and *C. bonariensis* from Spain (Dinelli *et al*., 2008).

1.4.5 Inheritance of Glyphosate Resistance

The resistant allele in weeds can be inherited by maternal or nuclear gene inheritance (Jasieniuk *et al*., 1996). For nuclear gene inheritance, the herbicide resistance expression can be dominant or semi-dominant. According to Lorraine-Colwill *et al*. (2001), glyphosate resistance in *L. rigidum* is semi dominant and encoded by a single gene. The F_1 progenies from a cross between susceptible and resistant parental lines showed intermediate resistance to glyphosate. In *E. indica* (Ng *et al*., 2004a) and *C. canadensis* (Zelaya *et al*., 2004) glyphosate resistance was also reported as having a similar pattern of inheritance. However, resistant populations of *L. rigidum* from Australia show variation between dominant and semi-dominant genes controlling glyphosate resistance (Wakelin and Preston, 2006b). In contrast, a study on *L. rigidum* from California suggested the involvement of multiple genes contributing to glyphosate resistance (Simarmata *et al*., 2005).

1.5 *Lolium rigidum* Gaud.

L. rigidum is a species native to Europe, North Africa and Asia (Kloot, 1983), and has 14 chromosomes (2n=14) (Simarmata *et al*., 2005). This species was introduced as a pasture grass in the 19th century and wide spread in cropping area in Australia (Tardif *et al*., 1997; Preston and Powles, 2002). It is cross pollinated by wind (Kloot, 1983). *L. rigidum* is an annual grass that can grow up to 1 m tall with a spike to 30 cm long (Hussey *et al*., 1997). The leaf sheaths are green or purple and glabrous with 3 to 12 cm long and 0.5 to 2 mm wide (Marchant *et al*., 1987). *L. rigidum* is a prolific seed

producer. In competition with wheat crop, the plants can produce between 31,000 and 45,000 seeds m^{-2} (Rerkasem *et al*., 1980a).

L. rigidum seeds germinate in Australia following the autumn rains. According to McGowan (1970), 80% of *L. rigidum* seeds germinate by the end of May (late autumn) and another 5% seeds germinate at late July and August (mid-winter). Some of the seeds do not germinate because of seed dormancy and sometimes the germination of seeds is delayed. Factors controlling the dormancy of *L. rigidum* seeds have been documented in several studies. The temperature during the development of seeds affects seed dormancy, with higher dormancy when the temperatures are lower (Steadman *et al*., 2004). The temperature and moisture during the after-ripening period also influences the dormancy of *L. rigidum* seeds (Steadman *et al*., 2003).

According to Gill (1996), *L. rigidum* is one the most important components of a rotation phase in cattle or sheep farming and cereal cultivation in Australia. It is a major pasture feed for sheep or cattle grazing, but during the wheat cropping phase, *L. rigidum* is a major weed. Competition between wheat and *L. rigidum* has an impact during the early stage of the crop's growth, resulting in a large reduction of yield (Reeves, 1976). Competition for nitrogen also occurs as early as the 2-leaf stage of wheat (Monaghan, 1980). This competition will reduce the production of tillers, ear formation and grain filling of wheat (Smith and Levick, 1974). However, the effect of *L. rigidum* on wheat yield is not as large if germination of *L. rigidum* is later than wheat (Rerkasem *et al*., 1980b).

1.5.1 L. rigidum Resistance to Herbicides

L. rigidum in Australia was first reported resistance to the herbicide diclofop-methyl in 1982 (Heap and Knight, 1982). After that, the number of *L. rigidum* populations reported resistant to various herbicide modes of action has increased. In Australia, *L. rigidum* has evolved resistance to inhibitors of acetyl-coenzyme A carboxylase (ACCase) (Heap and Knight, 1982; Tardif *et al*., 1993), acetolactate synthase (ALS) (Christopher *et al*., 1992), photosystem II, carotenoid biosynthesis, tubulin elongation (Burnet *et al*., 1991) and EPSP synthase (Powles *et al*., 1998). From a survey in the cropping area of Western Australia, the majority of *L. rigidum* populations were resistant to ACCase and ALS (Owen *et al*., 2007). In 1996, *L. rigidum* was first reported resistant to glyphosate (Powles *et al*., 1998; Pratley *et al*., 1999).

Since then, more than 87 locations, including fields, vineyards, orchards, irrigation channels and fence lines, with glyphosate resistant *L. rigidum* have been reported (Preston *et al*., 2009).

1.6 Conclusion

The most reliable and economic way to control weeds is by herbicide application. Therefore, growers will use herbicides every year contributing to the evolution of herbicide resistance in weeds. Resistance has occurred in many species of weeds and to most herbicide modes of action. In some cases, cross and multiple resistance to herbicides occur. However, the level of resistance and time taken for weeds species to evolve resistance to herbicides are different from one place to another.

Resistance to glyphosate was predicted to be unlikely in weeds because of the unique mode of action of the herbicide and the lack of metabolism of glyphosate in plants (Bradshaw *et al*., 1997). There were no reports on glyphosate resistance for more than 20 years after it was introduced in 1974. Glyphosate resistance was first reported in weeds in 1996 (Powles *et al*., 1998; Pratley *et al*., 1999). In some cases, weed resistant to glyphosate only takes around 3 years of intensive herbicide application (Lee and Ngim, 2000; VanGessel, 2001). So far, the only two mechanisms are known to be involved in resistance to glyphosate are target-site mutation and reduce herbicide translocation. The two mechanisms produce different, although modest, levels of resistance in weeds.

L. rigidum resistance to glyphosate is expected to increase if growers continue to use the herbicide. Recently, two populations of *L. rigidum* have been discovered with much higher levels of resistance to glyphosate than populations previously discovered. This much higher level of resistance may have appeared due to both mechanisms (target-site mutation and reduced herbicide translocation) being in the same plant. This could happen when a plant with one mechanism crosses with a plant with the other mechanism. Such a situation is likely where growers continue to rely on glyphosate even after resistance has evolved. Alternatively, another mechanism of resistance might be responsible.

1.7 Objectives of the Project

This project will investigate recently discovered populations of *L. rigidum* from Australia with high levels of glyphosate resistance. This will include detailed dose response studies to determine the levels of glyphosate resistance that can be achieved. The potential mechanisms of glyphosate resistance for each population will be determined. The final part of this study assessed the mode of inheritance of resistance through cross pollination of resistant and susceptible populations. This information will help understand the causes of evolution of *L. rigidum* populations with high levels of resistance and may help develop strategies to minimise evolution of this type of resistance.

CHAPTER TWO

Screening of *L. rigidum* Populations for Resistance to Glyphosate

2.1 Introduction

The continuous use of herbicides with the same mode of action results in evolution of resistance in weed populations. Glyphosate resistant populations of *L. rigidum*, a well established and major weed species have been reported from various locations and different agricultural areas, such as cereal crops fields, orchards and vineyards (Powles *et al*., 1998; Pratley *et al*., 1999; Wakelin *et al*., 2004; Wakelin and Preston, 2006a). The levels of resistance reported vary from low to moderate resistance. Recently, two new resistant populations had been discovered from South Australia potentially showing much higher resistance to glyphosate. Although these two populations were from different locations, both populations exhibited a much higher resistance level compared to previously report glyphosate-resistant populations in preliminary screens.

Reduced herbicide translocation and target-site mutations are mechanisms so far shown to occur in glyphosate resistant weed populations. The reduced herbicide translocation mechanism usually produces a higher resistance level population compared with target-site mutations. So far, only one resistant population of *L. rigidum* from South Africa has been shown to have of both mechanisms (Yu *et al*., 2007). The occurrence of very high resistant levels in two populations in South Australia warrants a study of the mechanisms involved.

Dose response experiments are normally used to determine the effect of different doses of herbicides on weed populations. Pot dose response experiments are a reliable means of determining resistance in weed populations. Plants can be grown under realistic conditions and treated with rates of herbicide similar to those used in practice. Here a pot dose response experiment was carried out to determine the resistant level of these new resistant populations comparing them with the existing populations with known mechanisms of resistance.

2.2 Materials and Methods

2.2.1 Plant Materials

The *L. rigidum* populations used in this experiment were originally collected from various parts of Australia where glyphosate resistance had been reported. The resistant populations used were NLR70, SLR77, SLR80 and SLR88; while one susceptible population, VLR1 was used as a control. The original source population of

NLR70 was from an apple orchard in Orange, New South Wales (Powles *et al*., 1998) and SLR77 was from vineyard in Eden Valley, South Australia (Wakelin and Preston, 2006a). Population SLR80 was from a vineyard near Clare, South Australia and population SLR88 from a vineyard near Coonawarra, South Australia. These populations of *L. rigidum* showed very high survival to glyphosate applications in initial screening tests (Preston 2008, pers. comm.) and were chosen for study because their response was qualitatively different to other glyphosate resistant populations tested. The glyphosate susceptible population, VLR1 was from an established pasture near Serviceton, Victoria. These plant materials were maintained as seeds at the Waite Campus, University of Adelaide.

2.2.2 Seed Germination

Seeds were germinated on 0.6% agar as described by Lorraine-Colwill *et al*. (2001). The seeds then were kept in an incubator with 12 hours dark period at 15^{o}C and 12 hours light period at 20^{o}C at 30 μmol $m^{-2}s^{-1}$. The seedlings were transplanted to Masrac Taglok punnet pot (9.5 x 8.5 x 9.5 cm) with standard potting mix after 7 days. Every pot consisted of 12 seedlings of the same biotypes with 3 replicates. The plants were maintained at normal growing condition (with approximate temperature ranged between 24 to 34^{o}C) during the growing period. The plants were watered 1 to 2 times a day, depending on the weather conditions.

2.2.3 Herbicide Application

Two weeks after transplanting, when the plants were at the 2 to 3-leaf stage, the plants were treated with glyphosate. The doses applied were: 0, 56.25, 112.5, 225, 450, 900, 1800, 3600 and 7200 g a.e ha^{-1} of glyphosate isopropylamine (Roundup Powermax®), Monsanto, Melbourne, Victoria, Australia. Non-ionic surfactant (Wetter TX®, Nufarm, Australia) was added at 0.2% V/V. The usual recommendation dose of glyphosate for *L. rigidum* is 450 g a.e ha^{-1}. From the preliminary test (data not shown), it had been determined that the resistant populations were not killed when treated with doses below the recommended rate. Therefore, the dosage treatment for NLR70 and SLR77 was started at 112.5 g a.e ha^{-1}; SLR80 and SLR88 at 225 g a.e ha^{-1}. This experiment was carried out at Waite Campus in October, November and December of 2008.

