ASPECTS OF HERBICIDE RESISTANCE IN THREE NEW ZEALAND WEED SPECIES

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Abstract

Herbicide resistant weeds have become a challenge in agricultural systems globally. In this thesis, aspects have been studied of three weed species which have evolved resistance to herbicides from different chemical families within New Zealand.

Dicamba-resistant fathen (*Chenopodium album*) was recently reported by researchers in Waikato. In this thesis, the level of resistance to dicamba in two of these populations of fathen was investigated using a whole plant dose-response experiments and it ranged from 5- to 20-fold. Also, a seed-test for rapidly and reliably detecting dicamba resistant fathen has been developed. Seed tests have seldom been used for detecting resistance within weeds to auxinic herbicides.

The thesis also investigated aspects of the first reported cases of glyphosate resistance in New Zealand, found in both Italian ryegrass (*Lolium multiflorum*) and perennial ryegrass (*Lolium perenne*) from vineyards. Resistance to glyphosate in two populations of Italian ryegrass (Populations A and P) and two populations of perennial ryegrass (Populations J and N) was found to be almost 10-fold, whereas it was almost 30-fold for one perennial ryegrass population (Population O). Three different quick tests (seed assays, excised tiller bioassays and shikimic acid assays) were developed for detecting glyphosate resistance in Italian ryegrass and perennial ryegrass.

Of the five populations of ryegrass studied, only Population O had a target site modification at Codon 106 of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Translocation of radiolabelled glyphosate was studied in four of the populations (Populations A, J, O and P), and movement from treated leaves was significantly reduced in them all compared with susceptible populations (non-target site mechanism of resistance). Therefore, Population O had two mechanisms of resistance, possibly explaining the 30-fold resistance.

The studied glyphosate-resistant ryegrass populations were all found to be resistant to glufosinate. Populations A, J and O were also found to be resistant to amitrole. Genetic studies showed that the restricted glyphosate translocation trait is incompletely dominant and can be transmitted via pollen. The restricted herbicide translocation was suppressed under cool conditions in experiments, suggesting that application of

glyphosate during winter might improve control of glyphosate-resistant Italian ryegrass and perennial ryegrass infestations.

KEYWORDs: *Chenopodium album*, dicamba, glyphosate, *Lolium multiflorum*, *Lolium perenne*, amitrole, glufosinate, glyphosate mechanisms of resistance, target site mechanism of resistance, restricted herbicide translocation.

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Dedication

I dedicate this work to my respected mother, with gratitude for her love, encouragement and support.

Table of contents

List of Tables

List of figures

Abbreviations

µCi	microcurie
ABC	ATP-binding cassette
ABP	auxin binding-protein
a.e.	acid equivalent
a.i.	active ingredient
Ala	alanine
ALS inhibitors	acetolactate synthesis inhibitors
AMPA	aminomethylphosphonic acid
ANOVA	analysis of variance
Arg	arginine
Asn	asparagine
ATPase	adenylpyrophosphatase
bp	base pair
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
drc	dose-response curve
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
Gly	glycine
GR_{50}	dose that caused growth reduction of 50%
IAA	indole-3-acetic acid
kBq	kilobecquerel
LD_{50}	lethal dose for 50% of population
Leu	leucine
LMP	low melting point
MBq	megabecquerel
Met	methionine
NAA	2-naphthoxyacetic acid
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
Pro	proline
rcf	relative centrifugal force

RNA	ribonucleic acid
rpm	revolutions per minute
S3P	shikimate-3-phosphate
Ser	serine
Thr	threonine
Trp	tryptophan
UV	ultraviolet

Chapter 1

Introduction, objectives and thesis structure

1.1 Introduction

Weeds are plants that grow where they are unwanted (Monaco *et al.*, 2001). Herbicide application is a weed control method that has been used for over 70 years to control weeds in agricultural and non-agricultural situations (Dodge, 2008). However, a phenomenon called herbicide resistance has been creating difficulties for weed management strategies throughout the world (Powles & Yu, 2010). The number of herbicide-resistant weeds has been increasing gradually with new cases of herbicide-resistance being reported every year globally (Heap, 2015). The term herbicide resistance was first noted 60 years ago and the idea was developed due to appearance of resistance to other pesticides like insecticides (Harper, 1957). The appearance of herbicide-resistant weeds is the adaptive response of populations of weeds to herbicide application (Délye *et al.*, 2013). It is generally accepted that evolution of herbicide resistance is due to "selection pressure" or "selection intensity" (Jasieniuk *et al.*, 1996) created by persistent applications of herbicides involving the same mode of action. Repeated application of herbicides involving the same mode of action allows individuals that benefit from mutations which enable them to survive herbicide applications, to increase in size and spread (Neve, 2007).

In New Zealand, since the first case of a herbicide-resistant weed was reported in the late 1970s (Rahman *et al.*, 1983), the number of herbicide-resistant weeds has increased and the majority of them are resistant to auxinic herbicides or photosystem II inhibitors (Heap, 2015). In this PhD study we firstly studied some aspects of herbicide resistance in *Chenopodium album* L. (fathen). Fathen populations have evolved resistance to both atrazine herbicides and dicamba (James *et al.*, 2005; Rahman *et al.*, 1983). Although evolution of resistance to dicamba had been reported in New Zealand prior to commencement of my work (James *et al.*, 2005), no studies had reported on the exact magnitude of this dicamba resistance in fathen. One objective of this PhD research was to quantify the level of resistance in two dicamba-resistant fathen populations.

The conventional method for evaluating the evolution of herbicide resistance is to conduct a dose-response test using potted plants which provides results that are similar to the plant response likely to be found in the field. However, this method is time consuming and requires a reasonable amount of glasshouse space for every population tested (Burgos *et al.*, 2013). Successful management of herbicide resistance requires rapid confirmation of herbicide-resistant biotypes especially when one deals with a large number of samples (Koger *et al.*, 2005). However, although quick tests have been developed for this purpose elsewhere, they have never been studied in New Zealand. It is desirable to develop a reliable test that can evaluate a large amount of samples in a short period. The distribution of dicamba resistance fathen in New Zealand is unknown. By developing a quick test, it would be possible in a short time to investigate how widespread dicamba resistance is in New Zealand. Hence, the reliability of a quick test using seeds for detecting dicamba resistance in fathen was investigated.

In 2012, for the first time ever, it was noted that glyphosate had failed to control *Lolium perenne* (perennial ryegrass) and *Lolium multiflorum* (Italian ryegrass) in some vineyards. Glyphosate is an important agrochemical because it controls a wide range of annual and perennial weeds, becomes inactive on contact with soil and has minimal environmental impact (Nandula, 2010). It was once thought that development of resistance to glyphosate would be unlikely (Bradshaw *et al.*, 1997), but since the first case of glyphosate resistance was reported in Australia (Powles *et al.*, 1998), the development of glyphosate-resistant weeds has been reported from over 20 countries (Heap, 2015). The evolution of glyphosate resistance in Italian ryegrass and perennial ryegrass could be of significant concern as these two species are important pastoral plant species in New Zealand. As *Lolium* spp. species is consisted of obligate out-crossing plants, the resistance gene could be easily spread from properties in which resistant plants develop to neighbouring areas with pasture. The spreading of glyphosate resistance genes to pastures could have consequences for New Zealand as glyphosate is the main herbicide that is used for pasture renewal. Lack of efficacy of glyphosate due to development of glyphosate resistance in ryegrass populations in pastures would mean farmers would need to use herbicides with different modes of action that are not as convenient to use as glyphosate due to leaving residues in the soil and costing more. Although different aspects of glyphosate resistance have received extensive study for cases in other countries (reviewed by Sammons and Gaines (2014), almost nothing was

known (*e.g.* mechanisms of resistance, resistance genetic basis, etc.) about the glyphosate-resistant populations of *Lolium* ssp. from New Zealand, and these aspects needed investigating in order to develop strategies to manage glyphosate resistance in this genus.

1.2 Objectives

The broad objectives of this PhD were to add to the knowledge already documented for dicamba-resistant fathen and also to obtain information about the first cases of glyphosate-resistant weeds in New Zealand. Because of the importance of being able to detect herbicide-resistant weeds in New Zealand, one of the aims of this study was to develop some quick tests for detecting both dicamba and glyphosate resistance within the weeds of interest. As the timing of this PhD programme coincided with the discovery of the first case of glyphosate resistance within New Zealand, it was decided to focus attention on glyphosate resistance for much of the programme because of its importance to the country. Also funding for the research was only available for investigating aspects of glyphosate resistance. Therefore, many of the objectives listed below relate more to aspects of glyphosate resistance in ryegrass, including the mechanisms of resistance and the inheritance of glyphosate resistance. Also, the response of glyphosate-resistant weeds to other herbicides was evaluated, and the influence of temperature on the level of resistance. Implications of the results from this PhD study for management of the glyphosate and dicamba-resistant weeds within New Zealand are then discussed.

The specific objectives of this study relating to dicamba-resistance in fathen were:

1- To evaluate fathen populations for herbicide resistance by conducting conventional dose-response tests.

2- To develop a quick test for detecting dicamba-resistant fathen using a seed assay.

Objectives relating to the glyphosate-resistant Italian ryegrass and perennial ryegrass were:

1- To confirm glyphosate resistance in populations of Italian ryegrass and perennial ryegrass by conducting conventional dose-response tests.

2- To develop quick-tests for detecting glyphosate-resistant biotypes using seed assays, excised tiller bioassays and *in vitro* (*i.e.* shikimic acid) assays.

3- To investigate whether alterations in glyphosate absorption/translocation or target site (EPSPS) modification were mechanisms of glyphosate resistance in Italian ryegrass and perennial ryegrass.

4- To investigate whether glyphosate-resistant populations of Italian ryegrass and perennial ryegrass are also resistant to other herbicides.

5- To determine whether a glyphosate-resistant weed population becomes less resistant to glyphosate when treated under cooler conditions.

6- To investigate the inheritance of glyphosate resistance in Italian ryegrass and perennial ryegrass

1.3 Thesis structure

This thesis consists of 12 chapters. Chapter 1 is an introductory chapter discussing why this research was undertaken. Chapter 2 is a literature review that provides some details on the two herbicides, dicamba and glyphosate, then outlines knowledge on dicamba and glyphosate-resistant weeds globally, biology of fathen, Italian ryegrass and perennial ryegrass and the status of herbicide-resistant weeds in New Zealand prior to this research. In Chapter 3, several populations of fathen are tested for herbicide resistance. In Chapter 4, the potential ability of a quick test using seeds for detecting dicamba-resistant fathen is investigated.

The remainder of the thesis deals with some aspects of glyphosate resistance in Italian ryegrass and perennial ryegrass. In Chapter 5, the evolution of glyphosate resistance in five putative glyphosate-resistant Italian ryegrass and perennial ryegrass populations is studied. In Chapter 6, the usefulness of three different quick tests for screening glyphosate-resistant Italian ryegrass and perennial ryegrass is evaluated. In Chapter 7, the target site-mechanism of resistance to glyphosate at position Pro-106 in the EPSPS enzyme is studied via genotyping assays. In Chapter 8, the presence of the non-target site mechanism of glyphosate resistance (restricted herbicide translocation) is investigated. The response of glyphosate-resistant Italian ryegrass and perennial ryegrass populations to other herbicides as well as the development of glufosinate and amitrole resistance in three populations of Italian ryegrass and perennial ryegrass is investigated in Chapter 9. In Chapter 10, the response of two populations of perennial ryegrass (one glyphosate-resistant and one susceptible to glyphosate) to different rates of glyphosate is compared at cold versus warm temperature conditions. In Chapter 11,

the inheritance of glyphosate resistance in Italian ryegrass and perennial ryegrass is investigated. Chapter 12 summarises the main findings of this PhD project and discusses the implications of the results for management of dicamba and glyphosate resistance.

Chapter 2

Literature review

2.1 Introduction

Developing herbicide resistance management strategies requires a better knowledge of biological aspects of herbicide resistant weeds. To date over 200 weed species have developed resistance to one or more herbicide family groups (Heap, 2015). In New Zealand, 12 weed species had been reported resistant to one or more herbicide groups by 2012 when this PhD project began. With this PhD, I have studied various aspects of three herbicide resistant weed species (*Chenopodium album*, *Lolium multiflorum* and *Lolium perenne*) which have evolved resistance to herbicides from different chemical families. The following literature review comprises three parts. In the first part, information is presented regarding the biology of fathen (*Chenopodium album*), the problems of fathen in maize cropping in New Zealand, chemical options for controlling weeds in maize cropping in New Zealand and information regarding auxinic herbicides in general and dicamba as one of the case studies in this PhD project. In the second part, information regarding the biology of Italian ryegrass (*Lolium multiflorum*) and perennial ryegrass (*Lolium perenne*) is discussed, and also information about glyphosate, to which some populations of these two species have now become resistant. In the third part, herbicide resistance is defined, mechanisms of herbicide resistance are discussed and a review of the current situation with herbicide resistant weeds in New Zealand is provided. Finally at the end of the third part, the current knowledge of mechanisms of resistance and modes of inheritance regarding auxinic herbicide resistant and glyphosate resistant weeds in the world is reviewed.

2.2 Fathen (*Chenopodium album*)

2.2.1 Biology of fathen

Fathen (*Chenopodium album*), formerly a member of the *Chenopodiaceae* family and now a member of the *Amaranthaceae* family (Rahiminejad & Gornall, 2004), is considered to be one of the 10 worst weeds in agricultural cropping systems (Holm *et al.*, 1977). It is a common annual weed species in temperate regions and is ranked as the most important weed in spring-planted crops such as maize (*Zea mays*) and sugar beet

(*Beta vulgaris*) (Aper *et al.*, 2010). Fathen can be found in most regions as it grows in a wide range of soil types from strongly acid to alkaline (Williams, 1963).

Fathen grows from 10 to 250 cm tall and has green or reddish parallel stripes on ridged, angular branched stems (Bassett & Crompton, 1978). Leaves are deep to light green, petiolated, alternate, 1-12 cm long, and 0.5-8.0 cm wide without stipules (Bassett & Crompton, 1978). The inflorescence consists of very small (1.0-1.5 mm) grey or grey-green flowers clustered on spikes in the forks of upper leaves (Mitich, 1988). The flowers of fathen are perfect with 5(4-3) anthers and protruded stigmas (Bassett & Crompton, 1978). Fathen is self-compatible (Bassett & Crompton, 1978) and is able to reproduce via cross- or self-pollination (Darmency & Gasquez, 1990). Every plant can produce 72,000-500,000 smooth reticulate black or brown seeds. The morphology and dormancy condition of seeds is determined by the photoperiod that mother plants experience during seed development. Under long-day length, seeds are small and black with a thicker testa and these types of seeds are dormant, whereas non-dormant seeds which are produced under short-day length are brown with thin seed coats and are heavier than black coloured seeds (Bradford & Nonogaki, 2007).

The seeds of fathen are able to germinate over a wide range of conditions (Cumming, 1963). Dormant seeds are a key factor in the success of fathen as a weed. A high proportion of fathen seeds is innately dormant and can remain viable in the soil for many years (Conn & Deck, 1995). Germination of fathen seed may be influenced by a number of factors like soil moisture, soil temperature, nitrate content of soil and light (Holm *et al.*, 1977). Freshly harvested seeds of fathen are dormant (Saini *et al.*, 1985). However, germination rates up to 64% can be obtained following temperature treatments of 0 to 5°C for 21-28 days (Williams, 1963). Depending on temperature and light quality (low red/far-red ratio of light inhibits fathen seed germination), seed emergence usually begins in early spring and continues throughout summer (Ogg & Dawson, 1984; Schuster *et al.*, 2007).

Seedling vigour is greatly influenced by factors such as light penetration through the canopy, nutrient availability for parent plants, and interactions between these factors (Mahoney & Swanton, 2006). Seeds which were collected from high nitrogen and low light areas had higher seedling vigour (Mahoney & Swanton, 2006). It was also reported that fathen exhibited some of the adaptive strategies that make it a persistent weed in agricultural and natural systems (Mahoney & Swanton, 2006). For instance, the

ability of fathen to germinate at lower temperatures allows it to establish its seedling before crop emergence, and therefore, it will be able to have a competitive advantage over crops (Wiese & Binning, 1987). Fathen is also a shade-avoiding species that adapts its morphology to shade other plants (Rohrig & Stutzel, 2001). These above-mentioned adaptive strategies of fathen increase fitness and persistence of this weed species within agricultural cropping systems, especially when planting pattern, crop density and fertilisation management are used as weed management strategies.

2.2.3 Problems caused by fathen

Fathen is one of the most troublesome weeds associated with crops such as maize in New Zealand (James *et al.*, 2005). Crop yield losses due to fathen competition can be dramatic. The competitiveness of fathen in maize has been investigated by several researchers who have determined a range of crop losses up to 100% due to varying environmental conditions and weed densities (Fischer *et al.*, 2004). Fathen has been shown to cause economic yield losses in crops such as sugarbeet (*Beta vulgaris*) (Schweizer, 1983), barley (*Hordeum vulgare*) (Conn & Thomas, 1987), tomato (*Solanum lycopersicum*) (Bhowmik & Reddy, 1988), peanut (*Arachis hypogaea*) (Wilcut *et al.*, 1991) and soybean (*Glaycine max*) (Crook & Renner, 1990).

The application of both pre-emergence and post-emergence herbicides has been reported to give satisfactory control of fathen (Chomas & Kells, 2004; Myers & Harvey, 1993). However, selection intensity toward the development of resistance in fathen populations has been increased as a result of repetitive usage of herbicides involving the same modes of action in consecutive growing seasons. Fathen was the first species known to have developed resistance to herbicides in New Zealand (Rahman *et al.*, 1983). In various parts of the world, fathen has also evolved resistance to many chemical groups including atrazines, substituted ureas, amides, acetolactate synthesis (ALS) inhibitors and auxinic herbicides (Heap, 2015).

2.2.3 Herbicides used for fathen control in maize

Maize (*Zea mays*) has been planted in parts of the North Island in New Zealand for many years. This crop is very sensitive to weed competition during the early stages of growth (Ghanizadeh *et al.*, 2010). The application of herbicides has become a popular method for weed control because it is cheaper and less time-consuming compared to other methods such as cultivation. In the early years of maize cropping, atrazine

herbicides like atrazine and cyanazine were the main pre-emergence herbicides for weed control in New Zealand (Harrington & James, 2005). Also these herbicides can be applied as post-emergence herbicides in the early stage of maize growth. They control a range of broad-leaf weed species but provide only weak control of many grass weeds. However, repeated application of atrazine herbicides in maize fields in New Zealand has resulted in appearance of atrazine resistance in weeds like fathen (Rahman, 1990). Occurrence of resistant weeds reduced the usefulness of atrazine herbicides for weed control in maize and led to investigations of the efficacy of other selective herbicides.

At post emergence, the benzoic acid group herbicide dicamba was then suggested as a useful alternative herbicide mainly to control atrazine-resistant fathen in maize cropping in New Zealand (Rahman *et al.*, 1983). It was also suggested to use metolachlor and alachlor herbicides with chlorbromuron or metribuzin to improve the control of atrazine-resistant fathen (Rahman *et al.*, 1983). Rahman and James (1992) reported that pre-emergence application of dimethenamid followed by post emergence application of dicamba or atrazine provided desirable control of annual grass weeds and the majority of broadleaf weeds including fathen in maize. Application of mesotrione as a pre-emergence herbicide also provided good control of broadleaf weeds like fathen in maize but not annual grasses. However, a combination of atrazine, metolachor or acetochlor with mesotrione increased its efficacy and caused a reduction in the biomass of annual grass and broadleaf weeds in maize (James *et al.*, 2006). Trolove *et al.* (2011) noted using flufenacil (a herbicide from the pyrimidinedione chemical group) alone at the rates of 70 or 105 g ai ha^{-1} provided good control of annual broadleaf weeds like fathen but not grasses, however, a combination of flufenacil with acetochlor provided excellent control of broadleaf and grass weeds in maize. Nicosulfuron (ALS inhibitor), bromoxynil and mesotrione were also reported to give good post-emergence control of fathen in maize (Rahman & James, 1993; Rahman *et al.*, 2008) if they were applied with appropriate adjuvants (James & Rahman, 1994). Metribuzin and linuron are other registered post-emergence herbicides in maize crops in New Zealand that control fathen (Harrington & James, 2005).

2.2.4 Auxinic herbicides

Auxin is the general term for the phytohormone IAA (indole-3-acetic acid) which influences several aspects of plant growth and development including cell expansion, cell division, gravitropism, root initiation, apical dominance, fruit development and

phototropism (Srivastava, 2002). Auxinic herbicides are thought to affect the same binding and transport processes as the auxinic phytohormone (Grossmann, 2000), causing similar physiological manifestations (Kern *et al.*, 2005) and similar patterns of plant gene expression (Datta *et al.*, 1993). Supporting the idea that auxinic herbicides act similarly to phytohormone IAA, low doses of auxinic herbicides (*e.g.* 2,4-D and dicamba) are commonly utilised to replace auxin in plant cell tissue culture (Devine *et al.*, 1993). However, higher doses of auxinic herbicides induce uncontrolled growth, vascular tissue proliferation, cell membrane disruption and finally death (Devine *et al.*, 1993).

Auxinic herbicides, based on the location of the carboxylic acid moiety in relation to the aromatic group and the type of aromatic group, are divided into three major classes: 1) phenoxy acids (*e.g.* 2,4-DB, MCPA and mecoprop), 2) benzoic acids (*e.g*. dicamba and chloramben), and 3) pyridine carboxylic acids (*e.g.* picloram and triclopyr). Another developed class of auxinic herbicides is the quinoline carboxylic acids (*e.g.* quinmera and quinclorac) which have activity on some grasses unlike other classes of auxinic herbicides (Sterling & Hall, 1997). Phenoxy acid herbicides are also sub-grouped into phenoxy acetics (*e.g*. 2,4-D), phenoxy propionics (*e.g*. mecoprop) and phenoxy butyrics (*e.g*. 2,4-DB) (Monaco *et al.*, 2001).

The first auxinic herbicides, 2,4-D and MCPA, were discovered independently by British and American scientists (Monaco *et al.*, 2001). The phytotoxic effects of the auxinic herbicides were first discovered from basic research on plant growth regulators with the discovery that IAA and NAA (2-naphthoxyacetic acid) promoted cell growth (Monaco *et al.*, 2001). These compounds were found to affect cell growth more than IAA and were not as readily metabolised in plant cells (Monaco *et al.*, 2001). This early research on these herbicides was not reported due to World War II security regulations (Monaco *et al.*, 2001). However, immediately after the war, these two selective herbicides were introduced to control broadleaved weeds in cereals (Preston & Mallory-Smith, 2001).

The exact mode of action of auxinic herbicides is still unknown. It is suggested that auxinic herbicides bind to the auxin receptors and cause physiological and morphological changes (Reade & Cobb, 2002). These changes can be attributed to several biochemical responses namely, a rapid Ca^{++} influx, activation of membrane ATPase and increasing both protein and nucleic acid biosynthesis (Coupland, 1994).

2.2.5 Dicamba

Dicamba is applied as a post-emergence herbicide to control annual and perennial broad-leaf weeds in grain crops and pastures (Monaco *et al.*, 2001). The growth regulating effect of benzoic acids was first outlined in 1942 which led to extensive testing of these chemicals as herbicides (Monaco *et al.*, 2001). This screening led to the discovery of several herbicides including 2,3,6-TBA, PBA, dicamba, tricamba and amiben (Ashton & Crafts, 1980). Some brand names for the formulations of dicamba in New Zealand include Banvel, Kamba 500, Dicamba 500SL, Dicam 480, Cutlass and Buttress (Young, 2015). Dicamba is soluble in water and boils at 200°C (National Institute for Occupational Safety and Health, 2011). This herbicide bonds with soil poorly and its half-life is between 1-6 weeks (National Institute for Occupational Safety and Health, 2011).

Worldwide, dicamba is available in a variety of acid and salt formulations. The salt formulation of dicamba is either organic (dimethylamine) or inorganic (Petersen *et al.*, 1985). The dimethylamine salt is the formulation which is used in New Zealand (Young, 2015). Some researchers have reported that organic salt formulations are more volatile than inorganic ones (Behrens & Lueschen, 1979). Dicamba is commercially available in liquids, liquid concentrates, wettable powders, granules and water dispersible granules (Environmental Protection Agency, 2006). In New Zealand it is sold in as a soluble concentrate (Young, 2015). Dicamba has a low acute toxicity in oral, dermal, and inhaled routes of exposure. The oral LD_{50} for dicamba is 757 to 1707 mg kg^{-1} in rats (Extension Toxicology Network, 1996). However, exposing the skin and eyes to dicamba can cause redness, pain, and blurred vision. The inhalation of dicamba may cause coughing, vomiting, weakness and nausea (National Institute for Occupational Safety and Health, 2011).

Dicamba can be absorbed by both foliage and root systems (Al-Khatib *et al.*, 1992). Dicamba is highly phloem mobile which can move acropetally and basipetally (Sterling & Hall, 1997). Dicamba which is taken up by plant roots is translocated acropetally in the xylem to the top of the plants and then will redistribute into the phloem ((Sterling & Hall, 1997). This herbicide, like other growth regulator herbicides, causes epinasty, defoliation, swelling of stems, destruction of conductive tissues, death of growing points, loss of apical dominance, and ultimately, necrosis (Vencill, 2002). The specific mechanism of action of dicamba, like other auxinic herbicides, is still not well

understood. It is known that they mimic the plant hormone indole-3-acetic acid (IAA) action (DiTomaso, 2002). However, auxinic herbicides are applied at rates considerably higher than the concentration of IAA in plants. RNA synthesis and growth in meristematic tissues would be inhibited by high levels of auxins (DiTomaso, 2002). The abnormal stimulation of cell division by synthetic auxin treatment as well as the rapid cell wall loosening response, leads to uncontrolled growth and callus tissue production (DiTomaso, 2002)). Consequently, excessive cell division causes stem swelling and eventually cellular collapse, particularly in the phloem tissues (DiTomaso, 2002).

Degradation and metabolism of dicamba in plants is species dependent. A limited degradation of dicamba has been reported in *Cyperus rotundus* (Magalhaes *et al.*, 1968), *Fagopyrum tataricum* and *Sinapis arvensis* (Chang & Vanden Born, 1971). In contrast, it has been reported that dicamba has been metabolised rapidly and extensively in grass species like wheat, barley (Chang & Vanden Born, 1971), *Poa pratensis* (Broadhurst *et al.*, 1966), *Sorghum halepense* (Hull & Weisenberg, 1967) and maize (Ray & Wilcox, 1967). In a study of detoxification of dicamba in wheat and barley, a major metabolite was identified chromatographically as 5-hydroxy-3,6-dichloro-o-anisic acid and a minor metabolite, 3,6-dichlorosalicylic acid, was also found (Chang & Vanden Born, 1971). Ray and Wilcox (1967) found that maize roots converted dicamba into a 5-hydroxy derivative as a minor metabolite and 3,6-dichlorosalicylic acid as the major metabolite. Thus, differential metabolism of dicamba is apparently related to plant species. Dicamba can be degraded by soil microorganisms and the rate of degradation increases under warm, moist soil conditions (Monaco *et al.*, 2001).

2.3 Italian ryegrass (*Lolium multiflorum*) and perennial ryegrass (*Lolium perenne*)

2.3.1 Biology of Italian ryegrass

Italian ryegrass (*Lolium multiflorum*) is an annual or biennial cool season grass (Champion *et al.*, 2012). It is one of the most commonly grown grasses in temporary or short-term pastures in New Zealand (Hickey & Hume, 1994). Italian ryegrass is originally from Asia, Europe and North America (Beddows, 1973). This grass also occurs on roadsides and waste places and is a weed in gardens (Champion *et al.*, 2012), cereals (Rolston *et al.*, 2003) and orchards (Perez & Kogan, 2003). Italian ryegrass grows erect, vigorous shoots, and once flower spikes have formed, the length of shoots

can be up to 100 cm (Beddows, 1973). Mature leaves are up to 25 cm long and 10 mm wide with a short ligule (1-2 mm) and fairly narrow auricles (Beddows, 1973).

Italian ryegrass generally shares a similar appearance with perennial ryegrass (*Lolium perenne*), however, in Italian ryegrass the young emerging leaf is rolled and lemmas are awned in contrast to perennial ryegrass whose young leaves are usually folded and lemmas do not have awns (Champion *et al.*, 2012). Italian ryegrass is well-adapted to cool, moist climates with a range of temperatures between 20-25°C, and grows best at soil pH levels of 6-7 (Romani *et al.*, 2002). This grass is self-incompatible and allogamous (Cooper, 1951), and vernalisation is not usually required for inducing flowering in Italian ryegrass, however, vernalisation does advance flowering (Cooper, 1951). Seed dormancy of Italian ryegrass seeds can be overcome by chilling at 15°C or below (Rodriguez *et al.*, 1998). Italian ryegrass seeds are able to germinate over a range of temperatures from 10/2 to 30/25°C (day/night) (Young *et al.*, 1975). Adaptability of Italian ryegrass to a wide range of environmental conditions, its ability to produce high numbers of seeds and evolution of resistance to many herbicides have made this weed become more troublesome (Hulting *et al.*, 2012).

2.3.2 Biology of perennial ryegrass

Perennial ryegrass (*Lolium perenne*) is a perennial temperate grass that is widely grown as a pasture plant in New Zealand (Charlton & Stewart, 2006). This grass was introduced to New Zealand by Europeans in the early 19Th century (Lee *et al.*, 2012). Perennial ryegrass can also be found in roadsides, waste places, tracks, sand dunes and river beds (Champion *et al.*, 2012). This grass grows 25-75 cm high and has dark green hairless leaves (Champion *et al.*, 2012). The young leaves in this plant are folded and mature leaves are 6-20 cm long and 1.5-6 mm wide and sometimes with small auricles (Champion *et al.*, 2012). The membranous ligule is up to 2 mm long. Flower heads in perennial ryegrass are green or purple, 10-30 cm long. The lemmas of this species do not have awns (Champion *et al.*, 2012). Perennial ryegrass grows in a wide range of soils but is best adapted to fertile and well-drained soils with a pH range of 5.1 to 8.4 (Hannaway *et al.*, 1997). Perennial ryegrass grows well in spring and autumn but during hot summers this grass can become dormant (Hannaway *et al.*, 1997). Maximum growth of perennial ryegrass occurs in a range from 20 to 25°C (Hannaway *et al.*, 1997). Perennial ryegrass is able to tolerate cold winters, however, very harsh winters may kill this grass (Hannaway *et al.*, 1997). Exposure to several weeks of low temperature

(about 10°C) and short photoperiods effectively stimulates inflorescence production in perennial ryegrass (McWilliams & Jewiss, 1973). Perennial ryegrass is self-incompatible (McCraw & Spoor, 1983). It was reported that optimum germination for perennial ryegrass can be occurred over a range of 15/25, 20/25, 20/30 and 25/30°C (day/night) (Shen *et al.*, 2008). The Department of Conservation (DOC) in New Zealand has listed perennial ryegrass as an environmental weed (Champion *et al.*, 2012).

2.3.3 Problems caused by Italian ryegrass and perennial ryegrass

Ryegrass species can be very competitive with crops once they have established (Hall, 1992) and therefore can reduce crop yields dramatically. For example, a yield reduction up to 100% has been reported for broccoli due to adverse impacts of perennial ryegrass competition (Bell, 1995). Also, a significant reduction in yield has been reported for wheat when competed with Italian ryegrass (Abul *et al.*, 1998; Appleby *et al.*, 1976). The competitiveness of Italian ryegrass and perennial ryegrass has also been shown by several researchers in barley (Wanic *et al.*, 2013), maize (Nandula, 2014) and sugar beet (Heisel *et al.*, 2002). Ryegrass species have also been troublesome weeds in orchards and vineyards in the world (Tan & Crabtree, 1990), partcularly in Ne Zealand (Harrington *et al.*, 2014). Also, ryegrass species can be troublesome within roadsides, railways, amenity and waste areas (Hall, 1992; Nandula, 2014).

Herbicide application has been a major method for controlling ryegrass species especially within orchards and vineyards in New Zealand (Harrington, 2014). Evolution of herbicide resistance in ryegrass species had not been noted in New Zealand until 2012 when it was reported that glyphosate failed to provide good control of ryegrass species within some vineyards (Harrington *et al.*, 2014). Following this discovery, ryegrass species caught the attention of the public not as pasture plants but as herbicide-resistant weeds in New Zealand.

2.3.4 Herbicides used in vineyards and orchards in New Zealand

A variety of chemical, mechanical and cultural weed control methods have been used in vineyards and orchards in New Zealand (Dastgheib & Frampton, 2000). However, studies have shown that weed management strategies have relied heavily on chemical control methods (Harrington *et al.*, 2002). After many years of atrazine herbicide applications, when evolution of atrazine resistance in some species like *Solanum nigrum*

became evident (Harrington, 2014), applications of residual herbicides from different chemical families than atrazine herbicides were suggested as a good option for managing weeds in orchards and vineyards (Harrington, 2005a). However, the application of residual herbicides has been discouraged due to their detrimental impacts on environment (Dastgheib & Frampton, 2000).

A survey conducted by Dastgheib and Frampton (2000) showed that glyphosate, amitrole, glufosinate and paraquat were the most frequently applied herbicides among all chemical options available to growers. However, due to restrictions imposed by the wine-growing producer board, paraquat application is no longer permitted within most vineyards (Harrington, 2012). Also, with the decrease in use of residual herbicides due to restrictions by the producer board, glyphosate applications have increased over time (Pearson, 2003).

Several chemical strategies for avoiding and managing herbicide resistance in orchards and vineyards have been suggested (Harrington, 2012; Harrington, 2014; Harrington *et al.*, 2014). Generally, applications of herbicides with different modes of action in combinations or rotations have been suggested as a way to avoid evolution of herbicide resistance (Harrington *et al.*, 2002).

2.3.5 Glyphosate

Glyphosate is a non-selective, systemic and broad spectrum herbicide (Franz *et al.*, 1997). Glyphosate was first introduced by Monsanto Agricultural Products Company in 1974 (Franz *et al.*, 1997). Glyphosate (N-(phosphonomethyl) glycine) is a weak organic acid derivative of the amino acid glycine and phosphonic acid (Franz *et al.*, 1997). Glyphosate is a water soluble chemical with a vapour pressure of 1.84×10^{-7} mm Hg at 45°C (Franz *et al.*, 1997).

Glyphosate is generally formulated as salts (Franz *et al.*, 1997). In New Zealand, glyphosate is sold as soluble concentrates using many different brand names which differ in concentration of active ingredients and salts used. Examples include Agpro Glyphosate 360 (contains 360 g L^{-1} active ingredient as isopropylamine salt), Donaghys Grunt 600 (600 g L^{-1} as potassium and triethanolamine salts), Nufarm Glyphosate Gold (360 g L^{-1} as dual isopropylamine and ammonim salts), Polaris 450 (450 g L^{-1} as isopropylamine salt), Agro Green Glyphosate 510 (510 g L^{-1} as isopropylamine salt), Ravensdown Glyphosate 540 (540 g L^{-1} as isopropylamine salt) and Roundup Transorb

(540 g L^{-1} as potassium salt) (Young, 2015). In New Zealand, glyphosate is used for the control of annual and perennial grasses as well as broadleaf weeds in orchards, vineyards, market gardens, forestry, waterways and many other areas (Young, 2015). Glyphosate under normal conditions (-20 to 40°C) is very temperature stable, non-volatile and photostable (Ashton & Crafts, 1980). This herbicide has very low toxicity with an oral LD_{50} (rat) of > 5000 mg kg^{-1} (Ashton & Crafts, 1980).

Glyphosate is a post-emergence herbicide with very low soil residual activity and normally enters plants through foliage (stem and leaves) (Franz *et al.*, 1997). A broad spectrum of weeds (annual and perennial weeds) can be controlled by this herbicide including some of the most troublesome weeds with below-ground organs (rhizomes, tap-roots, etc.) (Cobb & Reade, 2010). Glyphosate efficacy depends on the dose of herbicide delivered to the living parts of the plant (Nandula, 2010). Glyphosate inhibits the enzyme EPSPS, the sixth enzyme in the shikimate pathway from which the aromatic amino acids tyrosine, phenylalanine and tryptophan are produced (Steinrucken & Amrhein, 1980). The shikimate pathway consists of seven metabolic steps. This pathway begins with the condensation of erythrose-4-phosphate and phosphoenolpyrovate (PEP) and ends with chorismate synthesis (Herrmann & Weaver, 1999). In the sixth step of this pathway, EPSPS catalyses the condensation of PEP and shikimate-3-phosphate (S3P) yielding 5-enolpyruvoylshikimate-3-phosphate (EPSP) (Herrmann & Weaver, 1999). Glyphosate competes with PEP for the binding site on the EPSPS-S3P complex which finally leads to EPSPS inhibition. The inhibition of EPSPS results in the excessive accumulation of shikimic acid in affected tissues (Amrhein *et al.*, 1980).

One of the reasons for broad-spectrum glyphosate activity is believed to be due to a lack of significant metabolism by plants (Franz *et al.*, 1997). In a study with several crops a negligible degradation of glyphosate to carbon dioxide and aminomethylphosphonic acid (AMPA) was reported (Coupland, 1985). Metabolism of glyphosate to AMPA was also reported in a few additional reports (Franz *et al.*, 1997). Sandberg *et al.* (1980) noted that glyphosate was metabolised to AMPA in a limited extent in Californian thistle (*Cirsium arvense*), field bindweed (*Convolvulus arvensis*) and tall morning-glory (*Ipomoea purpurea*). Although some weed species have showed some levels of glyphosate degradation, there is no evidence that metabolic degradation plays a

significant role in evolution of resistance to glyphosate in herbicide-resistant weed species (Duke, 2011).

2.4 Herbicide resistance

2.4.1 Herbicide resistance definitions and concepts

Prolonged usage of herbicides for controlling weeds in crops has resulted in development of herbicide resistance within weed species due to repeated selection of resistant individuals in field populations (Neve, 2007). The terms “resistance” and “tolerance” are often used interchangeably and different ideas have been noted about these two terms. For instance, Baucon and Mauricio (2004) defined tolerance to a herbicide as the ability of a plant to reduce the damaging effects of a herbicide so plants are able to reproduce even though they have been damaged by the herbicide. In contrast, resistant plants never experience damaging effects of herbicides as a result of involving special mechanism(s). The accepted definitions of herbicide tolerance and herbicide resistance were published by the Weed Science Society of America (WSSA) terminology committee in 1998. According to the accepted definitions, "herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis. Herbicide tolerance is the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant." (WSSA, 1998).

There are genetic variations among weed populations and repeated application of herbicides with a similar mode of action against the same weed population can result in appearance of resistant biotypes as a result of selection and genetic responses (Warwick & Black, 1994). There is no evidence that herbicide application causes the genetic mutation which leads to herbicide resistance. Since the first reported case of herbicide resistance in *Senecio vulgaris* (Ryan, 1970), the number of weed species which have evolved resistance to herbicides has been increasing at an exponential rate, and Heap (2015) reported that over 200 weed species have evolved resistance to most herbicide groups, globally.

Appearance of resistance to herbicides compared to other pesticides like insecticides and fungicides is thought to be slower. Gressel and Segel (1978) listed a number of reasons to explain the slower appearance of resistance to herbicides compare to other pesticides. They believed that generating new mutations in natural populations of plants for herbicide resistance are lower than other pesticides. Furthermore, they noted the plant's fitness as one of the main factors to delay the build-up of herbicide resistance (it was assumed that herbicide resistance mutations usually confers costs to a plant`s fitness). Therefore, if the resistant individuals are less fit than their susceptible counterparts, in the absence of herbicide the susceptible biotypes are able to out-compete the resistant ones. They also believed that selection intensity in plants is lower because of seed dormancy enables susceptible plants to geminate throughout the crop year; hence, those later germinated susceptible plants will have a chance to escape herbicide exposure. The escaped susceptible plants later will occupy available space and produce a large amount of seeds.

2.4.2 Mechanisms of herbicide resistance

According to Nandula (2010), there are four main mechanisms of herbicide resistance in weeds: 1) enhanced metabolism, which is the ability of plants to detoxify herbicides to non-toxic metabolites; 2) altered target site, in which herbicides no longer fit exactly in the point of action due to alterations in that site, so no blocking of the enzyme is possible by the herbicide; 3) compartmentalism/ sequestration, in which the herbicide is translocated to other parts of plant cells where the herbicide cannot be active therefore the amount of herbicide that reaches the site of action is reduced; 4) gene amplification/over-expression of the target site, by which the target protein can be produced in large quantities by the plant and hence, results in dilution of the herbicide in relation to the target site.

Multiple-resistance and cross-resistance are other important concepts in herbicide resistance discussions. Cross-resistance is defined as a single resistance mechanism which confers the ability to withstand herbicides from the same chemical class, or from classes with similar modes of action. Multiple-resistance means individuals possess two or more resistance mechanisms which enable them to withstand herbicides from different chemical classes (Nandula, 2010).

2.4.3 Current situation of herbicide resistance in New Zealand weeds

In New Zealand, the number of herbicide-resistant weeds has increased and in 2011 (before the start of this PhD project), 11 different types of herbicide resistance had been confirmed, involving nine different weed species (Heap, 2015). This compares with the situation in 1990, when four cases of herbicide-resistant species had been reported (Rahman, 1990).

2.4.3.1 Chenopodium album

Atrazine-resistant fathen (*Chenopodium album*) was first reported in late 1970s in some New Zealand maize fields. Initial evidence suggested that this weed may have evolved resistance due to the strong selection pressure of repeated treatments with atrazine herbicides in successive maize crops (Rahman *et al.*, 1983). Later, results of two field trials confirmed that fathen had developed atrazine resistance (Rahman *et al.*, 1983). Many studies on atrazine-resistant weeds have suggested that there is a fitness cost as a result of the most common mutation conferring atrazine resistance (Ser 264 to Gly mutation of the psbA gene) which causes a reduction in CO_2 fixation, quantum yield, and seed and biomass production (Parks *et al.*, 1996). Resistance management and prevention strategies for atrazine-resistant biotypes were proposed by Harrington and James (2005). They suggested that crop rotation and application of a combination of two or more herbicides with different modes of action but target the same weeds, would overcome the problem, with dicamba often being used to kill fathen plants surviving atrazine applications.

Dicamba-resistant fathen has now also been reported from these maize fields (James *et al.*, 2005). Greenhouse trials were conducted to compare a known susceptible fathen biotype with suspected resistant ones and results confirmed resistance had developed in fathen to dicamba. Fathen survived with dicamba applied at four times the normal field rate. The mechanism of dicamba resistance in fathen is unknown. In order to manage this dicamba-resistant biotype in maize fields, it was shown that post-emergence applications of nicosulfuron, pyridate, bromoxynil or mesotrione provided effective control (Rahman *et al.*, 2008).

2.4.3.2 Carduus nutans

Nodding thistle (*Carduus nutans*) is a problematic weed in pastures (Bonner *et al.*, 1998). The challenge of controlling this weed is the difficulty of finding selective

herbicides (Bonner *et al.*, 1998). Resistance to MCPA was reported in 1987 in some New Zealand pastures (Harrington, 1989; Harrington & Popay, 1987) and later this auxinic herbicide resistance was found in various parts of the North Island (Rahman, 1990). This biotype was six times more resistant than susceptible ones under field conditions (Harrington & Popay, 1987). Later, Harrington (1989) reported cross-resistance of this biotype to 2,4-D and MCPB. In this study, it was noted that clopyralid, mecoprop, picloram and glyphosate gave adequate control of this resistant biotype at recommended rates. However, these herbicides were too damaging to pasture species to be used for selective control (Bonner *et al.*, 1998).

In a study regarding the herbicide resistance mechanism(s) for nodding thistle, it was reported that herbicide absorption and translocation were not different between resistant and susceptible biotypes. However, resistant biotypes of nodding thistle were able to metabolise auxinic herbicide (2,4-D) more than susceptible ones (Harrington & Woolley, 2006). No difference was found by Harrington (1990) between phenoxy susceptible and resistant biotypes in fitness. In another fitness study on eight selected biotypes of nodding thistle which were different in resistance to the herbicide 2,4-D, again no evidence was found of a relationship between differential herbicide resistance and competitive ability across these biotypes (Bonner *et al.*, 1998).

2.4.3.3 Persicaria maculosa

Willow weed (*Persicaria maculosa*) was first reported resistant to atrazine herbicides in 1980 in maize fields (Rahman, 1990). The atrazine-resistant willow weed biotype was claimed by Bourdôt (1996) to be restricted to a few maize and asparagus fields where atrazines had been applied continuously for several years. A trial showed that these particular biotypes were resistant to atrazine and cross-resistant to other atrazine herbicides (Rahman & Patterson, 1987). Resistance management and prevention strategies for this atrazine-resistant biotype are the same as those which were mentioned for atrazine-resistant fathen (Harrington & James, 2005).

2.4.3.4 Ranunculus acris

Giant buttercup (*Ranunculus acris*) is an important weed in dairy pastures which occurs throughout New Zealand (Bourdôt & Hurrell, 1990; Popay *et al.*, 1984). A biotype developed resistance to MCPA in dairy pastures throughout New Zealand where this herbicide had been applied repeatedly over several years (Bourdôt & Hurrell, 1991).

Bourdôt and Hurrell (1988) found that the LD_{50} of a population (Silcock) which was little affected by MCPA was 4.8 times greater than the LD_{50} of a population (Jones) for which a very high level of control was obtained in pasture with a single application of MCPA at 1.0 $kg.ha^{-1}$. It was therefore assumed that herbicide resistance was genetically based (Bourdôt & Hurrell, 1988). Further studies showed that variations in resistance were correlated with exposure histories of MCPA, and populations of giant buttercup evolved resistance to MCPA due to selection intensity imposed by consistent herbicide application for several years in dairy pasture throughout New Zealand (Bourdôt *et al.*, 1990). Bourdôt *et al.* (1994) reported that the MCPA-resistance mechanism in *Ranunculus acris* also confers resistance to MCPB and 2,4-D and a lower temporary resistance to the sulfonylureas, chlorsulfuron and thifensulfuron.

The only study on fitness or competitiveness between MCPA-resistant biotypes of giant buttercup and susceptible ones has been conducted by Bourdôt *et al.* (1996). They reported that resistant biotypes were less fit than susceptible biotypes at high density. Resistance management and prevention strategies for this resistant biotype were reviewed by Harrington (2005b). He reported that two selective herbicides flumetsulam and thifensulfuron gave desirable control for this resistant biotype. Also, 1-5 days after applying MCPA, an application of bentazone provided desirable results. In a study investigating the efficiency of three herbicides (flumetsulam, thifensulfuron-methyl and MCPA) against giant buttercup, it was reported that flumetsulam was the best option for managing this weed in pasture because it gave good control of giant buttercup while causing the least pasture damage (Lusk *et al.*, 2011). Recently, evolving resistance to flumetsulam in a population with high previous exposure to this herbicide was reported. Further investigation indicated that flumetsulam-resistant giant buttercup was also cross-resistant to thifensulfuron-methyl (Lusk *et al.*, 2015).

2.4.3.5 Solanum nigrum

Black nightshade (*Solanum nigrum*) is an exotic weed that is widely distributed throughout New Zealand (Harrington *et al.*, 2001). This weed was first shown to have evolved resistance to atrazines within New Zealand in 1999 in pea and sweet corn fields (Harrington *et al.*, 2001). Research has shown that these particular biotypes are resistant to atrazine, cyanazine, prometryn, and terbuthylazine and they may be cross-resistant to other atrazine herbicides (Harrington *et al.*, 2001). In this work it was shown that even a 32-fold increase in the recommended application rates of herbicides caused no damage

to resistant biotypes. It has been shown in other countries that atrazine-resistant black nightshade biotypes are ecologically less fit than normal susceptible ones (Kremer & Kropff, 1999; Kremer & Lotz, 1998a; Kremer & Lotz, 1998b). In order to manage this resistant biotype, researchers suggested applying herbicides such as MCPB, methabenzthiazuron, pendimethalin and bentazone in peas, if applied early enough and dicamba is the best option to manage this biotype in sweet corn fields (Harrington *et al.*, 2001).

Recently, researchers reported populations of two paraquat-resistant *Solanum* spp. (*Solanum nigrum* and *Solanum nodiflorum*) in sweet potato (*Ipomoea batatas*) crops (Lewthwaite & Triggs, 2009). They noted that the paraquat concentration required for lethal dose (LD) thresholds of 99% death in *Solanum nigrum* seedlings was a 100-fold greater than for standard plants (a population collected from outside the sweet potato production region). They also indicated that a concentration increase of more than 18-fold was required to produce a similar response in the resistant population of *Solanum nodiflorum* compared to standard plants.

2.4.3.6 Soliva sessilis

In 1999, Onehunga weed (*Solvia sessilis*) was found to have developed resistance to auxinic herbicides (a picloram/2,4-D mixture) at the Helensville Golf Course (Harrington *et al.*, 2001). Harrington *et al.* (2001) reported that this biotype was cross-resistant to pyridine herbicides like clopyralid, triclopyr, picloram and a triclopyr/picloram mixture. They proposed bentazone, bromofenoxim and mecoprop/ioxynil/bromoxynil as alternative herbicides to control this resistant biotype.

2.4.3.7 Stellaria media

Chickweed (*Stellaria media*), a winter annual species, is an important weed of cereal crops in the South Island (Seefeldt *et al.*, 2001). Chlorsulfuron-resistant chickweed was discovered in 1995 in New Zealand (Bourdôt, 1996). Chlorsulfuron belongs to a group of herbicides known as ALS inhibitors, within the sub-group of sulfonylureas. This group of herbicide inhibits acetolactate synthase (ALS), which is a key enzyme in the metabolic pathway for formation of the branched chain amino acids, by binding to it and covering the active site of the enzyme. Later, researchers confirmed cross-resistance of this biotype to thifensulfuron but not to tribenuron (Seefeldt *et al.*, 2001). They also reported that this biotype was still susceptible to methabenzthiazuron, pendimethalin,

ioxynil, mecoprop and diflufenican. They recommended these herbicides as key elements to control resistant chickweed. Therefore, either replacing chlorsulfuron with one of these herbicides or in combination with chlorsulfuron would provide control for ALS-resistant chickweed (Harrington, 2005c).

2.4.3.8 Nassella neesiana

Chilean needle grass (*Nassella neesiana*) was reported to have evolved resistance to dalapon in 1992 in New Zealand (Hartley, 1994). Dalapon belongs to a group of herbicides are known as thiocarbamates, which inhibit lipid synthesis. Initially, glyphosate was used as an effective herbicide to control this weed in pasture swards (Hartley, 1994). Rapid re-infestation due to high seed viability of this biotype in the soil led to replacement of glyphosate with the more selective dalapon which was able to prevent flowering and reduce plant density to 90-99% without damaging pasture as badly as glyphosate (Hartley, 1994). However, after 3 years of successive applications of dalapon, a biotype had developed resistance to this herbicide (Hartley, 1994). The resistance level of resistant plants was six times greater than that of susceptible plants (Hartley, 1994).

