Investigating the potential for glyphosate resistance evolution in UK weedy species

Laura Rachel Davies

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Declaration

This thesis is submitted to the University of Warwick for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. All work presented has been undertaken by myself, except where otherwise stated.

Summary

Glyphosate is the world's most used herbicide. There are currently 32 weedy species with resistant populations in 25 countries, although at present there are no reported cases of glyphosate resistance in the UK. As glyphosate use and selection pressure increases in the UK there is an excellent opportunity to investigate the potential for glyphosate resistance, and the evolutionary processes that may lead to resistance. The variability in standing genetic variation to herbicide susceptibility between weed populations can affect the amount of selection pressure and generations needed for resistance to evolve. If herbicide doses act within this standing genetic variation there may be a reduction in sensitivity due to a buildup of minor alleles related to reduced sensitivity. This thesis has investigated the glyphosate response of three UK weedy species, *Alopecurus myosuroides* (blackgrass), *Anisantha sterilis* (sterile brome), and *Arabidopsis thaliana*. Dose-response experiments showed significant variation in susceptibility between populations of all three species. Glasshouse selection experiments tested if glyphosate sensitivity could be further reduced under directional selection with below field rate doses, in *Alopecurus myosuroides* populations. Following selection, ten of eleven selected lines showed significantly different ED_{50} and ED_{90} values compared to unselected control lines, demonstrating that there is potential for selection of reduced glyphosate sensitivity, which may result in compromised field efficacy. Fitness cost experiments for two glyphosate-selected lines showed no major fitness costs associated with decreased glyphosate susceptibility both with and without wheat competition. Analysis of multi-parent advanced generation inter-cross *Arabidopsis thaliana* lines highlighted an area on chromosome 2 of the *Arabidopsis thaliana* genome that may be associated with variation in glyphosate susceptibility. These results are discussed in the context of the possibility of glyphosate resistance evolution in the UK.

Chapter 1 : Introduction

1.1 Weed control in agriculture

The European Weed Research Society (2014) defines weeds as:

"*plants that impact adversely on economic, aesthetic or environmental aspects of any system*."

The economic costs of weeds can be huge, with estimated yield loss in the USA costing $15.5 billion/year in the absence of herbicide use (Gianessi and Reigner, 2007). Of all agricultural pests, year on year, weeds have the greatest potential to reduce yield (up to 34%). However, due to effective control methods (e.g. herbicides, mechanical weeding) actual yield losses caused by weeds are currently about 9% (Oerke, 2006) (Figure 1-1), highlighting the importance of weed management.

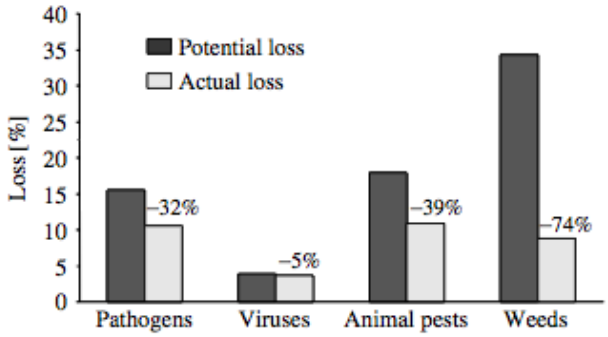

Figure 1-1:Potential yield loss of crops due to pests
Estimated yield losses when no control methods are used (dark grey) and actual yield loss with control methods (light grey), caused by the 4 main crop pests: pathogens, viruses, animals, and weeds. From Oerke (2006)

1.1.1 Weed control methods

In agriculture, various weed control methods have been used for hundreds, if not thousands, of years. In cereal crops there are four main weed control methods:

physical/ mechanical, cultural, chemical, and biological (Melander, *et al*. 2005; Oerke, 2006; Hatchet and Melander, 2003).

1.1.1.1 Physical/ mechanical control

Physical control methods can include harrowing, hoeing, and tillage (Melander *et al*. 2005). Harrowing can be carried out both pre- and post-emergence, with Brandsæter *et al*. (2012) reporting that in spring cereals pre-emergence harrowing could reduce weed density by 26%, post-emergence harrowing could reduce densities by 47%, and in combination densities could be reduced by 61%, with crop yields increased by 6.2%, 4% and 10% respectively.

Tillage is also a widely used practice in physical weed control, with two main types, inversion tillage where a seedbed is prepared by complete inversion of the soil incorporating all crop residues, and conservation/ non- inversion tillage (the main tillage used in the UK) where the upper 10cm of soil is inverted and 70% of crop residue is incorporated (Morris *et al*. 2010). By inverting the soil weed seed is distributed vertically in the soil profile, which significantly affects emergence, with seed buried deeper less likely to emerge (Morris *et al*. 2010). Changes in tillage systems can lead to weed species shifts, with the prevalence of perennial and grass weeds increasing under conservation tillage (Locke *et al*. 2002).

1.1.1.2 Cultural control

Cultural control methods include crop competition, crop rotation, delayed sowing, and stale seedbeds, amongst others (Melander *et al*. 2005). Crop rotation can have a significant effect on the weeds present in a field, with specific weeds associated with

certain types of crop in a sequence, for example cereal crops can produce low dicotyledon seed banks and low to medium monocotyledon seed banks, where as spring sown oil seed rape can be associated with high monocotyledon and dicotyledon seed banks (Bohan *et al*. 2011).

Delayed sowing, which can be used in conjunction with stale seedbeds, can also reduce weed biomass, with Rasmussen (2004) reporting upto a 40% reduction in weed biomass due to delayed sowing, however effects can vary. Stale seedbeds are commonly used in the UK (Morris *et al*. 2010), and are where a seedbed is prepared days, weeks or months before crop sowing (Johnson and Mullinix, 1995). Weed seeds are left to germinate before methods such as ploughing, tillage and, more recently, non-selective herbicides, such as glyphosate, are used to remove the weed seedlings before crops are sown (Dogan *et al*. 2009). Using herbicides as a weed control for stale seedbeds can be preferable to the farmer, as it can be more effective at weed control than other methods, such as mechanical weeding (Rasmussen, 2004), and it is faster and less costly (Gianessi and Reigner, 2007).

1.1.1.3 Chemical control

Weed control was revolutionised after the Second World War with the introduction of 2,4-dichlorophenoyxacetic acid (2,4-D), a selective auxinic herbicide used in cereal crops (Oerke, 2006). The introduction of herbicides meant that labour intensive weed control methods, such as hoeing, were replaced, saving time and money, and increasing yields (Cannell, 1985; Oerke, 2006). Herbicides are now commonly used for weed control in modern farming.