Treatments were applied to the plants by using a moving-boom laboratory sprayer with T-jet flat fan (015-110) nozzles (TeeJet 8001E, Spraying system Co., Wheaton, IL, USA). The output of the sprayer was 109 L ha^{-1} at 250 kPa pressure with 1 ms^{-1}. The distance between the plant samples and nozzles was 40 cm. The control plants were not treated with glyphosate, but were treated with surfactant.

The plants were returned outdoors after treatment. The number of plants surviving 21 days after treatment was recorded. Any plants showing severe chlorosis, extensive stunting and reduction in apical dominance were considered dead as they would not survive to produce seed. The effect of glyphosate on shoot growth was determined by cutting the shoots at ground level, drying the shoots at 40^{o}C for 3 days and measuring dry weight of shoots from each pot.

2.2.4 Statistical Analysis

The experiment was conducted in a Randomized Complete Block Design (RCBD) with 3 replicates and repeated 3 times. Mortality data were analysed using PriProbit version 1.63 to determine the herbicide dose-response relationships to the number of surviving plants. This provides the dose-response curves to the graph. The dry weight was analysed by log-logistic analysis (Seefeldt *et al*., 1995) using GraphPad Prism 5. LD_{50} (dose which controls 50% of the population) and GR_{50} (dose resulting in 50% growth reduction) were calculated for each population.

2.3 Results

Figure 2.1 shows the response of the five populations of *L. rigidum* to 450 g a.e ha^{-1} glyphosate. The susceptible population VLR1 was completely killed by this rate of glyphosate. Population SLR77 had some mortality and the survivors were stunted. Population NLR70 had little mortality, but survivors were stunted. In contrast, populations SLR80 and SLR88 were much less affected by this rate of glyphosate than the other populations tested.

The mortality responses of each *L. rigidum* population to different rates of glyphosate are shown in Figures 2.1 to 2.3. Each figure represents the data of a dose response experiment conducted in three different months of 2008. There were slight differences in the response to glyphosate between experiments; however, the pattern of responses for the five populations was similar across experiments. Environmental

conditions are known to influence the toxicity of glyphosate to target weeds (McWhorter and Azlin, 1978; Powles *et al*., 1998), which probably explains the differences between experiments seen here. In all the three experiments the most susceptible population was VLR1, which was well controlled by 450 g a.e ha^{-1} glyphosate. The next most susceptible population was SLR77 and followed by NLR70. Populations SLR80 and SLR88 were the most resistant to glyphosate. The survival rate of the resistant populations of SLR77, NLR70, SLR80 and SLR88 was 93.33 to 97.22%, 88.89 to 100%, 94.44 to 100% and 100% respectively. On the other hand, the survival rate of the susceptible population of VLR1 exhibited was 11.67 to 36.11% at the same rate of glyphosate.

Plate 2.1: Response of a susceptible and four resistant populations of *L. rigidum* 21 days after treatment with the recommended rate of glyphosate (450 g a.e ha^{-1}).

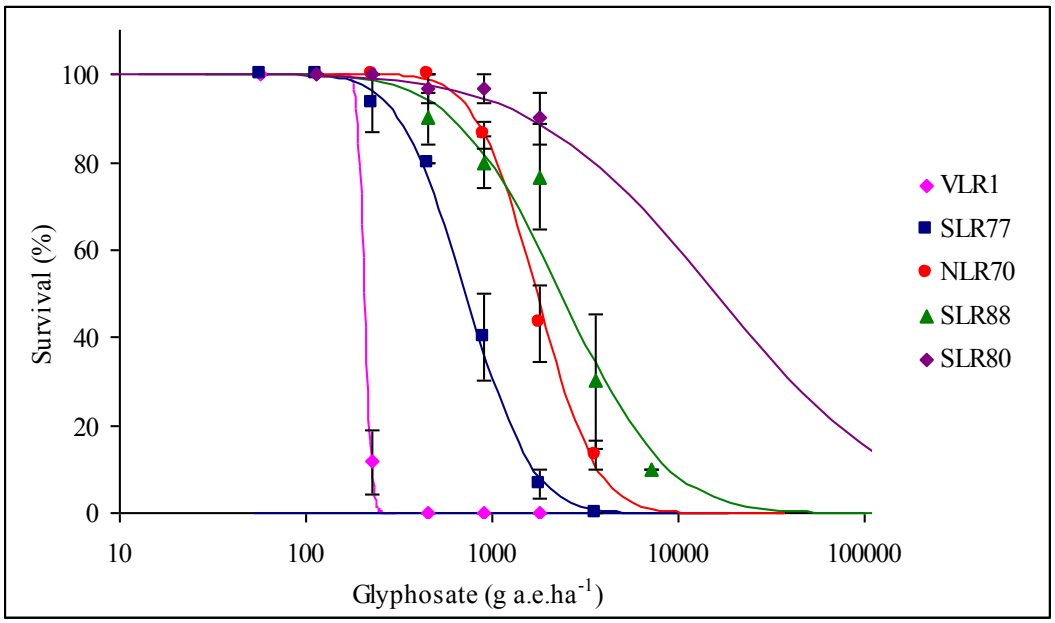

Figure 2.1: Dose response of a susceptible and four resistant populations of *L. rigidum* to glyphosate. This experiment was done in October 2008. Each point is the mean of 3 replicates ± SE.

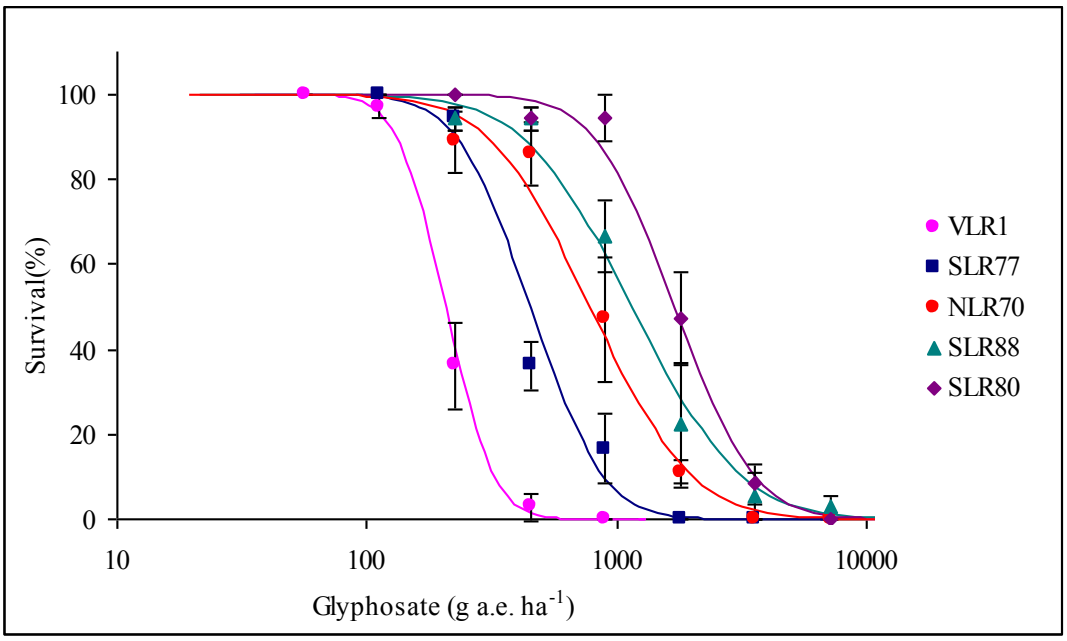

Figure 2.2: Dose response of a susceptible and four resistant populations of *L. rigidum* to glyphosate. This experiment was done in November 2008. Each point is the mean of 3 replicates ± SE.

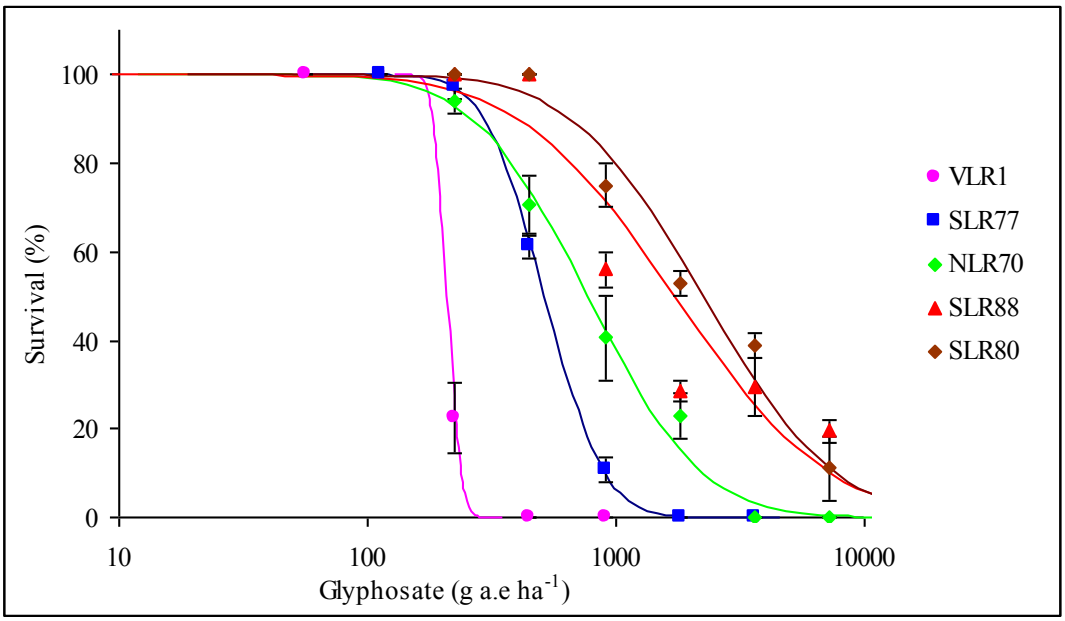

Figure 2.3: Dose response of a susceptible and four resistant populations of *L. rigidum* to glyphosate. This experiment was done in December 2008. Each point is the mean of 3 replicates ± SE.

The LD_{50}s for glyphosate calculated from the probit analysis for the five *L. rigidum* populations (Table 2.1) showed that the VLR1 population responded similarly across the three experiments with LD_{50}s between 206 and 209 g a.e. ha^{-1}. The LD_{50}s for the SLR77 population varied between 449 and 723 g a.e. ha^{-1} giving 2.2 to 3.5 fold resistance compared with susceptible population. The LD_{50}s for the NLR70 population varied between 767 and 1,737 g a.e. ha^{-1} giving 3.7 to 8.4 fold resistance. The LD_{50}s for the SLR88 population varied between 1,152 to 2,343 g a.e. ha^{-1} giving 5.6 to 11.4 fold resistance. The SLR80 population was the most resistant with LD_{50}s varying between 1,703 and an estimate of 15,762 g a.e. ha^{-1} for 8.2 to 76.7 fold resistance.