2.4.4 Auxinic herbicide-resistant weeds

The first case of evolved auxinic resistance in weeds was documented in a *Commelina diffusa* population from Hawaii a few years after introduction of auxinic herbicides (Hilton, 1957). However, in spite of using auxinic herbicides now for 60 years, few cases of resistance to this group of herbicides have been reported. Also, it is surprising that compared to other herbicide groups such as imidazolinones, atrazines and sulfonylureas, resistance to auxinic herbicides has been slower to develop and to date only about 30 different weed species have been reported as having evolved resistance to them (Heap, 2015). It is suggested that slower development of resistance to auxinic herbicide is due to their application in mixtures with other herbicides, their moderate selection pressure, multiple sites of action (Jasieniuk *et al.*, 1996), fitness penalties (Bourdôt *et al.*, 1996) and quantitative inheritance of the resistance traits (Cranston *et al.*, 2001).

Apart from the cases already reported above in New Zealand, some of the other cases of resistance that have been reported for auxinic herbicides include *Daucas carota* to 2,4-D in Canada, *Sphenoclea zeylanica* to 2,4-D in the Philippines, *Stellaria media* to

mecoprop in the United Kingdom, *Centaurea solstitialis* to picloram in USA and *Kochia scoparia* to dicamba also in USA (Heap, 2015). The characterization of mechanism of resistance to auxinic herbicide has only been studied in some of the reported resistant weed species. A combination of reduced absorption and increased herbicide detoxification was found to be responsible for resistance to MCPA in *Galeopsis tetrahit* (Weinberg *et al.*, 2006). In investigations of MCPA-resistance in wild radish (*Raphanus raphanistrum*), no differences were found in MCPA uptake and metabolism between resistant and susceptible populations but resistant plants translocated more MCPA to the root region, suggesting rapid root exudation by resistant plants might occur (Jugulam *et al.*, 2013). Lutman and Health (1989) suggested that herbicide detoxification or some form of compartmentalization at the site of action could explain mecoprop resistance in *Stellaria media*. Other researchers have reported that there were no differences in uptake, transportation and metabolism of auxinic herbicide between resistant and susceptible biotypes of *Sinapis arvensis* resistant to 2,4-D, dicamba or picloram (Peniuk *et al.*, 1993) and *Kochia scoparia* resistant to dicamba (Cranston *et al.*, 2001).

It has been suggested that resistance to several auxinic herbicides like dicamba, MCPA and picloram in wild mustard (*Brassica kaber)* could be due to differences in affinity of auxin binding-protein (ABP) between resistant and susceptible biotypes (Mithila & Hall, 2005; Webb & Hall, 1995). Webb and Hall (Webb & Hall, 1995) also noted that differences in biotype response to auxinic herbicides can be attributed to the levels of calcium and ATPase activity in protoplasts and their links to ABP activity. Deshpande and Hall (1995) observed that there was an alteration in the dynamics of intercellular Ca^{++} and H^{+} as a result of picloram effects in susceptible biotypes of wild mustard but not in their resistant counterparts. They suggested Ca^{++} levels have an important impact in different responses between susceptible and resistant biotype to picloram. Based on further observation of changes of H^{+} efflux as a result of herbicides induction (dicamba, mecoprop and picloram), it was hypothesized that the physiological basis for auxinic herbicide-resistance in plants is associated with altered binding to auxin binding sites (Deshpande & Hall, 2000).

The inheritance of resistance to auxinic herbicides in a few species has been found to be controlled by different genetic systems: a single partly dominant gene in *Sinapsis arvensis* resistant to dicamba (Jasieniuk *et al.*, 1996), a single dominant gene in

Brassica kaber resistant to picloram and 2,4-D (Jugulam *et al.*, 2005) and in *Kochia scoparia* resistant to dicamba (Preston *et al.*, 2009a), a single recessive gene in *Centaurea solstitialis* resistant to picloram and clopyralid (Sabba *et al.*, 2003) and in *Galium spurium* resistant to quinclorac (Eerd *et al.*, 2004) , and by two additive genes in *Galeopsis tetrahit* resistant to MCPA (Weinberg *et al.*, 2006). In MCPA-resistant *Raphanus raphanistrum,* a single incomplete gene was found to control the MCPA mechanism of resistance (Jugulam *et al.*, 2013). Different patterns of inheritance among these studies suggest that different mechanisms of resistance to auxinic herbicide have evolved in these different weed species. Those weed species for which resistance is conferred by a single recessive gene can only be established in homozygous conditions that would arise in highly self-pollinated weed species. On the other hand, if a single dominant gene determines resistance, resistance traits can be spread readily in weed species that are primarily cross-pollinated (Jasieniuk *et al.*, 1996).

2.4.5 Glyphosate-resistant weeds

The first case of glyphosate-resistant weeds was discovered in *Lolium rigidum* from Australia in 1996 (Powles *et al.*, 1998). So far, over 30 weed species have been reported as having evolved resistance to glyphosate globally (Heap, 2015). An important factor that has increased the evolution of glyphosate-resistant weeds has been transgenic glyphosate-resistant crops such as maize, soybean and cotton (Powles & Yu, 2010). In these crops, extensive applications of glyphosate instead of other means of weed control have increased selection intensity for glyphosate resistance in weeds (Powles & Yu, 2010).

Mechanisms of glyphosate resistance in weeds have been shown to be both target site and non-target site. In target site based resistance, either a modification of the site of action (EPSPS enzyme) or enzyme over-expression/gene amplification endows resistance to the weed species (Powles & Yu, 2010; Sammons & Gaines, 2014). The first case of target site based resistance (enzyme modification) for glyphosate was reported in *Eleusine indica* (Baerson *et al.*, 2002; Lee & Ngim, 2000). In this case, a modification resulted from a single nucleotide change (a serine substitution at Pro-106) to cause resistance (Baerson *et al.*, 2002; Lee & Ngim, 2000).

In another resistant biotype of *Eleusine indica*, another nucleotide substitution (Pro-106 to threonine) was noted to endow resistance to glyphosate (Ng *et al.*, 2003).

Subsequently, a target site based resistance mechanism to glyphosate has also been reported for other weed species such as *Lolium rigidum* from three different parts of the world: Australia (Bostamam *et al.*, 2012; Wakelin & Preston, 2006b), South Africa (Yu *et al.*, 2007) and USA (Simarmata & Penner, 2008), and another species, *Lolium multiflorum* from USA (Jasieniuk *et al.*, 2008) and Chile (Perez-Jones *et al.*, 2007). Nucleic acid substitution at Pro-106 endows a modest degree of glyphosate resistance to weed species (Powles & Yu, 2010).

A double amino acid substitution in the EPSPS gene was recently reported in *Eluesine indica* (Yu *et al.*, 2015). In this double amino acid substitution which is known as TIPS, modifications resulted from two nucleotide changes at positions Thr-102-Ile plus Pro-106-Ser (TIPS) (Yu *et al.*, 2015). The TIPS has been described in the first generation of Roundup Ready maize (Sammons & Gaines, 2014). However, this is the first example of the TIPS conferring glyphosate resistance to a weed species. In contrast to the single mutation Pro-106, the TIPS confers an extremely high level of glyphosate resistance (over 180-fold based on R/S LD_{50} ratio) (Yu *et al.*, 2015).

Recently, another mechanism of resistance was reported which conferred glyphosate resistance to *Amaranthus palmeri* (Gaines *et al.*, 2010). Studies of the mechanism of glyphosate resistance in one population of *Amaranthus palmeri* showed similar herbicide absorption and translocation between resistant and susceptible biotypes (Culpepper *et al.*, 2006). Furthermore, EPSPS from both resistant and susceptible biotypes was inhibited equally by glyphosate (Gaines *et al.*, 2010). However, the resistant biotype was 6- to 8-fold more resistant to glyphosate compared to the susceptible biotype (Culpepper *et al.*, 2006). Molecular investigations showed that resistant biotype of *Amaranthus palmeri* had a higher genomic copy number of the EPSPS gene which was positively correlated with EPSPS expression (Gaines *et al.*, 2010). Therefore, the authors concluded that gene amplification (over-expression) was the mechanism of glyphosate resistance for this biotype (Gaines *et al.*, 2010). This over-expression has also been documented for glyphosate-resistance within *Amaranthus tuberculatus*, *Lolium multiflorum*, *Kochia scoparia* and *Amaranthus spinosus* (Sammons & Gaines, 2014).

Restricted herbicide translocation is another mechanism of glyphosate resistance (non-target site) in glyphosate-resistant weed biotypes. In fact, non-target site based glyphosate resistance is more common among weeds species than target site

mechanisms (Powles & Yu, 2010). In a study of the cause of restricted glyphosate translocation using ^{31}P nuclear magnetic resonance (NMR), glyphosate was found to enter the cytoplasm of both glyphosate-resistant and susceptible *Conyza canadensis* at the same rate, but a large amount of glyphosate was sequestered in vacuoles shortly after spraying with glyphosate while this rapid sequestration of glyphosate was not observed in susceptible plants (Ge *et al.*, 2010). Also, there was a positive correlation between the levels of resistance to glyphosate and the extent of vacuole sequestration of glyphosate in resistant *Lolium multiflorum* from Brazil and Chile, and *Lolium rigidum* from Australian and Italy (Ge *et al.*, 2012). It has been suggested that the transporters associated with vacuolar membranes like ATP-binding cassette (ABC) transporters might have roles in the vacuolar sequestration of glyphosate (Yuan *et al.*, 2007).

Glyphosate non-target site based resistance has been reported in *Lolium rigidum* (Adu-Yeboah *et al.*, 2014; Wakelin *et al.*, 2004), *Conyza canadensis* (Feng *et al.*, 2004), *Lolium multiflorum* (Michitte *et al.*, 2007) and *Sorghum halepense* (Riar *et al.*, 2011b). However, it was reported that some weed species such as *Lolium rigidum* accumulated both target site and non-target site mechanism of glyphosate resistance and as a result, the studied populations of *Lolium rigidum* was more resistant to glyphosate compared to those *Lolium rigidum* populations in which resistance was only conferred by one mechanism of resistance (Bostamam *et al.*, 2012; Yu *et al.*, 2007).

A less known mechanism of resistance has also been observed for *Ambrosia trifida* in which the mature treated leaves of the glyphosate-resistant biotype show rapid necrosis 12 hours after glyphosate application (Sammons & Gaines, 2014). Further studies showed that glyphosate translocation was substantially restricted possibly due to this rapid leaf necrosis (Sammons & Gaines, 2014). The molecular and physiological basis of this mechanism has not been completely elucidated.

The inheritance of glyphosate-resistance has been investigated in a number of biotypes. Depending on the type of glyphosate resistance mechanism, varied modes of inheritance have been documented. It was reported that a target site mechanism of resistance was inherited as a single nuclear gene with partial dominance in an Australian *Lolium rigidum* population (Preston *et al.*, 2009b) and a population of *Eleusine indica* from Malaysia (Ng *et al.*, 2004). The mode of inheritance of glyphosate resistance in one population of *Lolium rigidum* with restricted herbicide translocation was reported to be due to a single incompletely dominant nuclear gene (Lorraine-Colwill *et al.*, 2001). This

was also reported for *Conyza canadensis* (Zelaya *et al.*, 2004) and *Lolium multiflorum* (Vargas *et al.*, 2007). Wakelin and Preston (2006a) reported that glyphosate resistance in four populations of *Lolium rigidum* was inherited as a single dominant gene. Okada and Jasieniuk (2014) reported that the glyphosate resistance in *Conyza bonariensis* was controlled by two different genes. In *Amaranthus palmeri* with the EPSPS gene amplification mechanism of resistance, the inheritance involves multiple nuclear genes (Mohseni-Moghadam *et al.*, 2013).

2.5 Conclusion

Herbicides have been the most common way to control weeds in agricultural systems in recent decades. Herbicide resistance is the evolutionary response of weed populations to continuous usage of herbicides with the same modes of action. In this chapter, several relevant issues regarding herbicide resistance, specifically resistance to auxinic herbicides and glyphosate, were reviewed. The number of herbicide-resistant weeds is increasing in New Zealand. The remainder of this thesis explores further aspects of resistance to two important herbicides in New Zealand. The focus is initially on dicamba-resistance in fathen, then moves on to glyphosate-resistance in ryegrasses as almost nothing was known about this for New Zealand when the research was initiated.

Chapter 3

Testing fathen populations for herbicide resistance[1]

3.1 Introduction

The evolution of herbicide resistance in fathen in New Zealand was reviewed in Section 2.4.3.1. However, information regarding the level of dicamba resistance in fathen populations in New Zealand was lacking. Determining the level of resistance to herbicides provides preliminary information regarding the nature of mechanisms of resistance (Burgos *et al.*, 2013). In a field trial conducted in Waikato, it was noted that fathen populations were able to survive a dicamba rate eight times higher than the recommended rate (Rahman *et al.*, 2008), but no dose-response curves had been developed to quantify the level of resistance more precisely. The conventional approach for determining the level of resistance is to conduct a dose-response trial in which plants are grown in pots then sprayed with several application rates (Streibig, 1988). In these sprayed potted plants experiments, a wide range of herbicide rates is often used by researchers in order to provide a good understanding of the level of resistance to a herbicide (Burgos *et al.*, 2013).

As mentioned in Section 2.4.3.1, in New Zealand, fathen initially developed resistance to atrazine herbicides, then dicamba was used to control atrazine-resistant fathen in maize crops (Rahman *et al.*, 1983). Although it had been shown that some fathen populations have developed resistance to dicamba (James *et al.*, 2005), it had not been shown whether the dicamba resistant plants were also atrazine-resistant. Therefore, in this chapter, the level of resistance to dicamba in populations of fathen which were collected from Waikato maize fields was investigated by comparing their dose response curves with those of standard susceptible populations. Also, each population was tested for resistance to atrazine herbicides.

[1] Some of the material in this chapter has been published in:

Ghanizadeh H, Harrington KC, James TK and Woolley DJ (2015) A quick test using seeds for detecting dicamba resistance in fathen (*Chenopodium album*). Australian Journal of Crop Science. 9, 337-343.

3.2 Materials and Methods

3.2.1 Seed material

In this study, seed materials were collected from fathen populations growing either in Waikato maize fields with long histories of herbicides use (Populations A, B, L and M) or from Palmerston North waste areas thought not to have been sprayed with herbicides in recent years (Populations C, P and Y). Plants of each of these seven populations were grown from seeds in a heated glasshouse. To establish each plant, planter bags (700 ml) were filled with potting mix (50% bark: 30% fibre: 20% Pacific Pumice (7mm)) and slow-release fertiliser (Woodace, Lebanon, PA), then five seeds were sown per bag and these were placed in a heated glasshouse (the average temperature was 18.2°C) with automated capillary irrigation on 20 December 2013 for the first experiment and 10 April 2014 for the second experiment. Over 80% of the seeds germinated after 2 weeks for all populations, and they were thinned to one plant per pot when plants were at the 3-4 leaf stage.

3.2.2 Investigating the susceptibility of populations to atrazine using floating leaf disc assay

The susceptibility of each population to atrazine was investigated using a method developed by Hensley (1981). Preliminary experiments showed that over 80% of the leaf discs of atrazine-resistant plants floated after 1 hour exposure to 1×10^{-4} M of concentration of atrazine while almost no leaf discs from susceptible plants were floating after 1 hour. In the first experiment, when plants were 9-10 cm tall, 21 out of the 35 plants available from each population were chosen randomly and 60 leaf discs were obtained from excised leaves (3-4 fully developed leaves) from each plant using a 2-mm diameter cork borer. For each of these plants, 30 leaf discs were treated with herbicide by placing them in three separate beakers (10 leaf discs per beaker) each containing 20 ml of phosphate medium (10 mM potassium phosphate buffer (pH 6.5), 0.02% sodium bicarbonate, 0.1% (v/v) Tween 20). Before adding atrazine (4 ml of Gesaprim 500 FW) to each beaker, leaf discs were vacuum infiltrated with the phosphate medium. The remaining 30 leaf discs were put in three other beakers (10 leaf discs per beaker) containing 20 ml of phosphate medium, after they had been vacuum infiltrated, to provide the untreated control (no Gesaprim was added). The beakers were exposed to light (110 μmol $m^{-2}s^{-1}$) supplied by four 40-W fluorescent lamps within a growth cabinet at 24±1°C. The number of floating leaf discs was determined after 1

hour. According to Hensley (1981) the reason that leaf discs float is due to oxygen production within the leaf as a result of photosynthesis. The oxygen increases the buoyancy of leaf discs and therefore the sunken leaf discs float to the surface of the solution. However, the photosynthesis inhibitory effect of atrazine herbicides stops oxygen production thus leaf discs remain sunk if plants are susceptible. The same method was used for the second experiment, however the test was conducted on every single plant of each population (n = 35).

3.2.3 Dose-response experiments using dicamba

Preliminary experiments showed that Populations A and B from Waikato maize fields and Populations C, P and Y from Palmerston North were susceptible to dicamba, and that Populations L and M from Waikato maize fields were resistant to dicamba. When the plants used for the first floating leaf disc assay experiment mentioned above were 13-14 cm tall, several rates of dicamba (Kamba 500, as dimethylamine salt) were applied on 25 January 2014 using a laboratory track sprayer calibrated to deliver 227 L ha^{-1} of spray solution at 200 kPa. The susceptible populations (A, B, C, P and Y) received 0, 50, 100, 200, 400, 800 and 1600 g ae ha^{-1} of dicamba. The resistant populations (L and M) were treated with 0, 400, 800, 1600, 3200, 6400 and 12800 g ae ha^{-1} of dicamba.

The experiment was conducted as a randomised complete block design with five replicates of each rate. The average temperature in the 2 weeks after spraying was 20.1°C. Measuring the effect of different rates of herbicides on biomass production of treated plants relative to untreated plants is a common and accepted technique for evaluating herbicide resistance and estimating the level of resistance (Burgos *et al.*, 2013; Knezevic *et al.*, 2007). Thus, all above-ground plant tissue was harvested 7 weeks after spraying, oven dried for 48 hours and weighed. This experiment was then repeated, with seeds were sown on 10 April 2014, grown in the glasshouse (the average temperature was 16.4°C) and sprayed on 16 May 2014 (plants were 15-16 cm tall) after they had been examined for atrazine sensitivity in the second floating leaf disc assay. The average temperature in the 2 weeks after spraying was 17.5°C. In the second dose-response experiment, daylight was supplemented using two 500 W hydrogen gas lamps to maintain a 14 h day length to try to stop the plants from flowering immediately.

3.2.4 Statistical analysis

The data from the two sprayed potted plant experiments were not pooled but analysed separately. These data were checked for normality (Shapiro-Wilk test), independence of residuals (Durbin-Watson test), and homogeneity of variance (Levene`s test) in order to ensure that basic assumptions for regression models were satisfied (Burgos *et al.*, 2013; Onofri *et al.*, 2010). A three-parameter logistic model was fitted to the data from the sprayed potted plant studies using the following equation:

$$Y = \frac{d}{1+\exp(b\times(\log(x)-\log(GR_{50})))}$$

where Y was plant biomass as a percentage of control, d was the upper limit, x was herbicide rate, GR_{50} was the rate of herbicide corresponding to 50% reduction in plant biomass and b was the slope around the GR_{50}. The data were fitted to this model using the statistical software R (Version 2.15.2) with its dose-response curve (*drc*) package (Knezevic *et al.*, 2007). A one-way ANOVA was performed to compare parameters estimated from the sprayed potted studies for the fathen populations using Graphpad Prism v.5 and means were separated using Tukey's tests at 5% probability (Ademola & Eloff, 2011).

3.3 Results

The results of the leaf disc assay showed that Populations A, B, L and M were atrazine-resistant while Populations C, P and Y were susceptible to atrazine as over 95% of the leaf discs for Populations A, B, L and M were floating after being exposed to 1×10-4 M of atrazine for 1 hour (Table 3.1). In contrast, none of the leaf discs were floating for Populations C, P and Y after 1 hour of exposure, signifying that the atrazine was effectively inhibiting photosynthesis in these leaves. These results confirmed that all populations from Waikato maize fields (A, B, L and M) were resistant to atrazine (Table 3.1).

Table 3.1 The percentage of leaf discs that were floating for seven fathen populations after 1 hour exposure to 1×10^{-4} M of atrazine.

Populations	Location	First assay(n* = 21)	Second assay (n = 35)
A	Waikato	96.5	95.9
B	Waikato	98.0	96.9
C	Palmerston North	0	0
L	Waikato	97.3	95.9
M	Waikato	96.9	96.7
P	Palmerston North	0	0
Y	Palmerston North	0	0

*n = number of tested plants.

When the seven fathen populations were sprayed with different rates of dicamba, a general trend of reduction in shoot dry weight was recorded for every population with increasing dicamba rates. The three-parameter log-logistic model provided a good fit of the data as indicated by coefficient of determination (R^2) values greater than 0.95 (Table 3.2). Only Populations L and M were found to be resistant to dicamba (Figures 3.1a and b) as different responses were observed between the susceptible populations (A, B, C, P and Y) and the dicamba-resistant populations (L and M) to dicamba rates (Figures 3.1a and b).

The plants of susceptible populations were all dead at the dicamba rate of 1600 g ae ha^{-1}, whereas all plants from Population L were completely controlled only at a dicamba rate of 12,800 g ae ha^{-1} and plants of Population M survived even 12,800 g ae ha^{-1}, though they were severely damaged at this rate. The shoot dry weight of plants from all populations except Populations L and M was reduced by 50% (compared with untreated plants) at a dicamba application rate of approximately 800 g ae ha^{-1}, whereas much higher rates were required to cause a 50% reduction in shoot dry weight for Populations L and M (Figures 3.1a and b). Interestingly, it was noted that the GR_{50} in Populations A and B was slightly lower than for Populations C, P and Y, though this difference was not significant at a 5% level of probability (Table 3.2).

Table 3.2 Parameters estimated for the nonlinear regression analysis of the dicamba dose–response experiment for seven fathen populations (A, B, L and M from Waikato maize fields and C, P and Y from Palmerston North) in the two sprayed potted plant experiments at 7 weeks after application of dicamba.

Population	d	b	GR_{50*} ($g\ ha^{-1}$)	R/S (A) GR_{50}	R/S (B) GR_{50}	R/S (C) GR_{50}	R/S (P) GR_{50}	R/S (Y) GR_{50}	R^2
First sprayed potted study									
A	96.4	1.3	579 **c**	-	-	-	-	-	0.96
B	96.6	2.1	648 **c**	-	-	-	-	-	0.97
C	93.5	3.1	882 **c**	-	-	-	-	-	0.96
L	96.7	1.4	10081 **b**	17.4	15.6	11.4	12.2	11.3	0.97
M	95.8	3.0	11595 **a**	20.0	17.9	13.1	14.1	13.0	0.99
P	95.2	2.5	822 **c**	-	-	-	-	-	0.97
Y	92.8	4.1	891 **c**	-	-	-	-	-	0.97
P values	0.18	0.1	<0.001						
Second sprayed potted study									
A	101.	1.1	520 **c**	-	-	-	-	-	0.99
B	98.5	0.9	481**c**	-	-	-	-	-	0.98
C	102.	1.1	766 **c**	-	-	-	-	-	0.95
L	100.	1.1	5385 **b**	10.4	11.2	7.0	4.9	7.8	0.98
M	96.7	1.1	7766 **a**	14.9	16.1	10.1	7.1	10.9	0.96
P	100.	1.1	1092 **c**	-	-	-	-	-	0.99
Y	101.	1.3	711 **c**	-	-	-	-	-	0.97
P values	0.99	0.8	<0.001						

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide ($g\ ae\ ha^{-1}$) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within each column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

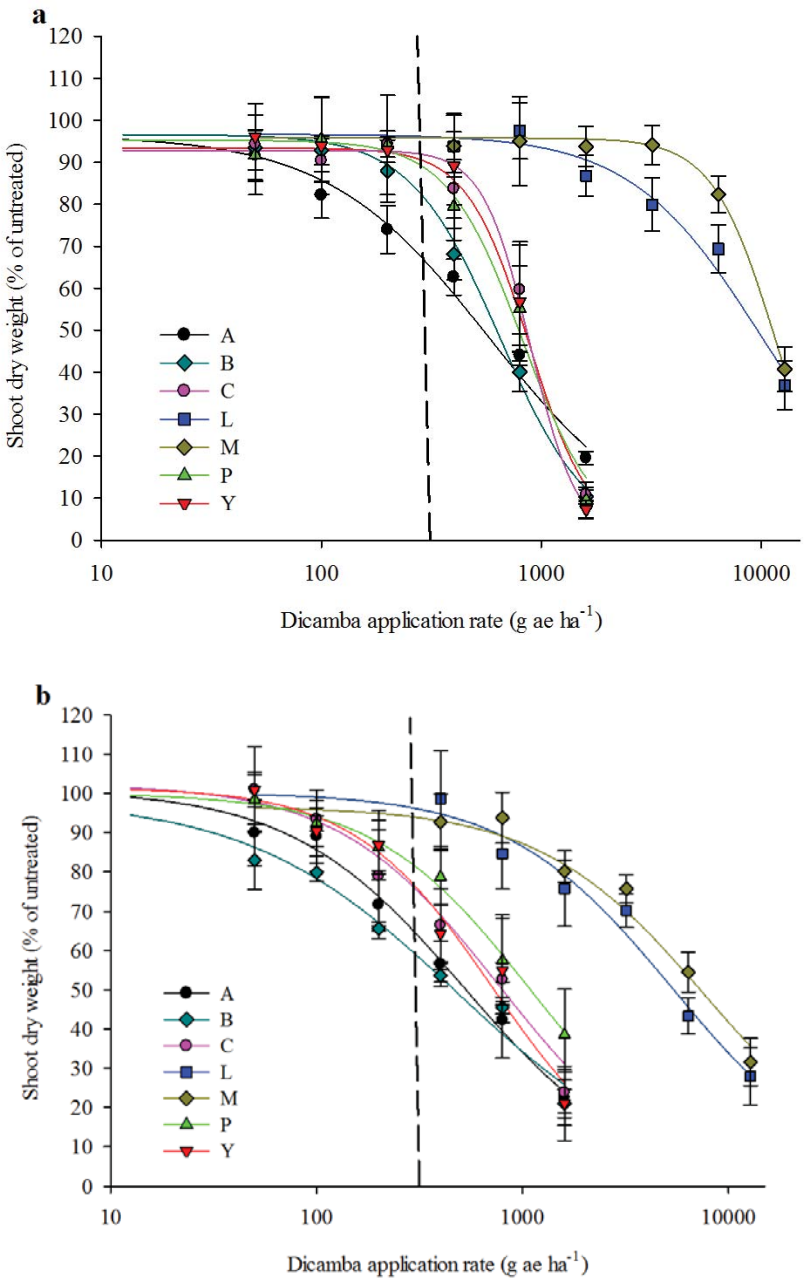

Figure 3.1 Fitted dose response curves (on a logarithmic dose scale) for seven populations of fathen populations (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) for dicamba, expressed as reduction in shoot dry weight, for (**a**) the first and (**b**) second sprayed potted plant experiments at 7 weeks after application of dicamba. Vertical bars represent ± standard error of the mean. The vertical dashed line indicates recommended field rate (300 g ae ha^{-1}) (Young, 2012).

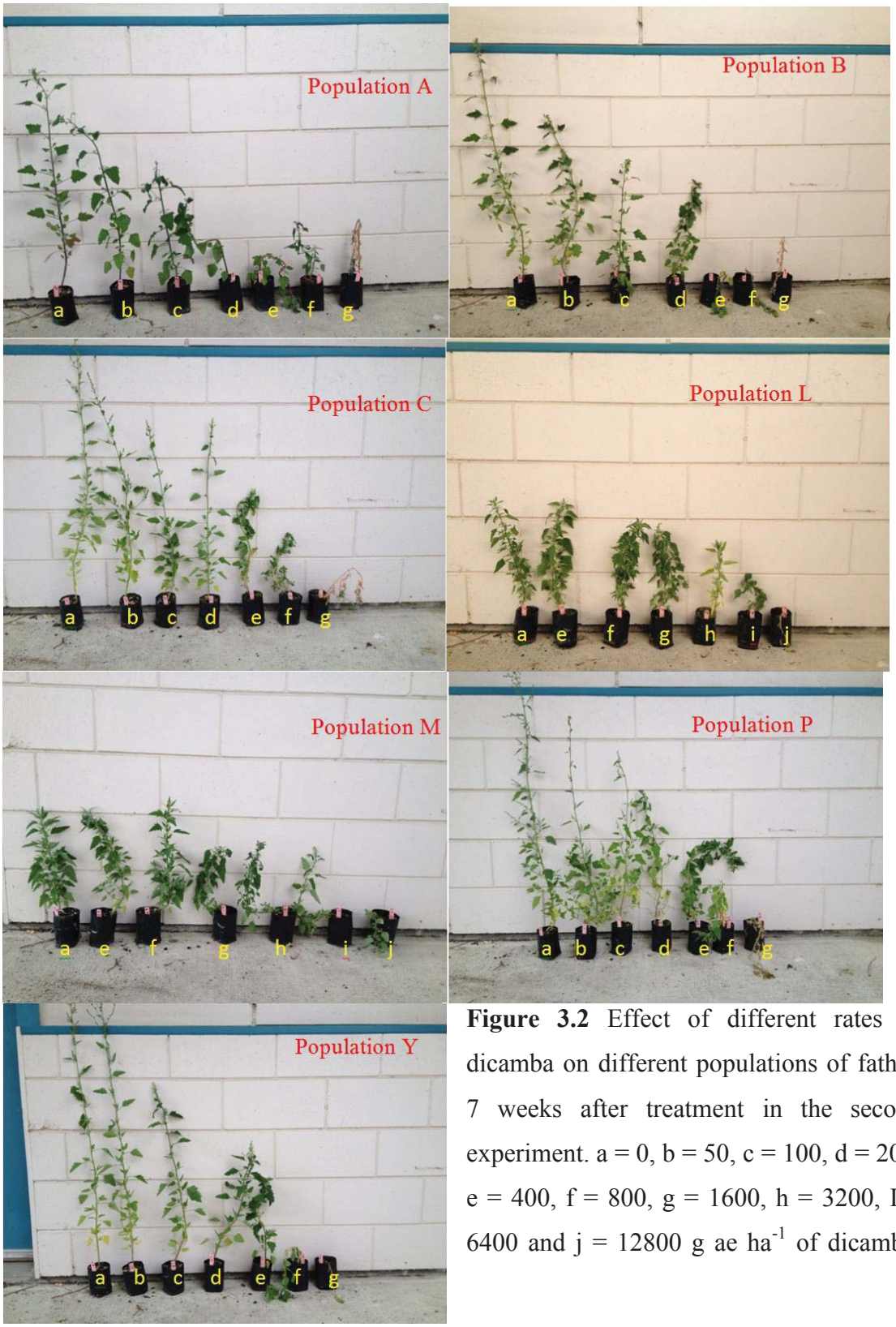

Figure 3.2 Effect of different rates of dicamba on different populations of fathen 7 weeks after treatment in the second experiment. a = 0, b = 50, c = 100, d = 200, e = 400, f = 800, g = 1600, h = 3200, I = 6400 and j = 12800 g ae ha^{-1} of dicamba.

When the level of dicamba resistance was calculated using the ratio of GR_{50} values of Populations L and M against those for the susceptible populations (A, B, C, P and Y), the level of resistance to dicamba in Population L in average ranged from 11- to 17-fold and the level of resistance in Population M ranged from 13- to 20-fold in the first sprayed potted plants study (Table 3.2). In the second sprayed potted plants study, the level of resistance to dicamba in Population L in average ranged from 5- to 11-fold and the level of resistance in Population M ranged from 7- to 16-fold (Table 3.2). However, the resistance level of dicamba-resistant populations was higher when the resistant/susceptible (R/S) GR_{50} ratios were assessed against atrazine-resistant Populations A and B. For instance, R/S GR_{50} ratios against Population A were 17.4 and 20.0 for dicamba-resistant Populations L and M, respectively, in the first sprayed potted plant experiment, whereas the levels of resistance to dicamba for Populations L and M were 11.4 and 13.1 times respectively, when R/S ratios were assessed against atrazine-susceptible Population C.

In this study, differential morphological characteristics were noted for dicamba-resistant fathen populations compared to susceptible ones. Dicamba-resistant fathen plants had different leaf shapes and colour from susceptible plants (Figure 3.3). The leaves of dicamba-susceptible plants were more dentate and had a darker green colour than dicamba-resistant plants (Figure 3.3). These morphological features were consistently observed for all the plants of Populations L and M in both studies. However, no apparent morphological differences were noted for other populations and the plants of Populations A and B (atrazine-resistant) looked similar to the plants of the atrazine-susceptible populations (C, P and Y).

Figure 3.3 Leaf shape of (a) dicamba-susceptible and (b) dicamba-resistant fathen.

3.4 Discussion

The results of this study confirmed the findings of previous published work regarding the presence of dicamba resistance within fathen populations on Waikato maize farms (James *et al.*, 2005). However, only two out of four fathen populations collected from the Waikato maize farms were found to be resistant to dicamba (Populations L and M). Other cases of dicamba resistance that have been reported elsewhere in the world are for *Kochia scoparia* in the USA (Cranston *et al.*, 2001) and *Sinapis arvensis* in Canada (Webb & Hall, 1995). The level of resistance to dicamba in Populations L and M estimated in this work based on reduction in plant biomass was between 5- and 20-fold. Preston *et al*. (2009a) reported a resistance level up to 30 times for dicamba-resistant *Kochia scoparia* lines compared to susceptible ones.

The results of this study also showed that atrazine-resistant fathen plants appear to be more susceptible to dicamba application than atrazine-susceptible ones as atrazine-resistant populations had slightly lower GR_{50} values than those of atrazine-susceptible ones in both sprayed potted plant studies. At a dicamba rate of 400 g ae ha^{-1}, which was above the recommended rate for controlling fathen in maize of 300 g ae ha^{-1} of dicamba (Young, 2012), the plants of Populations A and B (atrazine-resistant) were more damaged than those of Populations C, P and Y (atrazine-susceptible) (Figure 3.2). However, the plants of all dicamba-susceptible populations (both atrazine-resistant and atrazine-susceptible) were not completely dead at 7 weeks after application of dicamba. Recommended field rates are possibly more applicable to field conditions where weeds encounter biotic and abiotic stress whereas, in glasshouse conditions, optimum conditions are provided for plants and also, no intra- or inter-specific competition occurs among plants. Another possible explanation for the lack of complete control for susceptible populations at the recommended field rate at 7 weeks after dicamba application is that the duration of experiment was not long enough to allow plants that were severely damage to completely die.

The different sensitivity between Populations A and B (atrazine-resistant but dicamba-susceptible) and Populations C, P and Y (atrazine and dicamba-susceptible) to dicamba could be due to genetic variations between populations (Jasieniuk *et al.*, 1996). Variations in sensitivity to herbicides among different populations of weeds including fathen have been reported (Kohler *et al.*, 2004; Warwick & Marriage, 1982). Another possible explanation for the slight differences in GR_{50} values between atrazine-resistant

populations and atrazine-susceptible ones could be "negative cross-resistance" to dicamba in atrazine-resistant plants (Parks *et al.*, 1996). In evolutionary genetics, it is postulated that adaptation of a biotype to a new environment would often result in 'costs of adaption' for that biotype (Purrington, 2000). The 'costs of adaption' could influence the fitness of the biotype in the original environment (fitness cost) (Tian *et al.*, 2003). Many studies on atrazine-resistant weeds have suggested that there is a fitness cost as a result of the most common mutation conferring atrazine resistance (Ser-264 to Gly mutation of the *psb*A gene) which causes a reduction in CO_2 fixation, quantum yield, and seed and biomass production (Parks *et al.*, 1996). Fitness cost in atrazine-resistant plants might also influence the response of the plants to other herbicides and compared to atrazine-susceptible plants, atrazine-resistant plants might be more susceptible to other herbicides ("negative cross-resistance") (Parks *et al.*, 1996). It is suspected that almost all New Zealand maize fields are infected with atrazine-resistant fathen (Rahman *et al.*, 2013). Mechanisms of atrazine resistance in fathen populations from New Zealand have not been investigated. However, a high level of resistance to atrazine herbicides reported previously (up to 30-fold) (Rahman, 1990) suggests that the Ser-264 mutation of the *psb*A gene might have been evolved in fathen populations from New Zealand (Boger & Sandmann, 1989).

The "negative cross-resistance" has been well-documented in herbicide-resistant species including atrazine-resistant fathen. Park *et al*. (1996) also reported that *Chenopodium album* biotypes resistant to atrazine herbicides were up to 79% more susceptible to bentazon, bromoxynil, dicamba and pyridate than susceptible biotypes in the USA. The "negative cross-resistance" has also been reported for non-atrazine herbicide-resistant weeds. In other atrazine-resistant weed species, *Echinochloa crus-galli* and *Conyza canadensis*, it has been reported that biotypes resistant to atrazine herbicides were more sensitive to photosystem-II inhibitors such as bentazon and pyridate and some non-atrazine herbicides like glyphosate (Gadamski *et al.*, 2000). Poston *et al*. (2001) noted more sensitivity to cloransulam (an ALS-inhibitor herbicide) in an imidazolinone (another class of ALS-inhibitor herbicide) resistant population of *Amaranthus hybridus* compared to the susceptible population. Beckie *et al.* (2012) found that the individuals of one accession of ALS-resistant *Kochia scoparia* with a mutation at position Trp-574 were more sensitive to pyrasulfotole, mesotrione and carfentrazone than susceptible ones.

Although dicamba does not directly interfere with the electron transportation in photosystem II (Grossmann, 2000), the impact of dicamba on growth regulation could influence the growth rate of the atrazine-resistant populations that suffer from fitness costs. Thus having a atrazine-resistant population as a dicamba-susceptible standard population is likely to result in an over-estimate of the level of resistance to dicamba. This was shown in the current study where greater levels of resistance were estimated for dicamba-resistant Populations L and M when the atrazine-resistant Populations A and B were used as standard dicamba-susceptible populations.

Some unique morphological characteristics were recorded for dicamba-resistant populations in the current study. Rahman *et al.* (2014) had previously also noted some morphological differences between dicamba-resistant and susceptible fathen. They reported that dicamba-resistant fathen was shorter, produced less biomass compared to susceptible plants and also had less dentate leaves. They also confirmed that taxonomically, both resistant and susceptible populations were *Chenopodium album* (Rahman *et al.*, 2014). In our study we noted similar characteristics for our dicamba-resistant fathen populations (Populations L and M). As shown in Figure 3.3, dicamba-resistant fathen Populations L and M had different leaf shapes and colour than dicamba-susceptible populations. Different morphological characteristics have also been reported for *Sinapis arvensis* resistant to 2,4-D, dicamba, picloram and MCPA (Hall & Romano, 1995). Further studies would be interesting to investigate the genetic linkage between dicamba resistance and these morphological differences.

3.5 Conclusions

The results of both sprayed potted plant experiments confirmed the appearance of dicamba-resistance in two populations of fathen collected from separate maize fields in Waikato. The magnitude of resistance to dicamba for both populations was up to 20 times, although the magnitude of resistance was higher when the level of resistance in dicamba-resistant populations was compared against atrazine-resistant populations. Further investigations are probably needed in order to determine whether this greater sensitivity to dicamba in atrazine-resistant populations was due to the "negative cross-resistance" or it was simply due to genetic variations among populations, though the former explanation appears most likely.

The morphological features which were observed for dicamba-resistant populations need further investigations using more dicamba-resistant populations in order to confirm a correlation between these morphological features and dicamba resistance, and the genetic linkage between these characteristics and dicamba resistance. As a result of having two confirmed dicamba-resistant populations from this chapter, it was now possible to develop a quick test using seeds (Chapter 4) to distinguish dicamba-resistant populations from susceptible ones. Any difference in sensitivity to dicamba found in quick tests could be easily correlated back to the dose-response studies in this chapter.

Chapter 4

A quick test using seeds for detecting dicamba resistance in fathen (*Chenopodium album*)[1]

4.1 Introduction

To manage cases of herbicide resistance, it is useful to have an easy way of determining which weeds are resistant (Koger *et al.*, 2005). Screening for herbicide-resistant weeds usually either involves greenhouse studies in which plants are grown in pots then sprayed (sprayed potted plant experiments), or else laboratory assays, often known as quick tests (Beckie *et al.*, 2000). Developing quick tests for detecting herbicide-resistant weeds has an important role in managing herbicide resistance as these methods tend to be less expensive, quicker and not space-limiting compared with the sprayed potted plant experiments (Koger *et al.*, 2005). Herbicide resistance detection quick test techniques should also be easy to undertake and accurate (Moss, 1995).

The quick tests developed for screening samples collected from putative herbicide-resistant biotypes have included petri dish assays (Cirujeda *et al.*, 2001), chlorophyll fluorescence (Norsworthy *et al.*, 2008), leaf disc flotation (Hensley, 1981), pollen germination (Richter & Powles, 1993) and whole plant studies (Boutsalis, 2001). However, collecting seeds from a suspected resistant biotype, raising seedlings and comparing their responses to herbicide treatment is the most widely used method to detect herbicide-resistant biotypes as alternative techniques are often complicated and require expensive equipment. The objective of this work was to determine if dicamba-resistance in fathen could be reliably detected by germinating seeds within herbicide solutions.

[1]Some of the material in this chapter has been published in:

Ghanizadeh H, Harrington KC, James TK and Woolley DJ (2015) A quick test using seeds for detecting dicamba resistance in fathen (*Chenopodium album*). Australian Journal of Crop Science. 9, 337-343.

4.2 Developing a seed-based quick tests for detecting dicamba-resistant fathen

4.2.1 Developing a technique for breaking seed dormancy in fathen

4.2.1.1 Materials and Methods

Fathen seeds were collected in the autumn of 2011 from Palmerston North (Population C), dried and kept in cool room at 5°C till the beginning of this trial. As dormancy of the seeds initially hindered development of the seed-based quick test, an experiment was conducted using different treatments (Table 4.1) in order to develop a technique to overcome fathen seed dormancy. The perianth of all fathen fruits was removed in all treatments. Fifty seeds were placed on two layers of Steel Blue Seed Germination Blotters (Anchor Paper, Minnesota, USA) (hereinafter referred to as blotters) per each treatment. The blotters were put in plastic containers and sealed with plastic lids after receiving one of the treatments described in Table 4.1. These were then placed in a germinator under constant light at 25°C. Light was provided by four 40 W fluorescent white tubes giving a photon flux density of 30 μmol m^{-2} s^{-1}. The germination process was monitored weekly and germinated seeds were recorded and removed. The blotters were moistened with water when necessary. One untreated control (water) was included in this trial. The trial was conducted in a completely randomised design with four replicates per treatment. Data were subjected to an analysis of variance and means were separated using Fisher`s protected tests at 5% probability using SAS (SAS Institute Inc., Cary, NC, USA) (Version 9.3) software.

Table 4.1 The dormancy breaking techniques that were assessed on Population C from Palmerston North.

Treatment number	Description
Treatment 1	KNO_3[#] (0.02% v/v) + GA_3[*] (500 ppm)
Treatment 2	KNO_3 (0.02% v/v) + pre-chilling at 5°C for 7 days
Treatment 3	KNO_3 (0.02%)
Treatment 4	GA_3 (500 ppm)
Treatment 5	Pre-chilling at 5°C for 7 days
Treatment 6	Pre-chilling at 5°C for 14 days
Treatment 7	Untreated control

#Potassium nitrate, * Gibberellic acid.

4.2.1.2 Results

The effects of different treatments on fathen final germination are shown in Figure 4.1. Fathen seed germination was significantly improved to reach 88% using the KNO_3 (0.02%) + GA_3 (500ppm) treatment (Treatment 1) (Figure 4.1). Application of either

KNO_3 (Treatment 3) or GA_3 (Treatment 4) alone was not as effective as the combination of these. However, the application of KNO_3 when used with a period of 7 days pre-chilling at 5°C also increased seed germination significantly (Treatment 2) (Figure 4.1). In contrast, pre-chilling the fathen seeds for 7 and 14 days in the absence of KNO_3 (Treatments 5 and 6) did not improve germination, with the number of germinated seeds for these treatments significantly less than for other treatments and was not significantly different from untreated control treatment (Treatment 7) (Figure 4.1). Therefore, Treatment 2 (KNO_3 (0.02%) + pre-chilling at 5°C for 7 days) was selected for breaking fathen seed dormancy in later experiments as this treatment was not significantly different from the KNO_3 + GA_3 treatment and was less expensive.

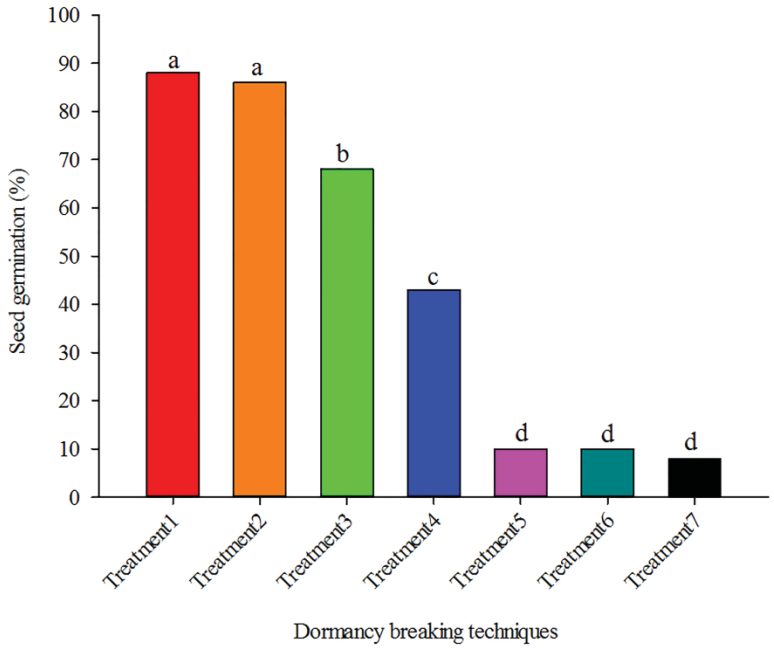

Figure 4.1 Effect of different dormancy breaking techniques on fathen Population C (from Palmerston North) seed germination. Mean values with the same letters were not significantly different at 5% probability according to Fisher`s protected tests.

4.2.2 The protocol for a seed-based assay for detecting dicamba resistance

4.2.2.1 Materials and Methods

In this study, the same seed materials and populations (A, B, C, L, M, P and Y) as those in the Chapter 3 were used to develop a quick test using seeds. Seeds were put on blotters saturated with KNO_3 (0.02% v/v) after the perianth had been removed, with 20

seeds used per replicate of each treatment. The blotters were placed in petri dishes which were kept for 7 days at 5°C in the dark to overcome seed dormancy. In Experiment 1, after this 7 day dormancy breaking treatment, all populations were treated with solutions of dicamba (Kamba 500, as dimethylamine salt) at concentrations of 0.02, 0.04, 0.08, 0.16 and 0.32 mg ae L^{-1}. Once herbicide had been added, the lids of petri dishes were sealed with cling wrap in order to decrease evaporation losses before the dishes were put into the growth chamber used in Section 4.2.1.1. An untreated control was included for each population and each treatment had three petri dishes of 20 seeds. After 14 days, the average radicle length and hypocotyl length of germinated seeds was determined using a scale printed on to an acetate sheet aided by a dissecting microscope and the difference in each length relative to those from the untreated control seedlings was determined, then the average decrease was calculated for each herbicide rate. This experiment was then repeated (Experiment 2).

In Experiment 3, the same procedure was used again, but this time a different set of herbicide concentrations was used for the two populations (L and M) which had been found to be resistant to dicamba (Chapter 3). The dicamba-susceptible populations (A, B, C, P and Y) were treated with the same concentrations used in Experiments 1 and 2, whereas the dicamba-resistant Populations L and M were treated with 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12 and 10.24 mg ae L^{-1} of dicamba. After 14 days, the hypocotyl length of germinated seedlings was measured using the same method as in Experiments 1 and 2, then this experiment was repeated (Experiment 4).

4.2.2.2 Statistical analysis

For each pair of identical seed-based assays (Experiments 1&2 and Experiments 3&4), a two-way analysis of variance compared the results from each assay and no significant interaction was found between data for quick tests ($P>0.05$) (Table 4.2), so the data were pooled. Data from the dose-response experiments were checked for the basic assumptions for regression models as described in Section 3.2.4. A three-parameter logistic model was fitted to the pooled data and the parameters were compared statistically using the method described in Section 3.2.4.

Table 4.2 P values from two-way ANOVA of fathen populations (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) quick test results.

	Fathen populations						
	A	B	C	L	M	P	Y
	P values						
Experiments 1 & 2							
Treatments	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001
Experiments	0.746	0.446	0.529	0.576	0.979	0.639	0.246
Treatment*Experiments	0.828	0.822	0.865	0.823	0.958	0.912	0.946
Experiments 3 & 4							
Treatments	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Experiments	0.994	0.828	0.843	0.746	0.906	0.879	0.344
Treatment*Experiments	0.898	0.893	0.773	0.917	0.829	0.997	0.696

4.2.2.3 Results

In all four assays of fathen seed germination within dicamba solutions, the herbicide caused inhibition of root elongation, radical swelling and shorter hypocotyls when compared to the untreated control seedlings. As dicamba rate was increased, hypocotyls and radicles were shorter compared to their untreated controls for all populations (Figure 4.2a and b). The effect of dicamba on radicle length was far more severe than on hypocotyl length, and was still detectable at the lowest dicamba concentration used in this study. The seven populations consistently responded differently in all four assays, with much shorter radicles observed for seedlings of susceptible populations (A, B, C, P and Y) compared with Populations L and M (Figure 4.2a and b).

Results from Experiments 1 and 2 showed that a dicamba concentration of 0.16 mg ae L^{-1} gave the best differentiation between resistant and susceptible populations (Figure 4.2a and b). The radicle length and hypocotyl length relative to the untreated control for the five susceptible populations (Populations A, B, C, P and Y) were significantly less than those of Populations L and M (dicamba-resistant) at this herbicide concentration (Figure 4.2a and b). Radicle length and hypocotyl length for resistant populations were almost unaffected at 0.16 mg ae L^{-1} of dicamba compared to susceptible populations. The radicle and hypocotyl length of both Populations L and M reduced slightly when dicamba concentration was increased to 0.32 mg ae L^{-1}.