Herbicides can be used with other weed control methods, such as tillage and stale seedbeds, and often herbicides with different modes of action are used in combination within a crop to obtain sufficient weed control, with different modes of action effecting different weeds, such as monocotyledonous and dicotyledonous weeds (Shaw and Arnold, 2002). Farmers may use herbicides with other more costly control methods, such as tillage, to increase the lifespan of the herbicide and increase the effectiveness of weed control by removing weeds missed by herbicide application (Dogan *et al*. 2009). However, the introduction of herbicides has changed farming methods, such as a shift towards minimum or non- tillage systems with the use of glyphosate (Woodburn, 2000), and has meant that weed management practices are no longer an integral part of cropping systems (Bhuler, 2002).

There are currently 25 herbicide modes of action (Heap, 2015) including acetolactate synthase (ALS) inhibitors, glyphosate, acetyl CoA carboxylase (ACCase) inhibitors, and photosystem inhibitors. However, no new modes of action have been discovered for over 20 years, with hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides the last mode of action to be introduced (Duke, 2012).

1.1.1.4 Biological control

Biological weed control is the use of biological agents, such as insects and plant pathogens (fungi, bacteria, viruses, and nematodes), to suppress weeds (Weed Science Society of America, 2015). Biological weed control agents are usually targeted at weeds in later life stages, and are used in conjunction with other weed control methods, such as mechanical control (Hatcher and Melander, 2003). It is often species specific, and therefore can be used to target specific weed species without damaging

the crop (Hatcher and Melander, 2003). Bioherbicides have been developed that are applied periodically to crops and release the bio-control agent over the entire weed population. Although there has been some success with bioherbicides, their use is not widespread, as conventional chemical herbicides provide much higher efficacy (Müller-Schärer and Collins, 2012).

1.1.1.5 Integrated weed management

Weed species can shift in response to control methods, for example herbicide resistance evolution, meaning that a more integrated approach to weed management is needed (Bhuler, 2002). The use of integrated pest management (IPM) is encouraged by the European Commission as a sustainable approach to reduce the use of pesticides and their associated risks to human health and the environment (European commission, 2014). IPM is a system based on three principles: using and integrating practices to prevent development of populations of harmful organisms, considering all available plant protection methods, and using these methods at economically and ecologically justifiable levels (Lefebvre *et al*. 2015).

Integrated weed management (IWM) is a part of IPM and involves the combination of multiple weed control methods (i.e. cultural, physical/mechanical, chemical, and biological), and the integration of weed biology knowledge into the management approaches (Bhuler *et al*. 2000, Müller-Schärer and Collins, 2012). IWM needs to be developed within the context of cropping systems, with crop rotations, long-term management practices, and the surrounding ecosystem, being taken into account (Bhuler *et al*. 2000). Furthermore, due to the plasticity and adaptability of weeds IWM also needs to be a continuous and adaptive process (Bhuler, 2002). When using IWM

the ubiquity of weeds in agricultural systems and weed seedbanks need to be taken into account, as the seedbank is the primary source of weed infestations. Furthermore, in IWM it is important to understand the processes relating to the emergence of the weed seedbank, such as dormancy levels, as these have a major impact on weed emergence and consequently the effectiveness of IWM strategies, as the success of these methods depends on affecting as many weed individuals as possible (Batlla and Benech-Arnold, 2007).

The practice of IWM is much less developed and used than those of other integrated pest management practices to control pests, such as insects, due to the reliance on herbicides to control weeds. However, due to the increasing spread of herbicide resistance and organic farming IWM practices are becoming more widespread (Müller-Schärer and Collins, 2012).

1.1.2 Lack of new herbicides

Globally, there is a growing herbicide resistance problem in weedy species, which is a major concern due to the potential yield losses caused by weeds (Oerke, 2006; Heap, 2015). Previously, herbicide resistant weeds may have been treated with a new mode of action, however, there have been no new herbicide modes of action introduced in the last 20 years, reducing the options for tackling herbicide resistance (Duke, 2012). The lack of new modes of action is mostly due to the introduction of genetically modified glyphosate resistant crops leading to the devaluation of other herbicides and, consequently, a disincentive to invest in research and development for new modes of action (Rüegg *et al*. 2007; Duke, 2012). Instead, industry resources have shifted away

from discovery of new herbicide modes of action and towards finding genes to use in genetically modified crops to make them resistant to existing herbicides (Clark, 2012).

Tightening toxicological and environmental restrictions, such as the EU Regulation 1107/2009, concerning the placing of plant protection products on the market and EU Directive 2009/128/EC establishing a framework for community action to achieve the sustainable use of pesticides, and the increasing cost of discovery and screening, have also contributed to the lack of new herbicide discovery and availability (Rüegg *et al*. 2007; EU, 2009a; EU, 2009b; Clark, 2012; Weis *et al*. 2012). New chemicals are subjected to more rigorous testing and are tested for bioaccumulation and toxicity to water fauna at an earlier stage (Clark, 2012). Tightening legislation has also meant that many existing chemicals have been removed from the market in the past decade and the risk of future tightening of regulation acts as a deterrent for investment in new chemicals (Chauvel *et al*. 2012).

1.2 Herbicide resistance

The Weed Science Society of America (1998) defines herbicide resistance as:

"the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type."

1.2.1 Global herbicide resistance

In 1957 the first cases of herbicide resistance were reported in populations of *Commelina diffusa* Burm.f. (spreading dayflower) in Hawaii, USA, and *Daucus carota* L. (wild carrot) in Michigan and Ohio, USA, and Ontario, Canada. These populations

had evolved resistance to the synthetic auxin herbicide 2,4-D (Heap, 2015). Currently, there are 246 weed species in 66 countries with populations resistant to 22 of the 25 available modes of action (Figure 1-2) (Heap, 2015).

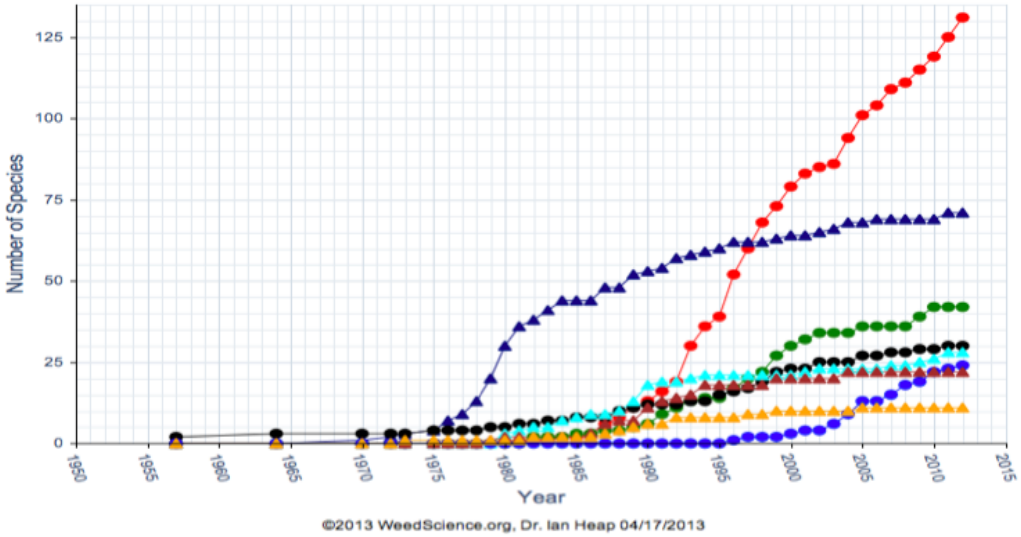

Figure 1-2: Chronological increase since 1950 in global number of species with evolved herbicide resistant populations
Resistant to 8 different herbicide modes of action: ALS (red), Triazines (dark blue), ACCase (green), Synthetic auxins (black), Bipyridiliums (light blue), Glycines (including glyphosate) (blue), Ureas and Amides (brown), and Dinitroanilines (orange) from Heap (2015)