Table 2.1: The rate of glyphosate required to control 50% of the population (LD_{50}) for susceptible and four resistant populations of *L. rigidum* and the resistance index for the resistant populations.

Population	LD_{50} (g a.e. ha^{-1})					
	October	**R/S**	November	**R/S**	December	**R/S**
VLR1 (S)	206		207		209	
SLR77 (R)	723	3.5	449	2.2	514	2.5
NLR70 (R)	1,737	8.4	783	3.8	767	3.7
SLR88 (R)	2,343	11.4	1,152	5.6	1,715	8.2
SLR80 (R)	15,762	76.7	1,703	8.2	2,251	10.8

In the quantitative assay, the dry weights of plant populations in the three experiments were analysed. Similar patterns were observed with dry weight response to glyphosate compared to mortality in the *L. rigidum* populations. Low rates of glyphosate had a marked effect on the dry weight of the susceptible population VLR1 (Figures 2.4 to 2.6). The resistant populations varied in their response with SLR77 being the least resistant population followed by NLR70, then SLR88 and SLR80. Generally, the recommended glyphosate rate of 450 g a.e. ha^{-1} reduced the dry weight of SLR77 and NLR70, but had much less impact on SLR88 and SLR80.

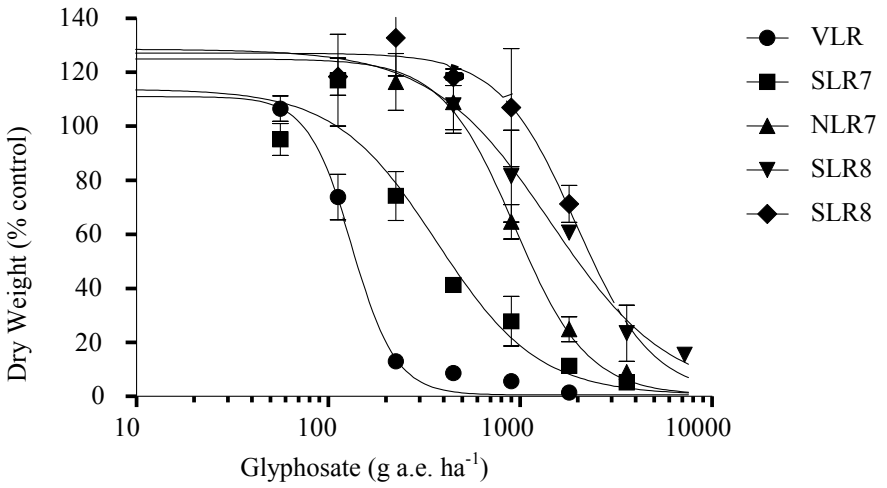

Figure 2.4: Dry weight of a susceptible and four resistant populations of *L. rigidum* to glyphosate. This experiment was done in October 2008. Each point is the mean of 3 replicates ± SE.

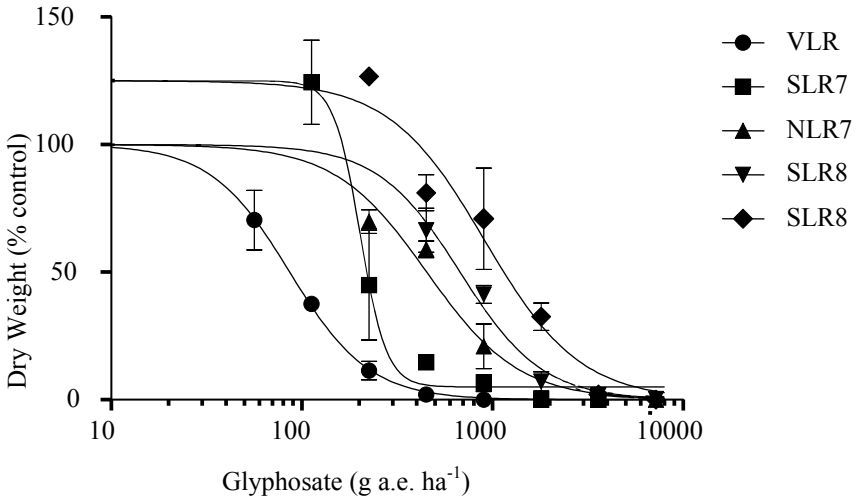

Figure 2.5: Dry weight of a susceptible and four resistant populations of *L. rigidum* to glyphosate. This experiment was done in November 2008. Each point is the mean of 3 replicates ± SE.

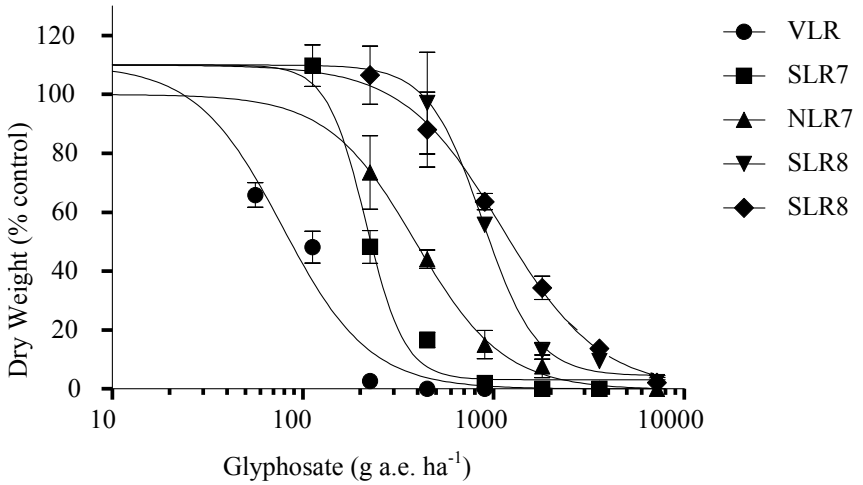

Figure 2.6: Dry weight of a susceptible and four resistant populations of *L. rigidum* to glyphosate. This experiment was done in December 2008. Each point is the mean of 3 replicates ± SE.

The resistant levels between each population were determined by comparing the GR_{50}s with the susceptible population (Table 2.2). The R/S between each experiment varied a little between experiments; however, the difference in the resistance level among the populations was similar. From the three analyses of dose response experiments, the GR_{50} of VLR1 population were between 78.3 to 135 g a.e ha^{-1}. The low-level resistant population SLR77 exhibited 2.4 to 2.8 fold resistance compared with susceptible population. The moderately resistant population NLR70 exhibited 5 to 7.2 fold resistance compared with VLR1. The two populations with the highest levels of resistance were SLR 88, with 7.8 to 11.2 fold resistance, and SLR80, with 10.5 to 15.2 fold resistance.

Table 2.2: The rate of glyphosate required to produce a 50% growth reduction (GR_{50}) for a susceptible and four resistant population of *L. rigidum*.

Population	GR_{50} (g a.e. ha^{-1})					
	October	**R/S**	November	**R/S**	December	**R/S**
VLR1 (S)	135		86.4		78.3	
SLR77 (R)	381	2.8	202.1	2.4	214.4	2.7
NLR70 (R)	977	7.2	452.5	5.3	390.4	5.0
SLR88 (R)	1,502	11.1	676.3	7.8	875.3	11.2
SLR80 (R)	2,048	15.2	909.3	10.5	1102	14.1

2.4 Discussion

The first documented case of *L. rigidum* resistant to glyphosate in Australia was NLR70 population, collected from an orchard near Orange, New South Wales. This population exhibited 7 to 11 fold resistant compared to susceptible population (Powles *et al*., 1998). The same population evaluated from LD_{50} in this study exhibited slightly lower resistance level with 3.7 to 8.4 fold resistance. The population SLR77 was also previously being reported to have a low resistance level of 1.9 to 3.4 fold resistance (Wakelin and Preston, 2006a). In this study, from LD_{50} population SLR77 exhibited 2.2 to 3.5 fold resistance. More surveys and collections have been done in Australia, especially in South Australia, and the glyphosate-resistant populations studied showed various resistances level from low to medium (Wakelin *et al*., 2004; Wakelin and Preston, 2006a; Preston, 2009).

In these dose response experiments, *L. rigidum* populations exhibited different resistance levels to glyphosate from low to high. Populations SLR88 and SLR80 were more resistant than previously characterised populations of *L. rigidum* (Table 2.1). The variation in resistance level between *L. rigidum* populations suggests that different resistance mechanisms may be present.

It was also believed that environment conditions influence the toxicity of glyphosate to target weeds (McWhorter and Azlin, 1978; Powles *et al*., 1998). A few studies had been conducted to determine the efficacy of glyphosate in relation to temperature, soil moisture and relative humidity (Jordan, 1977; Chase and Appleby, 1979; Chase and Appleby, 1979a). Higher relative humidity and temperature was found to increase the absorption, translocation and toxicity of glyphosate (Jordan, 1977). Similar environmental factors were also found to increase the toxicity of glyphosate to *Cyperus rotundus* (Chase and Appleby, 1979). In addition, prolonged humid conditions could also influence the efficacy of glyphosate, where it was more effective during rainy season than in dry season (Chase and Appleby 1979a).

The occurrence of resistant *L. rigidum* to glyphosate was also reported in other countries (Heap, 2009; Yu *et al*., 2007). Additionally, glyphosate resistance in other weeds species are also well studied. Typically, glyphosate resistance is not high, with resistance levels in the range of 3 to 13 fold. For example, *E. indica* populations from Malaysia were 2.1 to 11.8 fold resistant to glyphosate (Lee and Ngim, 2000; Ng *et al*.,

2004b). A population of *C. canadensis* from glyphosate-resistant soybean in Delaware, USA exhibited 8 to13 fold resistant (VanGessel, 2001). In Chile, a population of *L. multiflorum* from an orchard exhibited 4 fold resistant (Pérez and Kogan, 2003).

To date, there are two mechanisms identified that provide resistance to resistance to glyphosate in the weed populations; target-site mutations and reduced herbicide translocation. A target-site mutation mechanism occurs populations with a low level of resistance to glyphosate (Baerson *et al*., 2002) and reduced herbicide translocation mechanism occurs in resistant populations with a moderate level of resistance (Lorraine-Colwill *et al*., 2003). So far, there are no reports of the higher levels of resistance to glyphosate in *L. rigidum*, as seen in population SLR80. This suggests that population SLR80 may contain both mechanisms simultaneously in the population or contains an unknown resistance mechanism. This justifies the need for further investigation on the resistance mechanism in this population. Therefore, populations SLR80 and SLR88 were examined to determine whether they contained target-site mutations within EPSP synthase and/or decreased glyphosate translocation.