By increasing the concentrations applied to seeds from Populations L and M in Experiments 3 and 4, full dose response curves for all of the populations could be

produced based on reduction in hypocotyl length (Figure 4.4). The three-parameter logistic model provided a good fit to the data ($R^2 \geq 0.98$) (Table 4.3). The concentration of dicamba which caused 50% reduction in the length of hypocotyl (GR_{50}) differed between populations with the GR_{50} values for Populations L and M significantly higher than those of the other populations. The level of resistance to dicamba in Populations L and M when compared against the other populations was calculated based on relative GR_{50} values (Table 4.3). The apparent level of resistance to dicamba for Population L ranged from 22 to 31 times higher than the susceptible populations, with the variability due to differences in GR_{50} of the various susceptible populations. The average estimate was a 26-fold level of resistance for Population L, compared with a 55-fold level of resistance for Population M, with estimates of the level of resistance for Population M ranging from 47 to 65 times higher than the susceptible populations.

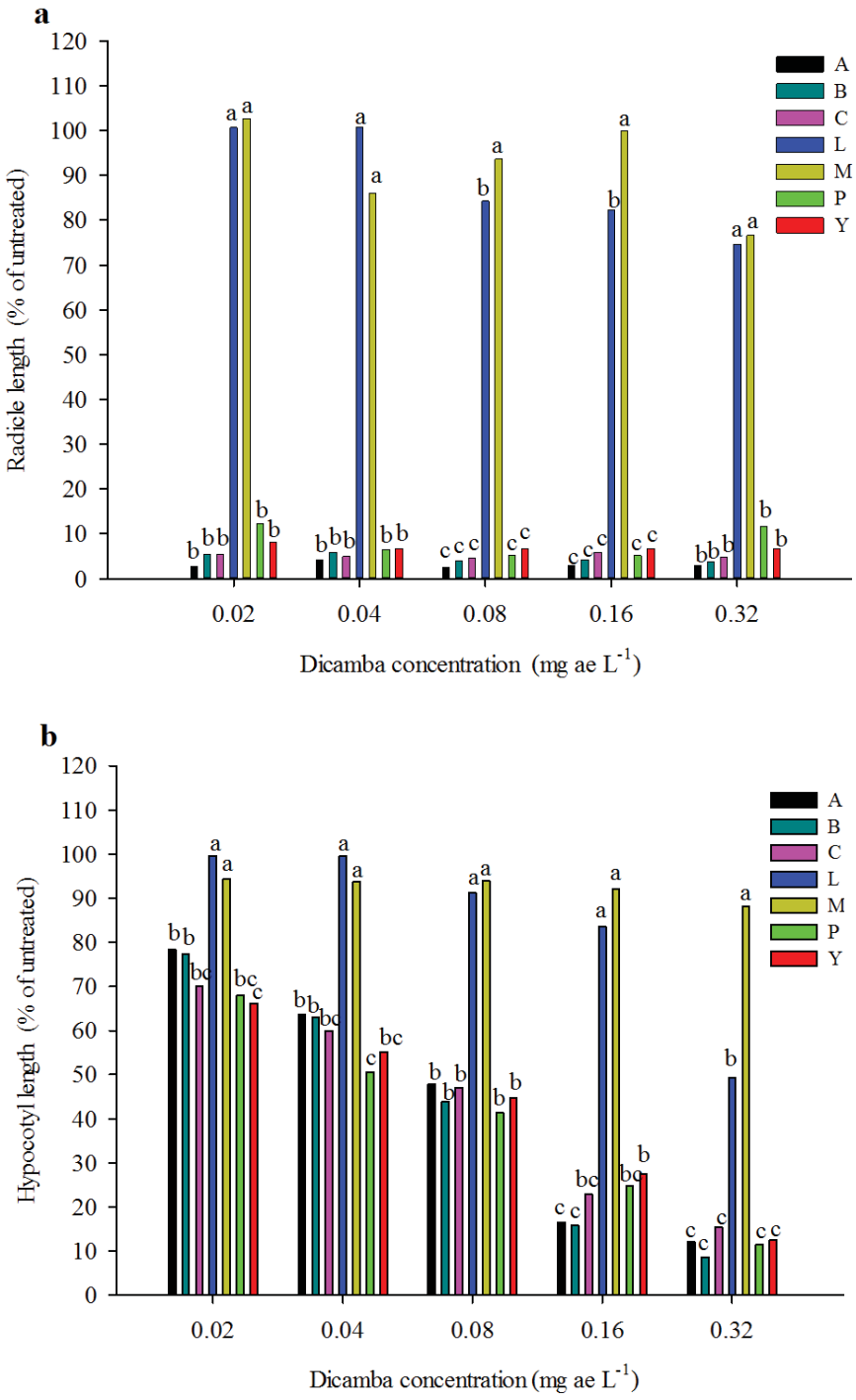

Figure 4.2 Effect of different dicamba concentrations on (a) radicle length and (b) hypocotyl length of germinating seedlings for seven populations of fathen (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) relative to untreated control in Experiments 1 and 2 (data pooled) measured 14 days after treatment. Mean values within each concentration of dicamba with the same letters were not significantly different at 5% probability according to Fisher`s protected tests.

Figure 4.3 Effect of the dicamba concentration of 0.16 mg L^{-1} on seedlings of (a) dicamba-susceptible fathen (Population C) and (b) dicamba-resistant fathen (Population L) 14 days after treatment.

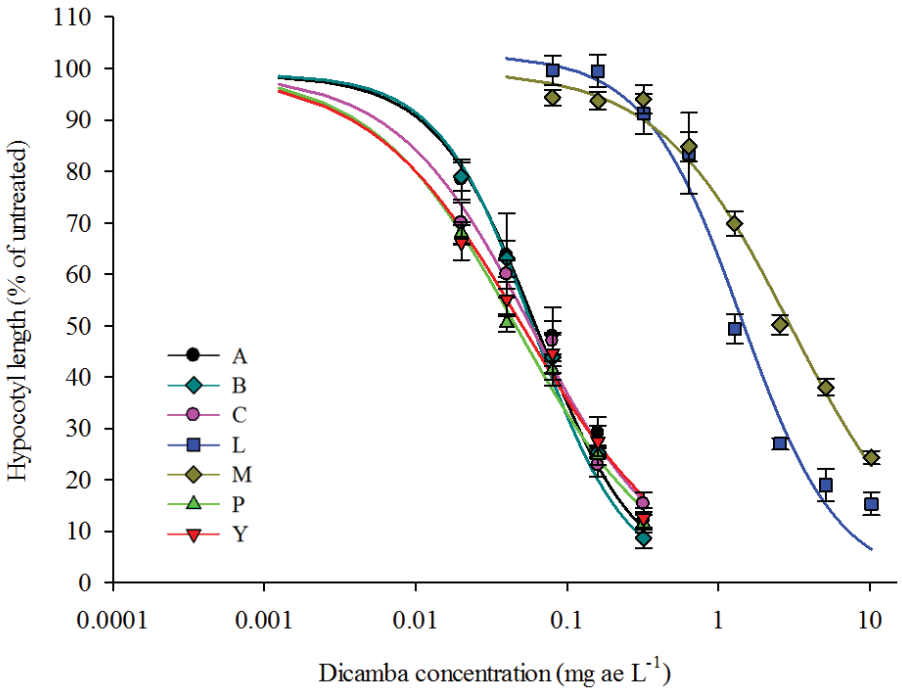

Figure 4.4 Fitted dose response curves (on a logarithmic dose scale) for seven populations of fathen (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) for effect of dicamba on the length of seedling hypocotyls in Experiments 3 and 4 (data pooled) measured 14 days after treatment. Vertical bars represent ± standard error of the mean.

Table 4.3 The parameters (see footnote) estimated for the nonlinear regression analysis of hypocotyl growth reduction caused by a range of dicamba concentrations on seedling germination of seven fathen populations (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) in Experiments 3 and 4 (data pooled). Each resistant population has been compared against each susceptible population for estimating level of resistance.

Population	d	b	GR_{50*} (mg L^{-1})	R/S (A) GR_{50} ratio	R/S (B) GR_{50} ratio	R/S (C) GR_{50} ratio	R/S (P) GR_{50} ratio	R/S (Y) GR_{50} ratio	R^2
A	98.8	1.3	0.063 **c**	-	-	-	-	-	0.98
B	98.9	1.4	0.059 **c**	-	-	-	-	-	0.99
C	99.2	1.0	0.058 **c**	-	-	-	-	-	0.99
L	102.7	1.3	1.415 **b**	22.4	23.9	24.4	30.7	27.7	0.98
M	99.7	1.0	3.004 **a**	47.7	50.8	51.7	65.2	58.8	0.99
P	99.8	0.9	0.046 **c**	-	-	-	-	-	0.99
Y	99.3	0.9	0.051 **c**	-	-	-	-	-	0.99
P value	0.99	0.50	<0.0001						

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the concentration of herbicide (mg ae L^{-1}) required to reduce hypocotyl length by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

4.3 Discussion

The petri dish experiments showed that the hypocotyls and radicles of fathen seedlings were reduced in length with exposure to increasing dicamba concentrations. Similar observations have been reported for the seedlings of *Sinapis arvensis* when treated with different solutions of various auxinic herbicides including dicamba (Wei *et al.*, 2000). However, the radicle of susceptible populations (A, B, C, P and Y) was particularly sensitive to dicamba compared with the hypocotyl, giving the impression that the resistant populations were many times more resistant than susceptible populations. Similarly, it has been reported that MCPA (an auxinic herbicide) caused a significant reduction in radicle length of the seedlings of *Brassica napus* (Polit *et al.*, 2014). As

measurements of hypocotyl length gave good indications of differences in susceptibility to dicamba between the populations tested, further experiments focused on just the hypocotyl rather than decreasing dicamba concentrations sufficiently to get dose response curves for comparing populations using radicle length.

The results of this work showed that the petri dish quick test could reliably detect dicamba resistance in fathen. So far, quick tests using seeds have mainly been used for detecting resistance to glyphosate (Perez & Kogan, 2003) and ACCase inhibitor herbicides (Tal *et al.*, 2000). The only assay found in the literature for resistance to auxinic herbicides that has used seeds involved *Papaver rhoeas* resistant to 2,4-D, in which use was made of different responses in the length of seedling shoots of resistant and susceptible populations when exposed to 2,4-D (Torra *et al.*, 2010). However, dose response curves were not calculated in that work to allow an estimate of the difference in resistance between populations.

Differences between dose response curves created using the seedling hypocotyl length suggested that Population L was about 26 times more resistant than susceptible populations to dicamba, and that population M was about 55 times more resistant. Yet with the results presented in Chapter 3, the levels of dicamba resistance for Populations L and M were far lower in the sprayed potted plant studies. During normal spraying of weeds in fields, seedlings are similar in size and growth form to what were used in the greenhouse dose-response tests, so presumably the level of resistance estimated in the greenhouse experiments is similar to what is found in the field (Burgos *et al.*, 2013).

So although the petri dish test could reliably detect which populations were resistant to dicamba, the magnitude of resistance to dicamba was over-estimated compared to sprayed potted plant tests (Chapter 3). Other workers who have also developed quick tests using seeds for detecting resistance to herbicides from different modes of action have also found that the quick tests tend to over-estimate the level of resistance. For example, Burke *et al.* (2006) reported that the level of resistance to clethodim and fluazifop in *Sorghum halepense* was over-estimated in their petri dish assays compared with greenhouse dose-response trials.

Despite over-estimating the magnitude of resistance, the petri dish assay remains a useful test for detecting resistance to dicamba within fathen populations as this test meets the features of a good quick test for screening herbicide-resistant weeds (Beckie

et al., 2000). By placing fathen seeds in petri dishes containing 0.16 mg ae L^{-1} of dicamba, presence or absence of resistance can be determined within 2 weeks. In contrast, the sprayed potted studies would take about four times longer and need sufficient space in a greenhouse for the plants to grow properly. The quick test needs only petri dishes and perhaps space in a seed germination cabinet, though it could probably be conducted on a window-sill. Therefore, this test enables easy testing of a large number of fathen populations to determine whether dicamba resistance has evolved. To our knowledge, this is the first use of an assay using seeds for detecting dicamba resistance. Further investigations are needed to study if this petri dish assay could be adapted for detecting other auxinic herbicide resistance, such as for the nodding thistle (*Carduus nutans*) (Harrington & Woolley, 2006) and giant buttercup (*Ranunculus acris*) populations that have developed resistance to MCPA in parts of New Zealand (Bourdôt *et al.*, 1990).

4.4 Conclusions

A quick test such as the seed-based test described in this chapter could be useful for monitoring for appearance of resistance. Currently, it is not known how widespread dicamba-resistant fathen is within New Zealand. Such information is useful for management of the problem and can be obtained in a short period using the petri dish assay developed in this study. By collecting seed from fathen plants within maize fields near harvest time, it would be possible to determine if they are present because they missed being sprayed by dicamba or whether resistance is developing. If resistance is detected, farmers could be warned to start using alternative herbicides to prevent the resistance getting worse.

Chapter 5

The response of *Lolium multiflorum* and *Lolium perenne* populations to glyphosate in dose-response experiments: confirmation of resistance and evaluation of resistance levels[1]

5.1 Introduction

Evolution of glyphosate resistance in several weed species from different parts of the world was reviewed in Section 2.4.5. In 2012, it was reported that glyphosate had failed to control an Italian ryegrass (*Lolium multiflorum*) population in a vineyard in Marlborough. Shortly after that, glyphosate was reported again to have failed to control Italian ryegrass or perennial ryegrass (*Lolium perenne*) on four other vineyards in Marlborough and Nelson. Although these two species are commonly grown as pasture species within New Zealand, they can also be troublesome weeds in vineyards. Glyphosate is the cheapest and most effective herbicide for controlling these and most other weed species within vineyards, so this has been applied continuously for years in many vineyards (Dastgheib & Frampton, 2000; Harrington, 2012).

As no glyphosate resistance had ever been recorded as evolving within New Zealand prior to these reports, the objective of this chapter was to determine whether resistance to glyphosate had evolved within these vineyards due to repeated use of glyphosate, and also to determine the magnitude of resistance if it was confirmed to exist.

5.2 Materials and methods

5.2.1 First experiment

Live plants (three to four plants) from each of the five populations suspected of being resistant were obtained in November 2012. All were obtained from vineyards that had been sprayed with glyphosate for at least 10 years, usually two to three times annually (Table 5.1), and now were no longer controlled by recommended rates of glyphosate.

[1] Some of the material in this chapter has been published in:

Ghanizadeh H, Harrington KC, James TK and Woolley DJ (2013) Confirmation of glyphosate resistance in two species of ryegrass from New Zealand vineyards. New Zealand Plant Protection 66, 89-93

Plants of perennial ryegrass (cv. Trojan) (Population SP) and Italian ryegrass (cv. Tabu) (Population SI) were also obtained from paddocks at Massey University in Manawatu for comparison as these plants were unlikely to have had a history of being sprayed with glyphosate.

Table 5.1 Summary of herbicide application history of Italian ryegrass and perennial ryegrass populations used in this work collected from five vineyards.

Population	Year of vineyard establishment	Glyphosate application history per year	Glufosinate application history per year	Amitrole application history per year
A (Italian ryegrass)	1982	3-4 times	Not available	Once since 2007
J (perennial ryegrass)	2001	2-3 times	Once	Once since 2003
N (perennial ryegrass)	1994	2-4 times	Once	Not applied
O (perennial ryegrass)	1991	2-3 times	Once	Once since 1991
P (Italian ryegrass)	2004	2-3 times	Once	Not applied

Information regarding the use of the infested areas prior to vineyard establishment is generally unclear due to changes in managers over time. Land used for the vineyards from where Populations O and P were collected had previously been in pasture grazed by sheep. Population J came from a property originally used for intensive arable production such as processed vegetables and small seed production, whereas Population N came from an area originally used as an apple orchard. The manager from where Population A was obtained was unclear about the history and was generally

uncooperative. Although details were often vague, glyphosate had been used extensively in all vineyards since the year of establishment. Glufosinate was mainly used near the time of fruit harvest in most years since the establishment of each vineyard (Table 5.1). Amitrole had been used less frequently (Table 5.1). Sheep grazing in winter had been used in all vineyards, as well as mowing of the vegetation between the crop rows during the growing season.

All plants were split into plantlets consisting of two to three tillers and each plantlet was established in separate polythene planter bags (700 ml) filled with potting mix (50% bark, 30% fibre, 20% Pacific Pumice (7mm) and slow-release fertilizer (Woodace, Lebanon, PA)), a similar technique to that used by Boutsalis (2001) to multiply up ryegrass plants for herbicide resistance testing in Australia. The plants were then left in an unheated glasshouse for 3 weeks (the daily maximum and minimum averaged 21.8°C and 12.6°C respectively) at Massey University to establish with automated overhead irrigation. When new leaves had emerged and plants were judged to be sufficiently robust to be sprayed (average of 4.7 tillers per plant), different rates of glyphosate (isopropylamine salt as Roundup 360 Pro) were applied using a laboratory track sprayer calibrated to deliver 230 litres ha^{-1} of spray solution at 200 kPa. Populations thought to be resistant were treated with 0, 180, 360, 720, 1440 and 2880 g ae ha^{-1} and those thought to be susceptible received 0, 22.5, 45, 90, 180 and 360 g ae ha^{-1}.

Plants were allocated to treatments within a randomised complete block design with 10 replicates (one pot as one replicate), and blocking was done based on plant vigour. All herbicide treatments contained 0.1% organosilicone surfactant (Pulse Penetrant). Spraying occurred on 16 December 2012, and the daily maximum and minimum temperatures in the 2 weeks following application averaged 26.8°C and 15.6°C respectively. Five weeks after treatment, the foliage of all plants was removed from pots at ground level, dried (at 70°C for 48 hours) and weighed. The effect of each herbicide treatment was calculated as a percentage of the dry weight of untreated plants for that population.

5.2.2 Second experiment

To confirm the results from the first experiment, the experiment was repeated, using the same techniques as described for the first experiment. Plants used for this experiment were taken from untreated plants that had been growing in pots within an unheated

glasshouse (the daily maximum and minimum temperatures averaged 25.8°C and 14.4°C respectively) from the original material obtained for Experiment 1, and were multiplied up using divided tillers prior to spraying. Treatments for this experiment were applied on 28 February 2013 when plants had an average of 5.0 tillers each. The only difference in treatments for the second experiment was that the highest rate of glyphosate applied to populations was increased to allow improved fitting of dose-response curves. Susceptible populations received rates of 0, 45, 90, 180, 360 and 720 g ae ha^{-1}; most of the resistant populations received rates of 0, 360, 720, 1440, 2880 and 5760 g ae ha^{-1}, while one of the perennial ryegrass populations (Population O) received rates of 0, 360, 720, 1440, 2880, 5760 and 11520 g ae ha^{-1} because in the first experiment it was not adequately controlled by any of the rates applied. All herbicide treatments contained 0.1% organosilicone surfactant (Pulse Penetrant). The daily maximum and minimum temperatures in the 2 weeks following application averaged 26.1°C and 12.2°C respectively.

5.2.3 Statistical analysis

The basic assumptions for regression models were checked using the method described in Section 3.2.4. Dose-response curves were fitted as described in Section 3.2.4.

5.3 Results

5.3.1 Italian ryegrass

The three-parameter logistic model provided a good fit to the data, indicated by coefficients of determination (R^2) values greater than 0.90 (Table 5.2). A consistent response was recorded for plants of each population within each replicate suggesting that plant materials provided from each vineyard were consistent in glyphosate resistance. Plants within each replicate had been randomised before spraying, therefore it was unlikely that all of the plants within each replicate were from the same mother plants. The herbicide rates calculated to cause a 50% reduction in growth (GR_{50}) for the Italian ryegrass populations in both experiments are shown in Table 5.2, and the dose-response curves for Experiments 1 and 2 are shown in Figure 5.1a and b.

The shoot growth of plants from the Manawatu population (Population SI) was significantly reduced by 360 g ae ha^{-1} of glyphosate and it was almost 22 and 35% of the untreated control in the Experiments 1 and 2, respectively (Figure 5.1a and b). However a 30% reduction in shoot growth for resistant Italian ryegrass populations (A

and P) required rates almost eight times higher (2880 g ae ha^{-1}) than for the susceptible population (Figure 5.1a and b). Thus the GR_{50} values for the two populations from Marlborough were significantly higher than for Population SI (Table 5.2). By comparing the GR_{50} of each Marlborough population against the GR_{50} of the Manawatu population for the first experiment, it was found that Population A was 13.4 times more resistant to glyphosate than Population SI, and Population P appeared to be 10.7 times more resistant. Results were very similar for Experiment 2. Although the recommended rate of glyphosate application is 450 g ae ha^{-1} (Young, 2012), at a glyphosate rate of 360 g ae ha^{-1}, the plants of susceptible population of Italian ryegrass (SI) were dead. However, for Populations A and P, plants were only completely controlled at glyphosate rates of 2880 g ae ha^{-1}.

Table 5.2 Parameters estimated for the nonlinear regression analysis of glyphosate sprayed potted plant experiments of three Italian ryegrass populations (A and P (glyphosate resistant), and SI (glyphosate susceptible)) at 5 weeks after application of glyphosate in both experiments.

Population	d	b*	GR_{50} (g ae ha^{-1})	R/S GR_{50} ratio	R^2
Experiment 1					
Manawatu SI	96.6	0.9 **b**	134 **b**	-	0.96
Marlborough A	95.8	1.1 **b**	1796 **a**	13.4	0.90
Marlborough P	99.6	1.2 **a**	1433 **a**	10.7	0.97
P value	0.19	0.009	<0.0001		
Experiment 2					
Manawatu SI	100.4	0.7 **b**	126 **b**	-	0.99
Marlborough A	99.2	0.8 **a**	1655 **a**	13.2	0.99
Marlborough P	99.3	0.8 **a**	1233 **a**	9.8	0.99
P value	0.58	<0.0001	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ae ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

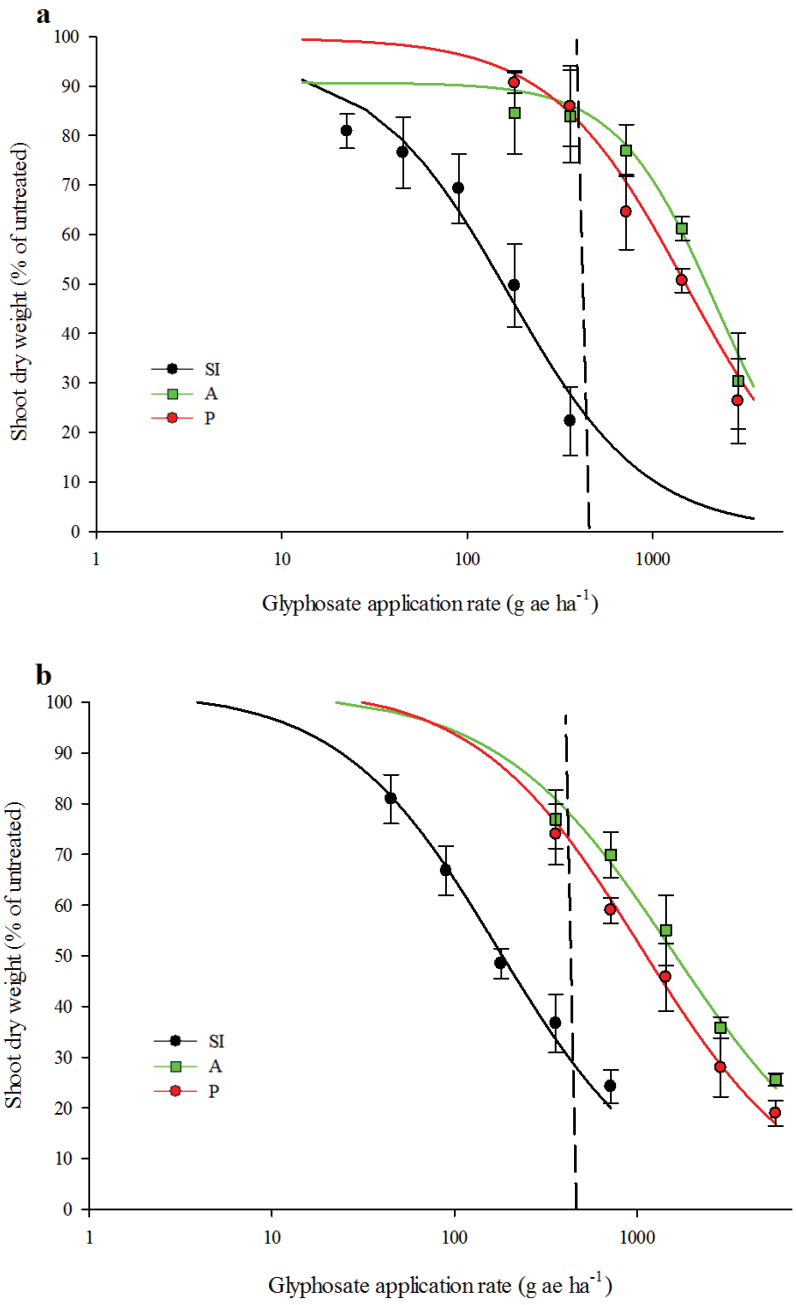

Figure 5.1 Fitted dose-response curves (on a logarithmic dose scale) for three populations of Italian ryegrass (SI from Manawatu pasture, A and P from Marlborough vineyards) following application of glyphosate to potted plants in (**a**) the first and (**b**) second experiments at 5 weeks after application of glyphosate. Vertical bars represent ± standard error of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

Figure 5.2 Effect of different rates of glyphosate on Italian ryegrass for (a) Population SI (glyphosate-susceptible) from Manawatu pasture and (b) Population A (glyphosate-resistant) from Marlborough in the second experiment at 5 weeks after application of glyphosate.

5.3.2 Perennial ryegrass

An assessment of goodness of fit and coefficients of determination revealed that the three-parameter logistic model described well the changes in plant dry weight at given glyphosate rates ($R^2 \geq 0.89$) (Table 5.3). The perennial ryegrass population from Manawatu (Population SP) had a very similar GR_{50} (Table 5.3) to the Italian ryegrass from Manawatu in Experiment 1, and once again the shoot growth of all plants was significantly reduced by 360 g ae ha^{-1} of glyphosate although the plants were not completely dead. Also, the response to glyphosate rates recorded for the plants within each replicate was similar to that for Italian ryegrass populations. However, as with the Italian ryegrass, the three populations from vineyards had much higher GR_{50} values. Population N from Nelson was estimated to be 14.5 times more resistant than Population SP, while one of the Marlborough populations (Population J) appeared to be 10.2 times more resistant (Table 5.3). The application rates used in the first experiment were not sufficiently high to allow a GR_{50} value to be estimated for Population O (Figure 5.3a), but as the population was less affected than Population N, the level of resistance was assumed to be greater than 15 times.

In the second experiment, the GR_{50} for each of the perennial ryegrass populations was slightly higher than the first experiment, although the ratios of resistant to susceptible GR_{50} values remained similar (Table 5.3). The higher rates used in the second experiment allowed a GR_{50} value to be calculated for Population O (Figure 5.3b), which was 30 times more resistant to glyphosate than Population SP. The plants of Population

SP were all dead at glyphosate rate of 360 g ae ha^{-1} whereas Populations J and N were not completely controlled until glyphosate rates reached 2880 g ae ha^{-1}. Plants of Population O were completely controlled at glyphosate rates of 11,520 g ae ha^{-1}.

Table 5.3 Parameters estimated for the nonlinear regression analysis of glyphosate dose-response experiments of four perennial ryegrass populations (J, N and O (glyphosate resistant), and SP (glyphosate susceptible)) at 5 weeks after application of glyphosate in both experiments.

Population	d	b*	GR_{50} (g ae ha^{-1})	R/S GR_{50} ratio	R^2
Experiment 1					
Manawatu SP	97.1	1.4 **a**	127 **b**	-	0.93
Marlborough J	99.2	0.8 **b**	1304 **a**	10.2	0.94
Nelson N	96.8	1.1 **ab**	1841 **a**	14.5	0.89
Marlborough O	100.6	0.9 **b**	> 2000#	> 15.0	0.97
P value	0.64	0.01	<0.0001		
Experiment 2					
Manawatu SP	101.0	0.1 **b**	161 **c**	-	0.99
Marlborough J	98.4	1.0 **a**	1533 **b**	9.5	0.98
Nelson N	99.3	0.1 **b**	2062 **b**	12.8	0.99
Marlborough O	100.7	0.1 **b**	4971 **a**	30.8	0.98
P value	0.24	<0.0001	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ae ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50}= resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent. # The analysis of the GR_{50} of Population O was not possible as the highest glyphosate rate used in the first experiment was lower than the rate needed to reduce the dry weight by 50%.

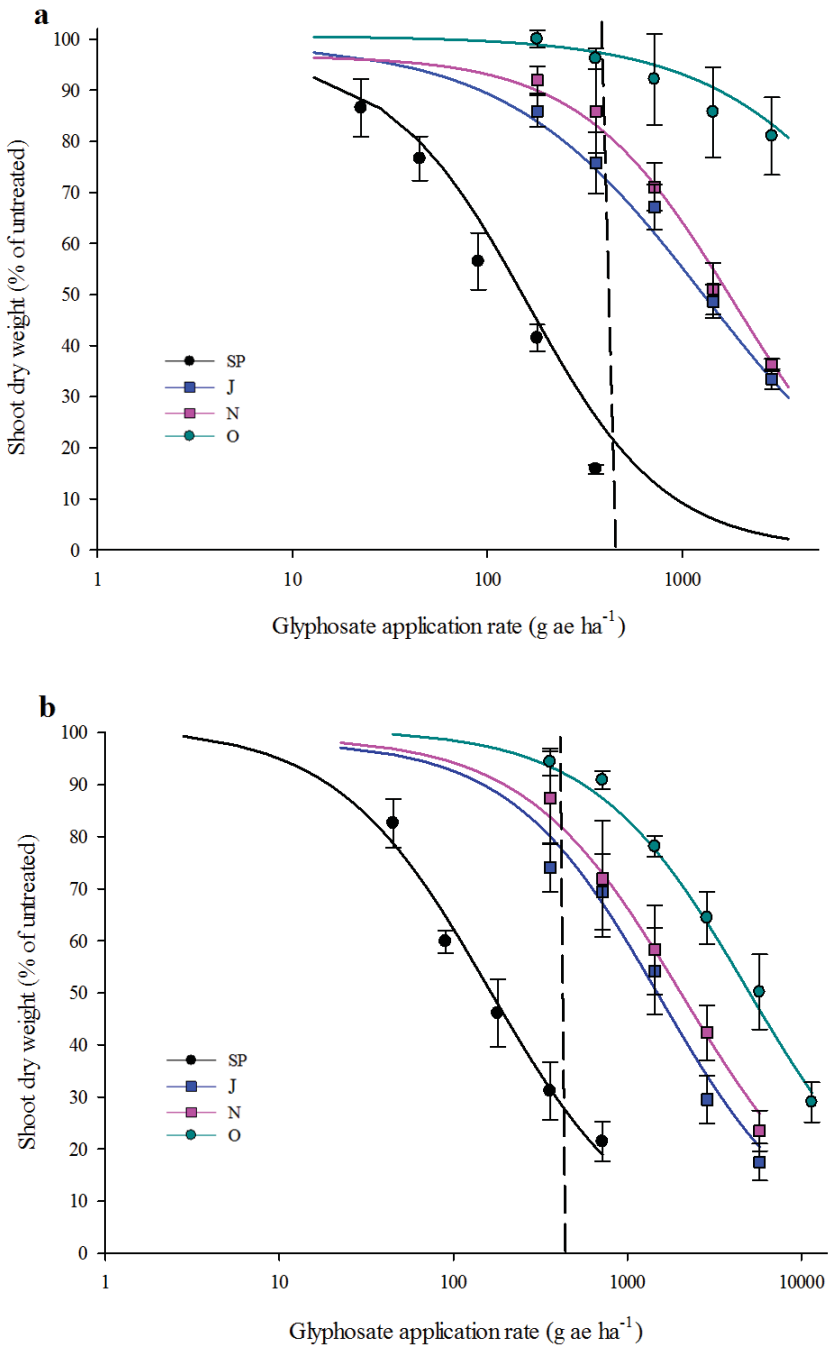

Figure 5.3 Fitted dose-response curves (on a logarithmic dose scale) for four populations of perennial ryegrass (SP from Manawatu pasture, N from Nelson vineyard, J and O from Marlborough vineyards) following application of glyphosate to potted plants in (**a**) the first and (**b**) second experiments at 5 weeks after application of glyphosate. Vertical bars represent ± standard error of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

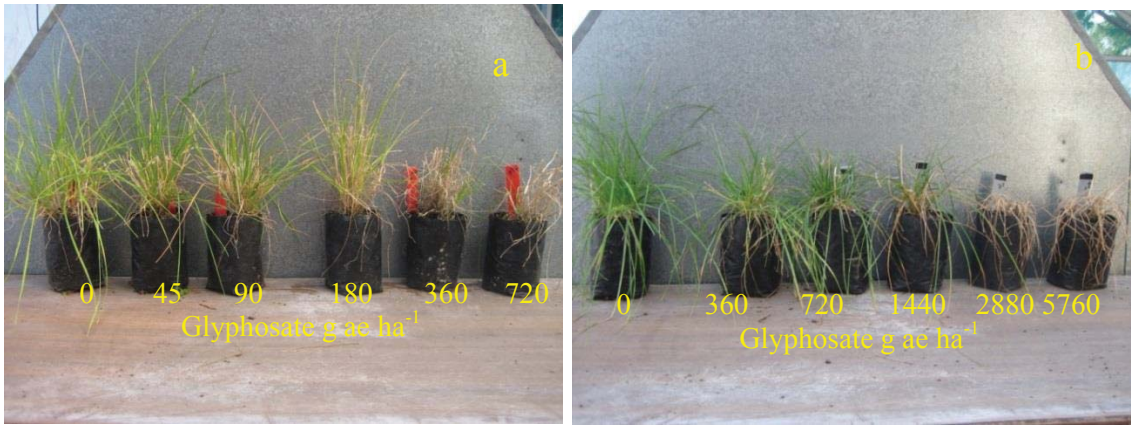

Figure 5.4 Effect of different rates of glyphosate on perennial ryegrass Population SP (glyphosate-susceptible) (a) from Manawatu pasture and Population J (glyphosate-resistant) (b) from Marlborough in the second experiment at 5 weeks after application of glyphosate.

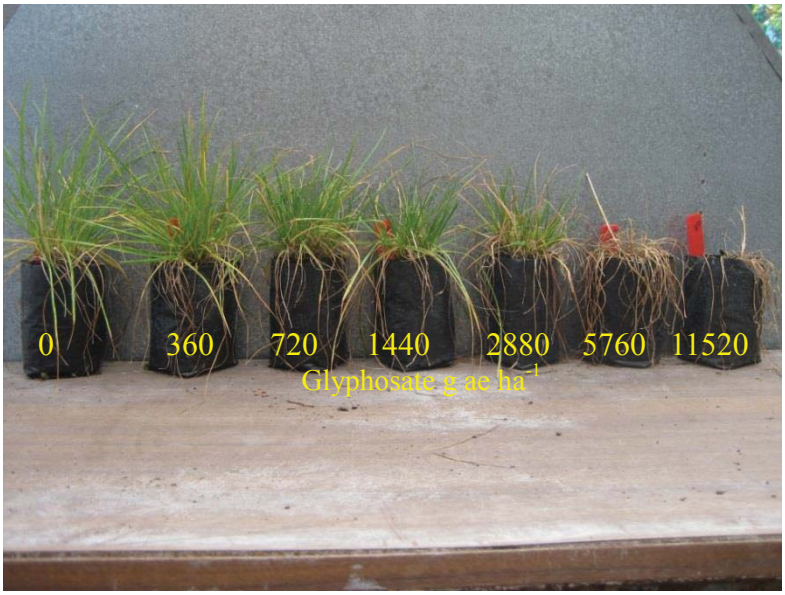

Figure 5.5 Effect of different rates of glyphosate on perennial ryegrass Population O (glyphosate-resistant) from Marlborough in the second experiment at 5 weeks after application of glyphosate.

5.4 Discussion

These results are consistent with the idea that glyphosate resistance has developed within New Zealand vineyards, for both perennial ryegrass and Italian ryegrass. These are the first known cases of glyphosate resistance developing within New Zealand. However, glyphosate resistance in both of these species has been recorded overseas

(Heap, 2015). Italian ryegrass has developed resistance to glyphosate which was used continuously for several years to control weeds in orchards, vineyards and crops (Jasieniuk *et al.*, 2008; Perez-Jones *et al.*, 2005; Perez & Kogan, 2003).

The first case of glyphosate-resistant perennial ryegrass in the world was reported from Argentina with an R/S LD_{50} ratio of 10.8, similar in magnitude to that found with most of the populations studied in this work (Yanniccari *et al.*, 2012). Many of the cases of resistance to glyphosate that have been reported worldwide have been for another member of the *Lolium* genus, i.e. *Lolium rigidum* (Heap, 2015).

Although four of the populations studied had approximately a 10-fold level of resistance, Population O had a significantly higher level of resistance, calculated as being 30-fold, suggesting that the mechanism of resistance in this population may differ from the other four. As glyphosate is one of the main herbicides used for weed control in New Zealand, these first occurrences of resistance suggest current practices may need to change if further resistance is to be avoided. A survey of herbicide practices in Marlborough vineyards in 1997 showed that almost all growers were using glyphosate at that time, though other herbicides were also used during the season, such as amitrole, glufosinate, paraquat and simazine (Dastgheib & Frampton, 2000). Since then, paraquat is no longer permitted to be used in vineyards within the sustainable winegrowing programme, residual herbicides are discouraged and amitrole can only be used in winter, so glyphosate is being increasingly relied on for weed control (Harrington, 2012). Improved weed management systems need to be developed both in vineyards and elsewhere in New Zealand to reduce the chance of more glyphosate resistance from developing.

Now that glyphosate resistance has been confirmed, and as glyphosate is such an important herbicide, it was decided to conduct further research as so little was known about these New Zealand cases of glyphosate resistance, such as the mechanisms of resistance. Some of these aspects were investigated for this PhD thesis and the results are presented and discussed in Chapters 6-11.

5.5 Conclusions

The results of both sprayed potted plant studies confirmed the appearance of the first cases of glyphosate-resistant weeds in New Zealand. Four populations had approximately 10-fold levels of resistance, and one of the perennial ryegrass

populations had a 30-fold resistance to glyphosate. The evolution of glyphosate-resistant Italian ryegrass and perennial ryegrass in New Zealand vineyards resulted from repeated application of glyphosate as the main herbicide for weed control.

Chapter 6

Quick tests for detecting glyphosate-resistant Italian ryegrass and perennial ryegrass[1]

6.1 Introduction

Various tests for screening collected samples from putative glyphosate-resistant biotypes have been developed overseas. One test involves seeds being germinated in petri dishes containing a range of glyphosate concentrations and measuring the length of root or shoot 7-8 days after treatment (petri dish assay) (Perez & Kogan, 2003). The shikimic acid assay is based on the inhibitory effect of glyphosate on EPSPS and the subsequent accumulation of shikimate in affected plant tissue (Bresnahan *et al.*, 2003). Shaner *et al.* (2005) described an assay in which the amount of shikimic acid accumulated in leaf discs from plants that had been incubated in different concentrations of glyphosate for 16 to 23 hours was measured with a spectrophotometer (shikimic acid assay). The assay has been used to differentiate between several susceptible and glyphosate-resistant weeds (Koger *et al.*, 2005; Nandula *et al.*, 2008; Nol *et al.*, 2012; Perez-Jones *et al.*, 2005). The leaf dip assay was developed by Koger *et al.* (2005) in which the excised leaves of *Conyza canadensis* biotypes resistant and susceptible to glyphosate were immersed in solutions with different concentrations of glyphosate for 48-72 hours and the percentage of leaf injury was then assessed visually.

Compared to traditional spraying tests (sprayed pot studies) for confirming glyphosate resistance, these alternative assays are quick with results being obtained within 1-8 days. As glyphosate resistance is now appearing in New Zealand, the objective of the work reported in this chapter was to assess the suitability of these quick tests for detecting glyphosate-resistant biotypes of Italian ryegrass and perennial ryegrass. A second objective was to ensure that these tests gave similar results for different formulations of glyphosate.

[1] Some of the material in this chapter has been published in:

Ghanizadeh H, Harrington KC, James TK and Woolley DJ (2015) Quick tests for detecting glyphosate-resistant Italian and perennial ryegrass. New Zealand Journal of Agricultural Research. 58, 108-120

6.2 Materials and methods

6.2.1 Seed-based assay

To obtain seeds from glyphosate-resistant populations, plants from the clones of each population assessed in the sprayed potted plant tests (Chapter 5) that survived 720 g ae ha^{-1} were grown together in a glasshouse with each population separated within pollen-proof cloth. Only seeds from glyphosate-resistant Populations A and J were assessed for the following assay as they were the only populations for which sufficient numbers of seeds were produced in time for the study. These seeds of Population A (Italian ryegrass) and Population J (perennial ryegrass) were then compared with commercially grown seeds of Italian ryegrass (cv. Jivet) (Population SJ) and perennial ryegrass (cv. Trojan) (Population SP), as they were unlikely to have had a previous history of exposure to glyphosate applications and had been shown in the preliminary experiments to be susceptible to glyphosate. The petri dish assay was conducted using 9 cm diameter petri dishes in which seeds from the four populations were exposed to different concentrations of glyphosate. For each petri dish, ten seeds of each population were placed onto three layers of filter paper (Whatman No. 1) then 5 ml of an appropriate glyphosate solution was applied. The glyphosate-resistant populations were treated with glyphosate concentrations of 0, 10, 20, 40, 80, 160 and 320 mg ae L^{-1} of glyphosate (Roundup Transorb, a potassium salt), whereas the susceptible populations were treated with 0, 1.25, 2.5, 5, 10, 20 and 40 mg ae L^{-1}. Each herbicide treatment was replicated three times for each of the four populations. The petri dishes were sealed using laboratory cling wrap to decrease evaporation loss then placed in a growth cabinet (30/20°C, 8 h light/16 h dark) (ISTA, 1996) with a light intensity of 40 µmol m^{-2} s^{-1} provided by two 40 W fluorescent white tubes. After 8 days the length of shoot and root was measured using a similar method to that described in Section 4.2.2.2. The petri dish assay was conducted twice.

6.2.2 Shikimic acid assay

The effect of glyphosate on shikimic acid accumulation in Italian ryegrass and perennial ryegrass was investigated using leaf segments following the method described by Perez-Jones *et al.* (2005). In this assay, a young rapidly expanding leaf (15-20 cm) of each tiller was excised from resistant and susceptible plants which were at the 5-7 tiller stage. The lowest 3-cm portion of the leaf was divided into three 1 cm leaf segments. The leaf segments were put in Eppendorf tubes containing 200 µl of different concentrations of

glyphosate. The herbicide concentrations were 0, 1.3, 2.6, 5.3, 10.6, 21.1, 42.3, 84.5, 169, 338 and 676 mg ae L^{-1} of glyphosate (Roundup Transorb, a potassium salt). Glyphosate solutions were diluted with 10 mM sodium phosphate (NaH_2PO_4) (pH 4.4) containing 0.1% v/v Tween surfactant. The Eppendorf tubes were kept for 16 hours in a growth cabinet at 25±1°C under continuous light (100 µmol m^{-2} s^{-1}) provided by four 40-W white fluorescent tubes. After that Eppendorf tubes were immersed in liquid nitrogen then thawed at 60°C for 20 minutes to disrupt the leaf tissue. Then each tube received 50 µl of 1.25 M HCl and samples were shaken for 5 minutes and incubated for 20 minutes at 60°C. Following centrifugation at 12000 g for 10 minutes, 25 µl aliquots of supernatant was transferred into Eppendorf tubes containing a 100 µl solution of 0.25% periodic acid and 0.25% metaperiodate. The samples were incubated at 37°C for 1 hour and then 100 µl aliquot of 0.6 M sodium hydroxide and 0.22 M sodium sulphite solution were added to each tube and mixed thoroughly. The optical density at 380 nm was measured using a spectrophotometer (Biochrom Libra S60PC, Cambridge, England). The shikimic acid experiment was conducted twice. The determination of the shikimic acid concentration was based on a shikimic standard curve. The standard curve was developed by dissolving 0.5, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5 mg of shikimic acid in 10 ml of HCl (1.25 M) and measuring the optical density at 380 nm (Figure 6.1).

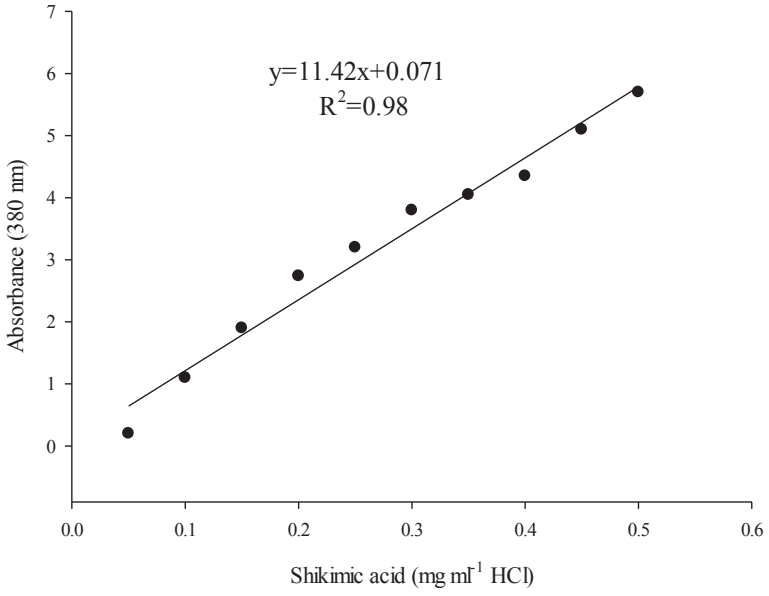

Figure 6.1 The shikimic acid standard curve, where x = shikimic acid (mg ml^{-1} HCl) and y = optical density at 380 nm.

6.2.3 Tiller dip assay

Koger *et al.* (2005) found that glyphosate resistance in *Conyza canadensis* could be detected using excised single leaves placed into solutions of glyphosate. However our preliminary work with this technique showed that using excised individual leaves of ryegrass was not satisfactory because untreated leaves would often begin deteriorating before symptoms of glyphosate phytotoxicity could express themselves in treated leaves. Kim *et al*. (2000) found that a quick test using plant parts of another grass species, *Echinochloa colona*, resistant to propanil and fenoxaprop, worked best using tillers with roots attached. Our testing with ryegrass tillers with roots attached showed they lasted much longer before deteriorating than single leaves placed in water. Therefore, an assay for glyphosate resistance in ryegrass was developed and tested using tillers with roots.

Two populations of Italian ryegrass (glyphosate-susceptible (SI) and glyphosate-resistant (A)), and two populations of perennial ryegrass (glyphosate-susceptible (SP) and glyphosate-resistant (J)) were compared. Seedlings of the four populations were germinated as described for the petri dish assay. On 2 May 2013, the germinated seeds were potted into separate polythene planter bags (700 ml) with potting mix and kept in a glasshouse in which the mean of daily maximum and minimum temperatures in the 8 weeks following planting were 23°C and 17°C, respectively. On 29 July 2013, plants had 10 to 15 tillers, and were divided into plantlets composed of three tillers plus some root material once potting material had been washed from the roots. Plantlets were then trimmed so that only rapidly expanding leaves remained and were allocated to treatments.

Plantlets were weighed before submerging their lower portions in 20 ml of glyphosate solution contained in a 25-ml plastic vial, one plantlet per vial. Treatments were 0, 10, 40, and 160 mg ae L^{-1} of glyphosate (Roundup Transorb, a potassium salt). Treatments were arranged in a completely randomised design with three replicates and the experiment was repeated. Vials were placed in a growth cabinet at 20$\pm$1°C temperature with a 12 hour photoperiod (100 µmol m^{-2} s^{-1}) provided by four 40-W white fluorescent tubes. Additional solution was added as needed to account for evaporation losses. Vials were removed from the growth chamber after 8 days and leaf injury was assessed visually using a scoring system based on severity of wilting and discoloration, with a scale from 0 (no visual injury) to 10 (severe necrosis). The fresh weight of plantlets was

also measured after 8 days and compared to their corresponding initial fresh weight (the weight before submerging the tillers into glyphosate solutions).

6.2.4 Investigating the efficacy of glyphosate as isopropylamine salt in all three quick tests

To determine whether each of the three quick tests assessed would give similar results if another formulation of glyphosate was used, all three tests were repeated using Roundup 360 Pro, an isopropylamine salt. In all assays, the investigation was done using one glyphosate-resistant perennial ryegrass (Population J) and one glyphosate-susceptible perennial ryegrass (Population SP). In the petri dish assay, the same technique as described above was used. In the shikimic acid assay, only four concentrations of glyphosate (0, 42.3, 84.5 and 169.1 mg ae L^{-1}) were used because earlier results showed that the leaf segments of resistant plants and susceptible plants accumulated different amounts of shikimic acid at these rates. The technique for the shikimic acid assay was similar to that described above though the test was done with both glyphosate formulations (isopropylamine salt and potassium salt). The tiller dip assay was conducted using the same method outlined above. All assays were conducted twice.

6.2.5 Statistical analysis

The basic assumptions of ANOVA/regression models for the data of each experiment were checked using a similar method to that described in Section 3.2.4. A two way ANOVA was conducted on the data of each experiment that was repeated, however no significant differences were observed between the results of any repeated experiment (Table 6.1), and therefore data were pooled. The seed based assay was conducted in a completely randomised design with three replicates of each concentration and each replicate consisted of 10 seeds. The dose response curves for seed-based assay were fitted as described in Section 3.2.4.

The shikimic acid assays were conducted in a completely randomised design with four replicates of each concentration and curves were fitted to a four parameters logistic model (Streibig, 1988):

$$Y = c + \left(\frac{d-c}{1+\exp\left(b\times(\log x - \log IC_{50})\right)}\right)$$

where *Y* was shikimic acid concentration (µg ml^{-1} HCl), *d* was the upper limit, *c* was the lower limit, *x* was the herbicide rate, IC_{50} was the concentration of herbicide required to inhibit enzyme activity by 50% and b was the slope around IC_{50}. Data were fitted to this model as described in Section 3.2.4. The ratio of IC_{50} values for resistant and susceptible populations was calculated.

The data of tiller dip assays were obtained from a completely randomised design with three replicates of each concentration and were subjected to an analysis of variance using SAS (SAS Institute Inc., Cary, NC, USA) (Version 9.3) software and means were separated using Fisher`s protected tests at the 5% level of probability. The data of shikimic acid assay using both glyphosate formulations (isopropylamine salt and potassium salt) were also analysed in SAS and mean separations within each concentration of glyphosate were tested using Student's t-tests. A one-way ANOVA was performed to compare parameters estimated from models used in each experiment using the method described in Section 3.2.4.

Table 6.1 P values from two-way ANOVA of results from petri dish assays, shikimic assays and tiller dip assays of Italian ryegrass populations (A (glyphosate-resistant), and SJ (glyphosate-susceptible)) and perennial ryegrass populations (J (glyphosate-resistant), and SP (glyphosate-susceptible)).