Weed populations can be resistant to one herbicide or multiple herbicides either through cross-resistance or the separate evolution of multiple herbicide resistance mechanisms. Cross-resistance is where a herbicide resistance mechanism that has evolved under the use of one herbicide provides resistance to other herbicides usually with in the same mode of action but occasionally between modes of action and is usually conferred by metabolic resistance mechanisms (Hall *et al*. 1997). Multiple herbicide resistance is where an individual has two or more different herbicide resistance mechanisms that have evolved separately of each other and confer resistance to two or more herbicides (Owen *et al*. 2007).

1.2.3 Herbicide resistance in the UK

The first case of herbicide resistance in the UK was reported in 1975 in a *Tripleurospermum inodorum* (L.) Sch. Bip. (scentless chamomile) population resistant to 2,4-D with cross-resistance to other synthetic auxins (Heap, 2015). Currently, there are 15 species with resistant populations to 9 herbicide modes of action in the UK (Table 1-1) (Heap, 2015). There are even some weed populations with resistance to multiple herbicide modes of action. For example, an *Alopecurus myosuroides* Huds. (blackgrass) population from Peldon in the UK has multiple resistance to ALS inhibitors (Marshall and Moss, 2008), ACCase inhibitors, Aryloxyphenoxypropionate (AOPP), Photosystem II inhibitors (Hall *et al*. 1997), and Photosystem I election diverters (Cummins *et al*. 1999) modes of action.

In a few situations herbicide resistance has become so severe there are no more cost- or technologically effective herbicide options left. Previously, problematic weeds may have been treated with a herbicide with a new mode of action, but due to the reduced availability of different modes of action this is becoming more difficult (Duke, 2012). It is therefore important to conserve the modes of action that are left.

Table 1-1: Weed species in the UK with herbicide resistant populations
Year of discovery and herbicide modes of action resistant to, adapted from Heap (2015)

Species	Common name	First discovered	Mode of action
Alopecurus myosuroides	Blackgrass	1982	ACCase inhibitors (A/1)
		1982	Photosystem (PS)II inhibitor (Ureas and amides) (C2/7)
		1984	ALS inhibitors (B/2)
		1987	Microtubule inhibitors (K1/3)
		-	AOPP (Hall *et al*. 1997)
		1999	PSI inhibitors (Cummins *et al*. 1999)
Arabidopsis thaliana	Mouse-ear Cress	1988	Photosystem II inhibitors (C1/5)
Avena fatua	Wild Oat	1994	Multiple Resistance: 3 Sites of Action
			ACCase inhibitors (A/1)
			ALS inhibitors (B/2)
			Antimicrotubule mitotic disrupter (Z/25)
Avena sterilis	Sterile Oat	1993	Multiple Resistance: 3 Sites of Action
			ACCase inhibitors (A/1)
			ALS inhibitors (B/2)
			Antimicrotubule mitotic disrupter (Z/25)
Chenopodium album	Common Lambsquarters	1989	Photosystem II inhibitors (C1/5)
Conyza canadensis	Horseweed	1982	Photosystem II inhibitors (C1/5)
Epilobium ciliatum	Fringed Willowherb	1981	Photosystem II inhibitors (C1/5)
		1989	PSI Electron Diverter (D/22)
Lolium perenne	Italian Ryegrass	1990	ACCase inhibitors (A/1)
ssp. multiflorum		1990	PSII inhibitor (Ureas and amides) (C2/7)
		2012	ALS inhibitors (B/2)
Matricaria discoidea	Pineapple-weed	1989	Photosystem II inhibitors (C1/5)
Papaver rhoeas	Corn Poppy	2001	ALS inhibitors (B/2)
Poa annua	Annual Bluegrass	1981	PSI Electron Diverter (D/22)
		1981	PSII inhibitors (C1/5)
Senecio vulgaris	Common Groundsel	1977	PSII inhibitors (C1/5)
Solanum nigrum	Black Nightshade	1983	PSII inhibitors (C1/5)
Stellaria media	Common Chickweed	1985	Synthetic Auxins (O/4)
		2000	ALS inhibitors (B/2)
Tripleurospermum perforatum	Scentless Chamomile	2002	ALS inhibitors (B/2)

1.2.4 Mechanisms of herbicide resistance

Understanding the mechanism for herbicide resistance can be important, enabling the evolutionary processes leading the appearance and spread of the mechanism to be determined and compared to other resistance cases (Neve, 2007). There are two types

of herbicide resistance mechanisms, target site resistance (TSR) and non-target site resistance (NTSR) (Délye *et al*. 2013a). TSR mechanisms are either the consequence of a mutation of the gene that expresses the targeted protein resulting in an amino acid and structural changes at the herbicide-binding site reducing herbicide affinity, or gene amplification and increased expression of the target protein. Most TSR mechanisms are dominant or semi-dominant nuclear traits, although there are a few cases of recessive TSR (Powles and Yu, 2010; Délye *et al*. 2013a).

NTSR mechanisms are any other mechanism of resistance not related to the target site (e.g. reduced translocation, reduced herbicide uptake, enhanced metabolism) and cause a reduction in the amount of herbicide reaching the target site (Petit *et al*. 2010; Powles and Yu, 2010; Délye *et al*. 2013a). NTSR is under complex genetic control, with it either being endowed by a single resistance allele, or by the accumulation of multiple minor alleles related to resistance, resulting in multiple resistance phenotypes (Petit *et al*. 2010; Délye *et al*. 2011; Beckie and Tardiff, 2012). Polygenic NTSR mechanisms may be diverse, reflecting the diversity of metabolic pathways and processes involved and inter- and intra-specific variation (Délye *et al*. 2013a).

Evolved resistance to one herbicide can also cause cross-resistance to other herbicides within the same group, and even other herbicide modes of action. The mechanism of resistance affects cross-resistance, with TSR and some NTSR mechanisms, such as translocation, usually restricting cross-resistance within mode of action, whereas other NTSR mechanisms, such as metabolism, can confer cross-resistance to multiple modes of action (Beckie and Tardiff, 2012). However, cross-resistance to some modes of action, such as glyphosate, can be rare (Beckie, 2011).