CHAPTER THREE

Glyphosate Translocation Patterns in Susceptible and Resistant Populations

3.1 Introduction

Glyphosate is a systemic herbicide that is translocated to the meristematic zone in plants via the phloem (Sprankle *et al*., 1975; Arnaud *et al*., 1994). Glyphosate has its main activity in shoot meristems. Therefore, a reduction on translocation of glyphosate to the meristem may reduce the efficacy of this herbicide. Studies with *L. rigidum* and *L. multiflorum* have shown that translocation of glyphosate was found to differ between resistant and the susceptible populations (Lorraine-Colwill *et al*., 2003; Yu *et al*., 2007; Michitte *et al*., 2007; Perez-Jones *et al*., 2007). In the resistant populations, glyphosate tends to accumulate in the leaf tips, whereas in susceptible plants glyphosate is translocated mostly to the shoot meristem (Lorraine-Colwill *et al*., 2003; Wakelin *et al*., 2004). This phenomenon also was found in *C. canadesis* and *C. bonariensis* (Feng *et al*., 2004; Koger and Reddy, 2005; Denelli *et al*., 2006).

Several later studies found that the occurrence of glyphosate resistance in weed populations was not due to reduced translocation of glyphosate to the meristematic system, but to mutations in the target site EPSPS. An altered target site was identified in *E. indica* from Malaysia (Baerson *et al*., 2002). In *L. rigidum* from California, it was suggested that insensitivity of EPSPS to glyphosate was the major factor of resistant in the population (Simarmata *et al*., 2003). Some of the resistant populations in Australia were also found to be caused by target-site mutations (Wakelin and Preston, 2006a). However, the resistant mechanism in several other resistant populations has not been identified.

The resistant levels of *L. rigidum* populations to glyphosate were determined in Chapter Two. There was considerable variation in response level between the populations. VLR1 was susceptible to glyphosate, SLR77 had low resistance, NLR70 moderate resistance and SLR88 then SLR80 the highest levels of resistance. This suggests that different mechanisms of resistance may operate in the different populations. The experiments here were to determine which of these populations of *L. rigidum* may be resistant to glyphosate as a result of reduced glyphosate translocation.

3.2 Materials and Methods

3.2.1 Seed Germination

Seeds of *L. rigidum* were germinated following methods described in 2.2.2. Seven days after germination, the seedlings were transplanted into hydroponic solution (Table 3.1).

3.2.2 Hydroponic Culture

Ten plants from each population of NLR70, SLR77, SLR80, SLR88 and VLR1 were transplanted into a 4 L plastic container (270 x 190 x 95 mm). The container was painted black to limit light reaching the nutrient solution. The container was covered with a lid to limit glyphosate spray reaching the nutrient solution. The container was filled with 3 L of 50% nutrient solution (Hoagland and Arnon, 1938) as listed in Table 3.1. The seedlings were planted through 8 mm diameter holes in the container lid and supported by 500 mL of black polypropylene beads. The plants were maintained in a growth chamber with 12 hours light period, 20^{o}C and 12 hours dark period, 15^{o}C at 300 µmol $m^{-2}s^{-1}$. Nutrient solution was topped up into the container to replace solution lost through evaporation.

Table 3.1: Concentration of nutrients in hydroponic nutrient solution used (Hoagland and Arnon, 1938).

Nutrient	Final concentration (µM)
$CaSO_4.2H_2O$	800
KH_2PO_4	500
$MgSO_4.7H_2O$	1000
$Ca(NO_3)_2.4H_2O$	1670
KNO_3	1670
K_2SO_4	400
$FeSO_4.7H_2O$	72
EDTA $Na_2.2H_2O$	64
H_3BO_3	23
$CuSO_4.5H_20$	0.16
$Na_2MoO_4.2H_2O$	0.25
$MnCl_2.4H_2O$	4.60
$ZnSO_4.7H_2O$	0.38

3.2.3 Glyphosate Treatment

When the plants had reached 2 to 3-leaf stage, they were sprayed with 225 g a.e ha^{-1} of glyphosate using a laboratory moving boom sprayer as described in 2.2.3. The plants were thinned to 5 for each population as replicates. Immediately after the glyphosate treatment, 0.5 µL radiolabelled ^{14}C-glyphosate, which contained 0.5 kBq of radioactivity and 0.0136 µmol of glyphosate, was applied to the lower half of the second leaf of each plant. Specific activity of ^{14}C-glyphosate (phosphonomethyl-^{14}C) (Sigma-Aldrich) was 0.167 GBq $mmol^{-1}$ (Wakelin *et al*., 2004). The treated plants were put back into the growth chamber after the treatment.

3.2.4 Radioactivity Determinations

The plants were harvested 48 hours after treatment and divided into four sections. The four sections were the treated leaf, the non-treated leaves, the stem and the roots (Figure 3.1). During harvesting, unabsorbed radioactivity on the treated leaf was removed by washing the leaf in 5 mL of 0.1% Triton X-100 (Sigma-Aldrich) solution in 20 mL glass vial. Each of four plant sections were kept separately in small envelopes and dried in an oven at 60^{o}C for 4 days.

The plant sections were combusted separately in a biological sample oxidiser (R.J. Harvey Instrument Corporation, Hillsdale, NJ, USA). The $^{14}CO_2$ released from the biological oxidiser was trapped in 14 mL of scintillation fluid [Carbo-Sorb E : Permafluor E^{+}, 1:1 (V/V), Canberra Packard, Groningen, The Netherlands] and the radioactivity was quantified by Liquid Scintillation Counter (Beckman Coulter, Fullerton, CA, USA). The radioactivity level in the leaf wash solutions was also quantified by Liquid Scintillation Counter following addition of 5 mL of Ultima Gold XR (Canberra Packard, Groningen, The Netherlands). The percentage of glyphosate absorbed was calculated as the sum of the amount in the various plant parts divided by the total amount recovered including leaf wash. The percentage of glyphosate in each plant part was calculated as the amount in that plant part divided by the amount of glyphosate absorbed.

3.2.5 Statistical Analysis

The translocation data was analysed by one-way Analysis of Variance (ANOVA) using InStat where the data was normally distributed following transformation. Where the data was not normally distributed following transformation the nonparametric Kruskal-Wallis Test. Means were separated by Tukey (ANOVA) or Dunn (Kruskal-Wallis) tests.

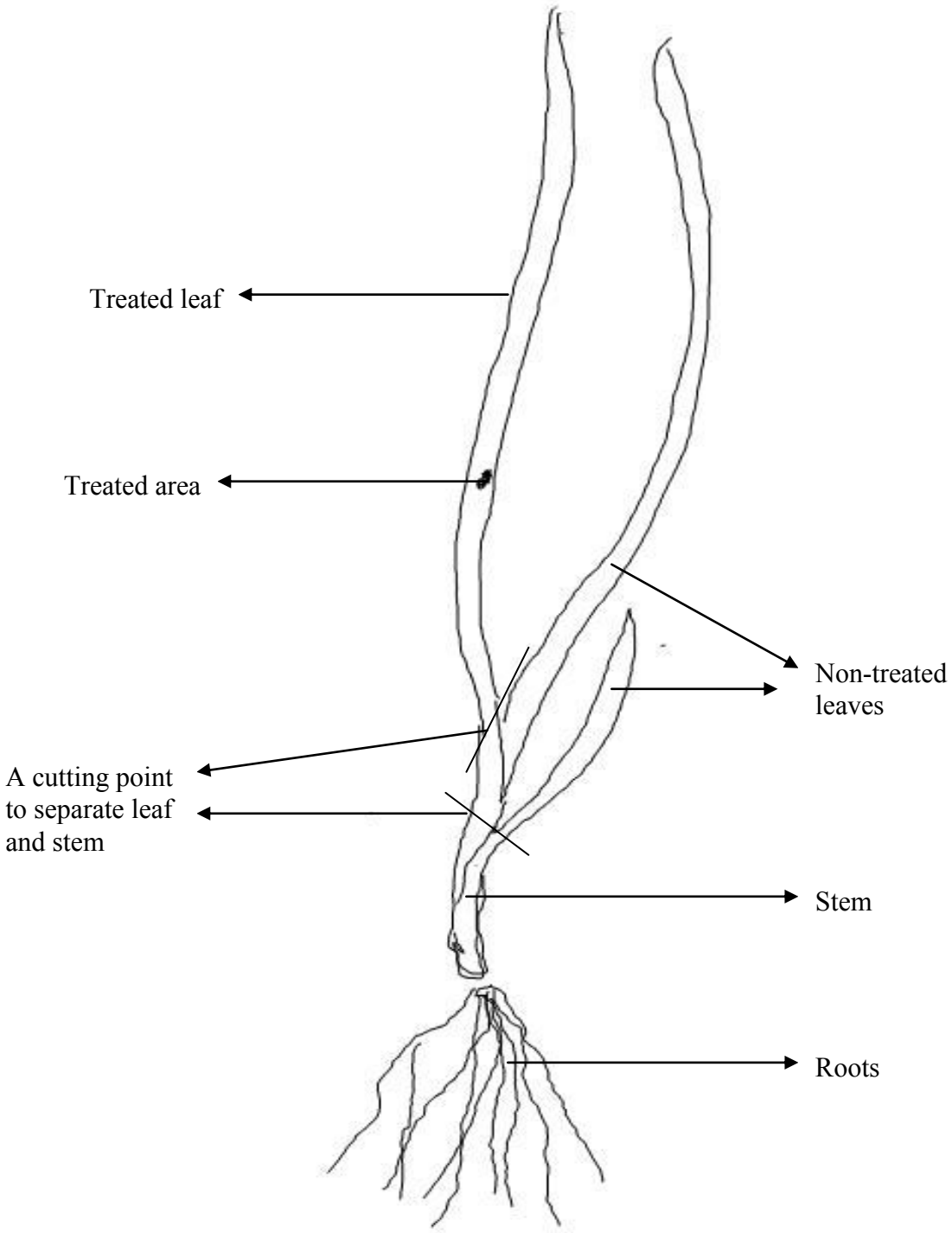

Figure 3.1: The four sections of *L. rigidum* during harvesting 48 hours after treatment (treated leaf, non-treated leaves, stem and roots).

3.3 Results

Table 3.2 shows the final distribution of ^{14}C-glyphosate in susceptible (VLR1) and resistant populations (SLR77, NLR70, SLR88 and SLR80) of *L. rigidum* 48 hours after treatment. There was no significant difference between populations in the amount of glyphosate absorbed with between 55.8 and 66.2% of the glyphosate absorbed. In the VLR1 and SLR77 populations less of the glyphosate was retained in the treated leaf 33.3% in VLR1 and 36.5% in SLR77, compared with the other populations. NLR70 had 69.6%, SLR80 had 54.7% and SLR88 had 56.4% of the glyphosate retained in the treated leaf.