	Italian ryegrass and perennial ryegrass populations			
	SJ	A	SP	J
	P values			
Petri dish assays (shoot)				
Treatments	<0.001	<0.001	<0.001	<0.001
Experiments	0.689	0.150	0.163	0.079
Treatments*Experiments	0.084	0.223	0.207	0.964
Petri dish assays (root)				
Treatments	<0.001	<0.001	<0.001	<0.001
Experiments	0.619	0.497	0.927	0.308
Treatments*Experiments	0.674	0.450	0.569	0.939
Shikimic assays				
Treatments	<0.001	<0.001	<0.001	<0.001
Experiments	0.139	0.608	0.065	0.261
Treatments*Experiments	0.978	0.980	0.319	0.861
Tiller dip assays				
Treatments	<0.001	<0.001	<0.001	<0.001
Experiments	0.729	0.902	0.078	0.085
Treatments*Experiments	0.472	0.396	0.2126	0.156

6.3 Results

6.3.1 Petri dish assay

As the concentration of glyphosate increased for seedlings of glyphosate-resistant (A) and susceptible (SJ) Italian ryegrass in the petri dish assay, the shoot and root length of both populations decreased relative to the untreated control (Figures 6.2a and b). Root growth was more sensitive to glyphosate than shoot growth, and also the relative response to glyphosate in root and shoot growth between two populations was different. The glyphosate rate that reduced the shoot growth of the susceptible population (SJ) by 50% (GR_{50}) was 23.0 mg ae L^{-1} compared with 99.5 mg L^{-1} for the resistant population (A) (Table 6.2). However, root GR_{50} values for Populations SJ and A were 3.2 and 34 mg ae L^{-1} of glyphosate, respectively. Based on 50% reduction in shoot growth (shoot

GR_{50}), Population A was 4.3 times more resistant to glyphosate, yet based on root GR_{50}, the level of resistance for Population A, was 10.8 times higher than Population SJ.

Table 6.2 Parameters estimated for the nonlinear regression analysis of seed-based experiments describing shoot and root length of two Italian ryegrass populations, SJ (glyphosate-susceptible) and A (glyphosate-resistant), and two perennial ryegrass populations, SP (glyphosate-susceptible) and J (glyphosate-resistant).

Population	d	b	GR_{50}* (mg L^{-1})	R/S GR_{50} ratio	R^2
Shoot length					
SJ	95	2.3	23.0 **b**	-	0.99
A	101	1.8	99.5 **a**	4.3	0.95
P value	0.43	0.45	0.0025		
Root length					
SJ	102	1.2	3.2 **b**	-	0.97
A	102	1.6	34.0 **a**	10.8	0.99
P value	0.64	0.19	<0.0001		
Shoot length					
SP	97	0.9	46.2 **b**	-	0.97
J	103	1.5	101.1 **a**	2.2	0.97
P value	0.64	0.13	0.0078		
Root length					
SP	105	1.7	3.9 **b**	-	0.97
J	102	2.2	34.8 **a**	8.9	0.99
P value	0.72	0.30	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (mg ae L^{-1}) required to reduce root/shoot length by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio, R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

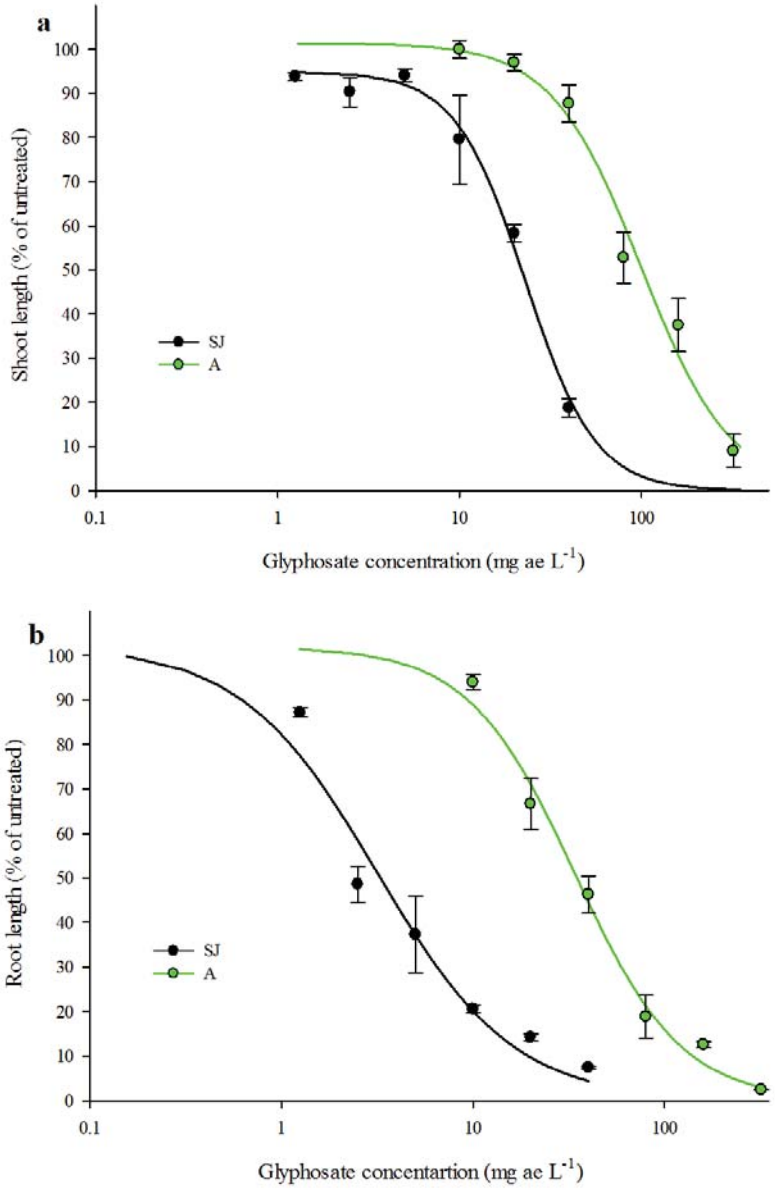

Figure 6.2 Fitted dose response curves (on a logarithmic dose scale) of the effect of increasing concentrations of glyphosate (potassium salt) on (a) seedling shoot and (b) root length for two populations, SJ (glyphosate-susceptible) and A (glyphosate-resistant), of Italian ryegrass. Data are the means of two experiments each with three replicates and ten seeds per replicate, measured 8 days after treatment. Vertical bars represent ± standard error of the means.

Similar results were obtained with perennial ryegrass (Figures 6.3a and b). Root growth for both populations was reduced by much lower concentrations of glyphosate than the concentrations that affected shoot growth. The glyphosate rate that reduced the shoot

growth of the susceptible population (SP) by 50% was 46.2 mg ae L^{-1}, compared with 101.1 mg ae L^{-1} for the resistant population (J) (Table 6.2).

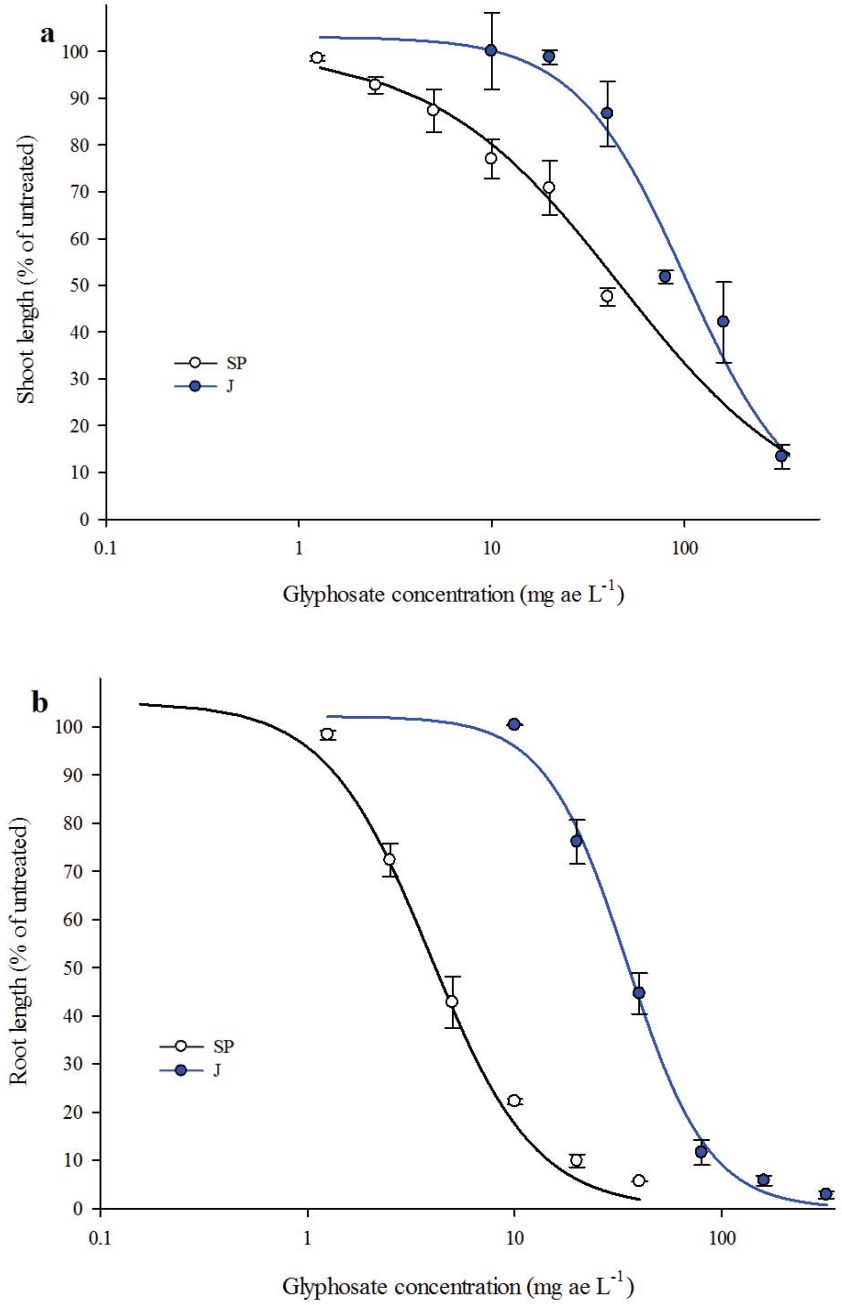

Figure 6.3 Fitted dose response curves (on a logarithmic dose scale) of the effect of increasing concentrations of glyphosate (potassium salt) on (a) seedling shoot and (b) root length for two populations, SP (glyphosate-susceptible) and J (glyphosate-resistant), of perennial ryegrass in the petri dish assay. Data are the means of two experiments each with three replicates and ten seeds per replicate, measured 8 days after treatment. Vertical bars represent ± standard error of the mean.

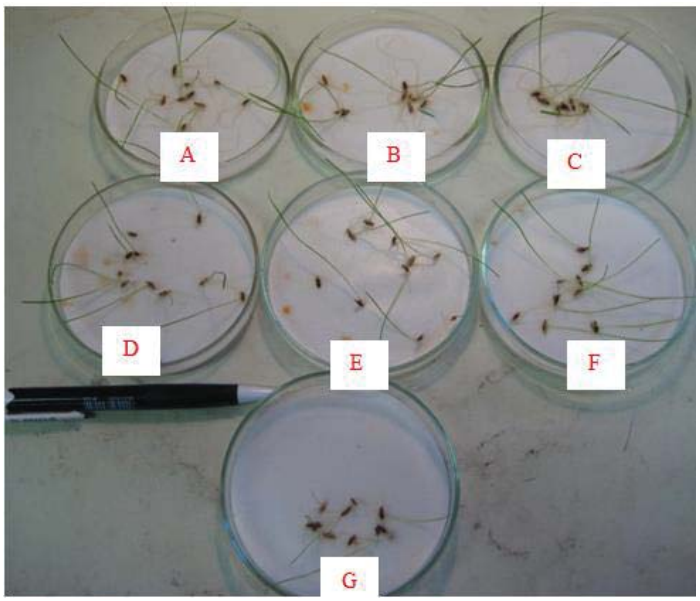

Figure 6.4 Effect of increasing glyphosate concentrations on shoots and roots of germinating seeds of glyphosate-susceptible perennial ryegrass (Population SP) after 8 days of being treated with different glyphosate concentrations. A = 0, B = 1.25, C = 2.5, D = 5, E = 10, F = 20 and G = 40 mg L^{-1} of glyphosate.

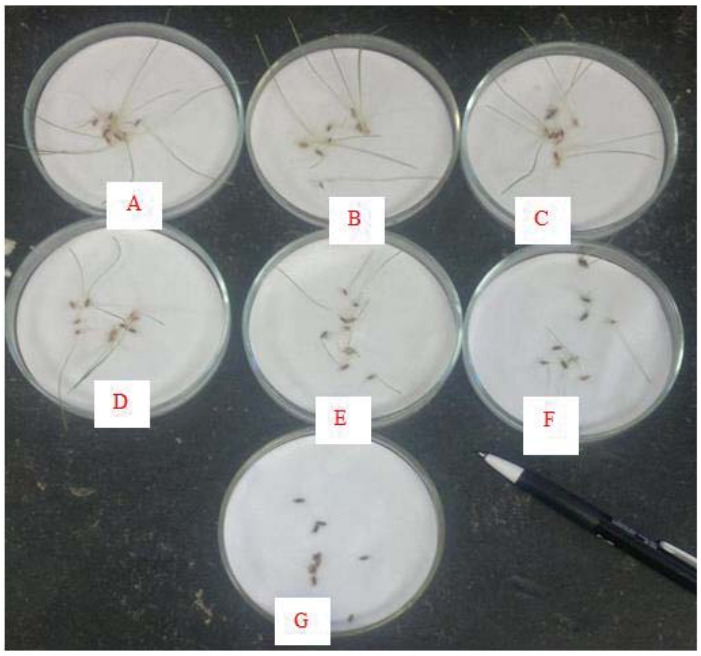

Figure 6.5 Effect of increasing glyphosate concentrations on shoots and roots of germinating seeds of glyphosate-resistant perennial ryegrass (Population J) after 8 days of being treated with different glyphosate concentrations. A = 0, B = 10, C = 20, D = 40, E = 80, F = 160 and G = 320 mg ae L^{-1} of glyphosate.

In contrast, the glyphosate rates that reduced the root growth by 50% in Populations SP and J were 3.9 and 34.8 mg ae L^{-1}, respectively. Based on shoot GR_{50}, Population J was 2.2 times more resistant to glyphosate while based on root GR_{50}, there appeared to be an 8.9-fold level of resistance.

6.3.2 Shikimic acid assay

Higher levels of shikimic acid accumulated in Italian ryegrass leaf segments treated with glyphosate for the susceptible population (SJ) compared to the resistant population (A) when exposed to glyphosate concentrations lower than 338 mg ae L^{-1} (Figure 6.6a). However, at the concentration of 338 mg ae L^{-1}, shikimic acid concentrations were similar for the two populations. The glyphosate IC_{50} (the concentration of herbicide required to inhibit enzyme activity by 50%) values for Populations SJ and A were estimated to be 41 and 309 mg ae L^{-1}, respectively (Table 6.3) Based on IC_{50} (the concentration of herbicide (mg ae L^{-1}) required to inhibit the enzyme activity by 50%), Population A was 7.5 times more resistant to glyphosate (Table 6.3).

Similar results were found for perennial ryegrass, with the susceptible population (SP) accumulating higher levels of shikimic acid compared to the resistant population (J) at glyphosate concentrations below 338 mg ae L^{-1} (Figure 6.6b). The glyphosate IC_{50} values for Populations SP and J were estimated to be 58 and 253 mg ae L^{-1}, respectively (Table 6.3). Based on $IC_{50,}$ Population J was 4.4 times more resistant to glyphosate (Table 6.3).

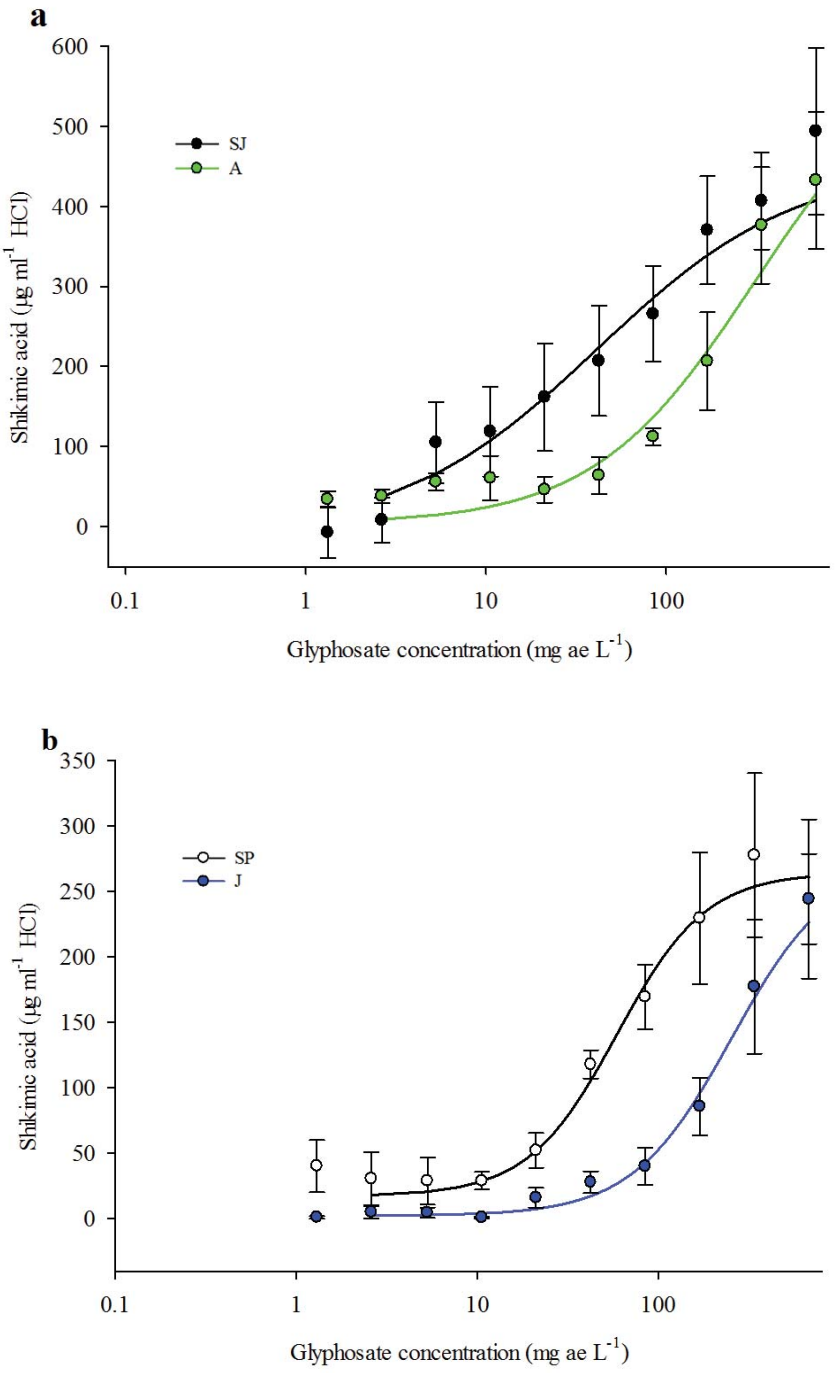

Figure 6.6 Shikimic acid accumulation in leaf segments of (**a**) two populations (SJ = glyphosate-susceptible and A = glyphosate-resistant) of Italian ryegrass, and (**b**) two populations (SP = glyphosate-susceptible and J = glyphosate-resistant) of perennial ryegrass at different glyphosate (potassium salt) concentrations in the shikimic acid assay. Vertical bars represent ± standard error of the means.

Table 6.3 Parameters estimated for the nonlinear regression analysis based on shikimic acid accumulation in leaf segments of two Italian ryegrass populations, SJ (glyphosate-susceptible) and A (glyphosate-resistant), and two perennial ryegrass populations, SP (glyphosate-susceptible) and J (glyphosate-resistant), after treatment with glyphosate (potassium salt).

Population	c	d	b	IC_{50} (mg L^{-1}) *	R/S IC_{50} ratio	R^2
Italian ryegrass						
SJ	10	463	-0.8	41 **b**	-	0.98
A	3	604	-0.9	308 **a**	7.5	0.98
P value	0.65	0.55	0.79	<0.0001		
Perennial ryegrass						
SP	17	247	-1.8	58 **b**	-	0.98
J	2	271	-1.6	253 **a**	4.4	0.99
P value	0.07	0.50	0.70	0.0007		

c = the lower limit, d = the upper limit, b = the slope around the IC_{50}, IC_{50} = the concentration of herbicide (mg ae L^{-1}) required to inhibit the enzyme activity by 50%, R/S IC_{50} = resistant/susceptible IC_{50} ratio, R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

6.3.3 Tiller dip assay

In the tiller dip assay, the level of injury to plantlets significantly increased as glyphosate concentrations increased in both resistant (A) and susceptible (SJ) populations of Italian ryegrass (Table 6.4). The glyphosate injury symptoms in the plantlets of both populations were wilting and necrosis. However, at glyphosate rates of 10 and 40 mg ae L^{-1}, the level of injury was either negligible or not evident for the resistant population compared to plantlets of the susceptible population which were severely injured (P<0.05). Also, plantlet fresh weight of susceptible plants was reduced significantly (P<0.05) after 8 days of exposure to glyphosate concentrations of 10 and 40 mg ae L^{-1}, whereas at these same concentrations the plantlet fresh weight of resistant plants was not reduced (Table 6.4).

Likewise, in perennial ryegrass the level of injury and reduction in fresh weight of the plantlets of the resistant population (Population J) were either not evident or significantly less ($P<0.05$) at glyphosate rates of 10 and 40 mg ae L^{-1} compared to susceptible ones (Population SP) (Table 6.4).

Table 6.4 Effect of glyphosate concentrations on plantlets of glyphosate-resistant (Population A) and glyphosate-susceptible (Population SJ) of Italian ryegrass and glyphosate-resistant (Population J) and glyphosate-susceptible (Population SP) of perennial ryegrass dipped in glyphosate (potassium salt) solutions in the tiller dip assay for 8 days.

Italian ryegrass	Susceptible	Resistant	Susceptible	Resistant
Glyphosate (mg L^{-1})	Visual score*		Plantlet FW (% of initial weight)**	
0	0.0	0.0	111.7	113.8
10	8.7	0.0	87.7	112.0
40	9.8	2.5	83.7	106.7
160	10.0	10.0	82.5	89.9
$LSD_{0.05}$	1.1		7.8	
Perennial ryegrass	Susceptible	Resistant	Susceptible	Resistant
Glyphosate (mg L^{-1})	Visual score*		Plantlet FW (% of initial weight)**	
0	0.0	0.0	108.0	112.2
10	8.7	0.7	86.5	109.1
40	10.0	4.8	83.8	93.8
160	10.0	10.0	79.6	86.4
$LSD_{0.05}$	1.2		9.5	

* Visual scoring was based on severity of injury on a scale of 0 (no visual injury) to 10 (severe necrosis).

** Plantlet fresh weight (FW) (%) = (a/b)*100, where a = FW after 8 days and b = initial FW.

Figure 6.7 Effect of different glyphosate concentrations on the tillers of two perennial ryegrass populations, J (glyphosate-resistant) and SP (glyphosate-susceptible) in the tiller dip assay after 8 days of being immersed in glyphosate. A, B, C and D represent glyphosate concentrations of 0, 10, 40 and 160 mg ae L^{-1} respectively.

6.3.5 Effect of glyphosate formulation on resistance quick tests

The results of the petri dish assay using the isopropylamine salt of glyphosate were similar to those observed for the potassium salt. In this investigation, the shoot and root length of both populations of perennial ryegrass decreased with increasing glyphosate concentrations (Figures 6.8a and b). The glyphosate rate that reduced the shoot growth of the susceptible Population (SP) by 50% was 42.2 mg ae L^{-1} compared with 177.6 mg ae L^{-1} for Population J (Table 6.5). However, root GR_{50} values for Populations SP and J were 6.5 and 61.3 mg ae L^{-1} of glyphosate, respectively (Table 6.5). Based on shoot GR_{50}, Population J was 4.2 times more resistant to glyphosate while based on root GR_{50}, a 9.4-fold level of resistance for Population J was observed (Table 6.5).

The results of the shikimic acid test showed that the level of shikimic acid was significantly greater for the susceptible population compared to the resistant one in both formulations, and test was able to discriminate the resistant population from the susceptible one for both formulations at all three concentrations of glyphosate (Figures 6.9a and b).

In the tiller dip assay using the isopropylamine salt, the same results were obtained as for the potassium salt (Table 6.6). There was a significant reduction recorded in the fresh weight of tillers for Population SP at the glyphosate rates of 10 and 40 mg ae L^{-1} while a negligible reduction was recorded for the tillers of Population J at these concentrations. Also, a good agreement was found between visual injury of the tillers and their fresh weight reduction. Severe visual injuries and a significant reduction in fresh weight were recorded for both populations at 160 mg ae L^{-1}.

A summary of all three quick tests using both formulations of glyphosate (isopropylamine vs. potassium) is shown in Table 6.7. Overall, the results of all three quick tests using the isopropylamine salt of glyphosate were similar to those observed for the potassium salt (Table 6.7).

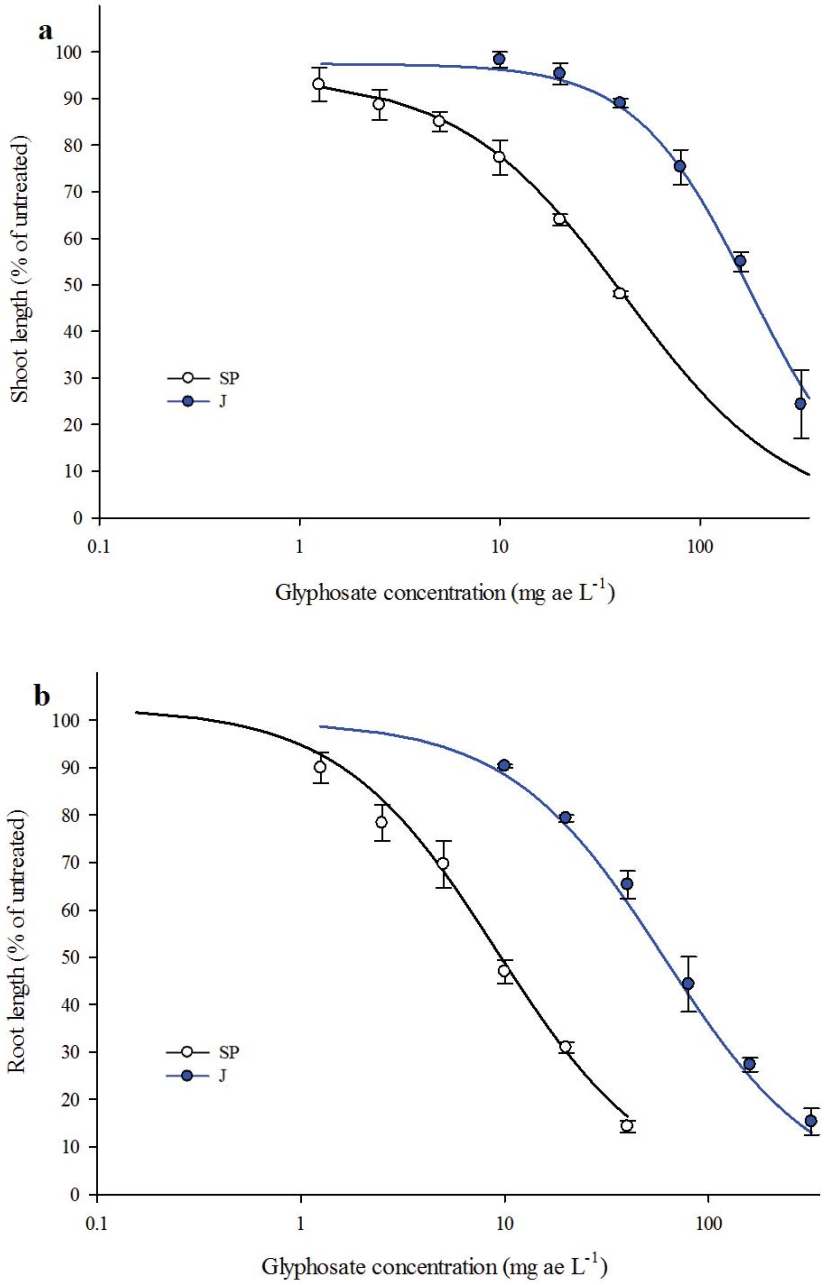

Figure 6.8 Fitted dose response curves (on a logarithmic dose scale) for two populations, SP (glyphosate-susceptible) and J (glyphosate-resistant) of perennial ryegrass for glyphosate (isopropylamine salt) concentrations using (**a**) shoot and (**b**) root length in the petri dish assay. Data are the means of two experiments each with three replicates and ten seeds per replicate, measured 8 days after treatment. Vertical bars represent ± standard error of the mean.

Table 6.5 Parameters estimated for the nonlinear regression analysis of petri dish assay using glyphosate formulated as an isopropylamine salt describing shoot and root length of two perennial ryegrass populations, SP (glyphosate-susceptible) and J (glyphosate-resistant).

Population	d	b	GR_{50} (mg L^{-1})*	R/S GR_{50} ratio	R^2
Shoot					
SP	94	1.0	42 **b**	-	0.98
J	97	1.5	177 **a**	4.2	0.99
P value	0.42	0.11	<0.0001		
Root length					
SP	102	0.9	6 **b**	-	0.99
J	100	1.1	61 **a**	9.4	0.98
P value	0.25	0.54	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (mg ae L^{-1}) required to reduce root/shoot length by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio, R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

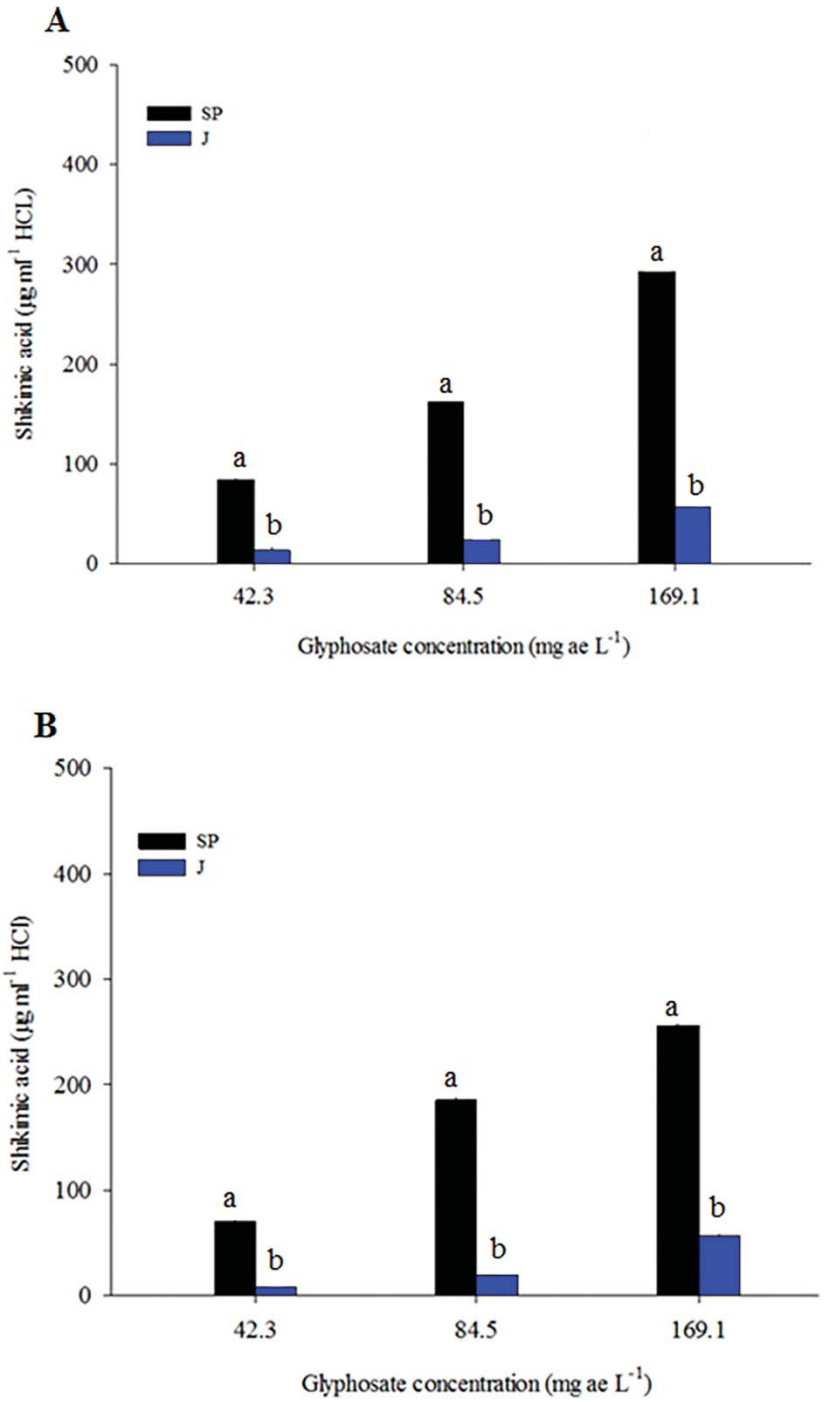

Figure 6.9 The level of shikimic acid accumulation in perennial ryegrass leaves from glyphosate-resistant (J) and glyphosate-susceptible (SP) populations when treated with three concentrations of (**A**) the isopropylamine salt (Roundup 360 Pro) and (**B**) the potassium salt (Roundup Transorb) of glyphosate in the shikimic acid assay. Bars with different letters within each glyphosate concentration were significantly different at 5% probability according to Student`s t-tests.

Table 6.6 Effect of glyphosate concentrations on plantlets of glyphosate-resistant (Population J) and glyphosate-susceptible (Population SP) of perennial ryegrass dipped in glyphosate (formulated as isopropylamine salt) solutions in tiller dip assay for 8 days.

	Susceptible	Resistant	Susceptible	Resistant
Glyphosate ($mg\ L^{-1}$)	Visual score*		Plantlet FW (% of initial weight)**	
0	0.0	0.0	120.9	129.2
10	4.8	1.1	84.5	106.9
40	9.1	3.3	82.1	91.5
160	10.0	9.5	75.3	78.9
$LSD_{0.05}$	2.0		10.0	

* Visual scoring was based on severity of injury on a scale of 0 (no visual injury) to 10 (severe necrosis).

** Plantlet fresh weight (FW) (%) = (a/b)*100, where a = FW after 8 days and b = initial FW.

Table 6.7 Summary of the comparison between two formulations of glyphosate (isopropylamine vs. potassium) for all three quick tests for two perennial ryegrass populations, SP (glyphosate-susceptible) and J (glyphosate-resistant).

Petri dish assay GR_{50} (mg L^{-1})				
	Isopropylamine		*Potassium*	
	J	SP	J	SP
Shoot	101.1	46.2	177.0	42.0
Root	34.8	3.9	61.0	6.0

Shikimic acid (µg ml^{-1})				
Glyphosate rate (mg L^{-1})	*Isopropylamine*		*Potassium*	
	J	SP	J	SP
42.3	13.4	84.5	8.1	70.2
84.5	23.8	162.5	19.7	185.7
169.1	56.7	292.5	57.1	256.4

Tiller dip assay								
Glyphosate rate (mg L^{-1})	*Isopropylamine*				*Potassium*			
	Visual score*		Plantlet FW (% of initial weight)**		Visual score*		Plantlet FW (% of initial weight)**	
	J	SP	J	SP	J	SP	J	SP
42.3	0.7	8.7	109.1	86.5	1.1	4.8	106.9	84.5
84.5	4.8	10.0	93.8	83.8	3.3	9.1	91.5	82.1
169.1	10.0	10.0	86.4	79.6	9.5	10.0	78.9	75.3

* Visual scoring was based on severity of injury on a scale of 0 (no visual injury) to 10 (severe necrosis).

** Plantlet fresh weight (FW) (%) = (a/b)*100, where a = FW after 8 days and b = initial FW.

6.4 Discussion

For management of herbicide resistance problems, it is important to have rapid and reliable techniques available to test weeds for resistance. The conventional method for detecting herbicide resistance is a sprayed pot study which involves growing resistant plants in pots alongside plants known to be susceptible then spraying with several application rates of a herbicide (Burgos *et al.*, 2013). In these dose response spraying tests, plants can be grown either from seeds or, in the case of grass species, can be obtained by splitting tillers from established plants according to the method described by Boutsalis (2001). However, these sprayed pot studies take several weeks to be completed and are thus expensive. Therefore, it was desirable to develop quicker tests for detecting herbicide resistance. Such quick tests have never been developed or used in New Zealand for any of the herbicide resistance problems.

The petri dish assay has been used by several researchers for detecting glyphosate resistance (Perez & Kogan, 2003; Zelaya *et al.*, 2004) and has good potential to be used as a rapid test because it can be completed in just a few days, with little labour. The results of the petri dish assay in our study showed that this test was able to provide a quick indication of the glyphosate resistance in perennial ryegrass and Italian ryegrass. They also showed that the shoot and root of Italian ryegrass and perennial ryegrass seedlings were reduced in length with exposure to increasing glyphosate concentrations. However, the root of susceptible populations (SP and SJ) was more sensitive to glyphosate compared with the shoot, at all glyphosate concentrations. A similar response for fathen seedlings exposed to different dicamba concentrations was also recorded (Chapter 4).

We found a close agreement between the magnitude of resistance from the sprayed potted plant study (Chapter 5) and the level of resistance in the petri dish assay by measuring reductions in root length. These results are in contrast to the results reported by Perez and Kogan (2003) who reported that Italian ryegrass root growth was less sensitive to glyphosate than shoots in their petri dish assay. However, Neve *et al*. (2004) also reported that measuring root length would provide a better indication of glyphosate resistance than shoot length. The results of our study showed that a glyphosate concentration of 10 mg ae L^{-1} could be used for discriminating resistant populations from susceptible ones when measuring root length.

The results of the shikimic acid assay using leaf segments also showed good potential for detecting glyphosate resistance in our biotypes. This assay was the quickest test for detecting glyphosate resistance as the results can be produced within 24-48 hours and a large number of samples can be tested because this test does not require much space (Shaner *et al.*, 2005). In our study, we found that young rapidly expanding leaves provided better results with the shikimic acid test, confirming the findings of other researchers who reported higher levels of shikimic acid in young tissues compared to older material (Singh & Shaner, 1998). In our study glyphosate concentrations of 42.3 to 169 mg ae L^{-1} were all good for discriminating resistant plants from susceptible ones.

The optimum glyphosate concentration for differentiating resistant plants using shikimic acid levels has been found in other work to vary considerably depending on species, tissue type, age of tissue and other factors like the type of resistance mechanism (Shaner, 2010). Thus the shikimic acid assay needs to be optimized for each new weed species, and is probably less useful than other quick tests assessed since specialist equipment and expertise are required to conduct them. Although the results can be obtained within 24-48 hours, the best results are obtained by using fresh rapidly expanding leaf materials. Therefore, if being used as a quick test for material posted in by growers, it might be necessary to grow up the overnight-posted plant samples for several days in order to obtain fresh rapidly expanding leaf material. Also, this test might be more expensive compared with other tests as the chemical compounds which are used in the test are expensive. For example, one should make up several shikimic acid standard curves in order to ensure a consistent response between the known concentrations and absorption values. However, 1 mg of shikimic acid costs almost $100.

The tiller dip assay was developed as a simple test for discriminating glyphosate-resistant Italian ryegrass and perennial ryegrass from susceptible ryegrass using field-grown plants and simple equipment. This test also showed good potential to detect resistance by using either visual scoring or measuring the effect of glyphosate on the fresh weight of treated tillers compared to their corresponding initial fresh weights prior to treatment. This test can be completed in 8 days and is non-destructive because only a few tillers need to be taken from suspected plants so the rest of the plant can be retained alive for further testing should resistance be detected. The test enables

researchers to complete a preliminary evaluation of suspected plants before further investigations such as spraying tests.

Koger *et al*. (2005) used excised leaves of *Conyza canadensis* immersed in different glyphosate solutions, and reported that it was possible to discriminate resistant plants from susceptible ones within 48 hours. However, our preliminary attempts showed poor correlations between glyphosate resistance and the level of injury in leaf segments because leaves began deteriorating before glyphosate could act. As glyphosate is a slow-acting herbicide (James & Rahman, 2005) and the symptoms of glyphosate injury can take up to 7 days to appear, we used rooted tillers in order to keep plantlets alive and healthy in the glyphosate solutions for at least 8 days. Our preliminary investigation showed that the rooted plantlets (consisting of three attached tillers) were able to remain healthy and keep growing for at least two weeks if they were kept in vials of water. Rooted plantlets in our assays could be used to discriminate glyphosate resistant perennial ryegrass and Italian ryegrass from susceptible plants at a glyphosate concentration of either 10 or 40 mg ae L^{-1} as the plantlets of resistant plants showed negligible visual injury and growth reduction compared to susceptible ones. Plants can be sent overnight by courier from around the country and can be tested immediately if their roots have been kept moist.

Glyphosate is sold in New Zealand in many different formulations, with different salts of glyphosate used, and also with many different surfactants (Young, 2015). Our comparison of two such formulations (the isopropylamine salt as Roundup 360 Pro and potassium salt as Roundup Transorb) suggested that all three quick tests discussed in this chapter should differentiate resistant biotypes from susceptible ones no matter which formulation was used.

As the objective of this work was to design quick tests for detecting glyphosate resistance, not all of the experiments had a sufficient range of rates used to allow complete dose response curves to be produced. Thus a limitation of the study was not being able to determine if all quick tests could correctly determine the magnitude of resistance, though the root measurements in the petri dish assay appeared to give similar estimates of the magnitude of resistance to the spray pot study. However, each test was able to detect resistance reliably and each has its own advantages over the sprayed potted plant experiments. For example, the petri dish assay only needs seeds, the tiller dip assay can use live plants taken from the field without needing to wait for seeds to be

produced, and the shikimic acid assay only needs live leaves and is the most rapid as long as plant samples have rapidly expanding leaves which can be treated with herbicide.

6.5 Conclusions

Three different quick tests for screening glyphosate-resistant Italian ryegrass and perennial ryegrass were evaluated and the results showed that all three methods could be used for detecting glyphosate resistance. The tiller dip assay and petri dish assay may be the most useful quick tests as they do not need the sophisticated equipment required for the shikimic acid test. The tiller dip assay does not even require plants to set seed before testing can be conducted.

Chapter 7

Target site mutation in glyphosate-resistant Italian ryegrass and perennial ryegrass[1]

7.1 Introduction

One of the mechanisms of glyphosate resistance in weed species is a target-site modification in EPSPS enzyme and this was reviewed in Section 2.4.5. The first cases of glyphosate-resistance in New Zealand have now been reported for Italian ryegrass and perennial ryegrass populations (Chapter 5). In this chapter, we investigate if the target-site modification in the EPSPS enzyme has occurred for these populations. The investigation was performed for all populations confirmed to be resistant to glyphosate.

7.2 Materials and methods

7.2.1 Plant material

Clones of the plants that had proven to be resistant to glyphosate in Chapter 5 were used for this study. The plant material of each population was potted and kept in a glasshouse until the beginning of testing. Plants of perennial ryegrass (SP) and Italian ryegrass (SI) populations known to be susceptible to glyphosate (Chapter 5) were included.

7.2.2 DNA extraction

For DNA extraction, the young expanding leaf of five plants of each population was harvested. A segment 10 mm in length was cut from each leaf and put in separate 1.5 ml Eppendorf tubes. DNA was extracted using a Dneasy Plant Mini Kit (Qiagen Inc.), following the method described by the manufacturer of the kits. The leaf tissue was macerated using a plastic pestle and to this was added 400 µl of buffer AP1 and 4 µl of RNAase. The samples were incubated at 65°C in a heating block for 10 minutes. The

[1] Some of the material in this chapter will soon be published in:

Ghanizadeh H, Harrington KC, James TK and Woolley DJ and Ellison NW (2015) Mechanisms of glyphosate resistance in two perennial ryegrass (*Lolium perenne*) populations. Pest Management Science. 71, 1617-1622.

Ghanizadeh H, Harrington KC, James TK and Woolley DJ and Ellison NW (2015) Restricted herbicide translocation was found in two glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) populations from New Zealand. Journal of Agricultural Science and Technology (in press)

samples were mixed two to three times during incubation by inverting the tubes. Next, 130 µl of buffer P3 was added to each tube and the samples were kept on ice for 5 minutes. The samples were then centrifuged at 14,000 rpm (revolutions per minute) for 5 minutes and the upper aqueous phase was transferred to QIAshredder Mini spin column placed in a 2 ml tube. The QIAshredder Mini spin column was centrifuged at 14,000 rpm for 2 minutes. The flow-fraction (450 µl) which accumulated in the 2 ml QIAshereder tube was transferred into a new 1.5 ml Eppendorf tube and 675 µl of buffer AW1 was added and mixed by pipetting. After that, 650 µl of this mixture was transferred to a Dneasy Mini spin column placed in a 2 ml tube and centrifuged at 8,000 rpm for 1 minute and the supernatant were discarded and the spin step was repeated. The Dneasy Mini spin column was transferred to a new 2 mL collection tube and 500 µl of buffer AW2 was added and the Dneasy Mini spin column was centrifuged at 8,000 rpm for 1 minute. Again, 500 µl of buffer AW2 was added to the Dneasy Mini spin column and centrifuged at 8,000 rpm for 2 minutes. In order to ensure that no residual ethanol would be carried over, the Dneasy Mini spin column was centrifuged at 8,000 for another 2 minutes. The Dneasy Mini spin column was then transferred to a 1.5 ml Eppendorf tube and 100 µl of buffer AE was directly pipetted on to the Dneasy membrane and incubated for 5 minutes at room temperature and then centrifuged at 8,000 rpm for 1 minute. The DNA solution in the tube was kept at -20°C until DNA amplification.

7.2.3 DNA amplification

For DNA amplification, forward and reverse primers for PCR (polymerase chain reaction) amplification and sequencing were designed according to Bostamam *et al*. (2012). Two µl of template genomic DNA was mixed with 11.6 µl molecular-grade water, 1.6 µL dNTP mix (2.5 mM each), 4 µL 5x PrimeStar® GXL buffer (PrimeStar® GXL, Takara Bio INC. Japan), 0.4 µL forward primer (5'-CAAAAAGAGCTGTAGTCGT-3') and reverse primer (5'-CAAGGAACTCAAGTATTGGC-3') (20 µM each) and 0.4 µL of PrimeStar® GXL (PrimeStar® GXL, Takara Bio INC. Japan). The tubes were placed in an automated DNA thermal cycler. PCR amplification was carried out with one cycle of 30 seconds at 98°C for denaturation, followed by 35 cycles of 10 seconds at 98°C for denaturation, 15 seconds at 55°C for annealing and 1 minute at 68°C for elongation,

followed by a final cycle of 7 minutes at 68°C. The mixture was kept at -20°C until the next steps.

7.2.4 Electrophoresis

7.2.4.1 Preparing agarose gel 1%

In order to prepare 1% agarose gel, 0.3 g of multipurpose agarose powder (LE agarose, HyAgarose^{TM}, ACTGene, USA) was added to 30 ml of 1x TAE (40 mM Trizma base, 1 mM Na_2EDTA, pH to 8 with glacial acetic acid) buffer in an Erlenmeyer flask. The flask (covered with a cap) was put in a microwave and heated for 1 minute. When the solution was completely clear (no suspension), the heating process was stopped. Before the gel solution was poured in a gel mould, 3 µl of ethidium bromide was added to the flask and mixed thoroughly.

7.2.4.2 Sample preparation for electrophoresis

To each tube, 4 µl of the DNA loading buffer was added to 20 µl of the PCR product (giving a 6-fold dilution of the dye). The samples were mixed thoroughly using a vortex shaker and 5 µl of each sample was loaded into a 1% agarose gel containing 1 µg ml^{-1} ethidium bromide. The ladder (DNA standard) was obtained by mixing 1 µl of 1-bp (base pair) ladder with 1 µL of the dye and 4 µl of molecular water.

7.2.4.3 Electrophoresis process

The gel mould was placed in an electrophoresis chamber containing buffer solution. First, 6 µl of 1-bp ladder was loaded into the first chamber. Then, the samples were loaded one by one in the rest of the cells. Electrophoresis was carried out for 30 minutes at 50 volts and the separated bands were visualised and photographed under UV light.

7.2.4.4 Low melting point agarose gel electrophoresis and gel extraction

Fifty ml of 1.2% low melting point (LMP) agarose gel was prepared by adding 0.6 g of low melting point analytical gel powder (UltraPure^{TM} LMP Agarose, Invitrogen, USA) to 1x TAE buffer. The mixture was then heated in a microwave for 1 minute or until a clear solution was obtained. Before pouring the solution into a gel mould, 5 µl of ethidium bromide was added to the solution and mixed thoroughly, then the gel was left to set. The gel mould was placed in an electrophoresis chamber containing buffer and the remainder of the dyed PCR products (almost 19 µl) from the previous electrophoresis step (Section 7.2.4.3) was loaded in each well. The electrophoresis

process was run for 30 minutes at 50 volts. The gel was visualised under long wavelength UV (ultraviolet) and bands were cut gently using a scalpel. The cut bands were put into separate labelled tubes.

A similar method to the technique described by Williams *et al.* (2001) was used for gel purification. For this, the tubes containing the gel bands were then incubated at 65°C in a heating block for 10 minutes or until the gel in each tube had completely melted. The tubes were put in racks and placed in an ice box containing liquid nitrogen. When the solution within each tube was completely frozen, the tubes were centrifuged at 14000 rpm for 10 minutes. Two phases consisting of the pure PCR products on the top (supernatant) and the agarose waste (pellets) at the bottom of the tubes were obtained using this method. Finally, the supernatant was transferred from each tube to fresh labelled tubes without disturbing the pellets and was used for sequencing.

7.2.5 Sequencing

7.2.5.1 Sequencing reaction

Sequencing reactions consisted of 2.5 µl of 5x TM buffer (400mM Tris-HCl, pH9.0, 10mM $MgCl_2$), 1 µl of DMSO (dimethyl sulfoxide ($(CH3)_2SO$) 99.9%, Aldrich USA), 0.5 µl of Big Dye sequencing mix (Applied Biosystems, Foster City, CA, USA), 1 µl of a 10 µl stock of forward (5'-CAAAAAGAGCTGTAGTCGT-3') or reverse primer (5'-ACATTCGCACCTAGTTGTTT-3') and 5 µl of gel-purified PCR product from Section 7.2.4.4 placed in a 96-well microtiter plate. The total volume for the sequencing reaction was 10 µl which was added to two separate 96-well PCR plates (one for the forward primer and the other one for reverse primer). The plates were sealed with an adhesive PCR seal (4titude, UK) and placed in an automated thermal cycle machine. The machine was programmed for 100 cycles with 10 seconds at 96°C for denaturation, 5 seconds at 50°C for annealing and 4 minutes at 60°C for extension.