1.3 Selection for herbicide resistance

1.3.1 Selection pressure

Herbicide use and consequently selection pressure varies from field to field and within regions, meaning that resistance usually evolves on a local scale and is not homogenous within a species (Délye *et al*. 2010). Selection for herbicide resistance traits can vary widely depending on initial allele frequency, whether the trait is monogenic or polygenic, and the rate at which selection occurs (Renton *et al*. 2011).

The herbicide dose applied to crops has a direct effect on herbicide resistance selection pressure and resistance evolution in weeds. High herbicide rates select for single gene mutations conferring high levels of resistance, whereas low rates can act within standing genetic variation and select for polygenic NTSR (Gressel, 2009). Furthermore, dose and, therefore, selection pressure will affect the rate and time taken for resistance traits to spread through a population. Monogenic traits will spread quickly under high doses and selection pressure, where as polygenic traits requires exposure to a low dose and selection pressure over a number of generations for resistance alleles to build up in individuals before resistance is conferred (Délye *et al*. 2013a). For example, in modeled populations, Renton *et al*. (2011) found that low herbicide rates accelerated resistance evolution in a polygenic resistance situation, whereas high rates increased herbicide longevity. In a monogenic resistance situation, alternating rates had no impact on the longevity of a herbicide and reducing rates increased the time taken for monogenic resistant weeds to evolve.

Application timing can also affect resistance evolution, as shown by Neve *et al*. (2003a), where later application exposed a higher proportion of modeled populations to glyphosate, increasing selection pressure and decreasing time taken for resistance evolution. Furthermore, later application of herbicide will expose individuals at later growth stages when herbicide efficacy is lower (Riemens *et al*. 2008), resulting in larger weed populations and the survival of less susceptible individuals that may not have survived at an earlier growth stage. Herbicides also apply different resistance selection pressures, as the chemical structure, mode of action, and residual activity of the herbicide influence resistance evolution (Powles and Yu, 2010).

1.3.2 De novo mutation

Monogenic TSR often arises as the result of a single nucleotide polymorphism causing an amino acid substitution on the protein targeted by the herbicide (Powles and Yu, 2010; Délye *et al*. 2013a). This can result in a change in structure of the target enzyme preventing the herbicide from binding to it. Depending on the structural change to the target protein, this leads to high or intermediate levels of resistance (Délye, 2013; Délye *et al*. 2013a).

Where monogenic resistance confers a high degree of resistance, enabling the survival and reproduction of individuals carrying the mutation, it will spread quickly throughout the population due to positive selection (Délye, 2013). The rate of monogenic TSR evolution depends on the rate of mutation, dominance, and fitness costs in the presence and absence of the herbicide (Délye *et al*. 2013a). The same TSR mechanism can occur independently in multiple populations of the same species (Délye *et al*. 2004).

The level of resistance conferred but mutation of the target site can vary with mode of action. The target site mutations of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) gene that confer glyphosate resistance do not usually confer high levels of resistance, for example, an Australian glyphosate resistant population of *Lolium rigidum* Gaudin (rigid ryegrass), with a proline-106 to threonine substitution, only has a 1.9-3.4 times higher LD_{50} compared to susceptible populations (Wakelin and Preston, 2006), whereas EPSPS gene amplification confers LD_{50} values in resistant individuals 7-13 times greater than susceptible individuals in a USA population of *L. rigidum* (Salas *et al*. 2012). The low-levels of resistance conferred by EPSPS gene mutation, and the presence of few target site resistant mutations that confer glyphosate resistance, is possibly due to there being few mutations at the EPSPS target site that can confer resistance whilst still maintaining protein function (Powles and Yu, 2010). For example, the EPSPS target site mutation threonine-102 to isoleucine cannot exist independently, as PEP binding affinity is extremely low with this mutation and impairs plant function, however, it can exist with the proline-106 to serine mutation that confers lower levels of resistance, but has a higher PEP binding affinity (Yu *et al*. 2015).

1.3.3 Extent of standing genetic variation

Standing genetic variation is the presence of different alleles at a locus in a population where the genetic variation is neutral, but can become beneficial under environmental change (Barrett and Schluter, 2008; Délye *et al*. 2013a). Standing genetic variation to herbicide tolerance can mean that a population can respond immediately to exposure

to a herbicide, rather than taking time for new mutations to appear (Orr and Betancourt, 2001).

Herbicide resistance from standing genetic variation can be the result of a resistance allele already being present within the population before herbicide application. For example, Délye *et al*. (2013b) found the Leu-1781 ACCase resistance allele in an *Alopecurus myosuroides* specimen collected in 1888, long before any exposure to ACCase herbicides could have taken place. Neve and Powles (2005a) found that progeny from survivors of five previously unexposed, susceptible, Australian populations of *L. rigidum* were resistant to the ACCase herbicide diclofop-methyl when subjected to field rate. However, lower field rates are used in Australia, which may have played a role in the resistance of the *L. rigidum* individuals, as the rates may have acted within the range of normal phenotypic variation. The resistance was found not to be a monogenic target-site resistance mechanism.

The frequency of resistance alleles present from standing genetic variation will greatly affect the rate at which resistance spreads once herbicide selection pressure is applied. Neve *et al*. (2003a) found that modeled *L. rigidum* populations with an initial glyphosate resistance allele frequency for a monogenic trait of 1×10^{-6} led to 100% resistance in all populations after 10 years. Whereas, in populations with an initial allele frequency of 1×10^{-8} with an incompletely dominant allele, resistance was predicted in <50% of the populations after 16 years.

Herbicide resistance from standing genetic variation can also be the result of the build up of multiple minor alleles related to resistance, leading to polygenic NTSR. Any

mechanisms that increase the chances of plant survival, either weak or strong, can increase in frequency in the population under herbicide selection pressure (Powles and Preston, 2006). When NTSR is the result of multiple resistant alleles from standing genetic variation accumulating in a plant, resistant phenotypes can be unpredictable, as progeny can inherit any number of resistance alleles from the parents its evolution depends on the amount and nature of available genetic variation, meaning that resistance levels may vary. However, under selection pressure in out-crossing species these alleles will increase in frequency and accumulate in individual plants over generations, resulting in an increase in the frequency of less susceptible individuals in a weed population (Hermission and Pennings, 2005; Petit *et al*. 2010; Délye, 2013). For example, Délye *et al*. (2011) found that an accumulation of NTSR alleles in *Alopecurus myosuroides* was required to confer resistance to a number of different herbicide modes of action and that the degree of resistance varied amongst progeny.

1.3.4 Variation in herbicide susceptibility

Standing genetic variation can potentially contribute to evolved herbicide resistance mechanisms within and between weed populations, therefore, initial variations in the level of herbicide susceptibility may be present. Natural variation to herbicides, such as glyphosate, can exist between different populations of the same weed species and the frequency of alleles related to resistance in a population can affect the amount of selection pressure and generations needed for resistance to evolve (Zeleya and Owen, 2005).