Table 3.2: Percentage of ^{14}C-glyphosate translocated from the treated leaf of susceptible and resistant *L. rigidum* population 48 hour after treatment.

Population	Mean percentage of ^{14}C-glyphosate				
	Absorption	**Treated leaf**	**Stem**	**Root**	**Non-treated leaf**
VLR1 (S)	62.0	33.3^{c}	25.9^{a}	25.6^{a}	15.2
SLR77 (R)	66.2	36.5^{c}	25.0^{a,b}	29.1^{a}	9.4
NLR70 (R)	62.0	69.6^{a}	9.9^{c}	8.9^{b}	11.6
SLR88 (R)	61.9	56.4^{a,b}	12.6^{c}	21.7^{a}	9.2
SLR80 (R)	55.8	54.7^{b}	16.6^{b,c}	19.3^{a,b}	9.4
P- value	n.s	< 0.0001	< 0.0001	0.0030	n.s

Values with the same letters in columns are not significantly different at P =0.05

The accumulation of ^{14}C-glyphosate in the stem region was higher in the susceptible VLR1 population (25.9%) and in SLR77 (25%) than the other populations. The resistant populations NLR70, SLR88 and SLR80 had about half the amount of glyphosate accumulating in the stem region than did the susceptible population VLR1. The resistant population NLR70 accumulated less glyphosate in the roots than the other populations. There were no differences in the amount of ^{14}C-glyphosate in the non-treated leaves among the populations.

3.5 Discussion

This study confirmed that translocation of glyphosate in the glyphosate resistant population NLR70 is very different to translocation of this herbicide in the susceptible population (Lorraine-Colwill *et al*., 2003; Wakelin *et al*., 2004). In this population, glyphosate is retained in the treated leaves rather than being translocated to the meristem. The study also confirmed that glyphosate translocation in the glyphosate resistant population SLR77 was similar to that of the susceptible population (Wakelin and Preston, 2006a). This latter population is resistant as a result of an alteration in the target site. The two highly resistant populations, SLR88 and SLR80 had glyphosate translocation patterns more similar to the resistant NLR70 than the susceptible VLR1. This indicates these populations have reduced glyphosate translocation as a resistance mechanism like NLR70.

There was no significant different in the absorption of glyphosate among the populations. In this experiment, the amount of glyphosate absorbed by the plant was around 55.8 to 66.2%. Lorraine-Colwill *et al.* (2003) found that the amount of glyphosate absorbed by the plants was around 40 to 80%. However, the range reported in this experiment was higher compared to the previously reported 39 to 45% by Wakelin *et al.* (2004). Glyphosate absorption is affected by formulation (Feng *et al*., 1998; Feng *et al*., 2000; Molin and Hirase, 2004) and environmental conditions (WcWhorter and Azlin, 1978; Caseley and Coupland, 1985; Westwood *et al*., 1997; Reddy, 2000). Some of the differences between absorption in this study and previous studies could be caused by the use of different glyphosate formulations or differences in the conditions under which they were treated.

According to Bariuan *et al.* (1999), roots and young leaves are physiologically active as a sink for the accumulation of glyphosate. Other plants species, such as *S. halepense* (Camacho and Moshier, 1991), *E. crus-galli* (Kirkwood *et al*., 2000) and *A. theophrasti* (Feng *et al*., 2000), also show high accumulation of glyphosate in the meristematic zones. Therefore, a reduction of glyphosate to the shoot meristem could result in plants surviving application of glyphosate.

Among the resistant populations, NLR70 showed the highest amount of glyphosate retained in the treated leaf and this also was reported by Wakelin *et al.*

(2004). However, the percentage of glyphosate reported here was higher compared with the percentage reported by Wakelin *et al.* (2004). This could be due to a difference in the concentration of glyphosate applied to the plants before treating with radiolabelled glyphosate. Wakelin *et al.* (2004) applied 450 g a.e ha^{-1} of glyphosate whereby in this experiment, the rate was only 225 g a.e ha^{-1}. Another possible reason could be a difference in formulation of glyphosate used in this experiment. Three formulations of glyphosate were found to affect the amount of glyphosate translocation in *A. theophrasti* (Feng *et al*., 1998). In contrast, Satchivi *et al.* (2000) found that the formulation of glyphosate did not greatly affect its absorption and translocation on *A. theophrasti* and *S. faberi*. A similar finding was also reported by Li *et al*. (2005) in *A. rudis*, *Ipomoea lacunosa* and *A. theophrasti* where the formulation of glyphosate did not influence its efficacy on these weeds.

Despite the NLR70 population having the highest amount of glyphosate retained in the treated leaf it is not the most resistant population tested (Table 1.1). Therefore, in populations SLR88 and SLR80 an additional resistance mechanism may account for their extra level of resistance.

CHAPTER FOUR

Target-site Mutations in EPSP Synthase in Resistant Populations

4.1 Introduction

It was determined in Chapter Three that the reduced herbicide translocation mechanism did not occur in the resistant population of SLR77, but did occur in all other glyphosate resistant populations. However, populations SLR88 and SLR80 are much more resistant to glyphosate than NLR70, despite all populations containing the reduced translocation resistance mechanism. The other mechanism that can contribute to glyphosate resistance in weed populations is a target-site mutation. This was identified in SLR77 and conferred low level resistance to the population (Wakelin and Preston, 2006a).

In other weed species such as *E. indica* and *L. multiflorum*, target-site mutations were found in resistant populations (Baerson *et al*., 2002; Ng *et al*., 2003; Ng *et al*., 2004b; Perez-Jones *et al*., 2007; Kaundun *et al*., 2008). These populations typically have low levels of glyphosate resistance. The change of proline 106 to serine and threonine were found in the resistance weed populations. A mutation in the same location was reported in a resistant *L. rigidum* population from South Australia (Wakelin and Preston, 2006a). Even though target-site mutation was suggested to confer low level resistance to the population, this chapter will investigate whether this mechanism is present in the highly resistant populations of *L. rigidum*. Therefore, DNA from all five populations was extracted, the conserved region of EPSP synthase amplified and sequenced to identify any mutations within this region of the EPSP synthase gene.

4.2 Materials and Methods

4.2.1 Seed Germination

Seeds of *L. rigidum* were germinated following methods described in 2.2.2. Seven days after germination, the seedlings were transplanted into 17 cm diameter pots with standard potting mix. Every pot consisted of 12 seedlings of the same populations. The plants were maintained at normal growing condition (with approximate temperature ranged between 24 to 34^{o}C) during their growing period. The plants were watered 1 to 2 times a day, depending on the weather condition.

4.2.2 Plant DNA Extraction

For extraction of DNA, healthy young leaves were harvested from five plants of each population. The leaf selected was the youngest fully-expanded leaf. Each leaf with

size around 50 mm was cut and put in a separate 1.5 mL eppendorf tube chilled on ice. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Australia), following methods described by the manufacturer. To each 1.5 mL eppendorf tube containing a leaf sample 400 µL of AP1 buffer and 4 µL of RNase were added. The samples were ground to a fine powder and then incubated at 60^{o}C for 10 minutes in water bath. The tubes were inverted 2 to 3 times during this period. Next, 130 µL of Buffer AP2 was added and the samples were kept on ice for 5 minutes. The mixture was then centrifuged by using Eppendorf tabletop centrifuge (Eppendorf, Germany) at 20,000 x g (14,000 rpm) for 5 minutes. The upper aqueous phase of the lysate were transferred to QIAshredder Mini spin column and centrifuged at 20,000 x g (14,000 rpm) for 2 minutes.

The flow-through fractions were transferred into new 1.5 mL eppendorf tube without disturbing the cell-debris pellets. To clear the lysate, 675 µL of Buffer AP3/E was added and samples were mixed by pipetting. Next, the mixture was transferred to a DNeasy Mini spin column and centrifuged at 6000 x g (8,000 rpm) for 1 minute and the flow through was discarded.

Each spin column, containing the DNA, was placed into a new 2 mL collection tube along with 500 µL of buffer AW. Samples were centrifuged at 6000 x g (800 rpm) for 1 minute and the flow-through discarded. To further wash the membrane-bound DNA, another 500 µL of buffer AW was added into the spin column and centrifuged at 20,000 x g (14,000 rpm) for 2 minutes. The spin column was transferred into a 1.5 mL eppendorf tube and 80 µL of buffer AE was pipetted directly onto the DNeasy membrane. It was incubated at room temperature for 5 minutes before centrifuging at 6000 x g (8000 rpm) for 1 minute. This step was repeated with another 30 µL of buffer AE.

4.2.3 DNA Concentration Determination

DNA samples from the stock were determined for their concentration by using a Nanodrop ND-1000 spectrophotometer (Nanadrop Technologies, Wilmington, DE, USA). The DNA solution in the tubes was kept at 5^{o}C until DNA amplification.

4.2.4 DNA Amplification

For PCR amplification, 1 µL of DNA solution from the DNA stock was put separately in each tube and mixed with 2 µL of 10x HiFi Buffer, (Invitrogen, Australia) 0.8 µL of 50 mM $MgSO_4$, 0.2 µL of 20 mM dNTPs, 0.4 µL of forward primer (LR-Fwd-FD) with a specific sequence of 5'-CAAAAAGAGCTGTAGTCG-3' and reverse primer (LR-Rev-FD) with a specific sequence of 5'-CAAGGAACTCAAGTATTGG-3', 15.1 µL sterile nanopure water and 0.1 µL of Hi Fi Tag DNA polymerase (Promega, Australia). A single tube without DNA template was included to serve as a negative control. The tubes were placed in an automated DNA thermal cycler (Effendorf Mastercycler® Gradient, Germany). The machine was programmed for 30 cycles with 2 minutes at 94°C for denaturation, 30 seconds at 55°C for annealing and 45 seconds at 68°C for elongation. The mixture was kept in fridge at 5°C until the next step.

4.2.5 Separation and Visualisation of DNA Fragments

PCR samples, 8 µL mixed with 1.6 µL of 6x Ficcol dye, were loaded into a 1.4% agarose gel (Gibco BLR) stained with 80 µL (1 mg/mL) of ethidium bromide. The product was separated by electrophoresis for 45 minutes at 100 volts and visualised under ultraviolet light. DNA products of the expected size (b.p.) were identified by comparison with low molecular weight mass ladder (Invitrogen, Australia).

4.2.6 DNA Sequencing

DNA sequencing was conducted at the Australian Genome Research Facility (AGRF) Ltd. using forward primer (LRO3f) with a specific sequence of 5'-AGCTGTA GTCGTYGGCTGYG-3' and reverse primer (LR-Rev-FDSeq) with a specific sequence of 5'-ACATTCGCACCTAGTTGTTT-3'. The DNA sequence data were assembled, compared and analysed by using ContiExpress from the Vector-NTi Suite 6 programs (Informex, USA).