7.2.5.2 Sequencing reaction purification and DNA sequencing

Sephadex fine DNA grade G-50 (G Health Care, UK) was loaded in a 96-well filtration plate (Multiscreen HV, Merck Millipore, USA) using a multiscreen column loader in order to obtain sephadex columns. Each well received 0.0325g of sephadex. Then the 96-well filtration plate was placed on a regular 96-well microtiter plate with a centrifuge alignment frame between two plates. After that, 300 µl of deionized water was added to each container well and the plate on the top (the filtration plate) was covered with a

Para-film before a lid was placed on it. The purification kit (96-well filtration plate plus the centrifuge alignment frame and regular 96-well microtiter plate) was kept in a fridge at 5°C either overnight or for 3 hours.

The purification kit was then centrifuged for 5 minutes at 910 rcf (relative centrifugal force) (7104 rpm) to remove any leftover water. Then, 10µl of 95% Cresol Red dye was added to each sequencing reaction well so each well now contained 20 µl of solution (10 µl Cresol dye plus 10 µl sequencing reaction product) and this solution was transferred to the sephadex columns. After loading all of the samples (10 µl Cresol dye plus 10 µl sequencing reaction product), the plate of sephadex columns was then placed on the top of a sequencing plate and these were centrifuged at 910 rcf for 5 minutes. The sequencing plate was then placed in a vacuum chamber for 90 minutes to dry the samples. Before putting the sequencing plate in the DNA sequencing machine, 10 µl of sequencing buffer (HiDi Formamide, Applied Biosystem, UK) was added to each well. The plate was sealed and vortexed for 1 minute then pulse centrifuged. The DNA sequencing was conducted using a Genetic Analyser (3130xl, Hitachi Applied Biosystem, Japan). DNA sequence data were assembled, compared with the consensus sequence (Bostamam *et al.,* 2012) and analysed using DNA Baser Sequence Assembler software (version.3.51).

7.3 Results

The EPSPS gene of glyphosate-resistant and susceptible populations of Italian ryegrass and perennial ryegrass was partially sequenced in order to investigate any changes in the EPSPS nucleotide sequence (Table 7.1). The predicted amino acid sequence of the susceptible populations of Italian ryegrass (SI) and perennial ryegrass (SP) was similar to the consensus sequence. A substitution of GGC to GGG at codon 98 and GCT to GCA were recorded for Population A (Italian ryegrass). A substitution of GGC to GGG at position 98 was also recorded for Populations J and O (perennial ryegrass). However, these nucleotide changes were silent changes and did not result in amino acid substitutions at these codons (Bostamam *et al.*, 2012). However, a single nucleotide substitution of CCA to TCA at codon 106 was observed in the perennial ryegrass glyphosate-resistant population O. The nucleotide change of C to T at the beginning of codon 106 has previously resulted to an amino acid substitution from proline to serine (Baerson *et al.*, 2002).

7.4 Discussion

Mutations in the EPSPS gene will cause target-site resistance to glyphosate (Padgette *et al.*, 1991). To determine the mechanism responsible for differences in the sensitivity to glyphosate in Italian ryegrass and perennial ryegrass populations, the EPSP synthase gene was partially sequenced in glyphosate-resistant and susceptible populations of Italian ryegrass and perennial ryegrass. DNA was extracted from leaves and the conserved active site was amplified by PCR from the DNA, and sequenced in five glyphosate-resistant populations (two Italian ryegrass and three perennial ryegrass populations), as well as two glyphosate-susceptible populations.

Table 7.1 Nucleotide sequence in 5-enolpyruvylshikimate-3-phosphate synthase DNA isolated from of Italian ryegrass populations (A and P (glyphosate-resistant), and SI (glyphosate-susceptible)) and perennial ryegrass populations (J, N and O (glyphosate-resistant), and SP (glyphosate-susceptible)). Changes to codons from the consensus sequence are bold underlined.

Amino acid number	97	98	99	100	101	102	103	104	105	106	107	108
Amino acid	Leu	Gly	Asn	Ala	Gly	Thr	Ala	Met	Arg	Pro	Leu	Thr
Consensus sequence	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
Italian ryegrass												
SI	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
A	TTG	**GGG**	AAC	**GCA**	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
P	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
Perennial ryegrass												
SP	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
J	TTG	**GGG**	AAC	GCT	GGA	ACT	GCA	ATG	CGG	CCA	TTG	ACG
N	TTG	GGC	AAC	GCT	GGA	ACT	GCG	ATG	CGG	CCA	TTG	ACG
O	TTG	**GGG**	AAC	GCT	GGA	ACT	GCG	ATG	CGG	**TCA**	TTG	ACG

In this study, four polymorphisms were observed in the glyphosate-resistant biotypes (Table 7.1). However, three of them were silent mutations that did not result in amino acid substitutions (Bostamam *et al.*, 2012). One change of a C (cytosine) to T (thymine) in the first codon at position Pro-106 in population O resulted in a change from proline

to serine which confirmed a target-site mutation in Population O. The target site mutation which led to a substitution of either serine or threonine for proline at position Pro-106 in EPSPS has been reported for glyphosate-resistant populations of *Lolium rigidum, Lolium multiflorum* and *Eleusine indica* (Baerson *et al.*, 2002; Jasieniuk *et al.*, 2008; Ng *et al.*, 2003; Wakelin & Preston, 2006b). The change of proline at position Pro-106 could result in alterations in both the structure and function of the EPSPS that confer some level of resistance to glyphosate in plants (Wakelin & Preston, 2006b). It has also been reported that this substitution of amino acids (either serine or threonine) for proline at position Pro-106 would provide a low level of resistance, approximately 2- to 4-fold, to glyphosate (Baerson *et al.*, 2002).

In the case of Population O, we previously demonstrated that the level of resistance to glyphosate in this population was almost 30-fold (Chapter 5). Therefore, a target site mutation at position Pro-106 was unlikely to be the only factor endowing this level of resistance. Individual plants sampled for DNA sequencing were all confirmed resistant to glyphosate after spraying with several doses of glyphosate (Chapter 5). However, no nucleotide changes leading to an amino acid substitution at position Pro-106 were observed in any of the populations apart from in Population O (Table 7.1). Thus the presence of another mechanism of glyphosate resistance such as restricted herbicide translocation appeared the most likely explanation for the New Zealand case of glyphosate resistance and this is explored in Chapter 8.

7.5 Conclusions

The possible presence of the target site mechanism of glyphosate resistance in the EPSPS gene was investigated for all glyphosate-resistant Italian ryegrass and perennial ryegrass populations identified in Chapter 5. The results indicated that only one of the perennial ryegrass populations (Population O) possessed a mutation at position Pro-106 which resulted in an amino acid substitution from proline to serine. This mutation was absent from the other four glyphosate-resistant populations.

Chapter 8

Non-target site mechanism of glyphosate resistance in Italian ryegrass and perennial ryegrass[1]

8.1 Introduction

Glyphosate is able to translocate in the plant from its site of application to the meristematic zone (Shaner, 2009). Translocation of glyphosate to the meristematic zones is important as the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the primary site of action of glyphosate, is highly active in meristematic zones such as apical meristems (Weaver & Herrmann, 1997). The neutral charge characteristic of the glyphosate molecule enables it to translocate in phloem from source leaves to sink tissue following photo-assimilate movement (Franz *et al.*, 1997). Any alteration in the molecule structure of glyphosate could affect its neutral characteristics and reduce glyphosate ability to move in the plant (Shaner, 2009). Glyphosate efficacy strongly depends on its ability to reach its site of action.

One of the known mechanisms of resistance to glyphosate is a non-target site mechanism in which the herbicide molecule is prevented from reaching its target site due to reduced uptake and/or restricted translocation, often due to sequestration in vacuoles (Sammons & Gaines, 2014). The role of restricted herbicide translocation in glyphosate resistant weed species was reviewed in Section 2.4.5. In Chapter 7, we looked into the existence of a possible mutation at position Pro-106 of EPSPS and no such mutations were detected in any of the studied populations except Population O. This suggested that a different mechanism of resistance was involved in our glyphosate-resistant populations. Therefore, the objective of this study was to determine if a non-target site mechanism of resistance was present in four of the five populations of

[1] Some of the material in this chapter has been accepted for publication in:

Ghanizadeh H, Harrington KC, James TK and Woolley DJ and Ellison NW (2015) Mechanisms of glyphosate resistance in two perennial ryegrass (*Lolium perenne*) populations. Pest Management Science. 71, 1617-1622.

Ghanizadeh H, Harrington KC, James TK and Woolley DJ and Ellison NW (2015) Restricted herbicide translocation was found in two glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) populations from New Zealand. Journal of Agricultural Science and Technology (in press).

glyphosate-resistant Italian ryegrass (Populations A and P) and perennial ryegrass (Populations J and O) which were studied in Chapter 7. Due to insufficient seeds being available, the non-target site mechanism of resistance for Population N (perennial ryegrass) was not studied.

8.2 Materials and methods

8.2.1 Seed materials

The clones of original plants of glyphosate-resistant Italian ryegrass populations (Populations A and P) and perennial ryegrass populations (Populations J and O) were grown in a glasshouse within pollen-proof cloth (a separate enclosure for each population) in order to obtain seeds using the method described in Section 6.2.1. Seeds of a susceptible Italian ryegrass population (cv. Tabu) (Population SI) and a susceptible perennial ryegrass population (cv. Trojan) (Population SP) were also obtained from a commercial source and these had been shown in the preliminary experiments to be susceptible to glyphosate.

8.2.2 Seed germination and seedling growth

The seeds of each population were germinated on blotters saturated with water within plastic containers. The plastic containers were sealed with lids and placed in a growth cabinet at 30/20°C and 8 h light/16 h dark (a light intensity of 40 μmol m^{-2} s^{-1} provided by two 40 W fluorescent white tubes). After 8 days, the germinated seedlings were planted in 230 ml plastic containers and grown hydroponically. Each seedling was planted through an 8 mm hole in the container lid, with four seedlings per container. The containers were each filled with 60 glass marbles to support the roots and with 130 ml nutrition solution (Hoagland & Arnon, 1938) (Table 8.1) then sealed with a lid. The pH of the nutrient solution was 5.6 with an EC (electrical conductivity) of 3.2 dS m^{-1}. The containers and their lids were painted black in order to limit light reaching the nutrition solution, thus discouraging algal growth. The containers were placed in a growth cabinet at 21$\pm$4°C temperature with a 12 hour photoperiod (100 μmol m^{-2} s^{-1}) provided by four 40-W white fluorescent tubes.

Table 8.1 Concentration of nutrients in hydroponic nutrient solution.

Nutrient	Concentration (µM)
$Ca(NO_3)_2.4H_2O$	838.5
KNO_3	1301.6
$MgSO_4.7H_2O$	403.3
$MnSO_4.4H_2O$	4.5
KH_2PO_4	399.7
Fe.EDTA.NA	16.3
$ZnSO_4.7H_2O$	0.2
$CuSO_4.5H_2O$	0.2
$Na_2B_8O_{13}.4H_2O$	0.9
$(NH_4)2MoO_4.4H_2O$	0.1
$Na_2B_8O_{13}.4H_2O$	0.9

8.2.3 Radiolabelled glyphosate preparation

Glyphosate, ^{14}C-labelled at the phosphonomethylene site, was obtained from PerkinElmer (940 Winter St., Waltham Massachusetts, 02451, USA). The specific activity of the radiolabelled glyphosate was 50 µCi (microcurie) (1.85 MBq (megabecquerel)). The radiolabelled glyphosate used in this study was prepared by mixing 0.34 µmol of the original radiolabelled glyphosate solution with 3.45 µmol glyphosate solution (Roundup 360 Pro, an isopropylamine salt) with 0.1% organosilicone surfactant (Pulse Penetrant) and stored at 5°C.

8.2.4 Unlabelled and radiolabelled herbicide treatments

When plants were at the three-leaf stage, they were thinned to one plant in each container. The apical half of the second leaf of each plant was marked using a fine point marker pen and covered with aluminium foil. In our preliminary experiments, glyphosate at 180 g ae ha^{-1} was found to be a suitable rate for discriminating between glyphosate resistant and susceptible plants. Therefore, plants were sprayed with 180 g ae ha^{-1} glyphosate (Roundup 360 Pro, an isopropylamine salt) and 0.1% organosilicone surfactant (Pulse Penetrant) using a laboratory track sprayer calibrated to deliver 250 L ha^{-1} of spray solution at 200 kPa (unlabelled herbicide treatments). The covered part of the second leaf of each seedling was then uncovered approximately 15 minutes after application of the unlabelled herbicide and treated with 1µL of radiolabelled ^{14}C-

glyphosate solution (Section 8.2.3) with 0.7 kBq (kilobecquerel) of radioactivity and 0.004 μmol of glyphosate. The application was made to the midpoint of the second leaf using a micropipette. The ^{14}C-glyphosate was smeared on the adaxial (upper) side of the treated leaf. Plants were then returned to the growth cabinet.

8.2.5 Radioactive quantification

Plants were harvested 48 and 72 hours after treatment (HAT) because significant differences in glyphosate translocation patterns were recorded in previous studies at 48 and 72 HAT between glyphosate-resistant and susceptible populations of *Lolium rigidum* (Wakelin *et al.*, 2004; Yu *et al.*, 2007) and *Lolium multiflorum* (Nandula *et al.*, 2008). The plants were each then divided into four sections: the treated leaf, the root, the pseudostem region (defined as the region of leaf sheaths in a vegetative grass (Beecher *et al.*, 2015)) and untreated leaves (i.e. the leaves that had not been treated with ^{14}C-glyphosate). The treated leaves were placed in 20-ml plastic tubes containing 5 ml of 0.1% Triton X-100 solution (Sigma-Aldrich) and agitated for 10 seconds in order to remove any non-absorbed radioactivity from the leaf surface. The leaf wash was then repeated with an additional 5 ml of 0.1% Triton X-100 solution. The solutions obtained from both leaf washes were then mixed, giving a total of 10 ml leaf wash for each treated leaf. Radioactivity in the 10-ml leaf wash solution was quantified by mixing 1 ml of the solution with 10 ml of Ultima Gold scintillation cocktail (PerkinElmer) in 20-ml scintillation vials. The ^{14}C-glyphosate in each divided plant section was extracted using sodium hypochlorite (Alister *et al.*, 2005; Perez *et al.*, 2004). Previous research has found mainly methanediol and only a trace amount of $^{14}CO_2$ upon reaction of ^{14}C-glyphosate (labelled at the phosphonomethylene position) and sodium hypochlorite (Mehrsheikh *et al.*, 2006). Therefore, it was assumed that a high recovery of ^{14}C-glyphosate occurred using sodium hypochlorite with negligible losses of radioactivity as $^{14}CO_2$. Each section was placed in a plastic tube (20 mL) containing 0.5 mL of sodium hypochlorite solution (100 g L^{-1}). The tubes were placed in an oven at 58°C for 4 h for tissue digestion, then the tubes were ventilated to eliminate sodium hypochlorite vapour and cooled to room temperature before the supernatant in each tube was diluted 20-fold with distilled water. A 1 ml aliquot from each tube was mixed with 5 mL of liquid scintillation cocktail (Optiphase HiSafe 3, Wallac, Finland) in 20 mL scintillation vials. The radioactivity of each sample (leaf wash and plant sections) was quantified using a liquid scintillation counter (Tri-Carb 2900TR, PerkinElmer).

The percentage of glyphosate in each plant section was calculated proportional to the sum of radioactivity in all plant sections (treated leaves, root, the pseudostem region and untreated leaves). The percentage of glyphosate absorbed was calculated using the following equation (Equ. 1):

$$\left[\frac{\text{the sum of radioactivity in all plant sections}}{\text{the sum of radioactivity in all plant sections + leaf wash}}\right] \times 100 \qquad \text{Equ. (1)}$$

^{14}C-glyphosate recovery was calculated using the following equation (Equ.2):

$$\left[\frac{\text{the sum of radioactivity in all plant sections + leaf wash}}{\text{the radioactivity of 1}\mu\text{L drop of } {}^{14}\text{C}-\text{glyphosate}}\right] \times 100 \qquad \text{Equ. (2)}$$

The absorption and translocation experiments were conducted in a completely randomised design with five replicates and were repeated a month later for each population.

8.2.6 Statistical analysis

A two-way ANOVA was conducted and the results showed that the data from the two runs were not significantly different (Table 8.2) and were therefore pooled. The basic assumptions for ANOVA were checked using the methods described in Section 3.2.4. Except for leaf absorption, sheath and untreated leaf data which were normally distributed, the remaining data (treated leaf and root) were $\log_{10}$ transformed prior to analysis. A one-way ANOVA was conducted for the transformed and non-transformed data. Means were separated using Fisher`s protected tests at a 5% level of probability.

Table 8.2 The P values from two-way ANOVA of ^{14}C-glyphosate absorption and translocation experiments results of Italian ryegrass populations (A and P (glyphosate-resistant), and SI (glyphosate-susceptible)) and perennial ryegrass populations (J and O (glyphosate-resistant), and SP (glyphosate-susceptible)).

	Italian ryegrass and perennial ryegrass populations					
	P values					
	Italian ryegrass			Perennial ryegrass		
	SI	A	P	SP	J	O
	48 hours after treatment					
Treatments	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Experiments	0.774	0.944	0.868	0.975	0.897	0.889
Treatments*Experiments	0.374	0.607	0.841	0.879	0.397	0.792
	72 hours after treatment					
Treatments	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Experiments	0.989	0.893	0.790	0.917	0.656	0.767
Treatments*Experiments	0.832	0.877	0.903	0.811	0.382	0.832

8.3 Results

8.3.1 Absorption and translocation in perennial ryegrass

The average ^{14}C-glyphosate recovery for each perennial ryegrass population in this study is shown in Table 8.3. The amount of glyphosate absorption among perennial ryegrass populations ranged from 28 to 31% at 48 HAT and 35 to 40% at 72 HAT, with no significant differences between populations (Table 8.4).

Table 8.3 Average percentage of ^{14}C-glyphosate recovered (Equation 2) in three perennial ryegrass populations (J and O (glyphosate-resistant), and SP (glyphosate-susceptible)), 48 and 72 hours after treatment (HAT).

Population	% recovery of applied ^{14}C-glyphosate	
	48 HAT	72 HAT
SP (susceptible)	94.8	96.7
J (resistant)	95.4	98.2
O (resistant)	96.3	98.1
P values	0.884	0.354

However, glyphosate translocation patterns were different in the glyphosate-resistant perennial ryegrass populations (O and J) compared with the susceptible one (SP) (Table 8.5). Almost 67% of the ^{14}C-glyphosate absorbed by Population SP plants was

translocated out of the treated leaf blade to the other parts of the plant by 48 HAT, compared with only 42 and 39% for Populations O and J respectively (Table 8.5). This significant difference was also measured at 72 HAT when only 29% of the absorbed ^{14}C-glyphosate still remained in the treated leaf blade for Population SP, whereas 49 and 54% of the ^{14}C-glyphosate was retained in treated leaf blades for Populations O and J respectively (Table 8.5).

By 72 HAT, approximately twice as much of the ^{14}C-glyphosate was found within the pseudostem region (the collection of leaf sheaths on a tiller) of the susceptible plants than for the resistant plants, with 48% of all the ^{14}C-glyphosate in the pseudostem region for Population SP, compared with 28% and 21% for Populations J and O respectively (Table 8.5). Smaller amounts of the ^{14}C-glyphosate were found in the roots and the leaf blades of untreated leaves than the pseudostem region, and the differences between susceptible and resistant plants were generally not significant, apart from Population J initially having less ^{14}C-glyphosate in the blade of untreated leaves than the other two populations (Table 8.5). A trace amount of radioactivity was detected in the nutrient solution (Table 8.6). However, no significant differences were observed between the glyphosate-resistant and susceptible populations in the amount of radioactivity detected in the nutrient solution (Table 8.6).

Table 8.4 Absorption of ^{14}C-glyphosate (Equation 1) in three perennial ryegrass populations (J and O (glyphosate-resistant), and SP (glyphosate-susceptible)), 48 and 72 hours after treatment (HAT).

Population	^{14}C-glyphosate absorption (% of applied)	
	48 HAT	72 HAT
SP (susceptible)	31.3	36.7
J (resistant)	30.5	40.1
O (resistant)	28.3	35.2
P values	0.12	0.13

Table 8.5 Distribution of ^{14}C-glyphosate in four plant sections (the treated leaf, the root, the pseudostem region and untreated leaves) in three perennial ryegrass populations (J and O (glyphosate-resistant), and SP (glyphosate-susceptible), 48 and 72 hours after treatment (HAT).

Population	^{14}C-glyphosate distribution (% of absorbed)							
	Treated leaf		Untreated		Pseudostem		Root	
	48	72	48	72	48	72	48	72
SP (susceptible)	33.3 **b**	29.7 **b**	10.8 **a**	8.2	43.7 **a**	47.6 **a**	11.9	14.5
J (resistant)	61.3 **a**	54.1 **a**	4.4 **b**	8.1	22.4 **b**	27.7 **b**	11.6	10.1
O (resistant)	58.3 **a**	49.8 **a**	10.3 **a**	12.3	17.9 **b**	20.6 **b**	13.5	17.3
P values	<0.001	<0.001	<0.01	0.095	<0.001	<0.001	0.51	0.65

Mean values within each column followed by the same letters are not different at 5% probability according to Fisher's protected tests.

Table 8.6 The amount of radioactivity detected in nutrient solution media for three perennial ryegrass populations (J and O (glyphosate-resistant), and SP (glyphosate-susceptible)), 48 and 72 hours after treatment (HAT).

Population	^{14}C-glyphosate (% of absorbed)	
	48 HAT	72 HAT
SP (susceptible)	0.21	0.28
J (resistant)	0.19	0.27
O (resistant)	0.25	0.32
P values	0.86	0.95

8.3.2 Absorption and translocation in Italian ryegrass

Similar results were recorded for the Italian ryegrass experiments. The average range of applied ^{14}C-glyphosate recovered from each Italian ryegrass population is shown in Table 8.7. The leaf absorption of ^{14}C-glyphosate between the glyphosate-resistant populations (Populations A and P) and the glyphosate-susceptible population (SI) was not different at 48 and 72 HAT. The percentage of ^{14}C-glyphosate absorption for Populations SI, A and P was 27.4, 28.1, and 32.8%, respectively at 48 HAT (Table 8.8).

At 72 HAT, the amount of glyphosate absorption for Populations SI, A and P was 30.7, 32.6 and 36.2% of applied ^{14}C-glyphosate, respectively (Table 8.8).

Table 8.7 Average percentage of ^{14}C-glyphosate recovered (Equation 2) in three Italian ryegrass populations (A and P (glyphosate-resistant), and SI (glyphosate-susceptible)), 48 and 72 hours after treatment (HAT).

Population	% recovery of applied ^{14}C-glyphosate	
	48 HAT	72 HAT
SI (susceptible)	89.1	88.1
A (resistant)	91.5	89.6
P (resistant)	95.1	92.9
P values	0.23	0.65

Table 8.8 Absorption of ^{14}C-glyphosate (Equation 1) in three Italian ryegrass populations (A and P (glyphosate-resistant), and SI (glyphosate-susceptible)), 48 and 72 hours after treatment (HAT).

Population	^{14}C-glyphosate absorption (% of applied)	
	48 HAT	72 HAT
SI (susceptible)	27.4	30.7
A (resistant)	28.1	32.6
P (resistant)	32.8	36.2
P values	0.56	0.23

As with perennial ryegrass populations, although glyphosate absorption was similar in all three Italian ryegrass populations, significantly different patterns of ^{14}C-glyphosate translocation were observed between the glyphosate-resistant and susceptible populations of Italian ryegrass. Significantly less of the ^{14}C-glyphosate was translocated out of the treated leaf blade section to the rest of the plant in the glyphosate-resistant populations (Populations A and P) compared to Population SI (glyphosate-susceptible). At 48 HAT, 59 and 56% of the ^{14}C-glyphosate absorbed by the plant remained in the treated leaf blade of Populations A and P, respectively in contrast to 31% for Population SI (Table 8.9). At 48 HAT, a greater percentage of ^{14}C-radiolabelled glyphosate absorbed by the plant moved to the pseudostem region for Population SI (39%)

compared to Population A and P in which only 15 and 18% of the ^{14}C-glyphosate, respectively, was translocated to the pseudostem region.

No significant differences were recorded between any of the three populations of Italian ryegrass for the amount of ^{14}C-glyphosate movement into the untreated leaves and roots at 48 and 72 HAT (Table 8.9). However, a greater amount of ^{14}C-glyphosate moved out of the treated leaf blade in the susceptible population (SI) compared to the resistant ones (A and P). Almost half of the ^{14}C-glyphosate was retained in the treated leaf of Populations A and P whereas, in Population SI, over 70% of the ^{14}C-glyphosate was translocated out of the treated leaf at 72 HAT (Table 8.9). The movement of the ^{14}C-glyphosate to roots and untreated leaves was not significantly different between the three Italian ryegrass populations, while a greater percentage of the ^{14}C-glyphosate accumulated in the pseudostem of Population SI compared to Populations A and P (Table 8.9). Similarly to perennial ryegrass, a negligible amount of radioactivity was detected in the nutrient solution of each population and no significant differences were recorded between the three populations of Italian ryegrass at both times this was studied (Table 8.10). Glyphosate-resistant populations of Italian ryegrass (Populations A and P) showed a similar pattern of glyphosate translocation in both experiments.

Table 8.9 Distribution of ^{14}C-glyphosate in four plant sections (the treated leaf, the root, the pseudostem region and untreated leaves) in three Italian ryegrass populations (A and P (glyphosate-resistant), and SI (glyphosate-susceptible)), 48 and 72 hours after treatment (HAT).

Population	^{14}C-glyphosate distribution (% of absorbed)							
	Treated leaf		Untreated leaves		Pseudostem		Root	
	48	72	48	72	48	72	48	72
SI (susceptible)	31.3 **b**	27.4 **b**	15.6	11.3	38.9 **a**	47.8 **a**	14.2	13.5
A (resistant)	59.1 **a**	50.9 **a**	13.1	15.2	15.1 **b**	23.1 **b**	12.7	10.8
P (resistant)	56.1 **a**	51.6 **a**	13.4	11.2	18.4 **b**	28.4 **b**	12.1	8.8
P values	<0.001	<0.001	0.29	0.36	<0.001	<0.001	0.77	0.59

Mean values within each column followed by the same letters are not different at 5% probability according to Fisher`s protected tests.

Table 8.10 The amount of radioactivity detected in nutrient solution media for three Italian ryegrass populations (A and P (glyphosate-resistant), and SI (glyphosate-susceptible)), 48 and 72 hours after treatment (HAT).

Population	^{14}C-glyphosate (% of absorbed)	
	48 HAT	72 HAT
SI (susceptible)	0.23	0.30
A (resistant)	0.20	0.23
P (resistant)	0.19	0.25
P values	0.85	0.95

8.4 Discussion

Reduced glyphosate absorption and restricted herbicide translocation would confer glyphosate resistance to weed species and the level of resistance typically associated with this mechanism of resistance is higher than the target-site mechanism of resistance (Preston *et al.*, 2009b; Yu *et al.*, 2007). In our study, the amount of glyphosate absorption was similar for both glyphosate-resistant and susceptible populations and ranged between 28-40% for perennial ryegrass and 28-33% for Italian ryegrass in the studied time courses. Similarly, Perez-Jones *et al.* (2007) and Carvalho *et al.* (2012) recorded nearly identical levels of ^{14}C-glyphosate absorption for *Lolium multiflorum* and *Digitaria insularis*. Thus the absorption observed in the current experiments appeared normal and differences in glyphosate absorption were unlikely to be the reason for resistance.

Results from previous studies with other cases of glyphosate resistance have shown that glyphosate metabolism was not the mechanism of resistance in *Lolium multiflorum* (González-Torralva *et al.*, 2012). Likewise, glyphosate was not metabolised to any significant extent in either resistant or susceptible plants of *Lolium rigidum* investigated by Lorraine-Colwill *et al.* (2002). As enhanced glyphosate metabolism had not been previously reported as a mechanism for resistance in *Lolium* spp., it was assumed that the radiolabelled materials extracted from different parts of the plants in this study were still the intact ^{14}C-glyphosate molecules prior to the processing of samples.

However, the translocation of absorbed radiolabelled glyphosate was significantly different between glyphosate-resistant and susceptible populations in this study. Adequate movement of glyphosate to active growth points (meristematic tissues) is

crucial for glyphosate efficacy. Within the leaf sheath region of vegetative grasses is where the meristematic tissues responsible for leaf and stem elongation are located, and accumulation of glyphosate within the meristematic tissue would perturb its function and this would finally lead to growth cessation and plant death. Almost half of the absorbed radiolabeled glyphosate had translocated to the leaf sheath region at 48 and 72 HAT in glyphosate-susceptible populations (Populations SP of perennial ryegrass and Population SI of Italian ryegrass) while over half of the ^{14}C-glyphosate was retained in the treated leaf of glyphosate-resistant populations (Populations J and O of perennial ryegrass and Populations A and P of Italian ryegrass).

Although this study was not able to show whether the ^{14}C-glyphosate within the pseudostem was in the meristematic tissue (where it can has its effect) rather than just the leaf sheath tissue of the treated leaf, similar observations for the pseudostem have been made in other studies involving glyphosate resistance. Greater accumulation of glyphosate mostly within the pseudostem region was reported for susceptible *Lolium rigidum* plants whereas for resistant populations, glyphosate was mainly retained in the treated leaf (Adu-Yeboah *et al.*, 2014; Bostamam *et al.*, 2012; Perez-Jones *et al.*, 2007). Restricted herbicide translocation has been reported as the most common mechanism of resistance to glyphosate in Australia (Preston *et al.*, 2009b).

Restricted glyphosate movement in resistant plants was postulated to be due to the existence of a cellular pump which actively pumped glyphosate to apoplastic spaces in resistant plants (Lorraine-Colwill *et al.*, 2002). However, in a study on a glyphosate-resistant *Conyza canadensis* (Canadian fleabane) population, it was noted that the glyphosate-resistant plants were able to rapidly shift a large amount of absorbed glyphosate from the cytoplasm to vacuoles while in susceptible plants, rapid transportation into vacuoles was not recorded (Ge *et al.*, 2010). Also, a positive correlation between the levels of resistance to glyphosate and extent of vacuole sequestration of glyphosate in glyphosate-resistant *Lolium spp.* from different countries has been documented (Ge *et al.*, 2012).

Yuan *et al*. (2007) suggested that transporters associated with vacuolar membranes such as ATP-binding cassette (ABC) transporters might have roles in the vacuolar sequestration of glyphosate. Ge *et al*. (2014) has since provided evidence for the existence of a tonoplast transporter which actively shifts glyphosate into vacuoles in Canadian fleabane. Also, Ge *et al.* (2013) showed that glyphosate vacuolar

sequestration is not restricted to the source leaves and that other plant parts such as stem and root tissue are also capable of vacuolar sequestration. This suggests that the portion of glyphosate that is translocated from the source leaves to the other parts of the plant like stem and root (sink tissues) could additionally be sequestered within the vacuoles of these sink tissues in the glyphosate-resistant plants which possess the restricted herbicide translocation mechanism of resistance. Although some studies suggested that the restricted herbicide translocation could be due to the existence of a tonoplast pump, it is unknown if the same mechanism is involved in our populations. However this hypothesis was indirectly examined on one of the glyphosate resistant populations (Population J) in a study that is described in Chapter 10 of this thesis.

In this study, both glyphosate-resistant Italian ryegrass populations (Populations A and P) and glyphosate-resistant perennial ryegrass populations (Populations J and O) showed the restricted glyphosate translocation mechanism of resistance. However, Population O also contains the target-site mechanism of resistance at Pro-106 (Chapter 7). Having both restricted translocation and the target site mechanism (Pro-106 substitution, Chapter 7) could explain the greater level of glyphosate resistance in Population O (see Chapter 5) compared to the other glyphosate-resistant populations (Bostamam *et al.*, 2012).

8.5 Conclusions

The non-target site mechanism of glyphosate resistance (restricted herbicide translocation) was investigated for Italian ryegrass and perennial ryegrass glyphosate-resistant populations. Restricted herbicide translocation was observed for all studied populations with glyphosate resistance. It appears that restricted herbicide translocation has a role in resistance to glyphosate within the currently known New Zealand resistant populations of Italian ryegrass and perennial ryegrass.

Chapter 9

The response of glyphosate resistant Italian ryegrass and perennial ryegrass populations to other herbicides[1]

9.1 Introduction

It is necessary to use existing herbicides efficiently since no major herbicides with new modes of action have been commercialized for the last two decades and the number of available herbicides has decreased owing to tighter environmental and registration regulations (Beckie & Tardif, 2012). A serious problem when designing management programmes for herbicide-resistant weeds is the appearance of multiple and cross-resistance to herbicides (Preston & Mallory-Smith, 2001). The concepts of cross-resistance and multiple-resistance to herbicides were defined in Section 2.4.2. Development of cross-resistance and multiple-resistance enables weed species to survive a wide range of selective and non-selective herbicides which reduces the options available to manage resistant populations (Burnet *et al.*, 1991; Hall *et al.*, 1994). To develop a good herbicide resistance management strategy, it is necessary to determine if a herbicide-resistant weed has also become less sensitive to the other herbicides.

The herbicide exposure records of vineyards from where the glyphosate-resistant populations of Italian ryegrass and perennial ryegrass described in Chapter 5 were collected, showed that glufosinate has been applied alternately with glyphosate in all vineyards (Table 5.1, Chapter 5). Amitrole has also been used in winter in some of these vineyards (Table 5.1, Chapter 5).

Glufosinate is a non-selective, broad-spectrum, contact post-emergence herbicide (Vencill, 2002) used for control of weeds in vineyards and orchards. As with glyphosate, the mechanism of action in glufosinate involves the inhibition of amino acid production, but the target enzyme for glufosinate is glutamine synthetase which is responsible for the production of the amino acid, glutamine (Cobb & Reade, 2010). The

[1] Some of the material in this chapter has been published in:

Ghanizadeh H, Harrington KC and James TK (2015) Glyphosate-resistant *Lolium multiflorum* and *Lolium perenne* populations from New Zealand are also resistant to glufosinate and amitrole. Crop Protection. 78, 1-4.

inhibition of glutamine synthetase leads to a build-up of free ammonia which is toxic to plants and is considered to be the primary cause of plant mortality (Cobb & Reade, 2010). Several biological processes in plants such as protein and nucleotide production and photosynthesis activity could be adversely affected by higher levels of free ammonia (Devine *et al.*, 1993). To date, resistance to glufosinate has only been recorded as evolving within *Eleusine indica* (Jalaludin *et al.*, 2010; Seng *et al.*, 2010) and *Lolium multiflorum* (Avila-Garcia & Mallory-Smith, 2011).

Amitrole is a non-selective systemic herbicide which is able to control a broad spectrum of weeds (Vencill, 2002). It is mainly used in vineyards during winter in New Zealand as it must not be used once fruit formation has begun (Harrington *et al.*, 2014). Amitrole has been classified as a pigment biosynthesis inhibitor because it has been found to inhibit ξ-carotene desaturase and lycopene cyclase (Cobb & Reade, 2010), which are two important enzymes in the carotenoid biosynthesis pathway (Zhao *et al.*, 2014). This inhibition of carotenoid biosynthesis causes bleaching symptoms in treated plants (Ashtakala *et al.*, 1989). So far, amitrole resistance has been documented in *Poa annua*, *Lolium rigidum*, *Polygonum aviculare* and *Agrostis stolonifera* (Heap, 2015).

Haloxyfop and fluazifop are two important herbicides for controlling grasses selectively within broad-leaved crops (Ashton & Crafts, 1980). They belong to a group of herbicides called ACCase inhibitors (Cobb & Reade, 2010) because they inhibit the activity of acetyl-coenzyme A carboxylase (ACCase), the enzyme which catalyses the first step in the synthesis of fatty acids (Powles & Yu, 2010). There are two types of ACCase in plant: a) cytosolic b) plastidic (Sasaki & Nagano, 2004). Also, ACCase can be found in two forms a) homomeric b) heteromeric (Sasaki & Nagano, 2004). In many grasses, the plastidic form is homomeric and sensitive to ACCase inhibitors whereas in broad-leaf weeds the plastidic form of ACCase is heteromeric and insensitive to ACCase inhibitors (Powles & Yu, 2010). Haloxyfop and fluazifop are another two chemicals with potential for managing glyphosate-resistant Italian ryegrass and perennial ryegrass if their activity has not been affected by the glyphosate resistance. However, haloxyfop has not yet been registered for used in vineyards in New Zealand.

The study described in this chapter was conducted to determine whether glyphosate-resistant Italian and perennial ryegrass were still sensitive to glufosinate, amitrole, haloxyfop and fluazifop. The results of this study were needed for developing strategies for managing glyphosate resistance in New Zealand.

9.2 Preliminary experiment

9.2.1 Materials and methods

9.2.1.1 Plant material and herbicide treatements

Clones of plants from each population of Italian ryegrass (A and P) and perennial ryegrass (J, N and O) that had proven to be resistant to glyphosate (Chapter 5) were used. Also plants of Italian ryegrass (Population SI) and perennial ryegrass (Population SP) known to be susceptible to glyphosate (Chapter 5) were included for comparison.

Table 9.1 The rates of herbicide and adjuvant applied in the preliminary cross-resistance experiment.

Herbicide (g ai L^{-1})	Commercial name	Rate g ha^{-1}(L ha^{-1})	Surfactant or adjuvant (w/w)
Glyphosate (360 g L^{-1}) (isopropylamine salt)	Roundup Pro	720 (2.0)	Pulse Penetrant (0.1%)
		1440 (4.0)	Pulse Penetrant (0.1%)
glufosinate (200 g L^{-1}) (ammonium salt)	Buster	1000 (5.0)	Contact (0.1%)
		2000 (10.0)	Contact (0.1%)
haloxyfop (100 g L^{-1}) (R-isomer as methyl ester)	Ignite	200 (2.0)	Uptake spraying oil (0.1%)
		400 (4.0)	Uptake spraying oil (0.1%)
fluazifop (150 g L^{-1}) (P-butyl ester)	Fusilade Forte	375 (2.5)	Uptake spraying oil (0.1%)
		750 (5.0)	Uptake spraying oil (0.1%)
amitrole (400 g L^{-1}) + ammonium thiocyanate (100 g L^{-1}) + sulphamic acid (230 g L^{-1})	Zelam Activated Amitrole	2400 (6.0)	Contact (0.1%)
		4800 (12.0)	Contact (0.1%)

The plant material for each population was divided into plantlets and potted on 20 August 2013 using a similar method to that described in Section 5.2.1. On 29 October 2013, when the potted plants had an average of 10 tillers per plants and all the plants had an average height of 10 cm, they were sprayed with the five herbicides shown in

Table 9.1. Five plants were sprayed with each rate and in total 10 plants were sprayed with each herbicide. Five plants from each population were also used as an untreated control.

Each herbicide was applied using a laboratory track sprayer calibrated to deliver 235 L ha^{-1} of herbicide solution at 200 kPa. All herbicides were applied with their recommended surfactants or adjuvants (Table 9.1). Plant materials were kept in an unheated glasshouse with an automated over-head irrigation system. The daily maximum and minimum temperatures in the 2 weeks following application averaged 22.2°C and 10.3°C respectively. Plants were removed from all pots at ground level 8 weeks after application, then dried and weighed in order to evaluate the effect of herbicides. The effect of each herbicide treatment was calculated as a percentage of the dry weight of untreated plants for that population.

9.2.1.2 Statistical analyses

The preliminary experiment was conducted using a randomised complete block design with five replicates of each rate. A one-way analysis of variance (ANOVA) was performed on the data of the preliminary experiment to determine if the differences in plant biomass between treatments were significant at 5% probability.

9.2.2 Preliminary experiment results

9.2.2.1 Italian ryegrass

Figure 9.1 illustrates the effect of five different herbicides applied at two rates on plant growth relative to untreated plants. Plants of Populations A and P again proved to be resistant to glyphosate, shown by differences in biomass of these populations compared with the susceptible population following treatment with glyphosate. At glyphosate rates of 720 and 1440 g ae ha^{-1}, the dry weight of the susceptible population (SI) was 13 and 7% of the untreated plants respectively, while both glyphosate-resistant populations had average dry weights of 50 and 35% of untreated plants at glyphosate rates of 720 and 1440 g ae ha^{-1} respectively.

Approximately 3 days after treatment with glufosinate, the treated plants of all populations turned yellow, then became necrotic by 2 weeks after treatment for all plants treated with both glufosinate rates, 1000 and 2000 g ai ha^{-1}. By 20 days after treatment, the plants of Populations A and P treated with the lower rate started

recovering and grew new green healthy leaf material, whereas the plants of Population SI treated with this rate remained completely necrotic until the end of the study (8 weeks after treatment). Thus Populations A and P had less reduction in dry weight compared to the susceptible population (SI) at 1000 g ai ha^{-1} of glufosinate. At 2000 g ai ha^{-1} of glufosinate, plants of Populations A and P also began regrowing following initial necrosis, though recovery only began 3-4 weeks after application. As with the lower rate, plants of Population SI treated with 2000 g ha^{-1} of glufosinate remained dead till the end of this assay.

Each of the three Italian ryegrass populations responded differently to amitrole (Figure 9.1). By 1 week after application of amitrole, the leaves of all three populations of Italian ryegrass had become bleached with some necrosis at both rates of amitrole assessed (2400 and 4800 g ai ha^{-1}). However, at 2400 g ai ha^{-1} of amitrole, plants of Population A were only bleached from the middle of the leaves to the leaf tips, whereas leaves of Populations SI and P had bleaching down their complete lamina length. By 25 days after treatment, plants from Population A had started producing new green leaves with no signs of bleaching, whereas plants from Populations SI and P continued to produce bleached leaves. The amitrole rate of 2400 g ai ha^{-1} reduced the dry weight of Populations SI and P to 20% and 31% respectively of untreated plant weight, whereas there was significantly less impact of this application rate on Population A, and the dry weight of Population A was 48% of the untreated because the plants of Population A started recovering 3 weeks after herbicide application.

Bleaching was also observed on the plants of Population A at 4800 g ai ha^{-1} of amitrole initially, with the entire lamina of treated leaves becoming bleached, in contrast to only parts of leaves when treated with 2400 g ai ha^{-1} of amitrole. However, four out of five plants of Population A treated with 4800 g ai ha^{-1} of amitrole started producing new green leaves with no signs of bleaching 3-4 weeks after treatment, while no plants from Populations SI and P recovered from this higher rate of amitrole by the end of the study (8 weeks after application).

In contrast to the results obtained for glufosinate and amitrole, there were no differences evident between the three populations in sensitivity to haloxyfop or fluazifop (Figure 9.1). A significant reduction in dry weight relative to untreated plants was recorded for the plants of glyphosate resistant and susceptible populations treated at both rates of

haloxyfop and fluazifop, and there were no differences between populations in how severely they were affected (Figure 9.1).

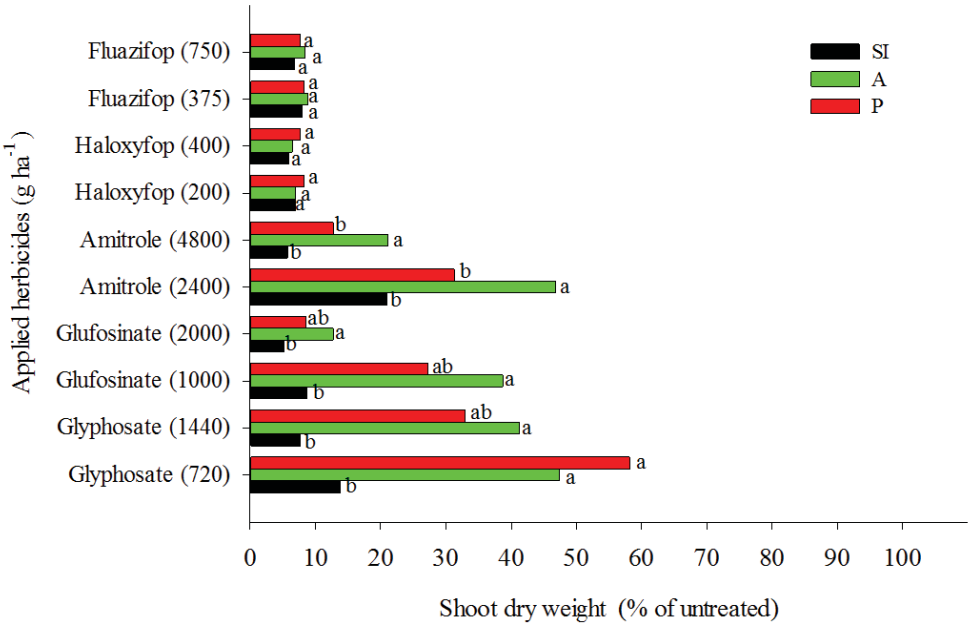

Figure 9.1 The reduction in plant shoot dry weight compared with untreated plants of three Italian ryegrass populations, A and P (from Marlborough), and SP (from Manawatu), 8 weeks after application of five different herbicides at two rates. For each herbicide rate, mean values showing the same letter are not statistically different at 5% probability according to Fisher`s protected tests.

9.2.2.2 Perennial ryegrass

Figure 9.2 illustrates the effects of the five herbicides at two rates on four perennial ryegrass populations in the preliminary experiment. Both rates of glyphosate tested (720 and 1440 g ae ha^{-1}) reduced shoot dry weight of the susceptible population (SP) significantly more than the plants from Populations J, N and O (Figure 9.2). Population O again appeared more resistant to glyphosate than Populations J and N, with only a 20% reduction in plant dry weight relative to untreated plants recorded for Population O at the glyphosate rate of 1440 g ae ha^{-1}, compared to 45 and 48% reductions in dry weight relative to untreated control for Populations J and N respectively, at this rate.

As occurred with the glyphosate-resistant Italian ryegrass populations, plants of glyphosate-resistant populations of perennial ryegrass had also survived 1000 g ai ha^{-1} of glufosinate to the end of the study (8 weeks after application), although the plants of all populations were initially affected within a few days of glufosinate application. At

1000 g ai ha^{-1} of glufosinate, the plants of Populations J, N and O did not become as necrotic as those of Population SP, and the plants of Populations J, N and O grew new green material 3 weeks after glufosinate application. The dry weight of plants of Populations SP treated with 1000 g ai ha^{-1} of glufosinate reduced by 80% relative to untreated control, whereas there was significantly less impact of this rate on Populations J, N and O which suffered only 46, 37 and 30% reductions respectively in plant dry weight relative to untreated plants (Figure 9.2). By the end of this study (8 weeks after application), only one out of five plants of Population SP treated with 1000 g ai ha^{-1} of glufosinate produced any new green leaf material while all plants of Populations J, N and O treated with 1000 g ai ha^{-1} of glufosinate produced new green material. The higher rate of glufosinate (2000 g ai ha^{-1}) also initially caused severe damage to all populations, and although Populations J, N and O did eventually recover, it took about 3-4 weeks before regrowth occurred. The susceptible plants never recovered and were totally necrotic when harvested 8 weeks after application.

Following application of the low rate of amitrole rate (2400 g ai ha^{-1}), plants from Populations J and O behaved much the same as the plants of Population A of Italian ryegrass described in Section 9.2.2.1. Plants of Populations J and O suffered some bleaching initially but had started recovering approximately 3 weeks after amitrole application in contrast to plants from Populations SP and N which continued to produce bleached leaves. For Populations J and O, no signs of bleaching were observed in new leaves of all treated plants from 4 weeks after amitrole application. Similarly, at 4800 g ai ha^{-1} of amitrole, the plants of Populations SP and N were severely damaged by the end of the study (8 weeks after application), whereas the plants of Populations J and O started recovering at 3-4 weeks after this treatment, though plant dry matter was lower than untreated plants (Figure 9.2). However, although the dry weight reduction was significantly less in Population J than for Populations SP and N at the lower amitrole rate, the plants of Population O appeared to be even more resistant compared to Population J, which was most evident at 4800 g ai ha^{-1} of amitrole (Figure 9.2).

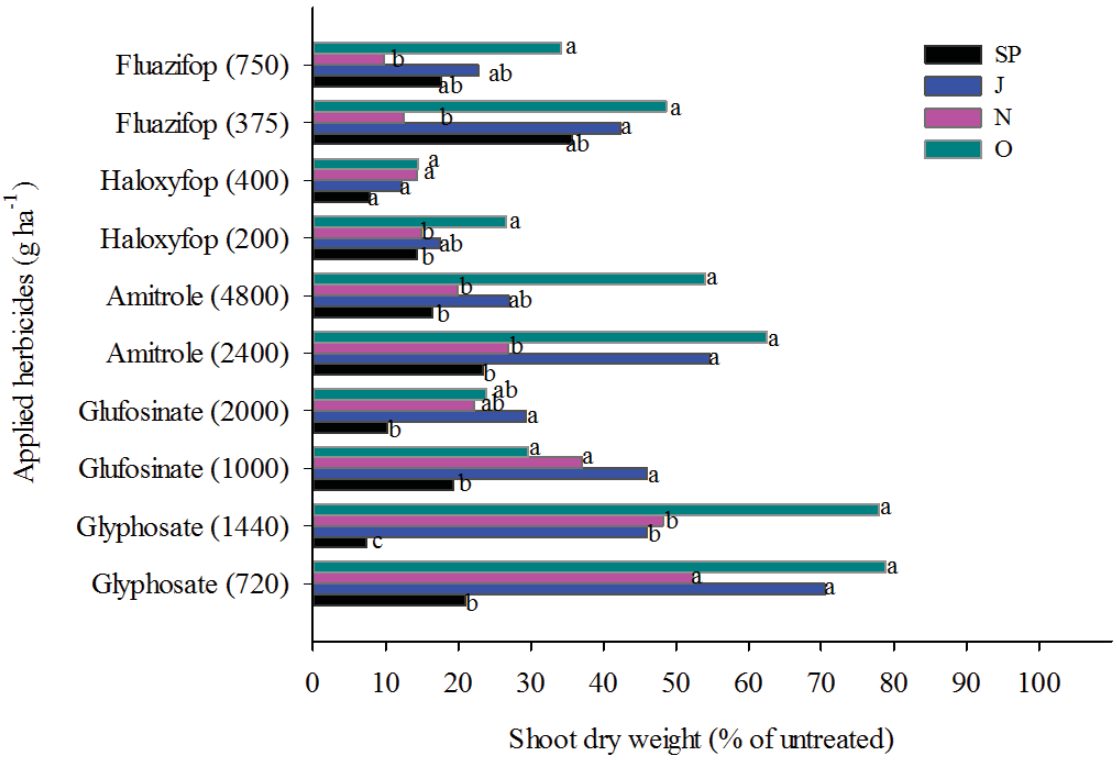

Figure 9.2 Dry weights obtained of the plants of four perennial ryegrass populations, J and O (from Marlborough), N (from Nelson), and SP (from Manawatu), when sprayed with five different herbicides at two rates. For each herbicide rate, mean values showing the same letter are not statistically different at 5% probability according to Fisher`s protected tests.