Susceptibility data can provide information on the state of inherent herbicide susceptibility and resistance in a species, and can potentially be used to identify

populations at risk of resistance evolution. Natural variation in herbicide susceptibility is often overlooked in herbicide resistance studies and true sensitivity data is not collected (Ulber *et al*. 2013). Baseline herbicide variability is the variability in susceptibility to herbicides in previously unexposed populations (Ulber *et al*. 2013). Baseline variability data can be used to assess the potential differences in responses to herbicides in susceptible weed populations and to detect any shifts in herbicide susceptibility/ tolerance in different populations (Espeby *et al*. 2011).

Random sampling of weeds in a field and a comprehensive background of farming practice can be used in herbicide sensitivity screening to measure the frequency of resistance or differences in tolerance and management strategies that contribute to resistance evolution in a field. Data collected from these surveys can be used to generate herbicide resistance evolution risk models (Burgos *et al*. 2013).

Variation in susceptibility to various herbicides, including glyphosate, has been found in populations of *L. rigidum* in Spain (Loureiro *et al*. 2010) and Australia (Neve and Powles, 2005a), *Bromus diandrus* Roth (ripgut brome) in Spain (Escorial *et al*. 2011), *Alopecurus myosuroides* and *Apera spica-venti* (L.) P.Beauv. in Sweden (Espeby *et al*. 2011), and *Conyza canadensis* (L.) Cronquist, (horseweed) in the USA (Main *et al*. 2004).

As well as standing genetic variation, variation in herbicide susceptibility and resistance can also be caused by gene flow. Busi *et al*. (2011) found that 2% and 37% of individuals in two *L. rigidum* populations from organic fields not exposed to herbicides were resistant to ACCase or ALS herbicides. These fields neighbored ones

where herbicides were used and identical herbicide resistant haplotypes were identified in both the exposed and unexposed populations, probably due to gene flow.

1.3.5 Creeping resistance

Standing genetic variation and initial variation in herbicide susceptibility may play a role in creeping resistance (Espeby *et al*. 2011). Creeping resistance can occur when weed populations are exposed to doses that are not 100% effective and act within the range of standing genetic variation present within the population, leading to some less susceptible individuals surviving. In outcrossing species these individuals may interbreed, resulting in recombination and accumulation of minor resistance alleles in the progeny leading to a gradual shift or creep in the mean level of herbicide resistance within the population. The rate at which creeping resistance can occur is influenced by the initial variation in standing genetic variation in herbicide susceptibility within and between populations, and the doses of herbicide applied (Gressel, 2009; Délye, 2013; Ulber *et al*. 2013). If creeping resistance occurs it can be predicted that differential tolerance to herbicides, which can be dependent on previous herbicide use, will increase between populations (Espeby *et al*. 2011).

Many herbicide resistance mechanisms are determined to be dominant or semi-dominant single gene nuclear traits (Powles and Preston, 2006). However, most studies focus on populations once high levels of resistance have evolved, so it is possible that the role of minor genes and creeping resistance, are being overlooked (Neve *et al*. 2009, Espeby *et al*. 2011). For example, Lorraine-Colwill *et al*. (2001) reported that at lower glyphosate doses multiple genes contributed to the survival of resistant plants in a *L. rigidum* population, but that a single incompletely dominant

gene conferred resistance at high doses. This oversight of early resistance evolution may occur because resistance conferred by single genes convey high levels of resistance that quickly becomes apparent in the field, whereas creeping resistance can go unnoticed as herbicide efficacy can vary year on year and temporal trends in efficacy are not well documented (Espeby *et al*. 2011).

Some studies have investigated the role of the accumulation of minor gene traits and creeping resistance in the evolution of herbicide resistance. Herbicide resistance can be selected for at low doses in populations that have never been exposed to the herbicide before. Busi and Powles (2009) found that after only three to four generations of selection at sublethal glyphosate doses in a susceptible population of *L. rigidum* led to an accumulation of minor gene traits and a shift towards resistance. Escorial *et al*. (2011) credited the additive effects of minor genes for the dose dependent glyphosate resistance in some populations of *B. diandrus*.

A *L. rigidum* population evolved resistance after fewer than three generations of selection at reduced rates of the ACCase inhibiting herbicide diclofop-methyl. The rarity of a single amino acid substitution resistance mechanism, the absence of resistance in the population that would be endowed by this mechanism, the shallow gradient of the dose response curves, and the increasing fitness of survivors (under herbicide application) between generations suggested that this resistance was polygenic (Neve and Powles, 2005b). It was later confirmed that three existing alleles, present from standing genetic variation, became stacked in individuals over the three cycles of selection and conferred resistance (Busi *et al*. 2013b). The same population also showed a shift towards resistance to a new herbicide, pyroxasulfone, over 3

generations of low dose selection, with some individuals showing cross-resistance to other herbicides (Busi *et al*. 2012).

In contrast to these examples where resistance has been selected for, Brotherton *et al*. (2007) found no increase in glyphosate tolerance after seven generations of selection of the Col-0 *Arabidopsis thaliana* (L.) Heynh. ecotype at low dose. The differences found between these selection experiments are likely due to *L. rigidum and B. diandrus* being out-crossing species, whereas *Arabidopsis thaliana* is selfing. Out-crossing is a significant factor in herbicide resistance evolution, as cross-pollination with other surviving plants enables the transfer of major single resistance genes to the progeny of susceptible plants and the additive enrichment of minor resistance genes, whereas this does not happen in selfing species (Busi and Powles, 2009).

Interestingly, but unsurprisingly, herbicide susceptibility can also be selected for in similar experiments by bulk-crossing clones of the most herbicide susceptible individuals, showing that excluding minor resistance alleles from a population can increase herbicide susceptibility (Manalil *et al*. 2012).

Reduced herbicide effectiveness as a consequence of selection for minor genes could lead to larger population sizes. The increased number of individuals in a population increases the amount of adaptive mutation and recombination in that population, which increases the rate of generation of genetic variation, in turn increasing the probability of the occurrence of herbicide resistant individuals and major gene selection, even at low rates of mutation (Jasieniuk *et al*. 1996; Neve *et al*. 2009).

1.3.6 Fitness costs and benefits

Fitness costs are where an adaptation to one environment (e.g. herbicide exposure) results in trade-offs in another environment (e.g. absence of herbicides) causing reduced fecundity (Jasienuik *et al*. 1996; Vila-Aiub *et al*. 2009a; Délye, 2013). Herbicide resistance traits confer a fitness benefit in the presence of the herbicide, but in its absence herbicide resistant individuals may be less fit than herbicide susceptible individuals. Fitness costs can result from a change in the effectiveness of the target enzyme's normal function, the herbicide resistance mechanism may divert resources away from growth and/ or reproduction, or there may be altered ecological interactions as a result of the resistance mechanism, for example with pollinators (Vila-Aiub *et al* 2009a).