4.3 Results

The partial DNA sequence of EPSP synthase of the susceptible and four resistant populations were amplified and sequenced to identify any changes in the nucleotide sequence. The predicted amino acid sequence from the susceptible population VLR1 was the same as the consensus sequence from other plant species in the conserved region sequenced. The resistant populations, SLR77, NLR70, SLR88 and SLR80, all showed polymorphisms within the nucleotide sequence in this region (Table 4.1).

Substitution of T (thymine) to C (cytosine) at codon 97, C to G (guanine) at codon 98, T to C at codon 100 and G to A (adenine) at codon 103 were observed in one or more of the resistant populations. However, none of these nucleotide substitutions changed the predicted amino acid sequence in this region. Therefore, they are all silent changes.

Table 4.1: Amino acid and change of nucleotide sequence in EPSPS DNA isolated from one susceptible and four resistant populations of *L. rigidum*.

Amino acid number	**95**	**96**	**97**	**98**	**99**	**100**	**101**	**102**	**103**	**104**	**105**	**106**	**107**	**108**
Amino acid	**Leu**	**Phe**	**Leu**	**Gly**	**Asn**	**Ala**	**Gly**	**Thr**	**Ala**	**Met**	**Arg**	**Pro**	**Leu**	**Thr**
Consensus Sequence	CTC	TTC	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
VLR1 (S)	CTC	TTC	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
SLR77 (R)	CTC	TTC	**CTG**	**GGG**	AAC	**GCC**	GGA	ACT	GCG	ATG	CGG	**ACA**	TTG	ACG
NLR70 (R)	CTC	TTC	TTG	**GGG**	AAC	**GCC**	GGA	ACT	**GCA**	ATG	CGG	CCA	TTG	ACG
SLR88 (R)	CTC	TTC	TTG	GGC	AAC	GCT	GGA	ACT	**GCA**	ATG	CGG	**TCA**	TTG	ACG
SLR80 (R)	CTC	TTC	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	**ACA**	TTG	ACG

In contrast, single nucleotide substitutions of A for C at codon 106 were observed in the resistant populations SLR77 and SLR80. This nucleotide change is predicted to substitute threonine for proline at position 106. In the resistant population SLR88, a nucleotide substitution of T for C was observed at the same codon. This nucleotide substitution is predicted to change the amino acid from proline 106 to serine.

4.4 Discussion

The result of this experiment showed target-site mutations were present in three resistant populations of *L. rigidum*: SLR77, SLR88 and SLR80, but not in NLR70. Target-site resistance is known to occur in resistant weed populations of *L. rigidum*, *L. multiflorum* and *E. indica* (Baerson *et al*., 2002; Ng *et al*., 2003; Wakelin and Preston, 2006a; Perez-Jones *et al*., 2007; Kaundun *et al*., 2008). In these populations, the target-site mutation produces a low resistant level around 2 to 4 fold (Baerson *et al*., 2002). Among the resistant populations tested, SLR77 was found to have 2.2 to 3.8 fold resistant to glyphosate (Table 2.1, Chapter Two). Therefore, a target-site mechanism as the sole resistance mechanism in this population is consistent with its level of resistance observed.

Target-site resistance to glyphosate occurs because of mutations within the EPSP synthase gene (Padgette *et al*., 1991). Most mutations occur at proline 106, although site directed double mutations at glycine 101 and proline 106 have been shown to provide resistance in crop species (Devine and Preston, 2000). Different substitutions at proline 106 are known to confer resistance to glyphosate. For instance, in *E. indica*, serine (Baerson *et al*., 2002) or threonine (Ng *et al*., 2003b) were substituted for proline at 106 in resistant populations. In *L. multiflorum*, an amino acid substitution of serine for proline at 106 has been observed (Perez-Jones *et al*., 2007). This suggests that proline 106 can easily mutate and is an important site for glyphosate target-site mutation (Yu *et al*., 2007). The substitution of other amino acids for proline reduces the sensitivity of the enzyme to glyphosate (Jasieniuk *et al*., 2008; Preston *et al*., 2009).

In addition to SLR77, target-site mutations were also present in two highly resistant populations of SLR88 and SLR80. Target-site mutations are not normally observed in weed populations highly resistant to glyphosate. In Chapter Two, the resistant levels for populations SLR88 and SLR80 were around 5.6 to 11.4 and 8.2 to 76.6 fold respectively. These populations also contain the reduced translocation mechanism of resistance. Therefore, SLR88 and SLR80 populations have multiple mechanism of resistance to glyphosate. It is likely that one or other of the resistance mechanisms evolved in the populations and then continuous selection pressure to the weed populations increased the resistant level of those populations to glyphosate through acquisition of a second resistance mechanism (Jasieniuk *et al*., 1996). Target-

site resistance was not present in NLR70 population, which was 5 to 7.2 fold resistant to glyphosate. This population only contains the reduced translocation mechanism of resistance (Lorraine-Colwill *et al*., 2003; Wakelin *et al*., 2004; Wakelin and Preston, 2006a). The accumulation of both target-site mutations and reduced glyphosate translocation, as observed here, can greatly increase the level of glyphosate resistance in weed populations.

CHAPTER FIVE

The Inheritance of Glyphosate Resistance in SLR88

5.1 Introduction

Several factors influence the evolution of herbicide resistance, such as mutation rate, selection pressure, gene flow and inheritance of resistance (Maxwell and Mortimer, 1994). As a cross pollination species, the spread of resistance in *L. rigidum* can occur through both pollen and seeds. If glyphosate resistance is a dominant trait it could be transferred between plants in pollen. The glyphosate resistance levels and their resistance mechanisms had been determined in Chapter Two, Chapter Three and Chapter Four respectively. SLR80 was identified having the highest level of resistance, while SLR88 had the second highest. Two resistance mechanisms were found present in these populations suggesting at least two genes would be contributing to resistance. With these findings, the need to further investigate the inheritance mechanism was considered important. Therefore, the objective of the experiment was to examine the inheritance of resistance in F_1 progeny, whether it is completely or incompletely dominant.

In this chapter, SLR88 was used as the parent material for the resistant population although in earlier experiments (Chapter Two) SLR80 was found to have the highest level of resistance than SLR88. This population (SLR80) was not used in the experiment described in this chapter due to time constraints.

5.2 Materials and Methods

5.2.1 Generation of First Filial Generation (F_1)

The seeds of the resistant population (SLR88) and susceptible population (VLR1) were germinated separately as described in 2.2.2. After 7 days, the seedlings were transplanted separately in two Masrac Taglok punnet pots (9.5 x 8.5 x 9.5 cm) with standard potting mix. Each pot consisted of 10 seedlings from the same population. After 2 weeks, a single and uniform plant of each population SLR88 and VLR1 were re-potted together into one large pot (26.5 cm diameter x 23.5 cm high). The total number of pots used was 20 pots. The plants were maintained at normal growing condition and watered 1 to 2 times a day, depending on the weather conditions.

When the plants started to flower, the pots were encased with 1.2 m high transparent plastic sleeve, supported by a mesh cage and open at the top. This was to reduce the chance of any pollen arriving from outside pollinating the two plants in the

pot. Mature seeds were collected separately from the maternal resistant and maternal susceptible plants and called F_1 progeny.

5.2.2 Dose Response on F_1 Progeny

The seeds collected from the F_1 progeny of maternal resistant and susceptible plants were germinated separately as described in 2.2.2. Twelve plants were planted in each pot. Two weeks after transplanting, when the plants were at the 2 to 3-leaf stage, the plants were treated with glyphosate using spraying apparatus as described in 2.2.3. The dosages were applied at 112.5, 225, 450, 900, 1800 and 3600 g a.e ha^{-1} of glyphosate isopropylamine (Roundup Powermax®), Monsanto, Melbourne, Victoria, Australia. Non-ionic surfactant (Wetter TX®, Nufarm, Australia) was added at 0.2% V/V. After treatment, the plants were maintained in the normal growing condition (with approximate temperature ranged between 24 to 34°C). Survival was assessed 21 days after treatment.

5.2.3 Statistical Analysis

The experiment was conducted with 4 replicates for parental resistant and susceptible populations, and one pot from each of four families for F_1 of maternal resistant and maternal susceptible populations for each dose of glyphosate. The data for the 4 F_1 families was pooled for analysis of dose response curves of the F_1 from maternal resistant and maternal susceptible families respectively. The experiment was repeated. Mortality data was analysed using PriProbit version 1.63 to determine the herbicide dose-response relationships to the number of surviving plants. This provides the dose-response curves to the graph and the LD_{50} (dose which controls 50% of the population).

5.3 Results

The dose response of the parental susceptible (VLR1), parental resistant (SLR88), F_1 maternal resistant and F_1 maternal susceptible populations in the two experiments are shown in Figure 5.1 and Figure 5.2. The susceptible parent was completely controlled with the recommended rate (450 g a.e. ha^{-1}) of glyphosate, whereas higher rates of glyphosate were required to control parental resistant and both F_1 progenies. From the graph, both F_1 progenies showed an intermediate and almost similar response to glyphosate compared with the parental populations. This indicates

that resistant in SLR88 to glyphosate is inherited not by maternal inheritance, but in the nuclear genome through the transfer of pollen during the cross pollination.

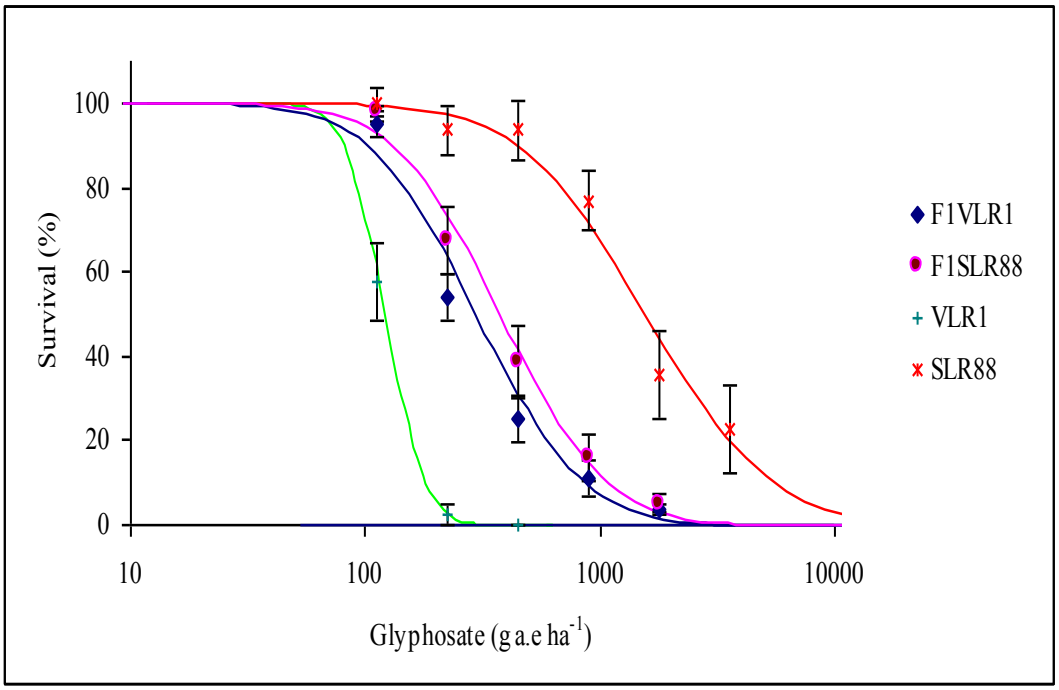

Figure 5.1: Dose response of susceptible, resistant, F_1 maternal susceptible and F_1 maternal resistant populations of *L. rigidum* to glyphosate in Experiment 1. Crosses were made between VLRI (susceptible) and SLR88 (resistant) populations of *L. rigidum*. Experiment 1 was conducted in early August 2009. Each point is the mean survival ± SE.