Haloxyfop at both rates reduced the biomass production of all perennial ryegrass populations in a similar manner to each other (Figure 9.2). Symptoms of haloxyfop damage such as chlorosis appeared about 2 weeks after application and continued to worsen. Although the dry weight reduction in Population O was slightly different from the other populations at the lower rate of haloxyfop (200 g ai ha^{-1}), at the higher rate of haloxyfop (400 g ai ha^{-1}), there were no differences between populations in how severely they were affected (Figure 9.2).

The lower rate of fluazifop (375 g ai ha^{-1}) had less of an effect on the biomass of plants from most of the perennial ryegrass populations when harvested after 8 weeks than the haloxyfop treatments or the higher rate of fluazifop, except for the plants of Population N which were affected severely (Figure 9.2). At 750 g ai ha^{-1} of fluazifop, although all populations were affected more than by the lower rate, plants had started recovering 6 weeks after herbicide application for all populations except Population N.

9.3 Dose-response experiments

9.3.1 First dose-response test for amitrole and glufosinate

In the next experiment, the populations that survived both rates of amitrole and glufosinate in the preliminary experiment were evaluated further. Unfortunately, only three of the glyphosate-resistant populations had sufficient plant material available for this study. The plants used for this study were from Population A (Italian ryegrass), and Populations J and O (both perennial ryegrass) that had all been shown to be resistant to amitrole and glufosinate in the preliminary assay. These resistant populations were compared against the same susceptible populations used in Section 9.2.1.1 (*ie* SI for Italian ryegrass, SP for perennial ryegrass). Plants of each population were established using the method described in Section 5.2.1 and kept in an unheated glasshouse (the daily maximum and minimum temperatures averaged 23.9°C and 12.2°C respectively) with an automated over-head irrigation system. After 4 weeks, when plants had an average of 7-8 tillers and all the plants had an average height of 10 cm, they were sprayed with different rates of amitrole and glufosinate (Table 9.2). Each herbicide was applied using a laboratory track sprayer calibrated to deliver 227 L ha^{-1} of spray solution at 200 kPa. The daily maximum and minimum temperatures in the 2 weeks following application averaged 24.1°C and 13.2°C respectively. All herbicides were applied with their recommended surfactants or adjuvants (Table 9.2) on 25 January 2014. All plants were harvested at ground-level 8 weeks after herbicide application, and the harvested materials were oven-dried at 70°C for 48 hours and weighed. There were 10 replicates (one pot per replicate) of each rate.

Table 9.2 The rates of herbicides that were used for each resistant (Population A of Italian ryegrass, and Population J and O of perennial ryegrass) and susceptible (Population SI of Italian ryegrass, and Population SP of perennial ryegrass) population.

Herbicides	Surfactant (w/w)	Characteristics	Herbicides rates (g ha^{-1})						
Glufosinate	Nonionic surfactant Contact (0.1%)	Resistant	0	125	250	500	1000	2000	4000
		Susceptible	0	62.5	125	250	500	1000	2000
Amitrole	Nonionic surfactant Contact (0.1%)	Resistant	0	600	1200	2400	4800	9600	-
		Susceptible	0	300	600	1200	2400	4800	-

9.3.2 Second dose-response test for amitrole and glufosinate

The second dose-response test for amitrole and glufosinate was conducted using the similar method and herbicide rates described for the first dose-response test. The plants for the second dose-response test were kept in an unheated glasshouse for 4 weeks (the daily maximum and minimum temperatures averaged 17.4°C and 8.3°C respectively) with an automated over-head irrigation system. Plants were sprayed with different rates of amitrole and glufosinate (Table 9.2) on 16 May 2014. The daily maximum and minimum temperatures in the 2 weeks following application of glufosinate averaged 16.2°C and 7.5°C respectively. The laboratory track sprayer was calibrated to deliver 230 L ha^{-1} of spray solution at 200 kPa, and plants were harvested 8 weeks after application as with the earlier experiments.

9.3.3 Third dose-response test for amitrole

The plants of perennial ryegrass Populations J and O did not show the same response to amitrole applications in the second amitrole dose-response experiment as in the first dose-response experiment, whereas results for amitrole susceptibility of Italian ryegrass Population A were consistent with those from the earlier experiment (see results). The effect of cold temperatures on the amitrole mechanism of resistance for perennial ryegrass populations was suspected to be responsible. Therefore, a third dose-response test for amitrole was conducted on 28 August 2014 using the same method and amitrole rates described for the first dose-response test. The plants used for this study were from Population A (Italian ryegrass), and Populations J and O (perennial ryegrass). Also, the

plants of known amitrole susceptible Population SI (Italian ryegrass) and Population SP (perennial ryegrass) were used for comparison. The plants of all populations were kept in a heated glasshouse (the daily maximum and minimum temperatures averaged 22.8°C and 16.5°C) and hand watered every day. The daily maximum and minimum temperatures in the 2 weeks following spraying of amitrole averaged 23.1°C and 16.1°C. The laboratory track sprayer was calibrated to deliver 223 L ha^{-1} of spray solution at 200 kPa, and plants were harvested 8 weeks after application as with the earlier experiments.

9.3.4 Statistical analyses

Dose-response experiments were conducted using a randomised complete block design with 10 replicates of each rate. The basic assumptions for ANOVA/regression models were checked using similar methods described in Section 3.2.4. Dose response curves for the other two experiments were fitted as described in Section 3.2.4.

9.4 Dose-response experiments results

9.4.1 Dose-response tests for amitrole in Italian ryegrass and perennial ryegrass

9.4.1.1 Italian ryegrass

The three-parameter logistic model described well the changes in plant biomass at increasing amitrole rates (R^2>0.97) (Table 9.3). The herbicide rates calculated to cause a 50% reduction in growth (GR_{50}) for the Italian ryegrass populations in first, second and third dose-response experiments are shown in Table 9.3, and the dose response curves for all three experiments are shown in Figures 9.3a-c. Plant shoot growth for the Manawatu population (Population SI) was significantly reduced by 2400 g ai ha^{-1} of amitrole, measured as 17, 19 and 23% of the untreated control in the first, second and third experiments, respectively (Figures 9.3a-c).

Thus the GR_{50} values for Population A were significantly higher than those of Population SI in all three experiments (Table 9.3). By comparing the GR_{50} of Population A against the GR_{50} of the Manawatu population for the first experiment, it was estimated that Population A was 2.9 times more resistant to amitrole than Population SI. In the second and third dose-response experiments, it was estimated that Population A was 4.6 and 3.3 times more resistant to amitrole respectively than Population SI (Table 9.3).

Table 9.3 Parameters estimated for the nonlinear regression analysis of amitrole dose-response experiments of two Italian ryegrass populations (A (glyphosate-resistant), and SI (glyphosate-susceptible)) at 8 weeks after application of amitrole in the first, second and third experiments.

Population	d	b	GR_{50} (g ha^{-1}) *	R/S GR_{50} ratio	R^2
First dose-response experiment					
Manawatu SI	100.7	1.5	703 **b**	-	0.99
Marlborough A	102.1	1.2	2027 **a**	2.9	0.97
P value	0.89	0.92	0.0006		
Second dose-response experiment					
Manawatu SI	100.3	1.1	662 **b**	-	0.99
Marlborough A	103.5	1.3	3089 **a**	4.6	0.97
P value	0.65	0.42	0.002		
Third dose-response experiment					
Manawatu SI	99.5	1.5	1216 **b**	-	0.99
Marlborough A	101.2	1.4	3996 **a**	3.3	0.97
P value	0.90	0.10	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ai ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

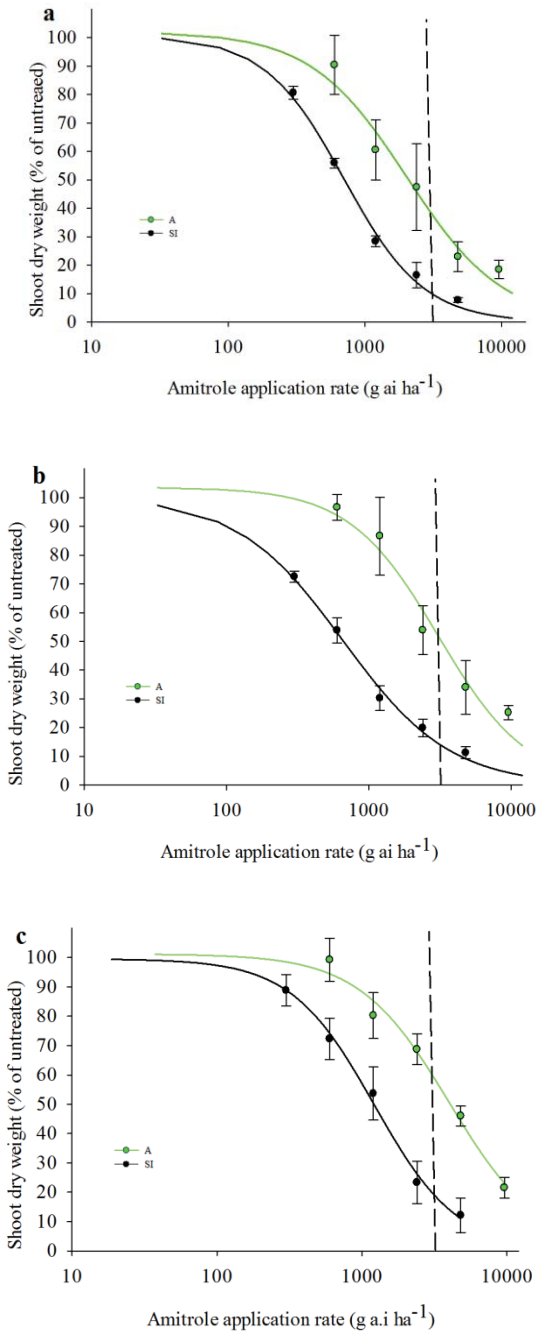

Figure 9.3 Fitted dose response curves (on a logarithmic dose scale) for two populations of Italian ryegrass (SI from Manawatu pasture and A from Marlborough vineyards) for amitrole estimated using shoot dry weight in the (**a**) first dose-response experiment, (**b**) second dose-response experiment and (**c**) third dose-response experiment at 8 weeks after application of amitrole. Vertical bars represent ± standard error of the mean. The vertical dashed line indicates the recommended field application rate (3200 g ai ha^{-1}) (Young, 2015).

Figure 9.4 Effect of different rates of amitrole on Italian ryegrass (a) Population SI (amitrole-susceptible) from Manawatu and (b) Population A (amitrole-resistant) from Marlborough in the first dose-response experiment.

9.4.1.2 Perennial ryegrass

The three-parameter logistic model also provided a good fit to the perennial ryegrass dose response data for amitrole as indicated by coefficients of determination (R^2) values greater than 0.95 (Table 9.4). The dose response curves for all three experiments are shown in Figures 9.5a-c. In the first dose-response experiment, the shoot growth of plants of Population SP was significantly lower than those of Population J and O at comparable rates of amitrole (Figures 9.5a).

However, when amitrole-resistant plants of Populations J and O were treated with amitrole in May, the treated plants showed a different pattern of sensitivity to amitrole from those of the first dose-response experiment (Figure 9.5b). In contrast to the first dose-response experiment, the plants of Populations J and O did not recover in the second dose-response experiment. They kept producing bleached plant material for a few weeks after amitrole application then died, thus resulted in a significant reduction in dry weight for the plants of Population J and O in the second dose-response experiment (Figure 9.5b).

Although the shoot growth of Population SP was significantly less than growth of Populations J and O at comparable rates of amitrole in the first experiment, in the second dose-response experiment, Populations J and O were as sensitive as Population SP to amitrole.

Table 9.4 Parameters estimated for the nonlinear regression analysis of amitrole dose-response experiments of three perennial ryegrass populations (J and O (glyphosate-resistant), and SP (glyphosate-susceptible)) at 8 weeks after application of amitrole in the three experiments.

Population	d	b	GR_{50} (g ha^{-1})*	R/S GR_{50} ratio	R^2
First dose-response experiment					
Manawatu SP	100.3	1.1	523 **c**	-	0.98
Marlborough J	98.6	1.0	2629 **b**	5.0	0.98
Marlborough O	102.9	0.9	6867 **a**	13.1	0.95
P value	0.85	0.78	<0.0001		
Second dose-response experiment					
Manawatu SP	99.9	0.9	556	-	0.99
Marlborough J	100.0	0.7	696	1.2	0.99
Marlborough O	99.9	0.8	593	1.1	0.99
P value	0.99	0.14	0.25		
Third dose-response experiment					
Manawatu SP	101.1	1.2	755 **c**	-	0.99
Marlborough J	100.1	1.2	3765 **b**	4.9	0.99
Marlborough O	103.7	1.5	7157 **a**	9.5	0.97
P value	0.86	0.40	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ai ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

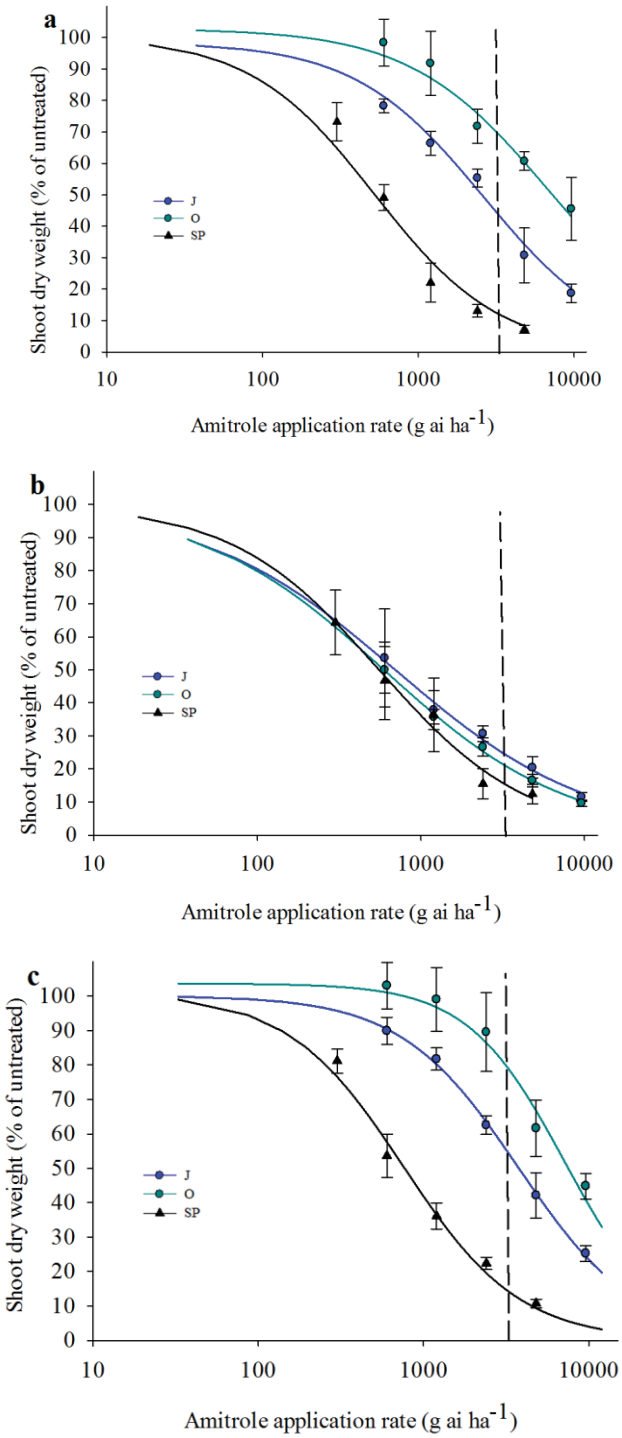

Figure 9.5 Fitted dose response curves (on a logarithmic dose scale) for three populations of perennial ryegrass (SP from Manawatu pasture, J and O from Marlborough vineyards) for amitrole estimated using shoot dry weight in the (**a**) first dose-response experiment, (**b**) second dose-response experiment and (**c**) third dose-response experiment at 8 weeks after application of amitrole. Vertical bars represent ± standard error of the mean. The vertical dashed line indicates the recommended field application rate (3200 g ai ha^{-1}) (Young, 2015).

However in the third dose-response experiment, which was conducted in a heated glasshouse, similar results to those for the first dose-response experiment were recorded for Populations J and O. The shoot growth rate of plants of Population J and O was significantly greater than that of Population SP at comparable rates (Figure 9.5c).

The GR_{50} values for the Populations J and O were significantly higher than those of Population SP in the first and third dose-response experiments (Table 9.4). However, the GR_{50} values of Populations J and O were not significantly different from that of Population SP in the second dose-response experiment. By comparing the GR_{50} values of Populations J and O against the one of the Manawatu population for the first dose-response test, it was estimated that Populations J and O were 5.0 and 13.1 times more resistant to amitrole than Population SP. Results were very similar for the third dose-response test in which Populations J and O were estimated to be 4.9 and 9.5 times more resistant to amitrole than Population SP (Table 9.4). These results were consistent with the findings from the preliminary test, with both dose-response experiments showing that Population O was more resistant to amitrole than Population J.

Figure 9.6 Effect of different rates of amitrole on perennial ryegrass (a) Population SP (amitrole-susceptible) from Manawatu and (b) Population O (amitrole-resistant) from Marlborough in the first dose-response experiment (applied in January).

Figure 9.7 Effect of different rates of amitrole on perennial ryegrass (a) Population J (amitrole-resistant) from Marlborough and (b) Population O (amitrole-resistant) from Marlborough in the second dose-response experiment (treated in May).

9.4.2 Dose-response tests for glufosinate resistance in Italian ryegrass and perennial ryegrass

9.4.2.1 Italian ryegrass

An analysis of variance of goodness of fit and also the coefficients of determination revealed that the three-parameter logistic model described well the changes in plant dry weight of both populations as glufosinate rates increased (Table 9.5). A significant difference in plant biomass relative to untreated control was recorded for Population SI compared with Population A at different rates of glufosinate in both experiments (Figures 9.8a and b). The shoot dry weight of plants of Population SI was 12 and 20% of the untreated control at 1000 g ai ha^{-1} of glufosinate in the first and second experiments, respectively (Figures 9.8a and b). In contrast, similar levels of reduction in shoot dry weight for the Marlborough population (Population A) only occurred once glufosinate rates rose to 4000 g ai ha^{-1} (Figures 9.8a and b). The glufosinate GR_{50} value for Population A was estimated to be 691 g ai ha^{-1} for the first experiment, whereas for the second experiment conducted under cooler conditions it was estimated to be 1065 g ai ha^{-1} (Table 9.5). Thus the GR_{50} values for the Population A were 3.9 and 5.5 times higher than those of the Population SI in the first and second experiments respectively (Table 9.5).

Table 9.5 Parameters estimated for the nonlinear regression analysis of glufosinate dose-response experiments of two Italian ryegrass populations (A (glyphosate-resistant), and SI (glyphosate-susceptible)) at 8 weeks after application of glufosinate in the first and second experiments.

Population	d	b	GR_{50} (g ha^{-1})*	R/S GR_{50} ratio	R^2
First dose-response experiment					
Manawatu SI	100.7	1.2	178 **b**	-	0.99
Marlborough A	103.6	1.3	691 **a**	3.9	0.99
P value	0.61	0.22	<0.0001		
Second dose-response experiment					
Manawatu SI	101.1	1.0	193 **b**	-	0.99
Marlborough A	102.1	1.4	1065 **a**	5.5	0.98
P value	0.76	0.1	0.0002		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ai ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

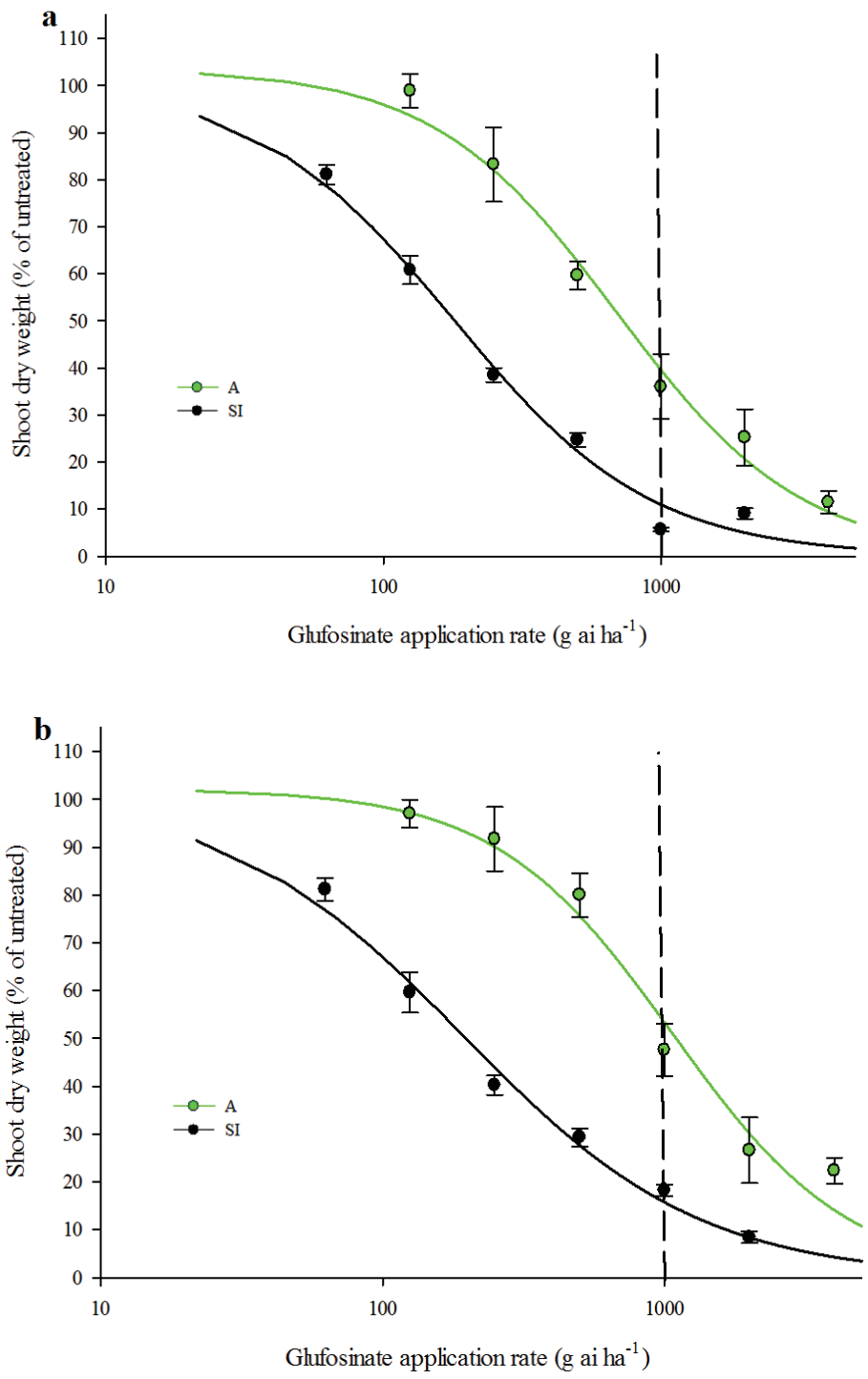

Figure 9.8 Fitted dose response curves (on a logarithmic dose scale) for two populations of Italian ryegrass (SI from Manawatu pasture and A from Marlborough vineyards) for glufosinate using shoot dry weight in (**a**) the first dose-response experiment and (**b**) second dose-response experiment at 8 weeks after application of glufosinate. Vertical bars represent ± standard error of the mean. The vertical dashed line indicates the recommended field application rate (1000 g ai ha^{-1}) (Young, 2015).

Figure 9.9 Effect of different rates of glufosinate on Italian ryegrass (a) Population SI (glufosinate-susceptible) from Manawatu and (b) Population A (glufosinate-resistant) from Marlborough in the first dose-response experiment.

9.4.4.2 Perennial ryegrass

As indicated by R^2 values greater than 0.97 (Table 9.6), once again the three-parameter logistic model provided a good fit to data for perennial ryegrass at different rates of glufosinate used in the first and second dose-response experiments. A different response

was recorded between the Manawatu population (SI) and Marlborough populations (J and O) at different rates of glufosinate in both experiments (Figures 9.10a and b). A significantly greater reduction in shoot dry weight of Populations SI relative to Populations J and O at each rate of glufosinate (Figures 9.10a and b) resulted in greater GR_{50} values for Populations J and O (Table 9.6).

The GR_{50} values of Populations J and O were 4.5 and 3.8 times greater, respectively, than that of Population SP for the first experiment. In the second experiment, Population J and O had 4.6 and 6.2 times higher GR_{50} values respectively than Population SP (Table 9.6). In the second experiment under cooler conditions, the level of resistance to glufosinate for Population O was almost double that estimated for the first dose-response experiment, whereas a similar level of resistance to glufosinate was recorded for Population J in both experiments.

Table 9.6 Parameters estimated for the nonlinear regression analysis of glufosinate dose-response experiments of three perennial ryegrass populations (J and O (glyphosate-resistant), and SP (glyphosate-susceptible)) at 8 weeks after application of glufosinate in the first and second experiments.

Population	d	b	GR_{50} (g ha^{-1})*	R/S GR_{50} ratio	R^2
First dose-response experiment					
Manawatu SP	100.9	1.1	164 **b**	-	0.98
Marlborough J	100.4	1.2	745 **a**	4.5	0.98
Marlborough O	99.3	1.9	630 **a**	3.8	0.99
P value	0.97	0.27	0.0005		
Second dose-response experiment					
Manawatu SP	101.7	1.1	206 **b**	-	0.99
Marlborough J	103.3	1.0	949 **a**	4.6	0.97
Marlborough O	103.4	1.0	1279 **a**	6.2	0.98
P value	0.94	0.27	0.0002		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ai ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

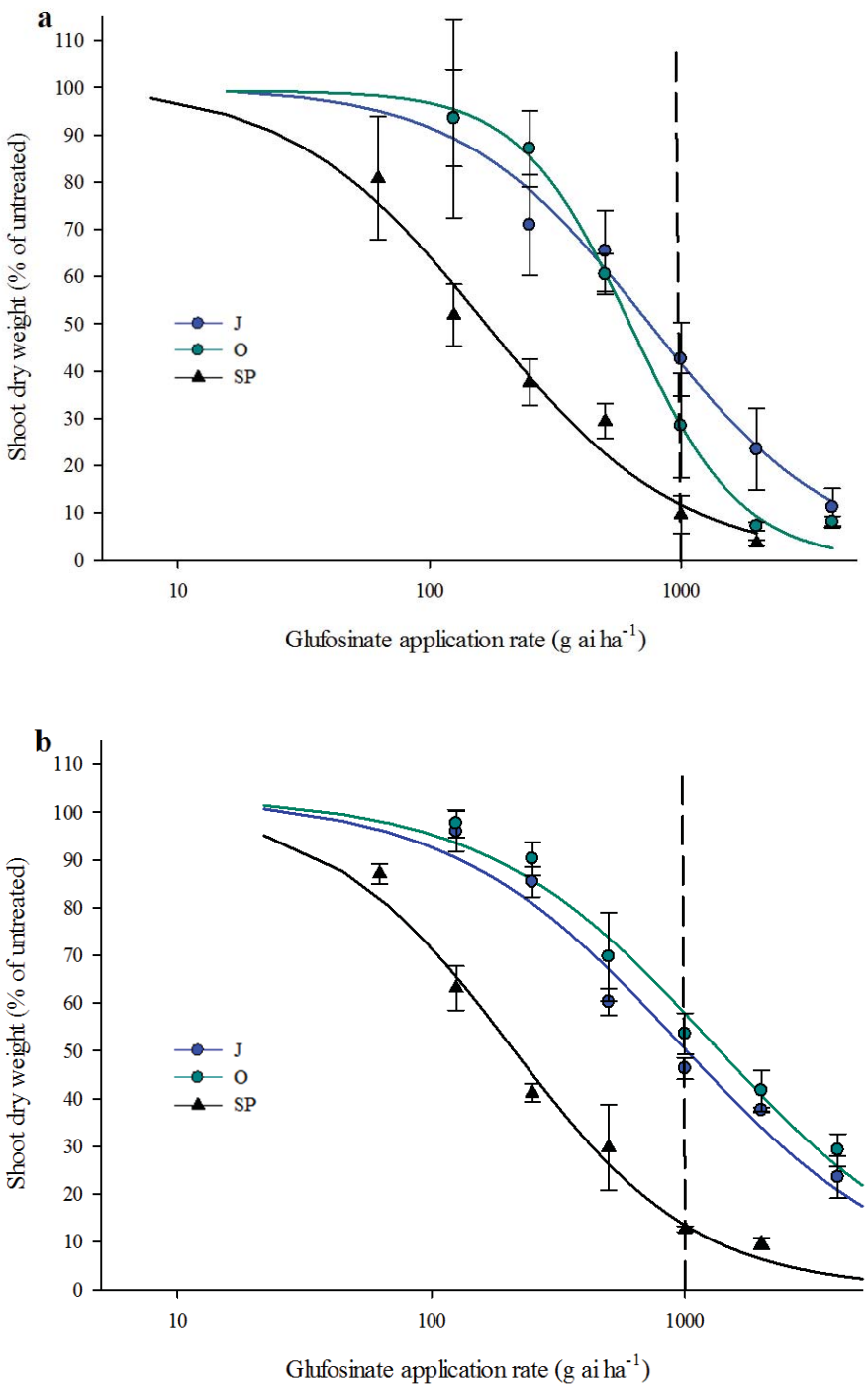

Figure 9.10 Fitted dose response curves (on a logarithmic dose scale) for three populations of perennial ryegrass (SP from Manawatu pasture, J and O from Marlborough vineyards) for glufosinate using shoot dry weight in (**a**) the first dose-response experiment and (**b**) second dose-response experiment at 8 weeks after application of glufosinate. Vertical bars represent ± standard error of the mean. The vertical dashed line indicates the recommended field application rate (1000 g ai ha^{-1}) (Young, 2015).

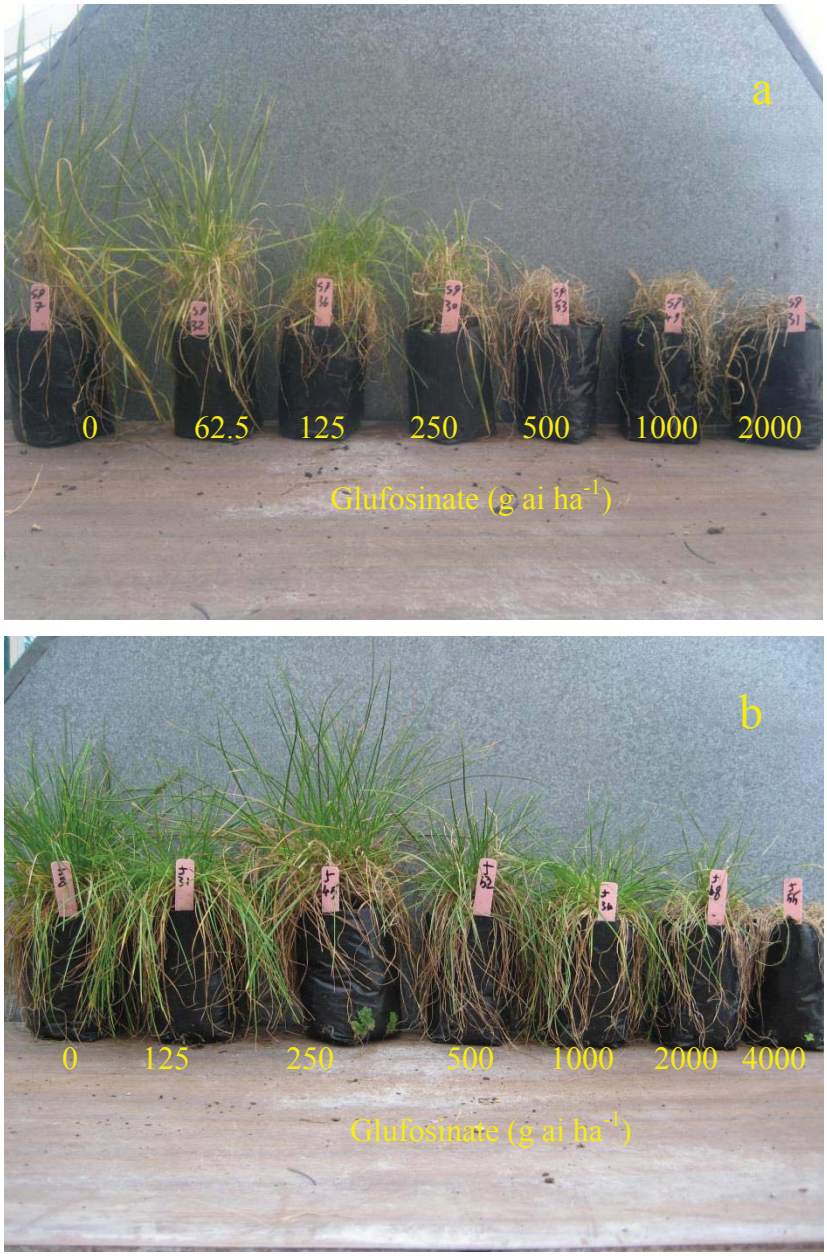

Figure 9.11 Effect of different rates of glufosinate on perennial ryegrass (a) Population SP (glufosinate-susceptible) from Manawatu and (b) Population J (glufosinate-resistant) from Marlborough in the first dose-response experiment.

9.5 Discussion

The responses of different glyphosate-resistant Italian ryegrass and perennial ryegrass populations to glufosinate, amitrole, haloxyfop and fluazifop were investigated in a preliminary experiment using two rates of each herbicide. All glyphosate-resistant populations were able to recover from both 1000 and 2000 g ai ha^{-1} of glufosinate whereas plants of Populations SI and SP (susceptible to glyphosate) were completely killed by both these rates of glufosinate.

Furthermore, in contrast to the glyphosate-susceptible populations of Italian ryegrass (SI) and perennial ryegrass (SP) which did not survive either rate of amitrole (2400 and 4800 g ai ha^{-1}) in the preliminary experiment, Populations A (Italian ryegrass) and Populations J and O (perennial ryegrass) survived both 2400 and 4800 g ai ha^{-1} of amitrole. However, two of the glyphosate-resistant populations (P (Italian ryegrass) and N (perennial ryegrass)) were susceptible. Based on the information provided by the growers of vineyards from where Populations N and P were collected, there had apparently been less use of amitrole by these growers than in the other vineyards (Chapter 5, Table 5.1).

Both haloxyfop and fluazifop appear to be good potential chemical alternatives for managing glyphosate-resistant Italian ryegrass as no cross-resistance was evident to these herbicides. However, only haloxyfop provided good control of glyphosate-resistant perennial ryegrass populations. At 200 g ai ha^{-1} of haloxyfop, Population O did have slightly higher dry matter than the other populations, but at 400 g of ai ha^{-1} of haloxyfop, a similar response was recorded for all perennial ryegrass populations and the dry weight of the treated plants were all significantly reduced compared to untreated plants. The slight differences recorded among perennial ryegrass populations were probably due to natural variations among populations to haloxyfop rather than any sign of resistance starting to develop.

Fluazifop appeared to be less effective for all the perennial ryegrass populations, including the glyphosate-sensitive population. It has been reported that although perennial ryegrass is sensitive to fluazifop as young seedlings (2-4 leaf stage), it becomes tolerant to fluazifop with increasing plant age (Warren *et al.*, 1989). In our study, plants of each perennial ryegrass population had an average of 10 tillers, so their age probably explains the observed tolerance to fluazifop in the perennial ryegrass.

Harker and Dekker (1988) showed that growth stage has an impact on fluazifop distribution in *Agropyron repens* and younger plants translocate more fluazifop from treated leaf to other parts than older plants. Unfortunately, due to time constraints, the response of glyphosate-resistant Italian ryegrass and perennial ryegrass to clethodim, another ACCase inhibitor which is thought to be effective against glyphosate-resistant Italian ryegrass and perennial ryegrass (Harrington *et al.*, 2014), was not studied here and this needs investigating.

The results of the dose-response experiments confirmed that all of the glyphosate-resistant populations of *Lolium multiflorum* and *Lolium perenne* from New Zealand vineyards were also resistant to glufosinate. As indicated on the label of glufosinate (Buster, 200 g ai L^{-1}), the registered rate for ryegrasses is 1000-1500 g ai ha^{-1}, with the lower rate (1000 g ai ha^{-1}) recommended for use when weeds are young, succulent and actively growing, or when the weed population is sparse and good coverage and penetration is possible. The higher rate (1500 g ai ha^{-1}) should be used when ryegrasses are mature or the population is dense (Young, 2015). The results of the preliminary experiment and dose-response experiments showed that glyphosate-resistant populations of *Lolium multiflorum* and *Lolium perenne* survived 2000 g ai ha^{-1} of glufosinate and only a rate of 4000 g ai ha^{-1} was able to provide complete control of resistant populations. This means over 20 L ha^{-1} of Buster would be needed in order to obtain good control of resistant plants of Italian ryegrass and perennial ryegrass, costing at least \$660 ha^{-1} (SprayShop, 2015), which would be unacceptable to growers.

Glufosinate-resistance has also been documented for *Eleusine indica* from Malaysia (Jalaludin *et al.*, 2010; Seng *et al.*, 2010) and glyphosate-resistant populations of *Lolium multiflorum* from USA (Avila-Garcia & Mallory-Smith, 2011). The present study showed that Populations A (*Lolium multiflorum*), J and O (*Lolium perenne*) had 4 to 6-fold levels of resistance to glufosinate, based on R/S GR_{50} ratio. Lower levels of glufosinate-resistance were reported by Avila-Garcia and Mallory-Smith (2011) (2- to 3-fold) and Seng *et al*. (2010) (3.4-fold) based on the R/S GR_{50} ratio. In vineyards where the resistant populations in the present study were collected, detailed records of glufosinate application were unable to be obtained, but it was thought the herbicide had been used once annually in most seasons close to the time of fruit harvest. Although Avila-Garcia and Mallory-Smith (2011) reported cross-resistance to glufosinate in glyphosate-resistant *Lolium multiflorum* with almost no glufosinate application history,

it is difficult in the present work to determine whether glufosinate resistance was simply due to cross-resistance from glyphosate resistance or if prior exposure to glufosinate had contributed.

In the present study, greater levels of resistance to glufosinate were recorded for Populations A (Italian ryegrass) and O (perennial ryegrass) when the glufosinate dose-response experiment was conducted under cooler conditions (the second dose-response experiment), whereas a similar level of resistance was recorded for Population J (perennial ryegrass) in both dose-response experiments. Poor control of *Raphanus raphanistrum* was recorded by Kumaratilake & Preston (2005) when glufosinate was applied under cooler temperatures (5/10 (night/day)) than warmer temperatures (15/20 and 20/25 (night/day)). They also noted that basipetal translocation of glufosinate was lower at cold temperatures than warmer temperatures, and they inferred that the poor control of *Raphanus raphanistrum* by glufosinate at low temperatures was likely due to reduced accumulation of glufosinate in the meristematic regions of the plant.

While a slightly greater GR_{50} value for Population J was recorded in the second dose-response experiment (cooler conditions), this did not result in a greater level of glufosinate resistance for this population because the lower efficacy for glufosinate under the cooler conditions also resulted in a higher GR_{50} value for the susceptible Population SP in the second dose-response experiment compared to the first dose-response experiment. However, in contrast to Population J, the cooler conditions resulted in greater levels of resistance to glufosinate for Populations A and O. One hypothesis is that Population J has evolved a different mechanism of resistance to glufosinate which is not temperature dependant, whereas the efficacy of the mechanism of resistance to glufosinate in Populations A and O might be temperature dependant. However, due to the lack of information on glufosinate mechanisms of resistance in weed species, it is difficult to draw a definite conclusion regarding the observed differences between the glufosinate resistant populations under warm and cool conditions in the present study.

Although the mechanisms of glufosinate-resistance in *Lolium multiflorum* and *Lolium perenne* here are unknown, a target site mechanism (target site modification) of glufosinate-resistance at position 171 in the glutamine synthetase gene (GS2) has been documented in a *Lolium multiflorum* population (Avila-Garcia *et al.*, 2012). This glufosinate-resistant *Lolium multiflorum* population was not glyphosate-resistant as this

population was controlled with the recommended field rate of glyphosate (Avila-Garcia *et al.*, 2012). However, a non-target site mechanism of resistance (restricted herbicide translocation) was suggested to be responsible for the other studied glufosinate-resistant populations of *Lolium multiflorum* as it had restricted glyphosate translocation (Avila-Garcia & Mallory-Smith, 2011). As discussed in Chapter 8, restricted glyphosate translocation has been shown to be present in Population A of *Lolium multiflorum* and Populations J and O of *Lolium perenne*.

Although differential glufosinate absorption and translocation appear not to have been investigated for documented cases of weed species in which resistance to glufosinate has evolved, differential glufosinate absorption and translocation have been observed for weed species that are poorly controlled by this herbicide. Steckel *et al*. (1997) noted that *Chenopodium album* is more tolerant than three other weed species that were studied, and it was found to absorb less glufosinate and also translocate less of the herbicide that was absorbed compared with the three more sensitive species (Steckel *et al.*, 1997). In another two weed species not controlled well by glufosinate, *Digitaria sanguinalis* and *Eleusine indica*, both restricted glufosinate translocation and enhanced glufosinate metabolism were found (Everman *et al.*, 2009). The regrowth of resistant plants following necrosis of treated foliage in our experiments was also reported for glufosinate-resistant *Eleusine indica* by Jalaludin *et al.* (2015). These observations might suggest that resistant plants have evolved a non-target site mechanism of resistance.

In addition to glufosinate-resistance, this study has also shown that Populations A, J and O were resistant to amitrole. The registered rate for amitrole (Zelam Activated Amitrole, 400 g L^{-1}) application in vineyards is 8-10 L ha^{-1} (Young, 2015). It is recommended to use the lower rate (3200 g ai ha^{-1}) for small weeds and the higher rate (4000 g ai ha^{-1}) for large weeds (Young, 2015). According to the results of the preliminary experiment and both dose-response experiments, plants of two amitrole-resistant populations (A (Italian ryegrass) and J (perennial ryegrass)) were able to survive 4800 g ai ha^{-1} (12 L ha^{-1}) of amitrole and one perennial ryegrass population (O) even survived 9600 g ai ha^{-1} (24 L ha^{-1}) of amitrole.

In one of the amitrole dose-response experiments, the amitrole-resistant perennial ryegrass populations (J and O) became sensitive to amitrole when the experiment was conducted in winter. In contrast, the amitrole-resistant Italian ryegrass population (A)

was resistant to amitrole in both winter and summer experiments. Italian ryegrass has faster initial growth during establishment, and a broader tolerance of temperatures than perennial ryegrass (Hill & Pearson, 1985). Winter growth rates of Italian ryegrass are generally higher than those for perennial ryegrass (Lamp *et al.*, 1990). One possible reason for the apparent disappearance of resistance in winter is that lower temperatures might have adversely affected the growth rate in perennial ryegrass, so they may not have had sufficient time to recover from the initial damage caused by the amitrole when plant material was harvested 56 days after treatment. In contrast, 56 days may have been sufficient time for the plants of Italian ryegrass to recover due to their better growth rates in winter.

Another likely reason for the different responses to amitrole at different temperatures is that the mechanism of resistance was suppressed by cold temperatures in amitrole-resistant perennial ryegrass. A longer persistence for amitrole activity was recorded in tomato when amitrole was applied under cool conditions (Muzik, 1965) and the author inferred that the persistence of amitrole activity under cooler conditions might have been due to lower detoxification of amitrole by plants at cooler temperatures (Muzik, 1965). Similarly, in the present study, the amitrole treated plants of Populations J and O under cooler conditions, persistently produced bleached leaf for 6 weeks and finally, except those plants which were treated with 600 g ai ha^{-1} of amitrole (the lowest amitrole rate which was used in the second dose-response experiment for Populations J and O), the plants of other treatments were severely necrotic and unable to regrow at harvest time (56 DAT) (Figure 9.7). This might suggest that 56 days might have been long enough for plants to regrow but because the plants were severely damaged, they were not going to regrow. Assuming the amitrole resistance in perennial ryegrass is due to an enhance amitrole metabolism hence (Muzik, 1965), it is likely that under cooler conditions amitrole degradation does not occur fast enough due to the impact of cold temperatures on catalytic activity of the enzyme(s) involve in amitrole degradation.

Different responses to low temperatures between amitrole-resistant Italian ryegrass and perennial ryegrass populations might suggest the evolution of different mechanisms of resistance to amitrole in these species. Unfortunately, an attempt to further investigate the effect of cooler temperatures on the response of amitrole-resistant populations of Italian ryegrass and perennial ryegrass was not successful as the plants kept in a cool temperature room became severely diseased (damping off), and the plants could not be

saved. Amitrole is mainly being used in winter in vineyards in New Zealand (Harrington *et al.*, 2014). If the mechanisms of resistance to amitrole in perennial ryegrass can be suppressed under cool conditions typical of winter in New Zealand, this has practical implications for weed management in vineyards. The effect of temperature on amitrole resistance in weeds has not been reported and needs further investigation.

Populations A and J were both almost five times more resistant to amitrole compared to the susceptible populations. However, Population O (*Lolium perenne*) was even more resistant than Populations A (*Lolium multiflorum*) and J (*Lolium perenne*), and it was estimated to be up to 13.5 times more resistant than Population SP. To date, resistance to amitrole has been confirmed in only a few weed species (Heap, 2015). The level of amitrole-resistance in a population of *Lolium rigidum* from Australia was over 10-fold (based on R/S LD_{50} ratio) compared to a susceptible biotype (Burnet *et al.*, 1991).

Population O came from a vineyard where amitrole had been used for almost 20 years, compared with approximately 6 years for Population A and 9 years for Population J (Chapter 5, Table 5.1). All of the populations had been sprayed with amitrole once annually and the populations were all from the same part of New Zealand but different vineyards. It has been suggested that differences in herbicide exposure history might account for variations observed in the level of resistance to herbicides among populations of a weed species (Bourdôt *et al.*, 1990). The longer history of amitrole applications may have allowed selection for several mechanisms of resistance to amitrole simultaneously in Population O.

The mechanism(s) of amitrole-resistance are not clearly understood (Burnet *et al.*, 1991). In our experiments, the plants of both resistant and susceptible populations treated with amitrole displayed herbicidal symptoms like bleaching within 7-14 DAT, however symptoms were shorter-lived for resistant plants and new emerging leaves of resistant plants did not display bleaching. Also, the plants of amitrole-resistant populations completely recovered by the end of the experiments (56 DAT) whereas the plants of susceptible populations continued producing new bleached leaf material for several weeks and finally died. The same observations have been reported by Burnet *et al.* (1991) who suggested that the mechanism of resistance to amitrole could be either herbicide compartmentalisation or metabolism.

Glyphosate, amitrole and glufosinate are the three main post-emergence herbicides used in New Zealand vineyards (Dastgheib & Frampton, 2000), especially now that paraquat is no longer permitted to be used by growers who abide by restrictions within the Sustainable Winegrowers New Zealand scheme. Thus apart from the unique nature of this multiple-resistance to glyphosate, amitrole and glufosinate, it also creates major weed control problems for the growers concerned. This study has reported the development of resistance in glyphosate-resistant Italian ryegrass and perennial ryegrass populations to amitrole and glufosinate each with different modes of action from glyphosate. Evolution of resistance to herbicides from several different modes of action (multiple-resistance) in a weed species has been reported from several countries (Heap, 2015; Yu *et al.*, 2007) and recently it was reported that a population of *Eleusine indica* was resistant to four different herbicide groups (glyphosate, paraquat, glufosinate and ACCase inhibitors) (Jalaludin *et al.*, 2015). The mechanisms of amitrole resistance and glufosinate in *Lolium multiflorum* and *Lolium perenne* need investigating in order to provide a better understanding of the biochemical and physiological basis involved in the evolution of resistance to these herbicides. Also, the possible link between the mechanism of resistance to glyphosate (restricted herbicide translocation) and either amitrole or glufosinate resistance in Italian ryegrass and perennial ryegrass needs investigating.

9.6 Conclusions

In this study it was shown that all of the glyphosate-resistant Italian ryegrass and perennial ryegrass populations were resistant to glufosinate and some were even resistant to amitrole too. This is a big concern as glufosinate and amitrole are two important alternative chemicals for glyphosate resistance management. Although all the studied glyphosate-resistant populations were resistant to glufosinate however, only three populations (Population A (Italian ryegrass), J and O (perennial ryegrass)) proved to be resistant to amitrole. However, although not assessed well enough in the present study, it is possible that the mechanism of resistance to amitrole in perennial ryegrass could be suppressed under cool conditions.

The results of this study also showed that the ACCase inhibiting herbicides, haloxyfop and fluazifop, could be recommended as chemical alternatives for managing glyphosate resistance in Italian ryegrass. However, fluazifop might not be suitable for existing populations of perennial ryegrass due to its poor activity on this species but haloxyfop

provided good control of glyphosate-resistant perennial ryegrass. Also, haloxyfop is not registered for use in vineyards, thus it can't be used under the rules of the New Zealand Winegrowers. The response of glyphosate-resistant Italian ryegrass and perennial ryegrass to another ACCase inhibitor, clethodim, needs investigating.

Chapter 10

Effect of cold temperature on glyphosate resistance in perennial ryegrass[1]

10.1 Introduction

The restricted herbicide translocation mechanism has been found to be associated with resistance to glyphosate in two perennial ryegrass populations from New Zealand, although one of the populations had both the enzyme modification at Pro-106 and the restricted herbicide translocation (Chapters 7 and 8). The restricted glyphosate translocation could involve more sequestration of glyphosate in vacuoles within biotypes resistant to this herbicide compared to susceptible ones (Ge *et al.*, 2010). Studies suggest that a unidirectional active transporter in tonoplasts probably assists vacuolar sequestration of glyphosate in resistant weeds with restricted herbicide translocation (Sammons & Gaines, 2014).

An interesting aspect of glyphosate vacuole sequestration is that Ge *et al*. (2011) have found it to be temperature dependant in *Conyza canadensis*, and so the restricted herbicide translocation mechanism of resistance does not operate efficiently at sub-optimal (cold) temperatures. Similarly, it has been shown that glyphosate resistance in *Sorghum halepense* and *Lolium rigidum* caused by the restricted herbicide translocation mechanism is also suppressed at cooler temperatures (Vila-Aiub *et al.*, 2013). Only weed species with the vacuolar sequestration mechanism of resistance appear to become sensitive to glyphosate at cool temperatures, whereas those weeds with the target site mutation at Pro-106 remain resistant to glyphosate when treated at sub-optimal temperatures (Sammons & Gaines, 2014).