Quantifying fitness costs is essential when trying to predict herbicide resistance evolution and spread. Under constant selection with a herbicide resistant individuals will have a higher level of fitness than susceptible individuals, therefore even if there is a fitness cost to resistance it will not be expressed, resulting in the fixation of the resistance allele (Jasienuik *et al*. 1996). If the fitness of resistant individuals is less of that of susceptible wild-type individuals, in the absence of the selection pressure the allele frequency will decrease and the evolution of resistance will slow (Jasienuik *et al*. 1996). Conversely, if there is no fitness cost to resistance in the absence of the selection pressure the frequency of the resistance allele will not change and the population will remain resistant even after the removal of the selection pressure (Vila-Aiub *et al*. 2014).

Fitness costs of resistance vary with species and the mechanism of resistance. For example, EPSPS gene amplification conveys no fitness cost in glyphosate resistant *Amaranthus palmeri* (Giacomini *et al*. 2014; Vila-Aiub *et al*. 2014), but does convey a fitness cost in *Amaranthus tuberculatus* populations (Cockerton, 2013). Additionally, the target site EPSPS threonine-102 to isoleucine mutation for glyphosate resistance confers such a high fitness cost due to reduced function it cannot exist independently, where as the proline-106 to serine mutation can (Yu *et al*. 2015).

1.4 Glyphosate and glyphosate resistance

1.4.1 Glyphosate mode of action

Glyphosate is the world's most used herbicide and the evolution of glyphosate resistance is currently of particular concern. Glyphosate is a non-selective herbicide that inhibits EPSPS in the shikimate pathway (Steinrücken and Amrhein, 1980). The shikimate pathway is essential in the synthesis of aromatic amino acids in plants, bacteria, and fungi. In the shikimate pathway EPSPS catalyzes the transfer of enolpyruvyl moiety from phosphoenol-pyruvate (PEP) to shikimate-3-phosphate, forming EPSP and inorganic phosphates (Schönbrunn *et al*. 2001). By acting as a competitive inhibitor to the PEP binding site and a non-competitive inhibitor for the shikimate-3-phosphate site, glyphosate prevents the formation of EPSP (Schönbrunn *et al*. 2001), causing excess carbon flow to shikimate-3-phosphate and the accumulation of high levels of shikimate in affected plants (Cereira and Duke, 2006) (Figure 1-3). It is thought that plant death is caused by the inhibition of EPSPS reducing levels of EPSP and its metabolic products, phenylalanine, tyrosine and tryptophan, to levels insufficient to maintain protein synthesis (Powles and Preston,

2006; Duke and Powles, 2008). Glyphosate phytotoxicity symptoms include chlorosis, pigmentation, stunting, reduction in apical dominance and eventually death (Baylis, 2000).

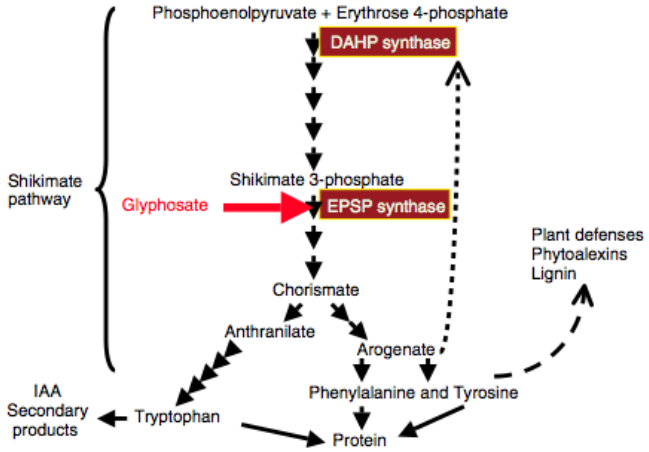

Figure 1-3: The shikimate pathway with the site of inhibition by the herbicide glyphosate
The shikimate pathway is essential for the production of aromatic amino acids in plants, bacteria, and fungi. Glyphosate inhibits the 6th enzyme in the process 5-enolpyruvly-shikimate-3-phosphate synthase preventing the production of aromatic amino acids, leading to plant death. Figure from Duke and Powles (2008)

Glyphosate is highly effective, as most plants are unable to metabolise it and, once absorbed, it is rapidly translocated throughout the plant via the phloem to metabolically active tissues (Cerdeira and Duke, 2006; Preston and Wakelin, 2008; Shaner, 2009). It is not known how glyphosate is translocated into the phloem, but it possibly occurs through mass diffusion into mesophyll cells then through the plasmodesmata into the phloem, or through active uptake by a phosphate transporter into the mesophyll or companion cells. However, once translocation has occurred, due to its hydrophilic nature it is unable to leave the phloem and is transported from source (treated leaves) to sink (roots, shoots, untreated leaves) (Shaner, 2009).

1.4.2 Global use of glyphosate

As a non-selective herbicide, glyphosate is usually applied to weeds resulting in their suppression and mortality before crop seeding (e.g. on stale seed beds), meaning that selection pressure for resistance is low as only a small proportion of a population is exposed at any one time (Powles, 2008). It is predicted that where glyphosate is used once a year in weed removal there is a low resistance risk, which increases with a second annual application (Neve, 2008). Selection pressure for glyphosate resistance is also lower than that of some other herbicide modes of action, as it is not active or residual in soil, meaning that glyphosate selection pressure events are short term and an intense (Powles, 2008). In addition, glyphosate use is usually followed by the application of other herbicide modes of action, such that initially rare resistant survivors are controlled by other herbicide modes of action (Neve, 2008).

In countries where genetically modified crops (GMCs) have been adopted, glyphosate is often used as the main or sole weed control method (Powles and Preston, 2006). Glyphosate is also commonly used on a frequent basis between rows of tree, nut and vine crops and for roadside weed control (Powles, 2008). The continual use of glyphosate in GMCs and orchards has imposed intense selection pressure for glyphosate resistance evolution and has led to weed species shifts and a shift towards glyphosate resistant individuals in these situations (Owen, 2008). For example, in soybean (*Glycine max*) crops in the Southern USA glyphosate was applied to <20% of crops between 1990 and 1995. By 2006, glyphosate was applied to 96% of soybean crops, 85% of which were glyphosate resistant GMCs under glyphosate only weed control regimes. In the same cropping systems between 1995 and 2009 *Amaranthus palmeri* S. Wats (Palmer amaranth), *C. canadensis* (L.) Cronquist, (horseweed) and

Richardia scabra L. (Florida pusley), all species that can be difficult to manage in glyphosate only systems, went from 23rd, 38th and 39th most problematic weeds to 2nd, 4th and 5th most problematic, respectively (Webster and Nichols, 2012). In Canadian glyphosate resistant oilseed rape (*Brassica napus* L.) systems, which have been in use since 1995, glyphosate resistance is yet to evolve in weeds, possibly due to lower selection pressure as a result of the additional use of alternative herbicide resistant oilseed rape and crop rotation (Harker *et al*. 2012).