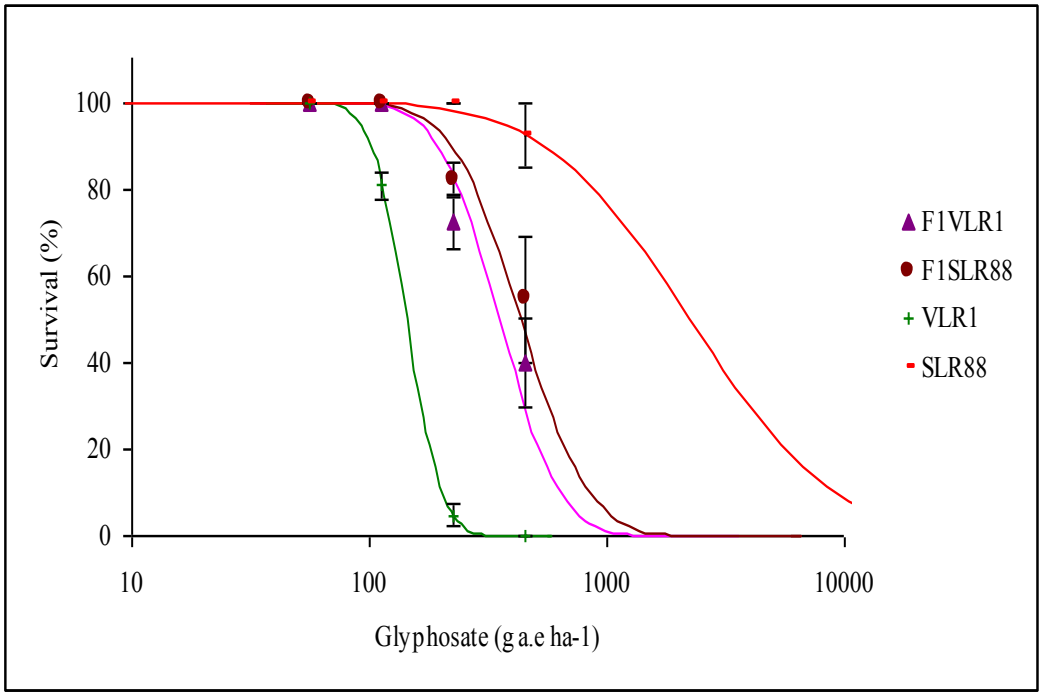

Figure 5.2: Dose response of susceptible, resistant, F_1 maternal susceptible and F_1 maternal resistant populations of *L. rigidum* to glyphosate in Experiment 2. Crosses were made between VLRI (susceptible) and SLR88 (resistant) populations of *L. rigidum*. Experiment 2 was conducted in late August 2009. Each point is the mean survival ± SE.

From the dose response, the LD_{50} of parental susceptible and resistant populations was calculated as 121 to 143 and 1,564 to 2,227 g glyphosate ha^{-1} respectively (Table 5.1). This gives the R/S of the resistant parent as 12.9 to 15.6 fold resistant to glyphosate compared with the susceptible parent. This result is within the range of R/S determined for the SLR88 population in Chapter Two. There was little difference in the LD_{50} for the F_1 maternal susceptible compared with the F_1 maternal resistant populations with LD_{50} values of 298 to 352 and 377 to 432 g a.e. ha^{-1} respectively. This gives the R/S for the F_1 progenies of 2.5 and 3 to 3.1 fold resistance compared with the susceptible parent. The intermediate resistance level for both F_1 progenies demonstrates that the inheritance mechanism of SLR88 population with glyphosate is incompletely dominant.

Table 5.1: The rate of glyphosate required to control 50% of the population (LD_{50}) for the susceptible, resistant, F_1 maternal susceptible and F_1 maternal resistant populations of *L. rigidum*; and the resistance index for the populations in Experiments 1 and 2.

Population	LD_{50} (g a.e. ha^{-1})			
	Experiment1	**R/S**	Experiment 2	**R/S**
VLR1 (S)	121		143	
F_1 VLR1	298	2.5	352	2.5
F_1 SLR88	377	3.1	432	3
SLR88 (R)	1,564	12.9	2,227	15.6

5.4 Discussion

In this inheritance study, resistance to glyphosate in SLR88 was found to be encoded by a nuclear gene and incompletely dominant. This is a commonly observed occurrence in resistant weed populations (Islam and Powles, 1988; Betts *et al*., 1992; Murray *et al*., 1995; Boutsalis and Powles, 1995; Volenberg and Stoltenberg, 2002; Preston, 2003). Only resistance to triazine herbicides is maternally inherited (Darmency and Gasquez, 1981; Scott and Putwain, 1981; Machado and Bandeen, 1982; Jasieniuk *et al*., 1996). Even though encoded by nuclear gene, resistance to dinitroaniline herbicides in *S. viridis* (Jasieniuk *et al*., 1994) and *E. indica* (Zeng and Baird, 1997), and resistance to triallate in *A. fatua* (Kern *et al*., 2002) were inherited as recessive alleles.

The result from this experiment was similar to previous reports on the inheritance of glyphosate resistance in *L. rigidum* populations from New South Wales, Australia and from California where resistance was encoded by a nuclear gene and incompletely dominant (Lorraine-Colwill *et al*., 2001; Simarmata *et al*., 2005). However, not all *L. rigidum* populations resistant to glyphosate show incompletely dominant inheritance of resistance. A study of several resistant populations from Australia showed only two of eight resistant populations had incompletely dominant inheritance (Wakelin and Preston, 2006b). In contrast, a resistant *L. rigidum* population from California and a population from Australia were reported to have multigenic inheritance (Simarmata *et al*., 2005; Pratley *et al*., 1999). This indicates that even within the same species, the number of genes controlling resistance can be different across resistant populations. On the other hand, resistance to glyphosate in a population of *E. indica* was reported as a single gene and incompletely dominant (Ng *et al*., 2004a) as was resistance to glyphosate in *C. canadensis* (Zelaya *et al*., 2004).

It can be important to determine the inheritance mechanism of resistant weed populations to aid management. The management options for resistance that is inherited as a recessive allele can be different to those where resistance is inherited as a dominant allele (Huang *et al*., 1999). As determined in this experiment, inheritance of glyphosate resistance SLR88 was incompletely dominant and nuclear encoded. Given there were two distinct resistance mechanisms present in this population, it is likely that two genes will be contributing to resistance; however, the pattern of inheritance of the F_2 progeny was not determined in this study due to the time constraints. The partial dominance of

glyphosate resistance in SLR88 and its dispersal by pollen suggest that resistance will increase rapidly in the field with continuing selection pressure by glyphosate on the population (Lorraine-Colwill *et al*., 2001; Wakelin and Preston, 2006b).

CHAPTER SIX

General Discussion and Conclusion

6.1 General Discussion

Since the first report of a *L. rigidum* population resistant to glyphosate from Australia (Powles *et al*., 1998; Pratley *et al*., 1999), the number of cases of weed species resistant to glyphosate throughout the world has increased (Heap, 2009). Resistance to glyphosate has occurred in both grasses and annual broadleaf species. The occurrence of weed species resistant to glyphosate has been documented from various locations, such as no-till cereal production, orchards, vineyards, fence lines, irrigation channels, firebreaks, and railway rights-of-way (Preston *et al*., 2009) as well as glyphosate-resistant field crops (VanGessel, 2001; Vidal *et al*., 2007). This is probably due to the widespread use of glyphosate as the major herbicide to control weeds. There is considerable variation among these resistant weed species in the level of resistance to glyphosate expressed.

C. canadensis from glyphosate-resistant soybean fields in Delaware, USA exhibited an intermediate resistant level of around 8 to 13 fold (VanGessel, 2001). *A. palmeri* from glyphosate-resistant cotton fields in Georgia, USA was reported 6 to 8 fold resistance (Culpepper *et al*., 2006). In Spain, *C. bonariensis* was reported to be 7 to 10 times more resistant to glyphosate than the susceptible populations (Urbano *et al*., 2007). Among grass species, *L. multiflorum* from Chile was 2 to 4 fold resistant to glyphosate (Pérez and Kogan, 2003), *L. mutiflorum* from Mississippi was 3 fold resistant to glyphosate (Nandula *et al*., 2007) and *S. halepense* from Argentina was 3.5 to 10.5 fold resistant to glyphosate (Vila-Aiub *et al*., 2007). In Malaysia, *E. indica* was reported to be 7.8 to 11.8 fold resistant to glyphosate (Lee and Ngim, 2000). Other resistant populations of *E. indica* also in Malaysia, exhibited low resistance level with 2.1 to 3.3 fold resistant (Ng *et al*., 2004b). According to Ng *et al*. (2004b), the variation in the levels of glyphosate resistance in different populations was probably due to the difference in selection pressure.

In general, the evolution of resistant weed species in glyphosate-resistant crops with no tillage seeding systems was faster compared to other systems (Powles, 2008). For example, *C. canadensis* from a glyphosate-resistant soybean field was found to evolve resistance to glyphosate 3 years after persistent use of the herbicide (VanGessel, 2001). *A. palmeri* from glyphosate-resistant cotton took 7 years to evolve resistance (Culpepper *et al*., 2006). The length of time taken for glyphosate resistant to evolve in a

L. rigidum population from a cereal crop field was 15 years (Pratley *et al*., 1999). The *L. multiflorum* population from orchard took slightly shorter time with 8 to 10 years to evolve resistant (Pérez and Kogan, 2003). In contrast, the occurrence of resistant *E. indica* from orchards in Malaysia took only 3 years with more frequency of glyphosate application in a year (Lee and Ngim, 2000).