Although glyphosate translocation was shown in Chapter 8 to be restricted in resistant perennial ryegrass from New Zealand vineyards, sequestration within vacuoles was not investigated. But if this glyphosate resistance was found to be reduced under cool conditions, this would provide some indirect evidence for vacuolar sequestration being

[1] The material in this chapter has been accepted for publication in:

Ghanizadeh H, Harrington KC and James TK (2015) Glyphosate-resistant population of *Lolium perenne* loses resistance at winter temperatures. New Zealand Journal of Agricultural Research (in press).

involved. It would also provide a strategy for controlling these resistant plants within vineyards. The objective of the work described in this chapter was to determine whether glyphosate-resistant perennial ryegrass with restricted herbicide translocation becomes less resistant when treated under cooler conditions.

10.2 Materials and methods

10.2.1 Dose-response experiments

Conventional dose-response studies were conducted using a glyphosate-resistant population of perennial ryegrass from a Marlborough vineyard (Population J) shown in Chapter 8 to have restricted herbicide translocation, to determine the magnitude of resistance compared with a susceptible population under a variety of conditions. Plants of a perennial ryegrass (cv. Trojan) population (Population SP) were obtained from grazed pastures at Massey University in Manawatu for comparison as they were unlikely to have been exposed to glyphosate in the past, and have previously been shown to be glyphosate-susceptible (Chapter 5). All plants were established using the technique described in Chapter 5. The plants were then left to develop in a heated glasshouse (the daily maximum and minimum temperatures averaged 24.1°C and 17.1°C) at Massey University and irrigated daily. When plants from each population had an average of 7-8 tillers, they were allocated to one of three groups. One group remained in the heated glasshouse which was covered in shade-cloth to give comparable light intensities to the other two environments. The average light intensity measured over several days between 11 am and 2 pm was 150±10 μmol m^{-2} s^{-1} in the heated glasshouse. The second group was shifted on 17 July 2014 to a growth chamber kept at a constant cool temperature of 8°C with a 10-h photoperiod and 100±2 μmol m^{-2} s^{-1} light intensity. The third group was shifted to an unheated shadehouse on the same day as the second group to grow in winter conditions, with a light intensity of 120±5 μmol m^{-2} s^{-1}.

The relocated plants were left in their new environments for one week before spraying with glyphosate to allow their physiology to adapt to the reduced temperatures. Plants of all three groups were then sprayed with several rates of glyphosate (Roundup Pro 360, an isopropylamine salt) using a laboratory track sprayer calibrated to deliver 249 L ha^{-1} of spray solution at 200 kPa on 24 July 2014 (Experiment 1). Both populations from each environment received rates of 0, 80, 160, 320, 640 and 1280 g ae ha^{-1}. All

herbicide treatments contained 0.1% organosilicone surfactant (Pulse Penetrant). After glyphosate treatment, plants were immediately put back into their allocated environments. The average daily temperature in the 2 weeks following application (with the daily maximum and minimum temperatures in brackets) was 20.1°C (25.6°C to 17.6°C) for the heated glasshouse, 9.1°C (16.2°C to 4.7°C) for the unheated shadehouse, and 8.6°C (10.9°C to 6.7°C) for the cool growth room. The foliage of all plants was removed from pots at ground level 10 weeks after treatment and dry weight of plant material was determined for each. The effect of the herbicide treatments was calculated as a percentage of the dry weight of untreated plants for that population and temperature regimes.

This experiment was then repeated except that only the heated glasshouse and the cool growth chamber were used as this experiment started on 29 October 2014 (Experiment 2). The daily average temperatures in the 2 weeks following application (with the daily maximum and minimum temperatures in brackets) was 20.3°C (26.9°C to 14.8°C) for the heated glasshouse and 9.7°C (11.8°C to 7.4°C) for the cool growth room.

10.2.2 Statistical analysis

Both experiments were conducted using a randomised complete block design with eight replicates of each rate. The basic assumptions for regression models were checked using similar methods described in Section 3.2.4. The dose response curves were fitted as described in Section 3.2.4. A three-way ANOVA was conducted using IBM SPSS v.20 to compare the effects of temperature, glyphosate resistance and glyphosate rates on biomass production of tested populations. The means were separated using Fisher`s protected tests (Experiment 1) or Student`s t-tests (Experiment 2) at 5% probability. A one-way ANOVA was performed to compare parameters estimated from the dose-response tests between the two perennial ryegrass populations using the method described in Section 3.2.4.

10.3 Results

The values of coefficient of determination (R^2) (Table 10.1) revealed that the three parameter model provided a good fit of the data for both populations regardless of temperature conditions. Figures 10.1 and 10.2 illustrate the effect of glyphosate application on the glyphosate-resistant Population (J) and glyphosate-susceptible Population (SP) at different temperatures in Experiments 1 and 2. In both experiments,

the untreated plants of both populations remained healthy until the end of the experiment for all growing conditions, thus plant health was not adversely affected by the lower temperatures.

However, in both experiments, significant differences in the response to glyphosate were recorded for Population J at different temperatures. In Experiment 1, when plants were grown in the heated glasshouse (ca. 20.1°C), the glyphosate rate that caused 50% reduction in growth (GR_{50}) for Population J was 749 g ae ha^{-1}, whereas the GR_{50} for this population dropped significantly to 154 g ae ha^{-1} when plants were kept in the cool growth room (ca. 9.1°C) and to 84 g ae ha^{-1} in the unheated shadehouse (ca. 8.6°C) (Table 10.1).

In contrast, there were no significant differences in GR_{50} values for Population SP for all three temperature regimes, with GR_{50} values of 147, 173 and 64 g ae ha^{-1} when plants were kept in the heated glasshouse, cool growth room and unheated shadehouse, respectively (Table 10.1). The results were very similar in Experiment 2 with GR_{50} values for Population J of 940 and 170 g ae ha^{-1} when plants were kept in the heated glasshouse (20.3°C) and the cool growth room (below 9.7°C) respectively, while the GR_{50} values for Population SP were 203 and 244 in the warm and cool conditions, respectively (Table 10.1).

Table 10.1 Parameters estimated for the nonlinear regression analysis of glyphosate dose-response experiments of two perennial ryegrass populations (J (glyphosate-resistant), and SP (glyphosate-susceptible)) treated at three different temperature regimes at 8 weeks after application of glyphosate in the first and second experiments.

	d	b	GR_{50} (g ha^{-1})*	R^2
Experiment 1				
Population J (glyphosate-resistant)				
Heated glasshouse	100.1	1.7	749 **a**	0.98
Cool growth room	101.5	1.3	154 **b**	0.97
Unheated shadehouse	100.1	0.8	84 **b**	0.99
P value	0.97	0.06	<0.0001	
Population SP(glyphosate-susceptible)				
Heated glasshouse	100.5	1.1	147	0.95
Cool growth room	102.0	1.1	173	0.96
Unheated shadehouse	100.1	0.8	64	0.99
P value	0.98	0.63	0.28	
Experiment 2				
Population J (glyphosate-resistant)				
Heated glasshouse	99.4	1.5	940 **a**	0.99
Cool growth room	101.1	1.0	170 **b**	0.98
P value	0.80	0.1	<0.0001	
Population SP(glyphosate-susceptible)				
Heated glasshouse	101.8	1.4	203	0.96
Cool growth room	100.1	1.2	244	0.99
P value	0.85	0.39	0.31	

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ae ha^{-1}) required to reduce dry weight by 50% and R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

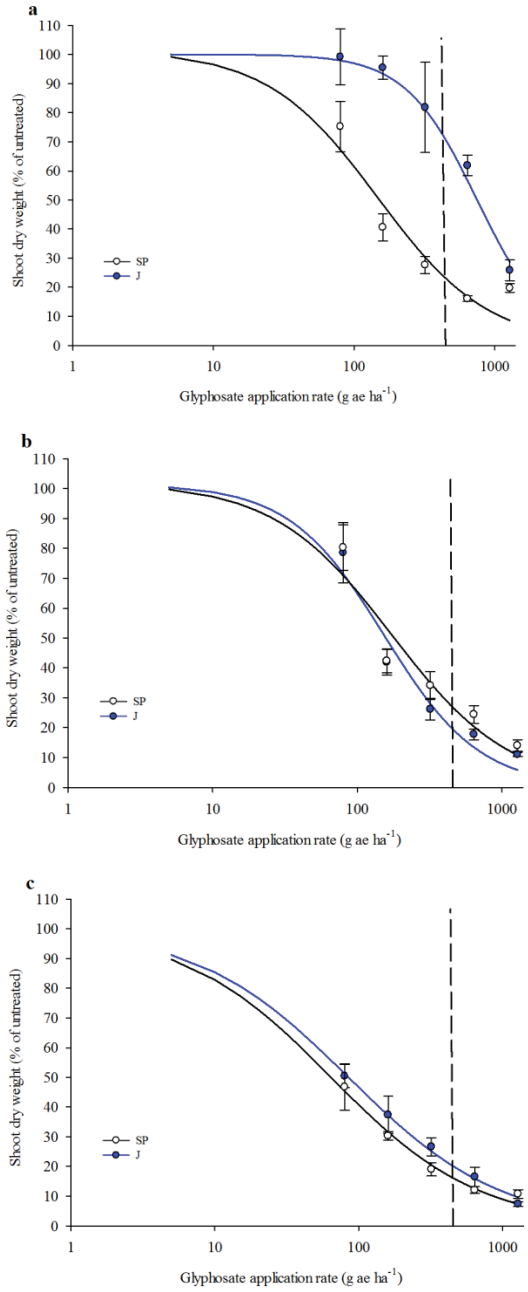

Figure 10.1 Fitted dose response curves (on a logarithmic dose scale) for two populations of perennial ryegrass in the Experiment 1 (SP (glyphosate-susceptible) from Manawatu pasture and J (glyphosate-resistant) from Marlborough vineyards) for the effect of glyphosate on shoot dry weight in (**a**) a heated glasshouse (20.1°C), (**b**) a cool growth room (below 9.1°C) and (**c**) unheated shadehouse in winter (8.6°C) at 10 weeks after application of glyphosate. Vertical bars represent ± standard errors of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

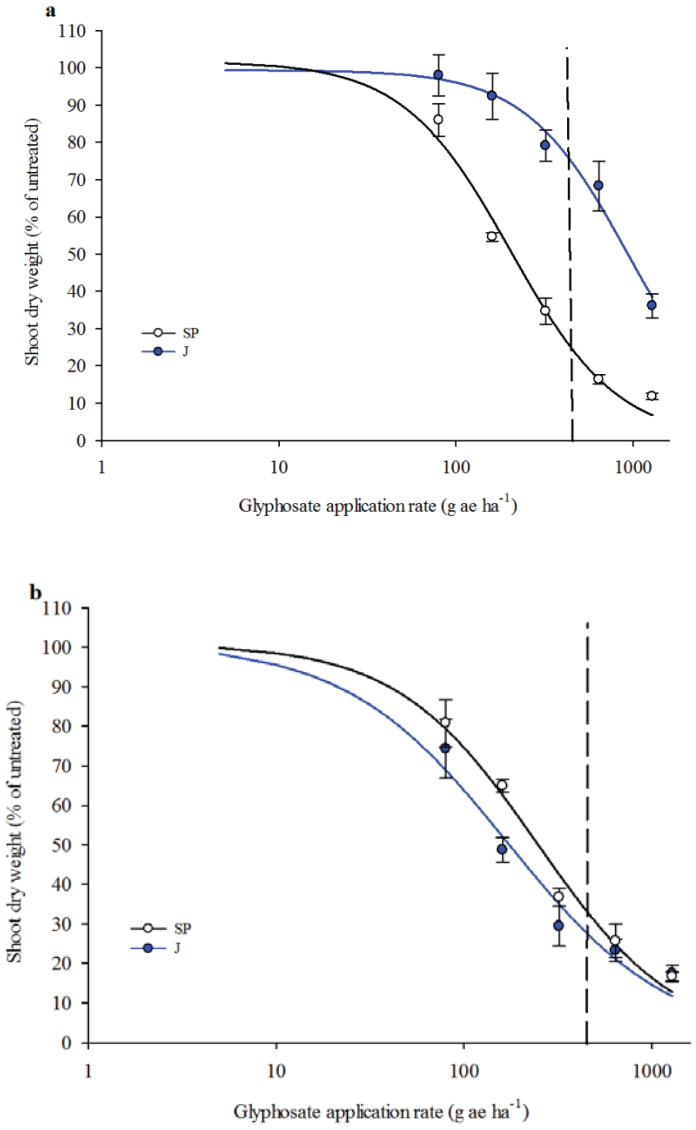

Figure 10.2 Fitted dose response curves (on logarithmic dose scale) for two populations of perennial ryegrass in the Experiment 2 (SP (glyphosate-susceptible) from Manawatu pasture and J (glyphosate-resistant) from Marlborough vineyards) for the effect of glyphosate on shoot dry weight in (**a**) a heated glasshouse (20.3°C) and (**b**) a cool growth room (below 9.7°C) at 10 weeks after application of glyphosate. Vertical bars represent ± standard errors of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

The results of a three-way analysis of variance for Experiments 1 and 2 (Table 10.2) also showed that temperature had a significant impact on the glyphosate resistance of population J ($P<0.0001$). A comparison of the means of dry weight attained by the plants of Populations J and SP at each rate of glyphosate under different conditions in Experiments 1 and 2 is shown in Figures 10.3 and 10.4. For each rate of glyphosate tested, plant biomass of treated plants of Population J in the cooler conditions was significantly different from those treated under warm conditions (Figures 10.3a and b). However, almost no significant differences in plant biomass were observed between treated plants of Population SP at every rate of glyphosate regardless of growing conditions (Figures 10.4a and b).

Table 10.2 A three-way ANOVA of the effect of glyphosate resistance, glyphosate rates and temperatures on dry weight of two populations of perennial ryegrass (J (glyphosate-resistant), and SP (glyphosate-susceptible)) in Experiments 1 and 2.

Experiment 1					
Source	*DF	Sum of Squares	Mean of Square	F-value	P-value
Corrected model	35	317774.9	9079.3	27.5	<0.0001
Intercept	1	697656.1	697656.1	2115.2	<0.0001
Glyphosate-resistance (R)	1	7541.9	7541.9	22.9	<0.0001
Glyphosate rate (G)	5	242752.4	48550.5	147.2	<0.0001
Temperature regime (T)	2	27654.9	13827.5	41.9	<0.0001
R*G	5	4805.2	961.1	2.9	0.014
R*T	2	15762.4	7881.2	23.9	<0.0001
G*T	10	11640.4	1164.0	3.5	<0.0001
R*G*T	10	7617.6	761.8	2.3	0.013
Error	252	83116.1	329.8		
Total	288	1098547.2			
Corrected Total	287	400891.1			
Experiment 2					
Source	DF	Sum of Squares	Mean of Square	F-value	P-value
Corrected model	23	190965.7	8302.6	57.4	<0.0001
Intercept	1	649384.2	649384.2	4487.4	<0.0001
R	1	6428.6	6428.6	44.4	<0.0001
G	5	150778.2	30155.6	208.4	<0.0001
T	1	8459.5	8459.5	58.5	<0.0001
R*G	5	3492.7	698.5	4.8	<0.0001
R*T	1	13641.6	13641.6	94.3	<0.0001
G*T	5	2887.4	577.5	3.9	0.002
R*G*T	5	5277.7	1055.5	7.3	<0.0001
Error	168	24311.5	144.7		
Total	192	864661.5			
Corrected Total	191	215277.2			

*DF = degree of freedom

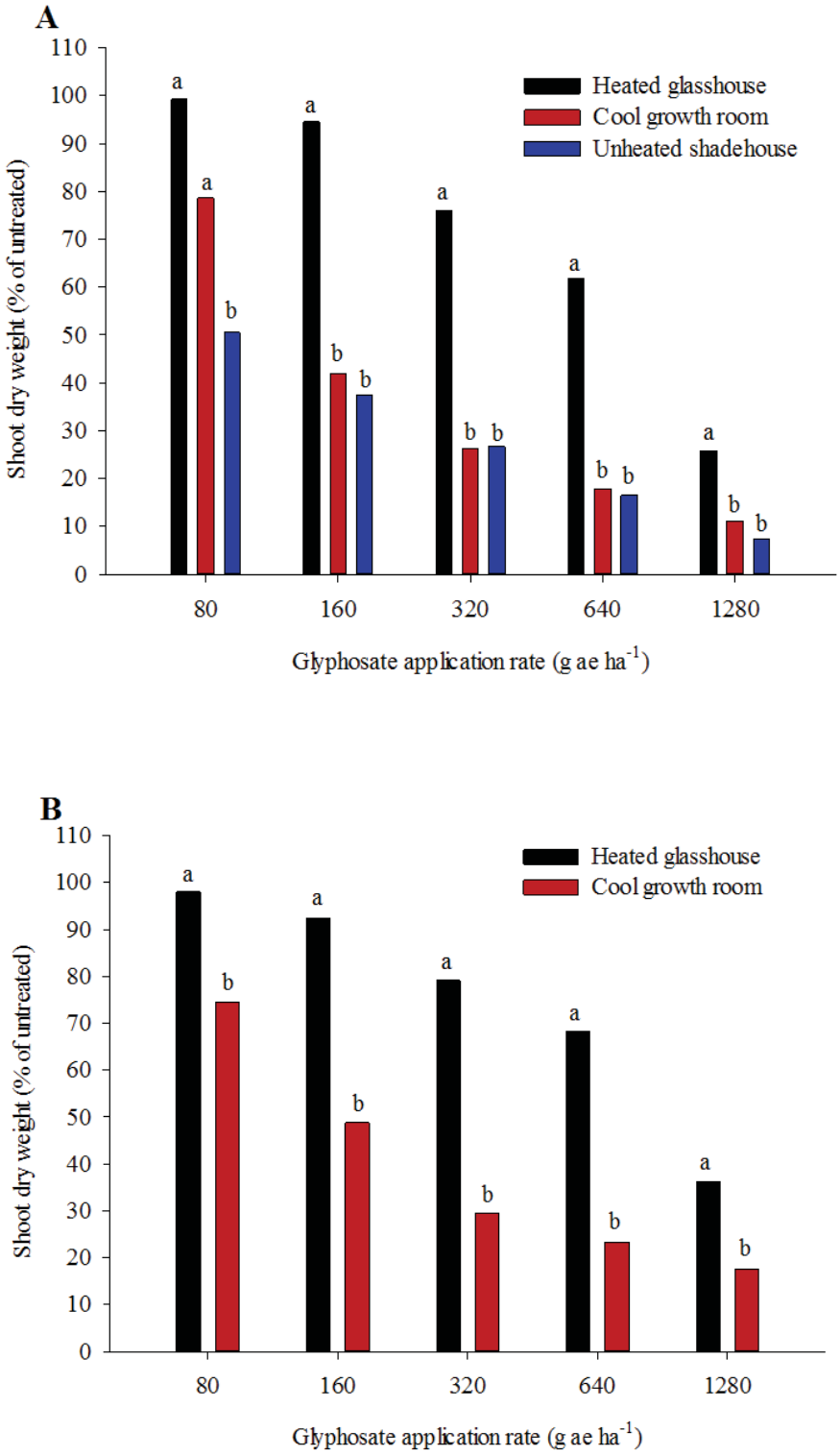

Figure 10.3 Biomass production of Population J treated with different rates of glyphosate at different temperature regimes in (**A**) Experiment 1 and (**B**) Experiment 2. Mean values within each glyphosate rate for each graph followed by the same letters are not different at 5% probability according to Fisher's protected tests (Experiment 1) or Student`s t-tests (Experiment 2).

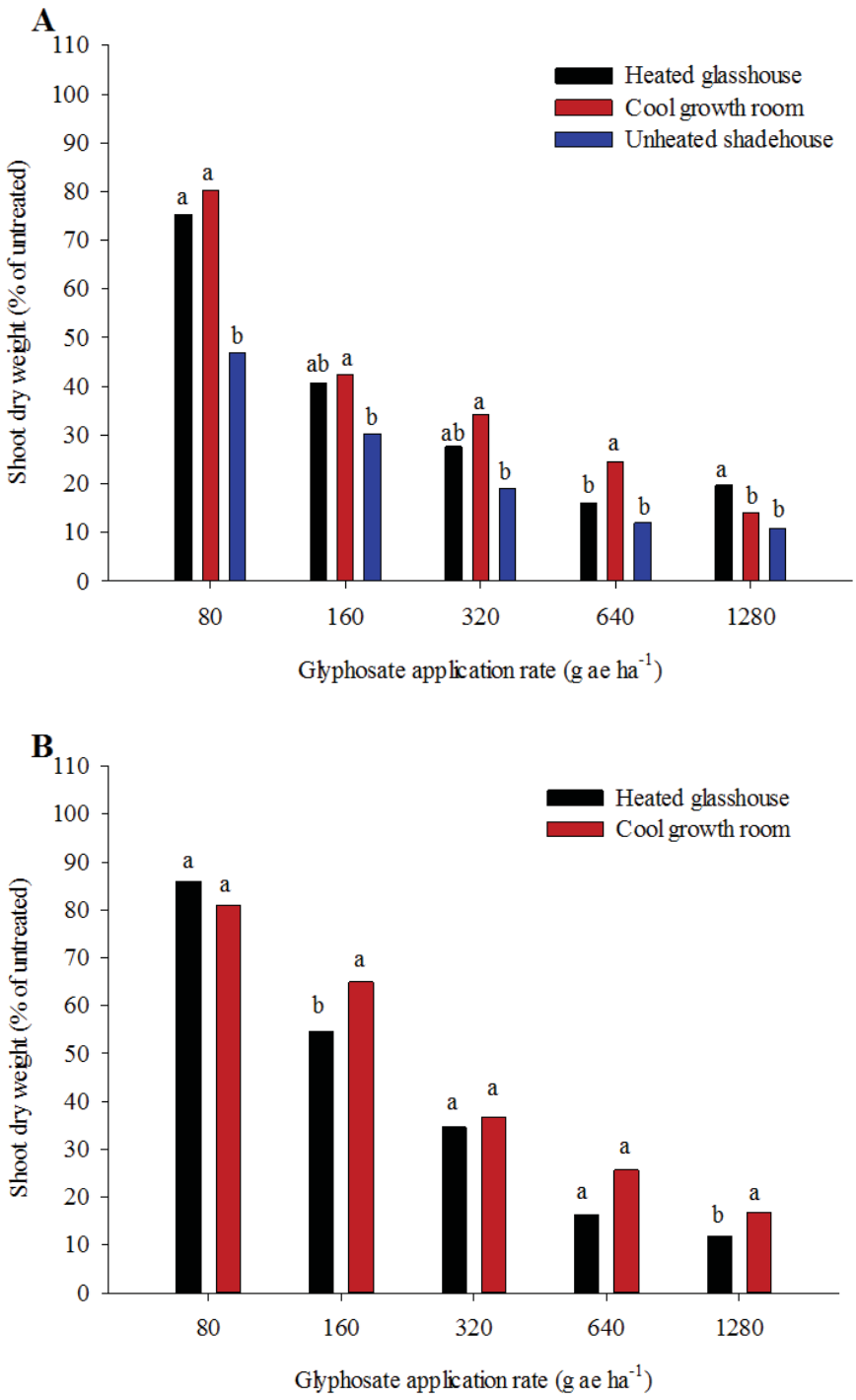

Figure 10.4 Biomass production of Population SP treated with different rates of glyphosate at different temperatures in Experiment 1 (**A**) and Experiment 2 (**B**). Mean values within each glyphosate rate for each graph followed by the same letters are not different at 5% probability according to Fisher's protected tests (Experiment 1) or Student`s t-tests (Experiment 2).

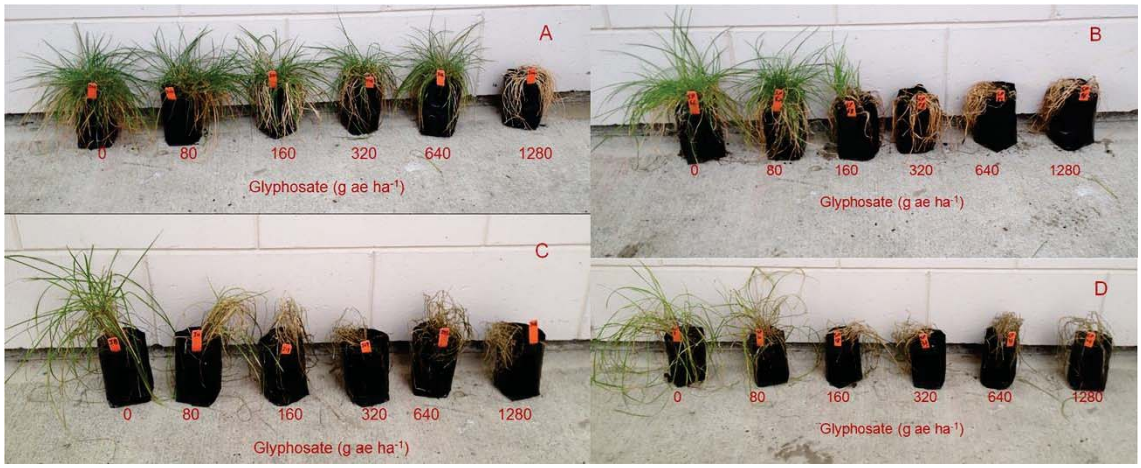

Figure 10.5 Effect after 10 weeks of different rates of glyphosate in Experiment 1 on glyphosate-resistant perennial ryegrass Population J (Panels A and C) and glyphosate-susceptible Population SP (B and D) kept in either a heated glasshouse (20.1°C) (A and B) or a cool growth room (below 9.1°C) (C and D).

In Experiment 1, Population J was estimated to be 5.1 times more resistant to glyphosate when plants were kept in the heated glasshouse, whereas the level of resistance for Population J decreased significantly in the cool growth room and the unheated shadehouse to become 0.9 and 1.3 times, respectively (Table 10.3). In Experiment 2, similar results were obtained for Population J which was 4.6 times more resistant than Population SP in the warm condition while in the cool condition Population J was once again just as susceptible as Population SP to glyphosate (Table 10.3).

Table 10.3 Estimates of the rate of glyphosate (g ae ha^{-1}) required to reduce dry weight by 50% (GR_{50}) after non-linear regression analysis of above-ground biomass for glyphosate-resistant (Population J) and glyphosate-susceptible (Population SP) perennial ryegrass plants grown at different temperature regimes.

Population	Temperature regime	GR_{50} (g ha^{-1})*	R/S GR_{50} ratio
Experiment 1			
Marlborough J	Heated glasshouse	749 **a**	5.1
Manawatu SP	Heated glasshouse	147 **b**	
P value		<0.0001	
Marlborough J	Cool growth room	154	0.9
Manawatu SP	Cool growth room	173	
P value		0.88	
Marlborough J	Unheated shadehouse	84	1.3
Manawatu SP	Unheated shadehouse	64	
P value		0.37	
Experiment 2			
Marlborough J	Heated glasshouse	940 **a**	4.6
Manawatu SP	Heated glasshouse	203 **b**	
P value		<0.0001	
Marlborough J	Cool growth room	170	0.7
Manawatu SP	Cool growth room	244	
P value		0.38	

GR_{50} = the rate of herbicide (g ae ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

10.4 Discussion

These two experiments showed that glyphosate resistance in Population J, which is known to involve the restricted herbicide translocation, was suppressed by sub-optimal growing temperatures (below 10°C (Hunt & Easton, 1989)). Similar results have been reported for glyphosate-resistant *Conyza canadensis* (Ge *et al.*, 2011), *Lolium rigidum* and *Sorghum halepense* (Vila-Aiub *et al.*, 2013). Glyphosate performance is temperature dependant and glyphosate efficacy increases at warm temperatures compared to cool temperatures (Adkins *et al.*, 1998; Tanpipat *et al.*, 1997).

This is consistent with our observations as treated plants of both populations did not show any symptoms (*e.g.* chlorosis) for almost 4 weeks after glyphosate treatment at cooler temperatures, whereas plants kept in warm conditions had symptoms appear 2 weeks after glyphosate application. Despite lower temperatures resulting in plants being affected more slowly by glyphosate, in Population SP the dose response curves were not significantly different between cool and warm conditions for either experiment. However, low temperatures had a much larger effect on the response to glyphosate in Population J, making the plants so susceptible to glyphosate that they became as sensitive as Population SP.

Although there were some differences in the light intensity between the different environments used in this study, these slight differences are unlikely to have caused the different responses to glyphosate recorded for Population J in the heated glasshouse compared with the cooler environments. Reducing the light intensity by 50% did not change the response to glyphosate in *Avena fatua* or *Urochloa panicoides* (Adkins *et al.*, 1998). Glyphosate sequestration was not light dependant in resistant plants studied elsewhere as identical amounts of glyphosate vacuolar sequestration have been measured under dark and light conditions (Ge *et al.*, 2010). Therefore, the substantial differences recorded for Population J under the different growing conditions were unlikely to have been caused by the small difference in the light intensity.

Suppression of glyphosate resistance in perennial ryegrass under cool conditions suggests that vacuolar sequestration may be responsible for restricted glyphosate resistance observed in this population (Ge *et al.*, 2011). The molecular, physiological and biochemical mechanisms that cause sub-optimal temperatures to reduce the vacuolar sequestration have not been completely elucidated. Ge *et al.* (2011) found that resistant plants of *Conyza canadensis* were still able to sequester glyphosate into vacuoles under cool conditions, but the amount of sequestration was significantly lower than in warm conditions. Interestingly, it was shown that plants with the vacuolar sequestration mechanism of resistance, treated with glyphosate at cool temperatures, were able to sequester the glyphosate rapidly into vacuoles once they were shifted to warmer conditions up to 14 days after glyphosate applications (Ge *et al.*, 2011). However, if the plants had already been damaged severely by the glyphosate before temperatures rose, resistant plants would presumably still be killed by glyphosate if conditions remained cool for several weeks. Glyphosate sequestration into vacuoles is

hypothesized to be mediated by an active transporter (Sammons & Gaines, 2014). Further studies are needed in order to investigate the role of transporters in vacuolar sequestration of glyphosate and also the effect of temperature on the transporters' activity.

Due to facility constraints, it was not possible to replicate the cold temperature treatments simultaneously within different growth chambers, which would have been the ideal method with respect to trial design. Despite this, the results clearly showed that the mechanism of glyphosate resistance in perennial ryegrass can be suppressed under winter temperatures. The suppression of the mechanism of resistance to glyphosate in Population J under cool temperatures has obvious practical implications. Application of glyphosate during winter while it is cold should provide greatly improved control of glyphosate-resistant perennial ryegrass infestations within vineyards. Field trials are now required to test this hypothesis. However, another perennial ryegrass population resistant to glyphosate that has been studied from Marlborough (Population O) has been found to have two mechanisms of resistance. In addition to the restricted glyphosate translocation (non-target site mechanism of resistance) (Chapter 8), the plants from this population also have a modified gene (target site mechanism of resistance) (Chapter 7) that affects the structure of the enzyme normally inhibited by glyphosate. Although the target site mechanism of resistance is temperature insensitive (Sammons & Gaines, 2014), it generally only makes plants 2-3 times more resistant to glyphosate (Baerson *et al.*, 2002). Thus if most of the resistance in Population O is because of restricted translocation, spraying in winter may be successful for these plants too if a higher rate is used.

10.5 Conclusions

Under cool conditions typical of winter in New Zealand, a population of perennial ryegrass that has evolved resistance to glyphosate in Marlborough vineyards becomes sensitive to the herbicide. This suggests that the restricted glyphosate translocation known to exist in this population could involve vacuolar sequestration because similar reductions in resistance under cool conditions have been reported previously where vacuolar sequestration causes glyphosate resistance. Thus control of glyphosate-resistant perennial ryegrass may be possible by spraying glyphosate during cool times of the year.

Chapter 11

The inheritance of glyphosate resistance in Italian ryegrass and perennial ryegrass

11.1 Introduction

The evolution of herbicide resistance in weeds is a function of several determinants, like mutation rate, initial frequency of the resistance gene, gene flow, selection pressure and the mode of inheritance (Maxwell & Mortimer, 1994). The process of genetic transmission of traits from one generation to the next is called inheritance (Nandula, 2010). In herbicide-resistant plants, the process of transmission of resistant alleles occurs through pollen and ovule (Jasieniuk *et al.*, 1996). Inheritance studies provide fruitful insights into understanding of genetic structures of herbicide-resistant weed populations and also providing data that can be used for modelling evolution of herbicide resistance and management of herbicide resistant weeds (Wakelin & Preston, 2006a). The inheritance of glyphosate resistance was reviewed in Section 2.4.5.

The study described in this chapter was conducted to determine the mode of inheritance in New Zealand glyphosate-resistant populations of Italian ryegrass and perennial ryegrass. As either Italian ryegrass or perennial ryegrass can be found in the majority of pastures in New Zealand, it was important to know whether the glyphosate resistance alleles can spread easily from properties with the problem. Previous publications on *Lolium* spp. suggested that the restricted herbicide translocation mechanism of resistance can be transferred via both pollen and ovule (as it is nuclear) and is controlled by a partially dominant gene (Preston *et al.*, 2009b). Therefore, the objective of this work was to examine the inheritance of resistance in F_1 progeny of Italian ryegrass and perennial ryegrass to determine if it behaves similarly to the inheritance reported for glyphosate-resistant *Lolium* spp. from other countries.

11.2 Materials and methods

11.2.1 Plant materials

In this study, the clones of the plants of Italian ryegrass Population A and perennial ryegrass Population J, that had proven to be resistant to glyphosate (Chapter 5) with the restricted herbicide translocation mechanism of resistance (Chapter 8), were each

crossed within the populations in order to produce seeds of each glyphosate-resistant population separately. To achieve this, plantlets from the same glyphosate-resistant populations were grown together in a glasshouse within pollen-proof cloth in order to prevent contamination by pollen from another population. Two known susceptible populations (Chapter 5) of Italian ryegrass (SI) and perennial ryegrass (SP) were also included in this study.

11.2.2 Generation of first filial generations (F1)

For each resistant and susceptible population, 25 seeds were pre-germinated using a similar method described in Section 8.2.2, potted in planter bags (700 ml) filled with potting mix (50% bark, 30% fibre, 20% Pacific Pumice (7mm) and slow-release fertilizer (Woodace, Lebanon, PA)), and were kept in a heated glasshouse in July 2013. The daily maximum and minimum temperatures in the following 4 weeks averaged 20.5°C and 16.2°C respectively.

Ten vigorous plants from each population were then selected and a leaf segment shikimic acid test was conducted in order to examine the glyphosate susceptibility of each plant, using the same method described in Chapter 6, for 84.5 mg ae L^{-1} of glyphosate. The results of the shikimic acid test confirmed that the plants from Populations SI and SP were all susceptible to glyphosate and those from Populations A and J were glyphosate-resistant. The plants were then shifted to a cool-room set at 8^{o}C on 28 August 2013 in order to get them vernalised. The vernalisation period for Italian ryegrass populations lasted 4 weeks, whereas the perennial ryegrass populations were vernalised for 8 weeks as perennial ryegrass is known to require a longer period to induce flowering (Cooper, 1951).

The vernalised plants were then shifted to a glasshouse with four supplementary lights to encourage seed-head formation. The flowering process of each plant was recorded and one resistant plant was crossed with one susceptible plant based on their flowering synchronisation. The tillers of susceptible and resistant plants were enclosed using pollen-proof bags and kept in the glasshouse within frames enclosed with cloth. Only four reciprocal crosses between four resistant and four susceptible plants for either Italian ryegrass or perennial ryegrass were successfully achieved (F_1 families 1 to 4). The schema of the reciprocal cross between four resistant plants with four susceptible plants is shown in Figure 11.1. For each of the F_1 families No. 1 to 4, one plant of the

resistant (R) population of Italian ryegrass or perennial ryegrass was crossed with a susceptible (S) plant from the same ryegrass species. The symbols R_1 to R_4 represent parents No. 1 to 4 of the resistant population of either Italian ryegrass (Population A) or perennial ryegrass (Population J). The symbols S_1 to S_4 represent parents No. 1 to 4 of the susceptible population of either Italian ryegrass (Population SI) or perennial ryegrass (Population SP).

The seeds were collected separately from each resistant and susceptible plant at maturity. The symbols R_1F_1 to R_4F_1 represent the first generation for resistant families No.1 to 4 as a result of the reciprocal cross between four resistant plants with pollen from four susceptible plants and these seeds were collected from the resistant plants. The symbols S_1F_1 to S_4F_1 represent the first generation for susceptible families No. 1 to 4 as result of the reciprocal cross between susceptible plants with pollen from four resistant plants and seeds were collected from susceptible plants at maturity (Figure 11.1).

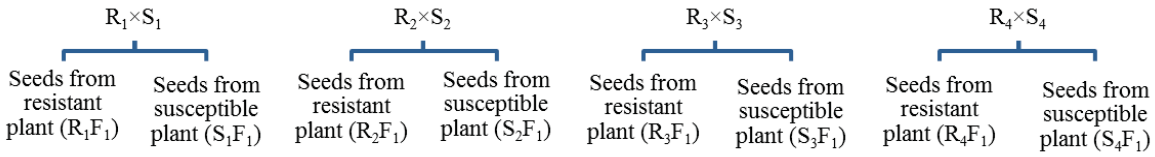

Figure 11.1 A schema of how each F_1 family was created for Italian ryegrass and perennial ryegrass.

In total, eight F_1 families were obtained for each species. For Italian ryegrass, four of these F_1 families had resistant maternal (pollen receptor) (R♀) and susceptible paternal (pollen donor) (S♂) parents (F_1 R(A)♀×S(SI)♂, denoted as IR_1F_1 to IR_4F_1) The other four had F_1 susceptible maternal (pollen receptor) (S♀) and resistant paternal (pollen donor) (R♂) parents (F_1 S(SI)♀×R(A)♂, denoted as IS_1F_1 to IS_4F_1). Likewise, there were eight F1 families for perennial ryegrass (denoted as PR_1F_1 to PR_4F_1 and PS_1F_1 to PS_4F_1). The parental resistant and susceptible plants were re-potted and used for the dose-response bioassays described in the next section (11.2.3).

11.2.3 Dose-response bioassay for the F1 families

The seeds collected from each F_1 family were pre-germinated and potted. Fifteen seeds of each F_1 family were germinated using the method described in Section 6.2.1. On 28 February 2014, the germinated seeds were potted and left to grow in a glasshouse for 8

weeks. The daily temperatures in the 8 weeks following seedling establishment averaged 20.5°C. After 8 weeks, when plants had on average 18 tillers each, the plants were divided further to create 75 potted plants for each F_1 family. Each of these plants consisting of three tillers which were trimmed back to 4 cm of root and 5 cm of shoot prior to repotting, as described in Section 5.2.1. Plants of similar size were also produced from the original parental resistant and susceptible plants that had been used for generating the F_1 families. Pots were then kept in a glasshouse for 4 weeks (daily maximum and minimum temperatures averaged 24.2°C and 11.5°C respectively) before treatment with glyphosate to produce dose-response curves.

On 27 May 2014, each of the F_1 families and the original parental plants were treated with five rates of glyphosate (0, 100, 250, 625 and 1562 g ae ha^{-1} as Roundup 360 Pro, an isopropylamine salt) using a laboratory track sprayer calibrated to deliver 254 litres ha^{-1} of spray solution at 200 kPa. All herbicide treatments contained 0.1% organosilicone surfactant (Pulse Penetrant). At the time of spraying, the plantlets each had an average of 7-8 tillers, and each treatment was applied to 15 plantlets of each F_1 family and of the parent plants. The daily maximum and minimum temperatures in the 2 weeks following application averaged 26.1°C and 12.2°C, respectively. Four weeks after treatment, the foliage of all plants was removed from pots at ground level and the dry weight of this plant material was determined. The effect of each herbicide treatment was calculated as a percentage of the dry weight of untreated plants for that population.

11.2.4 Statistical Analysis

These whole plant dose-response assays were conducted using a separate randomised complete block design for Italian ryegrass and perennial ryegrass, with five application rates and 15 replicates of each herbicide rate for each F_1 family (eight families in total for each species) and two parent populations of each species. The basic assumptions for regression models were checked using similar methods to those described in Section 3.2.4. Dose response curves were fitted as described in Section 3.2.4.

11.3 Results

11.3.1 Dose-response bioassay for F_1 families of Italian ryegrass

Dose-response experiments were conducted on F_1 families (the mean of all IRF_1 (F_1 R(A)♀×S(SI)♂) and ISF_1 (F_1 S(SI)♀×R(A)♂) families), and glyphosate-resistant and susceptible parental plants (Figure 11.2 and Table 11.1). The glyphosate rate required to

reduce the plant shoot dry weight by 50% (GR_{50}) for glyphosate-resistant Italian ryegrass (Population A) was 1095.1 g ae ha^{-1} compared with a significantly lower GR_{50} of 121.3 g ae ha^{-1} for the susceptible population, SI (Table 11.1). A resistance index was calculated by comparing the GR_{50} value of Population A against that of Population SI (R/S GR_{50} ratio) and it was estimated that Population A was nine times more resistant to glyphosate than Population SI.

The GR_{50} values from pooled data from all eight Italian ryegrass F_1 families (four F_1 R(A)♀×S(SI)♂ (IRF_1) and four F_1 S(SI)♀×R(A)♂ (ISF_1)) showed that higher rates of glyphosate were required to reduce shoot dry weight by 50% compared to Population SI (Table 11.1). A similar dose-response was observed for IRF_1 and ISF_1 (Figure 11.2). Also, by looking at the response of each F_1 family it can be seen that the progenies from the R(A)♀×S(SI)♂ and (SI)♀×R(A)♂ were substantially more resistant to glyphosate than the original susceptible Population SI (Figures 11.3 a-d)

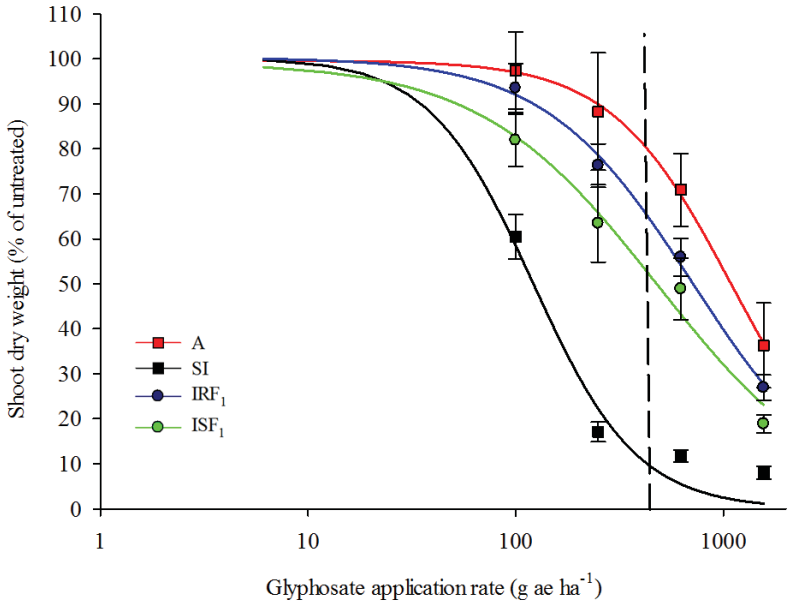

Figure 11.2 Fitted dose response curves (on a logarithmic dose scale) for parental Italian ryegrass Population A, glyphosate susceptible parental Population SI, family IRF_1 (A♀×SI♂) and family ISF_1(SI♀×A♂) for glyphosate using shoot dry weight at 4 weeks after application of glyphosate. Vertical bars represent ± standard errors of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

By dividing the GR_{50} values of IRF_1 and ISF_1 by that of the susceptible population (SI),

the IRF_1 and ISF_1 were estimated to be 5.9 and 4.0 times more resistant to glyphosate, respectively, than the susceptible population and thus showed an intermediate level of resistance to glyphosate compared to Population A (Table 11.1).

Table 11.1 Parameters estimated for the nonlinear regression analysis of glyphosate dose-response experiments of Italian ryegrass families at 4 weeks after application of glyphosate. A was the glyphosate-resistant parental population, SI was the glyphosate susceptible parental population, family IRF_1 corresponds to the cross A♀×SI♂ and ISF_1 corresponds to the reciprocal cross SI♀×A♂. R_1-R_4 and S_1-S_4 correspond to four F_1 resistant maternal and four F_1 susceptible maternal crosses respectively.

Families	d	b	GR_{50} (g ha^{-1})*	R/S GR_{50} ratio	R^2
A	99.6	1.5	1095 **a**	9.0	0.99
SI	100.3	1.7	121 **d**	-	0.98
IRF_1	100.3	1.2	712 **b**	5.9	0.99
ISF_1	99.3	1.2	484 **c**	4.0	0.98
P value	0.99	0.52	<0.0001		
A	99.6	1.5	1095 **a**	9.0	0.99
SI	100.3	1.7	121 **c**	-	0.98
IR_1F_1	101.9	1.3	606 **b**	4.9	0.97
IS_1F_1	98.2	1.1	567 **b**	4.7	0.97
P value	0.99	0.53	<0.0001		
A	99.6	1.5	1095 **a**	9.0	0.99
SI	100.3	1.7	121 **c**	-	0.98
IR_2F_1	93.5	1.6	965 **a**	7.9	0.99
IS_2F_1	99.2	1.1	464 **b**	3.8	0.99
P value	0.79	0.72	<0.0001		
A	99.6	1.5	1095 **a**	9.0	0.99
SI	100.3	1.7	121 **c**	-	0.98
IR_3F_1	103.8	1.2	469 **b**	3.9	0.95
IS_3F_1	99.6	1.1	462 **b**	3.8	0.98
P value	0.97	0.58	<0.0001		
A	99.6	1.5	1095 **a**	9.0	0.99
SI	100.3	1.7	121 **c**	-	0.98
IR_4F_1	96.3	1.6	994 **a**	8.1	0.98
IS_4F_1	99.8	0.9	453 **b**	3.7	0.97
P value	0.95	0.35	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ae ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

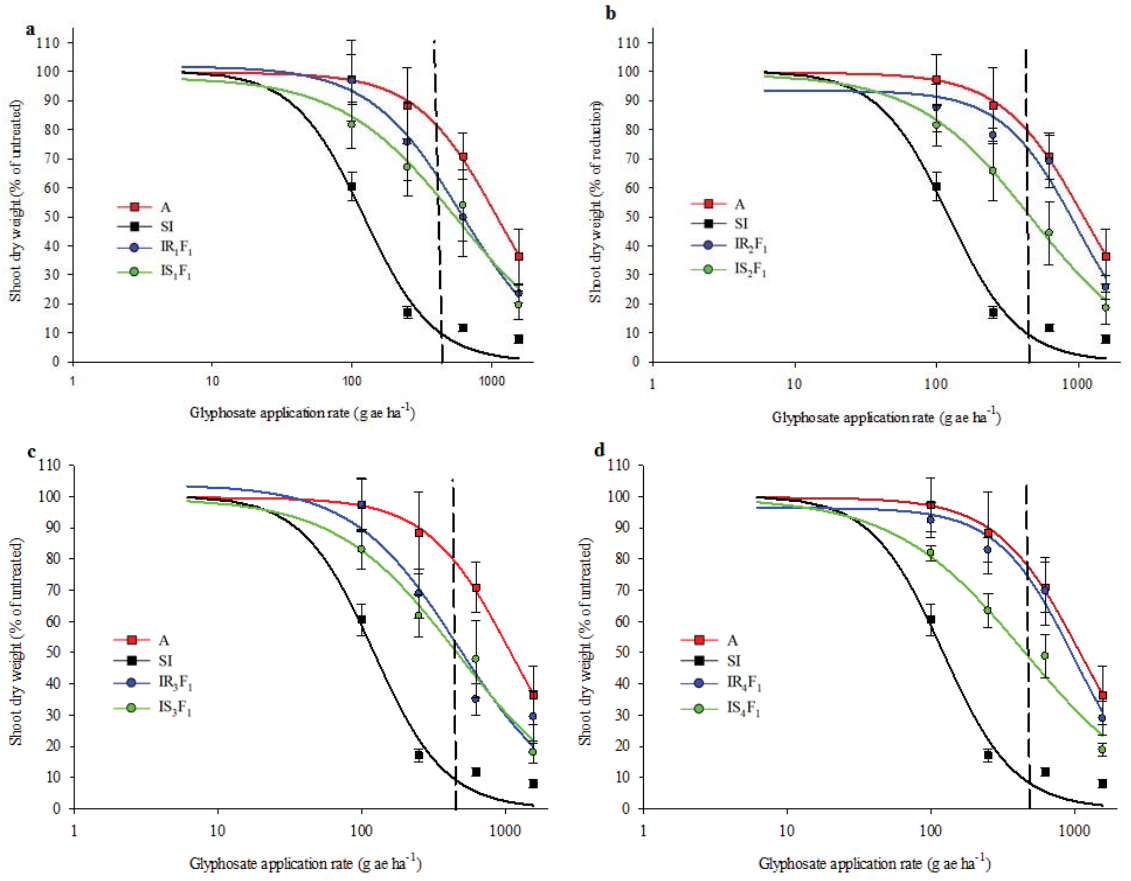

Figure 11.3 Fitted dose response curves (on a logarithmic dose scale) for parental Italian ryegrass Population A, glyphosate susceptible parental Population SI, families IR_1F_1 (A♀×SI♂) & IS_1F_1(SI♀×A♂) (**a**), IR_2F_1 & IS_2F (**b**), IR_3F_1 & IS_3F_1 (**c**) and IR_4F_1 & IS_4F_1 (**d**) for glyphosate using shoot dry weight at 4 weeks after application of glyphosate. Vertical bars represent ± standard errors of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

Varied GR_{50} values were estimated for resistant maternal F1 families (IR_1F_1-IR_4F_1) (Table 11.1). By comparing the level of glyphosate resistance of each individual resistant maternal F_1 family to Population SI, the progenies of resistant maternal plants of Population A (IR_1F_1-IR_4F_1) had levels of resistance ranging from 3.9 to 8.1 times. The level of glyphosate resistance for two out of four IRF_1 families (IR_2F_1 and IR_4F_1) was similar to that of Population A and the other two resistant maternal families, IR_1F_1 and IR_3F_1 had an intermediate level of glyphosate resistance compared to Population A.

However, GR_{50} values among susceptible maternal F_1 families (ISF_1-IS_4F_1) were similar. The levels of resistance for the progenies of susceptible maternal plants (IS_1F_1-

IS_4F_1) ranged from 3.7-4.7 times (Table 11.1) and thus the level of resistance to glyphosate for all ISF_1 families (ISF_1-IS_4F_1) was intermediate compared to Population A (Table 11.1).