Glyphosate-resistant GMCs are not the only cropping system where glyphosate selection pressure can be high. There are large areas of Australia infested with glyphosate-resistant *Lolium* (ryegrass) populations that evolved resistance in conjunction with the repeated and sole use of glyphosate for fallow weed control and weed removal before no-till crop seeding (Powles, 2008). This use of glyphosate as a weed control method is similar to that used in the UK, where it is predominantly used for weed control before crop sowing (Cook *et al*. 2010), which raises concerns of resistance evolution in the UK.

1.4.3 UK glyphosate use and resistance selection pressure

Over the past two decades glyphosate use has drastically increased in the UK, particularly in cereal crops (Figure 1-4). This increased use is partly due to the reduction in availability of other herbicide modes of action, both due to tightening regulations (Clark, 2012; Chauvel *et al*. 2012), increasing herbicide resistance to other modes of action in the UK (Moss *et al*. 2011; Heap, 2015), and glyphosate price decreasing since 1993 (Woodburn, 2000). With increasing glyphosate use comes

increased glyphosate selection pressure, as exposing more individuals to the herbicide increases the likelihood of resistance evolution (Neve *et al*. 2003a).

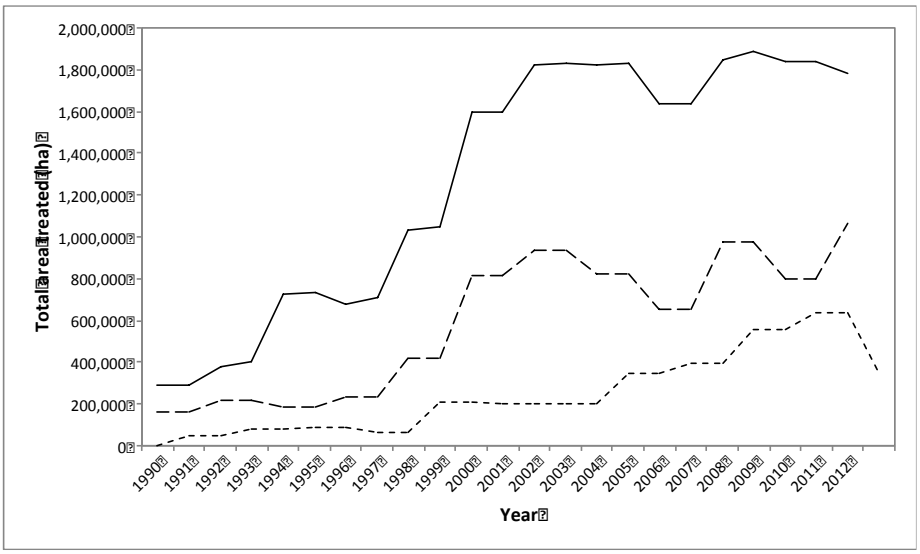

Figure 1-4: Total area treated with glyphosate in Great Britain 1990-2014
For all crops (solid line), cereal crops (dashed line) and oil seed rape (dotted line). Note the increase in use between 1999 an 2001, where glyphosate came off patent in 1999, Information from Fera (2014)

Currently, there are no reported glyphosate resistant weeds in the UK, however, with the prevalence of resistance to other herbicide modes of action it is important that it does not evolve, as in some cases glyphosate is one of the few chemical option left for weed control. If regulation and/or the evolution of resistance prevented glyphosate use in the UK, the cost across just three crops (wheat, winter barley, and oilseed rape) is estimated to be €633M per annum, with a reduction of yield of 12% in wheat and winter barley, and 10% in oilseed rape (Wynn *et al*. 2014). Furthermore, even with the absence of glyphosate-resistant GMCs in the UK, there is still the possibility that glyphosate resistance could evolve with its use in pre-sowing application, as has been the case in some glyphosate resistant populations in Australia (Neve, *et al*. 2004) and Italy (Collavo and Sattin, 2014).

1.4.4 Global glyphosate resistance

Glyphosate is a relatively low-risk herbicide for resistance evolution, particularly when compared to other modes of action, such as ACCase and ALS inhibitors, and glyphosate resistant weeds have been very slow to evolve. Glyphosate is a lower-risk herbicide as mutation rates for glyphosate resistance are relatively low compared to other herbicide modes of action (Jander *et al*. 2003) and there is also a high probability of glyphosate resistance mutations being lethal (Beckie, 2006). Furthermore, the way glyphosate is used also lowers the risk of resistance evolution, as when applied in conventional cropping systems on a stale seedbed only a proportion of the weed population is exposed to selection pressure, as some of the population will emerge after glyphosate application meaning that susceptible individuals remain in the population 'diluting' the proportion of any resistance genes possibly present (Neve *et al*. 2003a).

It was thought at one stage that due to the lack of glyphosate resistance evolution after 23 years of use and the complexity of developing glyphosate resistant crops, the evolution of resistant weeds would be unlikely. It was postulated that target site resistance had not evolved, as any changes in the EPSPS that would prevent glyphosate binding would also prevent PEP binding and so have a negative impact (Bradshaw *et al*. 1997). However, the first case of glyphosate resistance was reported by Powles *et al*. (1998) in an Australian population of *L. rigidum*, where glyphosate was used in an orchard two to three times a year for fifteen years. There are now 32 weedy species with resistant populations in 25 countries, with the USA particularly badly affected with 14 resistant species (Heap, 2015) (Figure 1-5).