In Australia, the number of *L. rigidum* populations resistant to glyphosate has increased since the first case reported in 1998. This is due to wide use of glyphosate to control weed populations. At present, glyphosate resistance has been documented in 98 populations of *L. rigidum* (Preston, 2009). As determined in Chapter Two, the resistant populations exhibited various levels of resistance from low to high. The considerable variation in the level of resistance to glyphosate in *L. rigidum* suggests that different mechanisms might contribute to resistance in the different populations. In Chapter Two, among four resistant populations evaluated, SLR80 had a much higher level of resistance than the others (Table 2.1). This population has the highest level of glyphosate resistance of any population in Australia (Preston *et al*., 2009).

The herbicide history for every population of glyphosate resistant *L. rigidum* in Australia is not known. A *L. rigidum* population resistant to glyphosate from an orchard took about 15 years of herbicide use at 2 to 3 applications per year before evolving resistance (Powles *et al*., 1998). In another *L. rigidum* population from a cereal crop field, glyphosate resistance was reported to evolve after 15 years of herbicide use at a frequency of one application per year (Pratley *et al*., 1999). Other occurrences of glyphosate resistance that evolved within the same time frame were reported in *L. rigidum* populations from South Australia (Mathews, 2002). Despite this relatively narrow variation in herbicide history, glyphosate-resistant *L. rigidum* populations in Australia range from 3 to 15 fold resistant (Preston *et al*., 2009). A similar level of glyphosate resistance was found in *E. indica* from Malaysia with 7.8 to 11.8 fold resistance, but the time taken for this weed species to confer resistance was 3 years with 6 to 8 applications of glyphosate per year (Lee and Ngim, 2000). A similar period of selection was reported to the resistant population of *C. canadensis* from glyphosate-resistant soybean field with 8 to 13 fold resistant (VanGressel, 2001). The level of glyphosate resistance in *L. rigidum* populations does not appear to be related to the length of exposure or the amount of glyphosate applied.

To date, two resistance mechanisms have been confirmed in populations of weed species resistant to glyphosate. These are target-site mutation (Baerson *et al*., 2002; Wakelin and Preston, 2006a) and reduced herbicide translocation (Lorraine-Colwill *et al*., 2003; Wakelin *et al*., 2004; Preston *et al*., 2009). The mechanisms of resistance to glyphosate were investigated in four resistant populations in this study. A target-site mutation was found alone in the resistant population with the lowest resistant level; SLR77 (Chapter Four). This finding was constant with reports by Baerson *et al*. (2002), Ng *et al*. (2003) and Wakelin and Preston (2006a) where target-site mutations in EPSPS result in low levels of resistance to glyphosate.

The other resistance mechanism, reduced herbicide translocation was identified as the sole resistance mechanism in population of NLR70 (Chapter Three). This population had the second lowest level of resistance of the four resistant populations tested (Table 2.1). Reduced herbicide translocation has been identified as a resistance mechanism in many population of *L. rigidum* (Lorraine-Colwill *et al*., 2003; Wakelin *et al*., 2004) and in other weed species, such as *C. canadensis* (Koger and Reddy, 2005; Dinelli *et al*., 2006), *C. bonariensis* (Dinelli *et al*., 2008; Ferreira *et al*., 2008) and *L. multiflorum* (Michitte *et al*., 2007). In all of these populations, the reduced herbicide translocation mechanism provided moderate resistance to glyphosate.

The other two glyphosate resistant *L. rigidum* populations, SLR88 and SLR80, contained both mechanisms of resistance. Each population had an amino acid substitution at proline 106 in the EPSP synthase gene, as well as reduced glyphosate translocation to the shoot meristem. These populations were much more resistant to glyphosate, probably due to the presence of both resistance mechanisms. Preston *et al*. (2009) crossed *L. rigidum* populations with the two different resistance mechanisms and produced progeny with a much higher level of resistance to glyphosate. The present study shows that the two mechanisms of resistance to glyphosate can be accumulated in the same individual from glyphosate application in the field.

The target-site mutations at EPSP synthase identified in this study was determined at proline 106. These involved a change of substitution to serine in SLR88 and to threonine in SLR80. Amino acid substitutions have been documented at this

location for other *L. rigidum* populations. For example, in glyphosate-resistant *L. rigidum* from South Africa, had a change from proline to alanine (Yu *et al*., 2007) and a population from USA had a change from proline to serine (Simarmata and Penner, 2008). In *L. multiflorum*, the change of amino acid to serine was determined in a population from Chile (Perez-Jones *et al*., 2007) and additional change to alanine in a population from USA (Jasieniuk *et al*., 2008). In resistant populations of *E. indica* from Malaysia the amino acid change was proline to serine (Baerson *et al*., 2002) or threonine (Ng *et al*., 2003).

To date, most investigations of glyphosate resistant weed populations found only a single mechanism of glyphosate resistant present. Several studies were unable to determine the resistance mechanism. Studies on glyphosate-resistant *L. rigidum* (Feng *et al*., 1999) and *E. indica* (Ng *et al*., 2003) failed to determine the resistant mechanism in the population. Possibly the low resistance level in the populations was due to target-site mutation mechanism (Powles and Preston, 2006); however, there was no target-site mutation observed in a very low resistant population of *E. indica* from Malaysia (Ng *et al*., 2004b). Therefore, other resistance mechanism may contribute to glyphosate resistance in these populations. There may be mutations in EPSP synthase at positions other than proline 106 (Powles and Preston 2006). The other possibility is an as yet unidentified resistance mechanism may be present.

In a cross pollinated weed species, such as *L. rigidum*, accumulation of glyphosate resistance mechanisms would be expected to occur. Cross pollination among the resistant individuals with different resistant mechanisms will produce progeny with both resistance mechanisms (Hall *et al*., 1995; Preston *et al*., 2009). This cross pollination is suggested to be a likely factor in the occurrence of multiple mechanisms of glyphosate resistance in the populations SLR88 and SLR80. These two populations may be progeny of cross pollination from individuals with the two different glyphosate resistance mechanisms. It should be expected that individuals with only one of the mechanisms would also be found in the same fields.

In Chapter Five, an inheritance study was carried out to determine the mode of inheritance in the resistant population. In this study, SLR88 was cross pollinated to the susceptible population to produce F_1 progeny. The progeny were resistant to glyphosate

showing the glyphosate resistant traits were nuclear encoded and partially dominant. Therefore, other cases of multiple glyphosate resistant *L. rigidum* should be expected in the future. It is expected that glyphosate resistance in this population would be encoded by two genes, one for each resistance mechanism. However, further research is required to determine this.

There may be some additional value in studying the behaviour of these multiple glyphosate-resistant populations in the field. There have been suggestions that glyphosate resistance carries with it a fitness penalty (Pedersen *et al*., 2007; Preston *et al*., 2009). If such a fitness penalty is present, multiple glyphosate resistant populations may be at a greater disadvantage and may decline in frequency faster in the absence of glyphosate selection. This may also affect gene flow of the resistance alleles. There are two mechanisms of gene flow; firstly by movement of resistant seeds and secondly by the transfer of resistant pollen. For management purposes, determining the relative importance of seed versus pollen movement would be useful.

L. rigidum is an obligate cross pollinated weed species present over a wide area, which contributes to a high level of genetic variation within populations. With a long history of control with different mode of action herbicides, *L. rigidum* has evolved resistance to herbicides with eight different modes of actions (Heap, 2009). *L. rigidum* populations have also evolved resistance to multiple herbicides (Llewellyn and Powles, 2001). The occurrence of multiple herbicide resistance of *L. rigidum* population creates more problems to control (Neve *et al*., 2004). This research demonstrates that *L. rigidum* populations can evolve multiple resistance to glyphosate as well as accumulating resistance to a wide range of other herbicides.

From this study, resistance to glyphosate in *L. rigidum* can reach a very high level, as found in the SLR88 and SLR80 populations. Glyphosate is no longer an effective herbicide to control those populations at any practical application rate. It was suggested that increasing the rate of glyphosate could overcome the problem of glyphosate resistance in the short term, but will also increase the frequency of glyphosate resistance in the population (Wakelin and Preston, 2008). Thus, weed management becomes more difficult and other strategies need to be used to control these resistant populations. Not only that, the transfer of resistant seeds to clean fields

also should be prevented to avoid the problem spreading. Alternative management, include other mode of action herbicides, such as paraquat (Neve *et al*., 2003), physical weed management, such as cultivation, crop competition, hay production and seed set control (Preston *et al*., 2009) needs to be implemented. Australian growers often try to control glyphosate resistant *L. rigidum* by using glyphosate mixtures with other herbicides. However, the mixing partners do not always control *L. rigidum* alone and the mixture relies on getting some herbicidal activity from glyphosate. These highly resistant *L. rigidum* populations will be less amenable to such tactics and will need other strategies to be employed.

The increased use of glyphosate and continuous selection pressure is likely to result in more resistant weed species in the future. In addition to this, the adoption of glyphosate-resistant crops and no till seeding systems in cereal and grain crops production also increase the number of resistant weeds (Powles, 2008). This makes strategies to mitigate resistance even more important. A good weed management strategy is needed to delay and reduce the occurrence of glyphosate resistant weeds. Besides this, increasing the diversity of crop species also was suggested to delay the evolution of resistant weed populations (Broster and Pratley, 2008). In no tillage seeding systems several techniques, such as replacing glyphosate with paraquat, were suggested (Neve *et al*., 2003). In glyphosate-resistant soybeans, ALS inhibitors are used to control glyphosate resistant populations of *C. canadensis* (Dill, 2005). Diversifying agroecosystems and the use of different herbicide modes of action in glyphosate-resistant crops system could reduce resistant weed populations (Powles and Preston, 2006; Powles, 2008). Continual use of glyphosate to control glyphosate-resistant weeds may lead to multiple glyphosate resistance, such as observed in populations SLR80 and SLR88.

6.2 Conclusion

In conclusion, the resistant populations of *L. rigidum* in this study exhibited various resistance levels to glyphosate. Among the resistant populations examined, SLR77 exhibited the lowest, NLR70 an intermediate, SLR88 high and SLR80 very high resistance level. From the resistance mechanism experiments, it was determined that the resistance mechanism of the intermediate to high resistance level in the population was due to reduced herbicide translocation and target-site mutation mechanism in the

population with low resistance level. Interestingly, SLR88 and SLR80 populations exhibited both resistance mechanisms to glyphosate. The high resistance level in these populations is suggested due to occurrence of both resistance mechanisms. The occurrence of multiple mechanisms of glyphosate resistance will make these populations harder to control with glyphosate.

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