11.3.2 Dose-response bioassay for F_1 families of perennial ryegrass

Very similar results were obtained for perennial ryegrass as with the Italian ryegrass. The pooled data from all eight perennial ryegrass families (four F_1 R(J)♀×S(SP)♂ (PRF_1) and four F_1 S(SP)♀×R(J)♂ (PSF_1)) showed a similar response between PRF_1 and PSF_1 families, and both families were significantly more resistant to glyphosate than the susceptible population (SP) (Figure 11.4). Each individual F_1 family showed an intermediate response to glyphosate between Population SP and the glyphosate-resistant Population J at comparable rates (Figures 11.5 a-b).

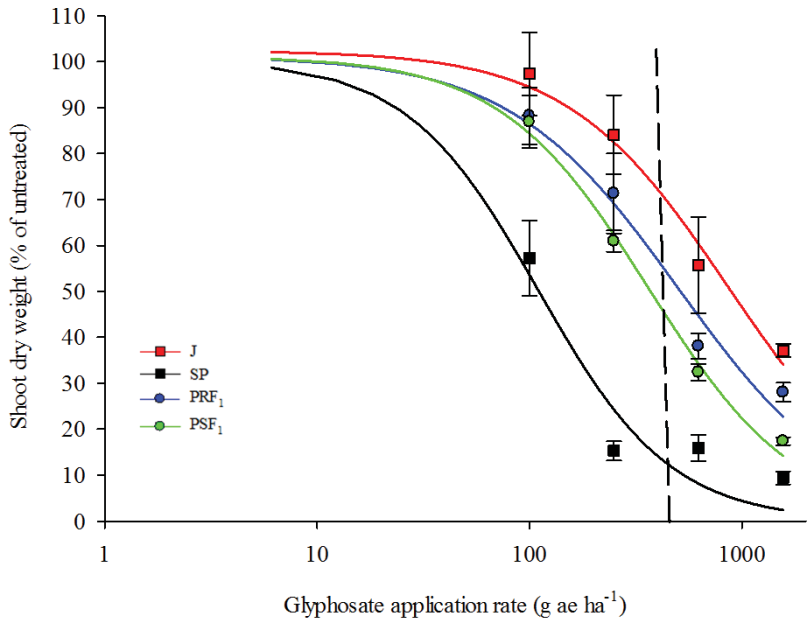

Figure 11.4 Fitted dose response curves (on a logarithmic dose scale) for perennial ryegrass parental Population J, glyphosate susceptible parental Population SP, family PRF_1 (J♀×SP♂) and family PSF_1(SP♀×J♂) for glyphosate using shoot dry weight at 4 weeks after application of glyphosate. Vertical bars represent ± standard errors of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

The glyphosate rate required to reduce shoot growth by 50% (GR_{50}) for Population J was 855 g ae ha^{-1} and this was almost 7.7 times higher than that for Population SP (110 g ae ha^{-1}) (Table 11.2). The GR_{50} values from the pooled data from all eight F_1 families showed that higher rates of glyphosate were also required to reduce shoot growth by 50% compared to Population SP (Table 11.2). By dividing the GR_{50} values of PRF_1 and PSF_1 by that of the susceptible population (SP), the level of resistance of PRF_1 and PSF_1 were 4.5 and 3.3 times higher, respectively, than that of Population SP suggesting an intermediate level of resistance to glyphosate compared to Population J. The GR_{50} values of each PRF_1 (PR_1F_1-PR_4F_1) were not significantly different from those of PSF_1 (PS_1F_1-PS_4F_1) families (Table 11.2) The level of resistance to glyphosate for the progenies of resistant maternal (PR_1F_1-PR_4F_1) ranged from 4.5-5.0 times more than Population SP based on R/S GR_{50} ratio. Also, the level of resistance to glyphosate for the progenies of susceptible maternal plants (PS_1F_1-PS_4F_1) ranged from 2.9 to 4.3 times more resistant than Population SP (Table 11.2). The level of glyphosate resistance of all PRF_1 (PR_1F_1-PR_4F_1) and PSF_1 (PS_1F_1-PS_4F_1) families were intermediate between Population SP and Population J (Table 11.2).

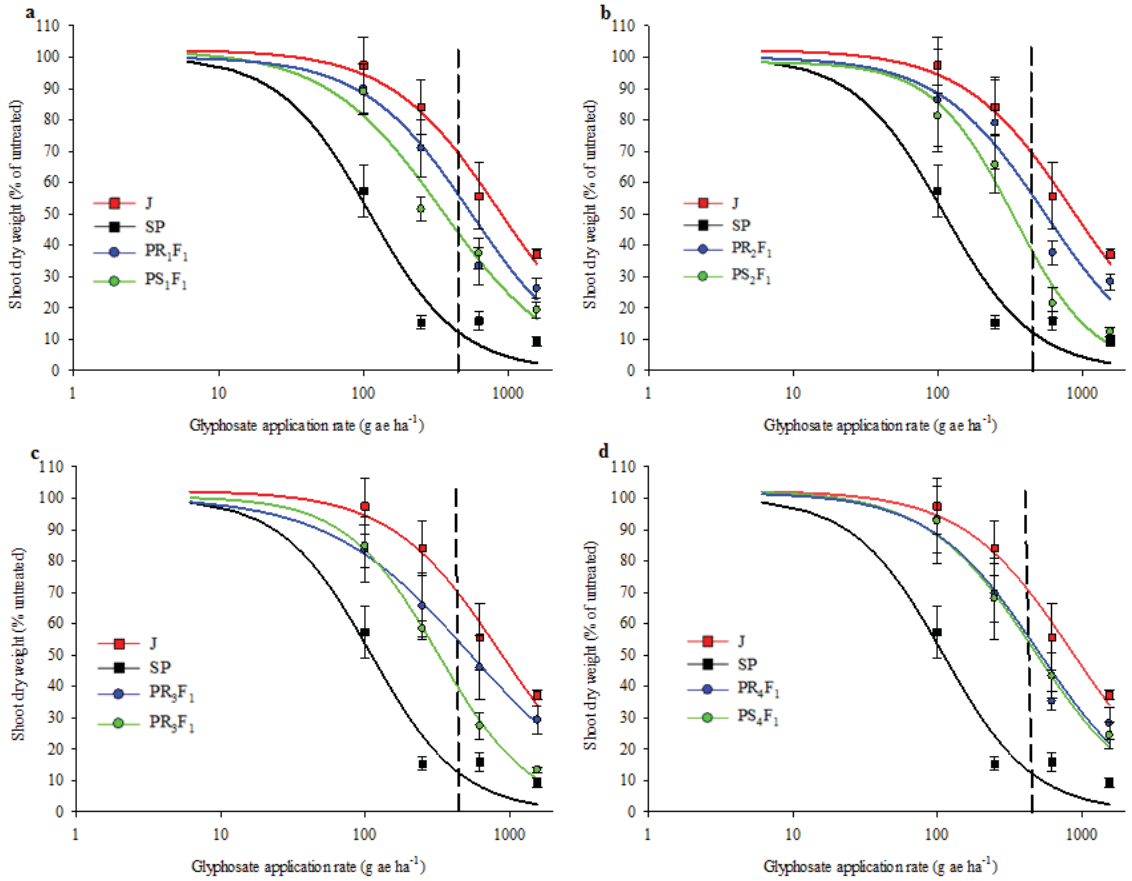

Figure 11.5 Fitted glyphosate dose response curves (on a logarithmic dose scale using shoot dry weight) for parental perennial ryegrass Population J, glyphosate susceptible parental Population SP, and (**a**) families PR_1F_1 (J♀×SP♂) & PS_1F_1(SP♀×J♂), (**b**) PR_2F_1 & PS_2F_1, (**c**) PR_3F_1 & PS_3F_1 and (**d**) PR_4F_1 & IS_4F_1 at 4 weeks after application of glyphosate. Vertical bars represent ± standard errors of the mean. The vertical dashed line indicates the recommended field application rate (450 g ae ha^{-1}) (Young, 2012).

Table 11.2 Parameters estimated for the nonlinear regression analysis of glyphosate dose-response experiments of perennial ryegrass families at 4 weeks after application of glyphosate. J corresponds to the glyphosate-resistant parental population, SP corresponds to glyphosate-susceptible parental population, family PRF_1 corresponds to the cross J♀×SP♂ and PSF_1 corresponds to the reciprocal cross SP♀×J♂. R_1-R_4 and S_1-S_4 correspond to four different F_1 resistant maternal and four F_1 susceptible maternal crosses respectively.

Families	d	b	GR_{50} (g ha^{-1})*	R/S GR_{50} ratio	R^2
J	102.4	1.1	855 **a**	7.7	0.98
SP	100.4	1.4	110 **c**	-	0.97
PRF_1	101.2	1.1	503 **b**	4.5	0.98
PSF_1	101.2	1.2	364 **b**	3.3	0.99
P value	0.99	0.91	<0.0001		
J	102.4	1.1	855 **a**	7.7	0.98
SP	100.4	1.4	110 **c**	-	0.97
PR_1F_1	100.1	1.2	552 **b**	5.0	0.96
PS_1F_1	102.3	1.1	347 **b**	3.2	0.98
P value	0.99	0.94	<0.0001		
J	102.4	1.1	855 **a**	7.7	0.98
SP	100.4	1.4	110 **c**	-	0.97
PR_2F_1	100.1	1.2	553 **b**	5.0	0.96
PS_2F_1	98.5	1.5	335 **b**	3.0	0.98
P value	0.98	0.88	<0.0001		
J	102.4	1.1	855 **a**	7.7	0.98
SP	100.4	1.4	110 **c**	-	0.97
PR_3F_1	100.7	0.9	535 **b**	4.9	0.97
PS_3F_1	100.6	1.4	321 **b**	2.9	0.99
P value	0.99	0.64	<0.0001		
J	102.4	1.1	855 **a**	7.7	0.98
SP	100.4	1.4	110 **c**	-	0.97
PR_4F_1	101.8	1.2	507 **b**	4.6	0.96
PS_4F_1	102.5	1.2	473 **b**	4.3	0.99
P value	0.99	0.95	<0.0001		

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ae ha^{-1}) required to reduce dry weight by 50%, R/S GR_{50} = resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. *Mean values for each population within a column followed by different letters are different at 5% probability. Where the mean values within a column were not different (P>0.05), lettering is absent.

11.4 Discussion

The mechanism of resistance to glyphosate-resistant Italian ryegrass Population A and perennial ryegrass Population J was found to be restricted herbicide translocation (Chapter 8). In the present study, first filial generations (F_1) were created from crossing glyphosate-resistant and susceptible populations of Italian ryegrass and perennial ryegrass, and then clones of the parent plants and also their corresponding F_1 families were sprayed with several rates of glyphosate. The results showed that glyphosate resistance in both Italian ryegrass and perennial ryegrass can be transferred via pollen and ovule because all of the F_1 families (progenies from both resistant maternal and susceptible maternal) in both species were more resistant to glyphosate than their corresponding susceptible population (SI (Italian ryegrass) or SP (perennial ryegrass)). Thus the gene responsible for the restricted glyphosate translocation mechanism of resistance was shown to be in the nuclear genome with no evidence of maternal effects.

While the level of dominance between the progenies of two Italian ryegrass resistant maternal (IR_2F_1 and IR_4F_1) and susceptible maternal (IS_2F_1 and IS_4F_1) families was slightly different, the level of dominance between the other two families IR_1F_1 and IR_3F_1 (resistant maternal), and IS_2F_1 and IS_4F_1 (susceptible maternal) was similar. This might reflect the variability among individual plants to the applied herbicide (Yu *et al.*, 2009). However, the level of dominance among the progenies of four perennial ryegrass resistant maternal and (PR_1F_1-PR_4F_1) and susceptible maternal (PS_1F_1-PS_4F_1) families was similar. The dose-response results of the pooled data from all F_1 families of Italian ryegrass (IRF_1 (resistant maternal) and ISF_1 (susceptible maternal)) and perennial ryegrass (PRF_1 (resistant maternal) and PSF_1 (susceptible maternal)) showed that glyphosate resistance in both species is an incompletely dominant trait as the progenies of both resistant maternal (RF_1) and susceptible maternal (SF_1) from both Italian ryegrass and perennial ryegrass had intermediate levels of resistance to glyphosate compared to the glyphosate-resistant and glyphosate-susceptible populations.

The nuclear inheritance found in this study for both glyphosate-resistant Italian ryegrass and perennial ryegrass is the same type of resistance inheritance pattern as that found in glyphosate-resistant *Lolium rigidum* from Australia with a restricted herbicide translocation mechanism of resistance (Lorraine-Colwill *et al.*, 2001; Wakelin & Preston, 2006a). The nuclear inheritance enables glyphosate resistance alleles to be transferred through both seed and pollen (Jasieniuk *et al.*, 1996). Nuclear coded genes

are the most common form of inheritance amongst the majority of weed populations resistant to herbicides like glyphosate (Jasieniuk *et al.*, 1994; Riar *et al.*, 2011a; Sabba *et al.*, 2003; Wakelin & Preston, 2006a). Cytoplasmic coded genes have been reported only for atrazine-resistant weeds with a target site mechanism of resistance (Darmency & Gasquez, 1981; Jasieniuk *et al.*, 1996). The results of this study also showed that glyphosate resistance in the studied Italian ryegrass and perennial ryegrass populations was controlled by genetic factors with incomplete dominance and this finding is consistent with previous results on inheritance of glyphosate resistance in *Lolium rigidum* from Australia with the restricted glyphosate translocation mechanism of resistance (Lorraine-Colwill *et al.*, 2001).

Due to time constraints in this PhD project, the number of genes that control glyphosate resistance within a species was not studied. The non-target site mechanism of glyphosate resistance for *Lolium rigidum* from Australia has been reported to be controlled by a single gene (monogenic) (Lorraine-Colwill *et al.*, 2001; Wakelin & Preston, 2006a). Similar results have been reported for *Conyza canadensis* (Canadian fleabane) (Zelaya *et al.*, 2004). *Lolium* spp. are self-incompatible (McCraw & Spoor, 1983) and pollen carrying the resistance gene from plants of *Lolium* spp. have been able to fertilise plants at some distance from the parent plant (3000 m) (Busi *et al.*, 2008). Therefore, it could be expected that glyphosate resistance gene would spread to nearby sites with Italian ryegrass and perennial ryegrass (*e.g.* pastures, orchards, *etc.*) from vineyards containing resistant plants.

11.5 Conclusions

In this study, an investigation of the heritability of restricted herbicide translocation in Italian ryegrass and perennial ryegrass revealed that the gene(s) controlling this mechanism of resistance in both species have incomplete dominance and can be readily transmitted via pollen.

Chapter 12

General discussion

12.1 Introduction

Herbicides play an important role in weed management systems (DiTomaso, 2002). Since no herbicides with a new mode of action have been commercialized in the previous two decades (Duke, 2012), efficient usage of available herbicides is necessary. Developing strategies for herbicide resistance management requires quick methods for detecting herbicide resistant weeds and a better understanding of physiological, molecular and genetic aspects of mechanisms of herbicide resistance. One of the main aims of this thesis was to investigate some quick techniques for detecting dicamba-resistant fathen, and glyphosate-resistant Italian ryegrass and perennial ryegrass. Also, some aspects of glyphosate-resistance (*eg* physiological and molecular mechanisms of resistance) were evaluated because of its potential importance to New Zealand. The results from experiments which were discussed in each chapter in this thesis provided further understanding of some aspects of resistance to dicamba and glyphosate in New Zealand. In this chapter, the major findings, implications of the results, suggested priorities for future research and possible management implications will be discussed.

12.2 Dicamba-resistant fathen (*Chenopodium album*)

12.2.1 The response of different fathen populations to dicamba

Although dicamba-resistant fathen was first reported in New Zealand in 2005 (James *et al.*, 2005), the level of resistance to dicamba in this weed species has never been properly quantified. In this thesis, dicamba resistance was evaluated in two fathen populations from Waikato and the results provided a better understanding of the level of resistance to dicamba in these resistant populations. Our results showed that the level of resistance to dicamba in Populations L and M was between 5-fold and 20-fold. Dicamba resistance has also been reported for *Centaurea cyanus*, *Galeopsis tetrahit*, *Kochia scoparia*, *Lactuca serriola* and *Sinapis arvensis* (Heap, 2015). A resistance level up to 30 times for dicamba-resistant *Kochia scoparia* has been reported by Preston *et al.* (2009a).

The mechanism of resistance to dicamba is currently not completely understood (Cranston *et al.*, 2001). Dicamba-resistant fathen has only been reported from New Zealand so far (Heap, 2015) and therefore, the mechanism of dicamba resistance in fathen needs investigating. The pattern of cross-resistance in dicamba-resistant fathen to other herbicides is still unknown. It is likely that dicamba-resistant fathen might be also cross-resistant to auxinic herbicides, especially those that share a very similar molecular structure with dicamba (Cranston *et al.*, 2001).

Chemical options for managing dicamba-resistant fathen in maize fields have been studied by Rahman *et al.*(2008) and they noted that dicamba-resistant fathen was susceptible to pyridate, bromoxynil, mesotrione and nicosulfuron. However, mesotrione and nicosulfuron were found to provide better control of dicamba-resistant fathen than pyridate or bromoxynil (Rahman *et al.*, 2014). It has also been reported that nicosulfuron also provided good control of some other weed species in maize like *Solanum nigrum*, *Polygonum persicaria*, *Amaranthus powellii* (Rahman *et al.*, 2014).

Nicosulfuron is a post-emergence herbicide which belongs to a group of herbicide called ALS-inhibitors (James *et al.*, 2006). Unfortunately, evolution of herbicide resistance to ALS-inhibitors occurs rapidly (Tranel & Wright, 2002) and over-reliance on nicosulfuron would lead to the evolution of resistance to this herbicide. Mesotrione belongs to a group of herbicides called inhibitors of HPPD (4-hydroxyphenylpyruvate dioxygenase) and has been developed for the selective pre- and post-emergence control of a wide range of grass and broad-leaved weeds in maize (Mitchell *et al.*, 2001). So far, evolution of resistance to mesotrione has been reported for *Amaranthus palmeri* and *Amaranthus tuberculatus* (Heap, 2015).

In New Zealand, fathen is the only species in maize fields to date which has been found to have developed resistance to more than one group of herbicides (multiple herbicide resistance) (Rahman *et al.*, 2014). Strategies like employing long term crop rotations and application of herbicides with different modes of action have been suggested for management of herbicide-resistant weeds like fathen in maize fields (Harrington & James, 2005).

In the present PhD project, some morphological differences were noted between dicamba-resistant and susceptible fathen similar to those reported previously for other dicamba-resistant fathen populations (Rahman *et al.*, 2014). Morphological differences

between auxinic herbicide-resistant and susceptible plants of *Sinapis arvensis* have also been reported (Hall & Romano, 1995). The link between leaf morphology and dicamba resistance is unknown and needs investigating. Recently, a close link between the shape of first true leaf and auxinic herbicide resistance has been reported for an auxinic herbicide-resistant *Sinapis arvensis*, and several molecular markers were produced using amplified fragment length polymorphism (AFLP) analysis techniques (Mithila *et al.*, 2012). Such molecular markers could be used in cloning and identification of auxinic resistance gene(s).

The morphological differences observed for fathen might be due to differential ploidy levels between dicamba-resistant and susceptible plants (Mukherjee, 1986). It has been noted that diploid plants of *Chenopodium album* had less dented leaves (similar to dicamba-resistant plants) than polyploid (hexaploid) plants which were dented (similar to dicamba-susceptible plants) (Mukherjee, 1986). There is little information regarding the effect of ploidy levels on evolution of herbicide resistance. In a study conducted by Yu *et al.* (2013), it was stated that the level of resistance to ACCase-inhibitors in hexaploid *Avena fatua* was less than diploid grasses like *Lolium rigidum* although the same ACCase resistance mutation was present in each biotype. The authors stated that lesser levels of ACCase-inhibitor resistance in *Avena fatua* could be due to the presence of the resistance mutation only at one out of three homologous ACCase genes. The authors also mentioned that polyploidy might minimize the effect of genes that endow resistance to a biotype. The level of ploidy in dicamba-resistant fathen should be investigated. Studying the level of ploidy might provide fruitful information regarding the evolution of dicamba resistance in fathen. If there is a mix of diploid and polyploid fathen plants in maize fields, then it is possible that the selection pressure imposed by frequent dicamba application has selected for the diploid type of fathen.

12.2.2 A quick test using seeds for detecting dicamba resistance in fathen

In this thesis, the potential for developing a quick test using seeds for detecting dicamba-resistant fathen was studied as quick tests for dicamba resistance in weeds appear to have never been developed (Chapter 4). The quick test using seeds (seed-test) was able to discriminate dicamba-resistant populations from susceptible ones successfully and the results of the quick test were in agreement with the results of sprayed potted plant experiments. In this study, it was shown that radicle expansion was more sensitive than hypocotyl elongation in dicamba treated seedlings. However,

simply looking at the obvious differential responses in seedling development between dicamba-resistant and susceptible populations when grown on dicamba treated media may be sufficient to distinguish resistant biotypes from susceptible ones (Figure 4.5, Chapter 4).

Developing quick tests like this seed-test enables one to monitor the evolution of resistance to a herbicide like dicamba in each season as the seed-test has specific features like not being time-consuming and also does not need much space as experiments in which potted plants are sprayed. When the level of resistance to dicamba was studied in seed dose-response assays compared to sprayed potted plant dose-response experiments, the level of resistance to dicamba was over-estimated in the seed assays. The estimate of level of resistance to dicamba in the seed assays was almost 10 times greater than estimates obtained in the sprayed potted plant experiments. This over-estimation of level of resistance to dicamba might enable one to detect dicamba resistance at an early stage. Evolution of resistance to auxinic herbicides like dicamba occurs slowly (Mithila *et al.*, 2011), therefore early detection of dicamba resistance enables the growers to shift to herbicides with different modes of action in subsequent growing seasons.

12.3 Glyphosate-resistant Italian ryegrass and perennial ryegrass

In this thesis, the first cases of evolved glyphosate-resistant weeds in New Zealand was reported and discussed in Chapter 5. Glyphosate is one of the main herbicides for managing weeds in New Zealand vineyards (Harrington, 2012). It is generally accepted that over-application of herbicides with the same mode of action for several years consecutively can result in the evolution of herbicide-resistant biotypes (Powles & Yu, 2010). In this thesis, we reported glyphosate resistance in Italian ryegrass (two populations) and perennial ryegrass (three populations) from different vineyards. This was the first confirmed report of glyphosate resistant weed populations having developed within New Zealand.

One of the main concerns about the evolution of glyphosate resistance in New Zealand is the spread of resistance genes to nearby sites, especially pastures where Italian ryegrass and perennial ryegrass plants are present. Glyphosate has been the main herbicide used to remove old plants in the pasture renewal process or for planting crops in a paddock as it is cheap, controls most species and has no residual activity. If

pastures in New Zealand become infested with glyphosate resistant plants, then pasture growers will have to shift to other herbicides which do not have the advantages of glyphosate, or else to cultivate their paddocks.

Although only five populations of *Lolium* spp. have so far been confirmed to be resistant to glyphosate, glyphosate resistance may well be present in other places where glyphosate has been used for several years. It is necessary to investigate the evolution of glyphosate resistance in other vineyards or any other places like orchards, railways and roadsides, to provide information on how widespread the glyphosate resistance is in New Zealand. As evolution of glyphosate resistance could become an important issue in New Zealand, a number of aspects (*eg* mechanisms of resistance) regarding glyphosate resistance were investigated in this thesis.

12.3.1 Developing quick tests for detecting glyphosate-resistant Italian ryegrass and perennial ryegrass

As mentioned for dicamba-resistant fathen, it is useful to develop some quick techniques for detecting resistance to glyphosate. Therefore, three different quick tests (seed test, shikimic assay and tiller dip assay) for detecting glyphosate resistance were assessed (Chapter 6). Although two of the investigated quick tests (the seed test and *in vitro* shikimic assay) had been developed previously by overseas researchers (Shaner, 2010), it was necessary to study the usefulness of these tests for detecting glyphosate-resistant Italian ryegrass and perennial ryegrass in New Zealand. Detailed protocols needed to be developed so the tests could be used for checking future suspected glyphosate-resistant Italian ryegrass and perennial ryegrass populations found elsewhere.

The seed test successfully distinguished glyphosate-resistant populations from susceptible ones by measuring root and shoot length. As with the fathen test, we found that the ryegrass root elongation was more sensitive to glyphosate than shoot elongation. Information is lacking on the differential physiological effects of glyphosate on seedling root elongation compared with shoot elongation of glyphosate-resistant and susceptible weeds. It can be hypothesized that the differential responses observed between root and shoot elongation to glyphosate application could be due to differences in triggering the mechanism of glyphosate resistance in different plant tissues. The results of root length provided better data points for estimation the level of resistance to

glyphosate and the level of glyphosate resistance obtained by measuring root length was in close agreement with the level of resistance obtained in the sprayed potted plant tests.

The shikimic acid assay also distinguished glyphosate-resistant populations from susceptible ones successfully. However, compared to the other tests (*eg* seed tests), the shikimic acid assay needs more sophisticated equipment and is also more expensive as the chemical compounds used in the test are expensive. Although the results from the shikimic acid assay could be obtained more rapidly than the other tests, the assay works best when using fresh rapidly expanding leaf material. Therefore, if used as a quick test for material posted in by growers, it might be necessary to grow plant samples for several days after being received through the postal system in order to obtain fresh rapidly expanding leaf material.

In the present PhD project, the ability of a test using excised tillers for detecting glyphosate resistance was investigated (tiller dip assay), and this technique was a new method that had not been published elsewhere. The tiller dip assay is a useful quick test for detecting glyphosate resistance in ryegrass as it does not need sophisticated equipment, does not require the plant to set seed before testing can be conducted and is faster than the Syngenta Quick Test (Boutsalis, 2001) as it can provide results within 8 days of obtaining plants from the field.

It was important to check that results from each of three quick tests would not be affected by the formulation used as glyphosate is sold in different formulations. The results of a study using two formulations (the isopropylamine salt versus potassium salt) suggested that all three quick tests discussed above differentiated resistant biotypes from susceptible ones no matter which formulation was used. As the resistance was to the active ingredient that all such formulations have in common, this was not surprising.

12.3.2 Mechanisms of glyphosate resistance in Italian ryegrass and perennial ryegrass

In Chapter 5, four populations (two Italian ryegrass populations and two perennial ryegrass populations) showed a level of resistance that ranged between 10 and 13-fold, but one population (Population O) had almost a 30-fold level of resistance compared to standard susceptible populations. The greater difference recorded for Population O compared to the other populations in the level of resistance to glyphosate suggested different mechanisms of resistance within Population O. In this thesis, two possible

mechanisms of glyphosate resistance were investigated based on previously published work overseas, namely a target site mutation at position Pro-106 of EPSPS and restricted herbicide translocation. In Chapter 7, the partial sequencing of the EPSPS genome revealed that only Population O had the target site mutation at the Pro-106 position while the other populations did not possess this mutation (Chapter 7, Table 7.1).

The second possible mechanism of glyphosate resistance, the restricted herbicide translocation was investigated on four populations (Populations A and P of Italian ryegrass, and Populations J and O of perennial ryegrass). Investigating the restricted herbicide translocation using a destructive radiolabelled study showed that over half of the absorbed radiolabelled glyphosate was retained in the blade of the treated leaf of all studied glyphosate-resistant populations of Italian ryegrass and perennial ryegrass in contrast to susceptible populations in which over half of absorbed radiolabelled glyphosate was translocated to the pseudostem region (Chapter 8, Tables 8.5 and 8.9).

Population O was shown to have both the target site mechanism of resistance and the restricted herbicide translocation mechanism. Having two mechanisms of glyphosate resistance appears to explain the higher level of glyphosate resistance within Population O compared to the other glyphosate-resistant populations.

In *Conyza canadensis* (Canadian fleabane) with the restricted herbicide translocation mechanism of glyphosate resistance, a massive glyphosate vacuole sequestration was recorded shortly after glyphosate application in contrast to susceptible plants (Ge *et al.*, 2010). Glyphosate vacuolar sequestration has also been observed for glyphosate-resistant *Lolium* spp. with the restricted herbicide translocation mechanism of resistance from Australia, Brazil, Chile and Italy (Ge *et al.*, 2012). It has been noted that some vacuolar membrane-associated transporters might have a role in shifting glyphosate from cytoplasm to vacuole (Ge *et al.*, 2014; Peng *et al.*, 2010; Yuan *et al.*, 2010). In glyphosate-resistant *Conyza canadensis* biotypes with the restricted herbicide translocation mechanism of resistance, it was observed that two ATP-binding cassette (ABC) transporter genes (M10 and M11) were up-regulated in glyphosate-resistant plants compared to susceptible ones upon glyphosate treatment (Nol *et al.*, 2012). The molecular, physiological and biochemical characteristics of the active transporters have not been elucidated completely.

It has been shown that vacuolar sequestration of glyphosate is suppressed at cool temperatures (Ge *et al.*, 2011). Although vacuolar sequestration in New Zealand populations was not investigated in the present PhD project, the results of Chapter 10 showed that glyphosate resistance in the one population of perennial ryegrass tested was temperature dependent and at cool temperatures, the plants of this population (J) became sensitive to glyphosate applications. Physiological and biochemical aspects of this suppression in vacuolar sequestration of glyphosate at cold conditions are not clear yet. Assuming the vacuolar sequestration is mediated by an active transporter, then sub-optimal temperatures may reduce the catalytic activity of the transporter (Yoshida & Matsura-Endo, 1991).

12.3.3 Evolution of glufosinate and amitrole resistance in glyphosate-resistant Italian ryegrass and perennial ryegrass

In order to develop a management system for controlling glyphosate-resistant Italian ryegrass and perennial ryegrass, it was important to know how these glyphosate-resistant populations respond to other herbicides with different modes of action. A preliminary experiment was conducted on all glyphosate-resistant populations of Italian ryegrass and perennial ryegrass (Chapter 9). The results indicated that all glyphosate-resistant populations survived applied rates of glufosinate. Further investigations using two dose-response tests on one population of Italian ryegrass (Population A) and two populations of perennial ryegrass (Populations J and O) confirmed that these glyphosate-resistant populations were also resistant to glufosinate.

Although the mechanism of resistance to glufosinate was not investigated in this thesis, we hypothesise that a non-target site mechanism of resistance might be associated with glufosinate resistance. We think this because glufosinate symptoms like chlorosis were observed 2-3 days after herbicide application for both resistant and susceptible plants but which suggests that the target site of glufosinate, glutamine synthetase, is still sensitive in both resistant and susceptible plants. However, the resistant plants started recovering approximately 3 weeks after herbicide application whereas susceptible plants remained chlorotic for the rest of the experiment period. This observation suggests that the glufosinate might have been metabolised or its translocation might have been restricted in resistant plants. So far, there is only one other report of a glufosinate-resistant Italian ryegrass which is also resistant to glyphosate, and a non-target site mechanism of resistance has been suggested for this glufosinate resistance (Avila-

Garcia & Mallory-Smith, 2011). A target site mechanism (enzyme modification) of resistance has been found for another Italian ryegrass population from USA resistant to glufosinate, but this glufosinate-resistant Italian ryegrass population was not resistant to glyphosate (Avila-Garcia *et al.*, 2012).

It has not been elucidated if glufosinate and glyphosate share the same non-target site mechanism of resistance (restricted herbicide translocation). The results of Chapter 10 showed that restricted glyphosate translocation is temperature dependant and can be suppressed at cool temperatures. However, all three glufosinate-resistant populations (Population A (Italian ryegrass) and Populations J and O (perennial ryegrass)) that we studied in Chapter 9 were still resistant to glufosinate even at cool temperatures. For a better understanding, it will be necessary to study the mechanism of resistance to glufosinate in Italian ryegrass and perennial ryegrass from New Zealand.

Investigating the response to amitrole of all five glyphosate-resistant populations showed that one Italian ryegrass population (Population A) and two perennial ryegrass populations (Populations J and O) were resistant to amitrole. The amitrole exposure records of these populations showed that amitrole has been continuously used for at least 6 years in vineyards where Populations A, J and O were collected (Chapter 5, Table 5.1). The mechanism of resistance to amitrole has yet to be understood. We hypothesise a non-target site mechanism of resistance to amitrole in our resistant weeds because: a) resistant plants showed similar herbicide effects like bleached leaves as susceptible plants after amitrole application which means the target site of amitrole is still sensitive to amitrole in resistant populations; b) in resistant plants, only the middle of the leaves to leaf tips were bleached (typical symptom for amitrole damage); c) the recovery process of resistant plants began with new green leaves growing without any signs of amitrole damage which means either amitrole was not completely translocated to meristematic points (restricted herbicide translocation) or had been deactivated before reaching the meristematic points (herbicide metabolism). To help understand this amitrole resistance, the physiological and molecular basis of the amitrole resistance mechanism in Italian ryegrass and perennial ryegrass need investigating as such information is lacking.

In one of the amitrole dose-response experiments, it was noted that amitrole-resistance in perennial ryegrass populations might be temperature dependant. When the amitrole dose-response experiment was conducted under cold temperatures, the amitrole-

resistant Populations O and J (perennial ryegrass) were as susceptible as the susceptible population (SP). However, for more conclusive proof regarding this temperature effect, it is necessary to conduct amitrole dose-response experiments at warm versus cold conditions using a similar method to the experiments described in Chapter 10 for glyphosate resistance.

12.3.4 The mode of inheritance in Italian ryegrass and perennial ryegrass with restricted glyphosate translocation

As mentioned previously, one of the greatest concerns about the evolution of glyphosate resistance in New Zealand is the consequence of spread of the resistant gene to nearby sites. In Chapter 11, therefore, genetic inheritance of restricted herbicide translocation in glyphosate-resistant Italian ryegrass and perennial ryegrass was investigated. The objective of this work was to examine if the reciprocal crossing between resistant plants and susceptible plants would result in transferring the resistant gene to the progenies of both resistant and susceptible maternal plants (reciprocal F_1 families). The results of the study showed that the progenies of both resistant and susceptible plants were resistant to glyphosate. It suggests that the glyphosate resistant gene can be transferred via both pollen and ovule because the progenies of reciprocal F_1 families from both resistant and susceptible populations were resistant to glyphosate. However, the glyphosate resistance gene in Italian ryegrass and perennial ryegrass was found to have incomplete dominance because the level of resistance to glyphosate in the reciprocal F_1 families was found to be intermediate between original resistant and susceptible populations (Chapter 11).

Given that Italian ryegrass and perennial ryegrass are outcrossing, wind-pollinated and self-incompatible species, outcrossing between glyphosate-resistant and susceptible plants could readily transfer the resistant trait to the offspring. However, the results of this study showed that glyphosate resistance gene is not completely dominant. It might suggest that the rate of spread of the resistant trait might be limited if there is no selection pressure from glyphosate and therefore, the portion of resistant individuals might be reduced in the population after several generations. By using the F_2 generation of crossing between one glyphosate-resistant and one glyphosate-susceptible population, Preston and Wakelin (2008) noted that under natural conditions and no selection pressure from glyphosate, the frequency of glyphosate resistance individuals of *Lolium rigidum* with restricted herbicide translocation significantly declined after three generations.

The gene flow from glyphosate-resistant Italian ryegrass and perennial ryegrass at the site of infection to pastures is another interesting aspect of herbicide resistance in both species that needs investigating. Gene flow studies, especially pollen-mediated gene flow which is more important than seed-mediated gene flow for wind-pollinated *Lolium* spp., could provide fruitful information regarding the future distribution of resistance traits and resistance gene frequency in populations.

12.3.5 Fitness cost

Fitness can be defined as the potential evolutionary ability of a biotype to survive, compete and reproduce where the fittest individuals leave the greatest number of offspring and contribute a greater proportion of its genes to the gene pool of the population (Warwick & Black, 1994). Organisms can change their patterns of resource allocation in response to biotic and abiotic stresses and divert more resources into organs or functions which reinforce their fitness (ecological success or adaptive strategies) against environmental stress over their lifetime (Lerdau & Gershenzon, 1997). However, diverting more resources into one organ or function decreases allocation to other organs or functions because environmental resources are limited (Lerdau & Gershenzon, 1997). Therefore, in the absence of stress, organisms which have evolved heritable resistance to environmental stress may possess an ecological disadvantage compared to their susceptible counterparts and thus resistant organisms may somehow suffer from fitness costs (Vila-Aiub *et al.*, 2009a).

Although the fitness cost of the glyphosate resistant population was not studied in this PhD project, we did notice some differences in plant growth between one of the perennial ryegrass populations (Population O) and other populations (either resistant or susceptible). It was observed that the plants of glyphosate-resistant Population O were always smaller than plants from the other populations. However, it is not possible at this stage to draw a definite conclusion and relate the existence of a fitness cost within this population to its herbicide resistance. Population O had multiple-resistance to three different herbicides (glyphosate, glufosinate and amitrole) and of all the resistant populations we studied, only Population O was found to be highly resistant to both glyphosate (Chapter 5) and amitrole (Chapter 9). Also, both target site and non-target site mechanisms of glyphosate resistant were found in Population O. Thus if a fitness cost was present, it would be most expected to occur in Population O.

By studying plant growth and seed production using a population of *Lolium rigidum* with the restricted herbicide translocation mechanism of resistance, Pedersen *et al.* (2007) did not find a major fitness penalty for the glyphosate resistant plants. However, in order to draw a definite conclusion on the fitness penalty for the resistant weeds, it is recommended to examine fitness costs at various life history stages and in many populations (Vila-Aiub *et al.*, 2009a).

Studying the relative fitness of herbicide resistant and susceptible biotypes provides fruitful information about biological attributes that differ between biotypes. This information can be useful for designing the best herbicide resistance management strategies because it is suggested that mutations which create herbicide resistance in a biotype are likely to affect its other phenotypic characteristics (Roux *et al.*, 2004). If resistant weeds are less fit than their susceptible counterparts, the cessation of herbicide applications allows natural selection to restore the predominance of susceptible biotypes. However, if the fitness of resistant weeds is the same as that of susceptible weeds, resistance may only decline slowly (Schweizer & Westra, 1991). Therefore, it is important to know the relative fitness and competitive ability of resistant and susceptible weeds before adoption of any management strategies of herbicide resistant weeds. There was insufficient time within this PhD project to investigate the fitness of the populations.

12.4 Steps to develop strategies for managing and preventing glyphosate-resistant Italian ryegrass and perennial ryegrass in New Zealand

The first step in developing strategies for managing glyphosate resistance is to identify the problem and provide information regarding how widespread the problem is. Also, programs should be developed in order to make growers aware of the evolution of glyphosate resistance and how that might affect them. The second step is to find solutions to manage the problem. In this regard, physiological, molecular and biochemical characteristics of herbicide resistance should be investigated initially (*eg* mechanism(s) of resistance, the pattern of cross-resistance to other chemicals, heritability of the mechanism of resistance). Information provided on each issue could assist in developing herbicide resistance management strategies. After developing management strategies, it is necessary to inform growers of these strategies. In this regards, growers and farmers could be invited to seminars, field days, meetings, etc. In the present PhD project, some aspects regarding the first and second steps were

investigated. In the next two sections, there is discussion of the implication of the results found in this PhD project to manage glyphosate resistance and the need for further investigations on glyphosate resistance management in New Zealand.

12.4.1 Identifying the problem

Identification of herbicide-resistant weeds requires reliable methods which are accurately able to detect the resistant weeds. Some quick methods for detecting glyphosate resistance in Italian ryegrass and perennial ryegrass were studied in this thesis. Evolution of glyphosate resistance has only been evaluated for five populations of *Lolium* spp. from different vineyards. However, it is important to determine if other populations of resistant ryegrass have evolved resistance to glyphosate elsewhere in New Zealand. There have been many anecdotal messages received during the previous 3-4 years that many other vineyards in Marlborough also have resistant ryegrass. Therefore, it is crucial to provide information regarding the extent of the glyphosate resistance issue in Italian ryegrass and perennial ryegrass in New Zealand. Also, there might be other weed species which have evolved resistance to glyphosate in New Zealand and this needs investigating. Methodical studies to survey the distribution of herbicide-resistant weeds have been conducted in other countries (Boutsalis *et al.*, 2012; Johnson *et al.*, 2014; Menchari *et al.*, 2006; Walsh *et al.*, 2004) but such studies have never been conducted in New Zealand.

12.4.2 Possible solutions for managing glyphosate-resistance in New Zealand vineyards

Strategies for managing herbicide resistance should consist of both chemical and non-chemical methods (Norsworthy *et al.*, 2012). In many regions in the world, the chemical approach has mainly been used for managing glyphosate resistance (Culpepper *et al.*, 2010; Preston, 2010; Steckel *et al.*, 2010; Valverde, 2010; Weller *et al.*, 2010). A sensible chemical approach for managing glyphosate resistance management is to use herbicides with different modes of action (Beckie, 2011; Jasieniuk *et al.*, 1996). Application of a mixture of herbicides with different modes of action is thought more likely to slow down the build-up of herbicide resistance than using herbicides in rotation (Diggle *et al.*, 2003).

Based on models, the application rate is considered to have a major role in the evolution of resistance to herbicides (Renton *et al.*, 2011). The application of herbicides at rates

lower than the recommended rates will increase the rate of evolution of polygenic mechanisms of resistance to herbicides (Renton *et al.*, 2011). However, reducing herbicide rates might delay the evolution of monogenic mechanisms of resistance to herbicides (Renton *et al.*, 2011). Results of reduced herbicide rates will also depend on factors like the biology of the weed species and the genetics of resistance (e.g. relative initial frequency of minor and major alleles). For example, allogamous weed species like *Lolium* spp. are more responsive to the recurrent selection with reduced rates of herbicides than autogamous species like *Avena fatua* (Busi & Powles, 2009). Also, if the frequency of the minor alleles is high compared to major alleles, then the application of reduced rates of herbicides will hasten polygenic mechanism of resistance to herbicides (e.g. diclofop-methyl metabolism in *Lolium rigidum* (Busi *et al.*, 2013)).

In the case of glyphosate resistance, it has been reported that both restricted herbicide translocation and enzyme modification mechanisms of resistance are single-gene traits (reviewed by Powles & Preston (2006)) and theoretically, based on the models (Renton *et al.*, 2011), the evolution of resistance to glyphosate could be delayed under reduced rates of glyphosate (Renton *et al.*, 2011). However, it has been shown that recurrent selections with reduced rates of glyphosate has led to evolution of resistance to glyphosate after three to four generations and these results suggest that one or two additive minor genes could be involved in resistance to glyphosate in the selected population (Busi & Powles, 2009). This result clearly indicates that application of reduced rates of herbicides might not be a good idea as it could lead to accumulation of minor gene traits (Busi & Powles, 2009). Herbicide applications at full doses have also been recommended by other researchers in order to delay the evolution of herbicide resistance (Busi *et al.*, 2013; Lagator *et al.*, 2013; Manalil *et al.*, 2011).

Chemical options could be different due to the situations where weeds have evolved resistance to glyphosate (*e.g.* fruit crop vs. waste areas). Not all the herbicides are registered for use in all situations. For example, paraquat is one of the chemical options that has been suggested to be used following glyphosate application to manage glyphosate-resistant weeds ("double knocks") in Australia (Walker *et al.*, 2004). However, paraquat application in vineyards is not permitted in New Zealand within the Sustainable Winegrowing programme (Harrington, 2012). There are other alternative herbicides that can be used for managing glyphosate-resistant *Lolium* spp. in New Zealand (Harrington, 2014) and some of them were evaluated in this study (Chapter 9).

The results in Chapter 9 showed that glyphosate-resistant weeds studied in this project were also resistant to glufosinate. Although glufosinate no longer appears to be a good option for managing glyphosate-resistant *Lolium* spp., it could still be useful for other weeds which are tolerant to glyphosate (Harrington *et al.*, 2014).

Amitrole is an alternative option that has been registered to be used in vineyards for many years. Although we have found amitrole resistance in populations that were from vineyards where amitrole had been sprayed for several years (Chapter 9), amitrole still can be used for situations where plants are still susceptible to this herbicide. However, it is important to note that relying solely on amitrole would result in the evolution of amitrole resistance. In vineyards, amitrole is only permitted to be used in winter (Harrington, 2012). Interestingly, in Chapter 10 it was shown that glyphosate resistance in one of the populations of perennial ryegrass with the restricted herbicide translocation was suppressed at cold temperatures (below 10°C). Therefore, it should be possible to provide better control of glyphosate-resistant weeds in vineyards if glyphosate and amitrole are applied in combination or in sequential applications during winter.

Application of ACCase inhibitors could be another alternative chemical option for managing glyphosate resistance (Harrington *et al.*, 2014). In our study it was shown that while fluazifop and haloxyfop provided good control of Italian ryegrass, only haloxyfop successfully controlled perennial ryegrass but not fluazifop. Although haloxyfop is not registered for use in vineyards in New Zealand, it would be a useful option for controlling glyphosate resistant Italian ryegrass and perennial ryegrass in situations other than vineyards where the herbicide is permitted to be applied. Clethodim is a closely related herbicide that could be used on perennial ryegrass in vineyards (Christoffoleti *et al.*, 2005). However, the evolution of resistance to ACCase inhibitors has been reported in many different grass species including *Lolium* spp. (Heap, 2015). Therefore, the sole application of ACCase inhibitors such as clethodim for a long period is not recommended.

Residual herbicides have also been recommended for managing glyphosate-resistant *Lolium* spp. as they have different modes of action from glyphosate (Bond *et al.*, 2014). The herbicidal activity of herbicides like terbuthylazine, oxyfluorfen and saflufenacil lasts for several months and provides long lasting control of weeds (Harrington *et al.*, 2014). These residual herbicides could be used as a part of weed management strategies in order to provide good long term control of weeds (Widderick *et al.*, 2013). However,

factors like rainfall, soil type, and the weed species (perennial vs. annual) can affect the efficacy of the herbicide mixture (Monteiro & Moreira, 2004). Field trials should be conducted in order to optimize management strategies using residual herbicides, though they would be useful for preventing a new flush of germination of resistant *Lolium* spp. once old plants had been successfully killed.

There are several non-chemical methods that can be adapted in order to manage glyphosate resistance. For instance, mowing, flaming and shallow cultivations could be used in vineyards as non-chemical methods (Harrington *et al.*, 2014). The results of our study showed that glyphosate resistance can be readily transferred via pollen in ryegrass. Also, Italian ryegrass and perennial ryegrass can only be spread via seed production (Beddows, 1973). In vineyards and orchards, mowing can be employed in order to prevent ryegrass from flowering (Harrington *et al.*, 2014).

An "integrated weed management" approach has been strongly advocated for managing weeds (reviewed by Buhler (2002)). In the "integrated weed management" system, a combination of chemical (herbicide applications) and non-chemical (*e.g.* grazing, biological control, cultivation) methods is employed to provide a better solution for weed control (Hartzler & Buhler, 2007). Using the "integrated weed management" approach could delay resistance enrichment within weed populations via reducing selection pressure (Beckie, 2006).

12.5 Strategies for reducing the risk of evolution of more glyphosate-resistant weeds in New Zealand

Recommendations for avoiding the evolution of new glyphosate-resistant weeds are similar to those mentioned for managing glyphosate resistance. Generally, diversity is the key factor for reducing the risk of herbicide resistance evolution. In this regard both chemical and non-chemical approaches should be employed. The strategies for preventing glyphosate resistance evolution have been reviewed by Harrington (2012). More diversified herbicide rotations are highly recommended in preventing the evolution of glyphosate-resistant weeds (Harrington *et al.*, 2014). Using herbicides with different modes of action in weed control strategies would greatly delay the evolution of resistance in weeds (Neve, 2008). However, depending on the cost, efficacy and the number of applications of the alternative herbicides, the benefit of using alternative chemical options will differ. Chemical methods alone are not sufficient and it is

preferable to manage and avoid the evolution of herbicide resistance. A combination of chemical and non-chemical strategies is the key to preserve the longevity of herbicides (Buhler, 2002).

Glyphosate application is the cheapest and most efficient way to control weeds, so it might be difficult to convince growers to adopt other methods of weed control. However, the evolution of glyphosate-resistant ryegrass is an example of over-reliance solely on glyphosate application. It is also important to note that over-reliance on glyphosate application increases the risk of evolution of glyphosate resistance in other weed species in New Zealand. Species like *Conyza* spp., *Poa annua*, *Sonchus oleraceus* and *Amaranthus* spp. are examples of weed species that are in New Zealand and have evolved resistance to glyphosate overseas (Heap, 2015). The risk of evolution of glyphosate resistant-weeds not only applies to vineyards but wherever glyphosate is being used heavily. Other places like orchards, railways and roadsides are also at risk of evolution of glyphosate-resistant weeds. In order to have sustainable weed management, it is necessary to employ a combination of different chemical and non-chemical methods.

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Appendices

DRC 16 forms - statement of contribution to doctoral thesis containing publications.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hossein Ghani Zadeh

Name/Title of Principal Supervisor: Dr Kerry C. Harrington

Name of Published Research Output and full reference:

H Ghanizadeh, KC Harrington, TK James and DJ Woolley (2015) A quick test using seeds for detecting dicamba resistance in fathen (Chenopodium album). Australian Journal of Crop Science. 9, 337-343.

In which Chapter is the Published Work: Chapters 3 & 4

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Name/Title of Principal Supervisor: Dr Kerry C. Harrington

Name of Published Research Output and full reference:

H Ghanizadeh, KC Harrington, TK James and DJ Woolley (2013) Confirmation of glyphosate resistance in two species of ryegrass from New Zealand vineyards. New Zealand Plant Protection 66, 89-93

In which Chapter is the Published Work: Chapter 5

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Name of Published Research Output and full reference:

H Ghanizadeh, KC Harrington, TK James and DJ Woolley (2015) Quick tests for detecting glyphosate-resistant Italian and perennial ryegrass. New Zealand Journal of Agricultural Research. 58, 108-120

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Name/Title of Principal Supervisor: Dr Kerry C. Harrington

Name of Published Research Output and full reference:

Ghanizadeh H, Harrington KC, James TK and Woolley DJ and Ellison NW (2015) Mechanisms of glyphosate resistance in two perennial ryegrass (Lolium perenne) populations. Pest Management Science (in press).

In which Chapter is the Published Work: Chapters 7 & 8

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Name of Published Research Output and full reference:

Ghanizadeh H, Harrington KC and James TK (2015) Glyphosate-resistant Lolium multiflorum and Lolium perenne populations from New Zealand are also resistant to glufosinate and amitrole. Crop Protection. 78, 1-4.

In which Chapter is the Published Work: Chapter 9

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Name/Title of Principal Supervisor: Dr Kerry C. Harrington

Name of Published Research Output and full reference:

Ghanizadeh H, Harrington KC and James TK (2015) Glyphosate-resistant population of Lolium perenne loses resistance at winter temperatures. New Zealand Journal of Agricultural Research (in press).

In which Chapter is the Published Work: Chapter 10

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