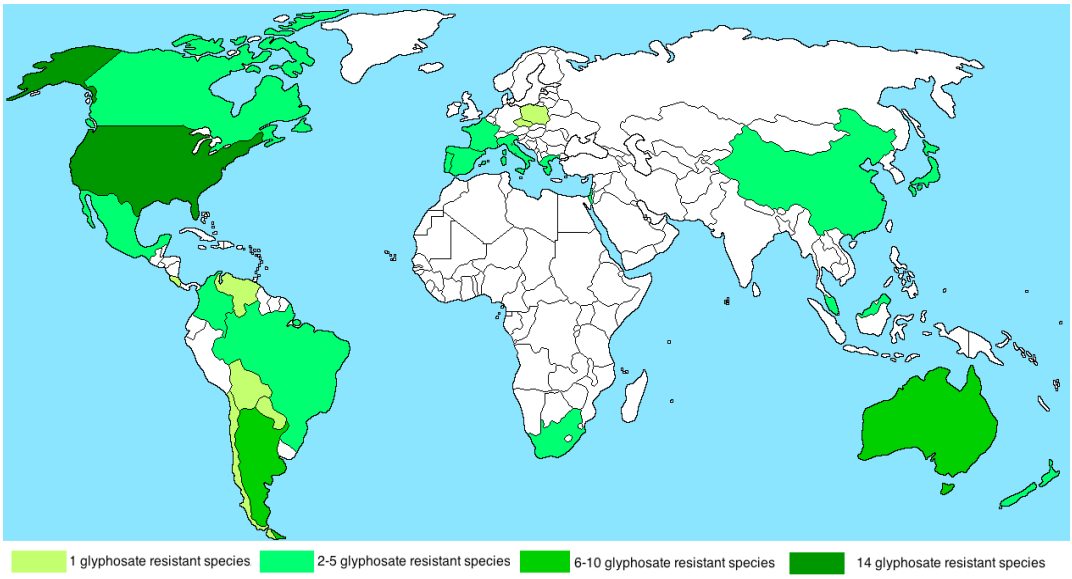

Figure 1-5: Map of countries with glyphosate resistant weed populations
The problem is particularly prevalent in countries where GM glyphosate resistant crops have been adopted (e.g. USA, Canada), but also in Australia where glyphosate is used heavily for weed control before crop sowing. Adapted from Heap (2015)

1.4.5 Glyphosate resistance mechanisms

Multiple TSR and NTSR glyphosate resistance mechanisms have evolved and the evolution of these is dependent on a number of factors, including use rates and weed species (Busi *et al*. 2013a).

Altered glyphosate translocation (a type of NTSR) confers resistance in some populations of *L. rigidum*, *Lolium multiflorum* Lam. (Italian ryegrass), *C. canadensis* (Preston and Wakelin, 2008) and *Conyza bonariensis* (L.) Cronquist (hairy fleabane) (Shaner *et al*. 2012). Reduced translocation can occur through glyphosate not loading into minor vascular bundles and subsequently remaining in the apical portion of treated leaves. Perez-Jones *et al*. (2007) suggested that the reduced translocation resistance mechanism in a glyphosate resistant *L. multiflorum* population was either due to glyphosate being trapped in the apoplast or through glyphosate movement into the xylem rather than the phloem with the glyphosate accumulating in the leaf tip as a

result of transpiration. However, the absolute mechanism underpinning reduced glyphosate translocation is still unknown (Shaner, 2009). Another NTSR mechanism is vacuolar sequestration. This has been reported *in C*. *canadensis* (Ge *et al*. 2010) and *Lolium* species, where glyphosate sequestration into the vacuole was unidirectional and possibly through active transport (Ge *et al*. 2012). There is also a glyphosate resistance mechanism in *Ambrosia trifida* (L.) (Giant ragweed) that has been reported to confer resistance through the rapid necrosis of mature leaves that have come into contact with the herbicide (NTSR), possibly preventing glyphosate translocation, and suppressing growth of immature tissue for a time (Brabham *et al*. 2011).

Mutation of the EPSPS gene can confer TSR. Proline 106 to serine and proline 106 to threonine substitutions have been found to confer resistance in *Eleusine indica* (L.) Gaertn. (Indian goosegrass) and *Lolium* species, by reducing the affinity of EPSPS for glyphosate binding (Perez-Jones *et al*. 2007; Ng *et al*. 2003). More recently a Malaysian *E*. *indica* population has been found to have two amino acid substitutions on the EPSPS gene, threonine-102 to isoleucine and proline-106 to serine, which confer high glyphosate resistance levels, 180-fold more than the susceptible wild type and 32-fold more than resistant biotypes with just the proline-106 to serine mutation (Yu *et al*. 2015).

Resistance can also be conferred through EPSPS gene amplification (a form of TSR). This has been found to be a resistance mechanism in *Amaranthus palmeri* (Gaines *et al*. 2010), and *L*. *multiflorum*, with one population having up to 25 copies of the EPSPS gene in resistant individuals (Salas *et al*. 2012). Increased gene expression and increase enzyme activity is another form of TSR. In a glyphosate resistant *L*. *rigidum*

population there was over-expression of the EPSPS gene and a 2.5- to 3.5-fold increase in EPSPS enzyme activity levels in resistant individuals when compared to that of susceptible individuals, with no evidence of gene amplification (Baerson *et al*. 2002).

Furthermore, some glyphosate resistant biotypes also have more than one resistance mechanism. For example, a *L. multiflorum* glyphosate resistant biotype in Spain and a *L. rigidum* biotype in Australia have resistance conferred both through a target site mutation and reduced glyphosate translocation mechanisms, with the *L. rigidum* biotype being more resistant than biotypes with a single resistance mechanism (González-Torralva *et al*. 2012; Bostamam *et al*. 2012). As yet, unlike resistance to other herbicide modes of action, no metabolic glyphosate-resistance mechanism is known to have evolved (Duke, 2011).

1.4.6 Potential for Glyphosate resistance evolution in the UK

It has been proposed that herbicide resistance research should focus more on the evolutionary process of resistance, integrating research into standing genetic variation and fitness costs, amongst others, rather than focusing on resistance once it has already evolved (Neve, 2007; Busi *et al*. 2013a, Neve *et al*. 2014). There is currently an excellent opportunity to implement this research in the UK, as selection pressure for resistance is increasing due to the increasing use of glyphosate in weed control. Currently, selection pressure for glyphosate resistance evolution in the UK is relatively low, as glyphosate is mainly used in a weed removal treatment before crop seeding. However, as mentioned above, glyphosate resistance has evolved in these situations in Australia (Powles, 2008) and Italy (Collavo and Sattin, 2014).

Glyphosate is such an important herbicide in the UK and worldwide, with its use in conjunction with GMCs and the possibility of the introduction of glyphosate resistant GMCs in the UK in the future, and resistance to other herbicides being so prevalent in the UK, where glyphosate is sometimes the only effective chemical option left. It is, therefore, essential that the potential for glyphosate resistance evolution in the UK is investigated. This information could potentially be utilized in management strategies to help prevent resistance evolution.

1.5 Study species

1.5.1 *Alopecurus myosuroides*

Alopecurus myosuroides Huds. (blackgrass) is the most problematic arable weed in Western Europe, with the largest problem in England (Moss *et al*. 2007). *Alopecurus myosuroides* in a genetically diverse, wind pollinated, out-crossing, self-incompatible grass, that is native to Europe and the Mediterranean (Chauvel and Gasquez, 1994; Menchari *et al*. 2007; Délye *et al*. 2010). *Alopecurus myosuroides* populations are prone to herbicide resistance evolution, with Délye *et al*. (2004) reporting multiple origins of the same ACCase resistance mechanisms in different populations. Evolution of herbicide resistance in *Alopecurus myosuroides* populations can vary from field to field and can depend on previous herbicide exposure and cultural weed control use (Délye *et al*. 2010).

There is high genetic diversity within *Alopecurus myosuroides* populations, but little genetic differentiation between populations, probably due to its recent expansion as a

weed. Herbicide resistance evolution appears to have had little impact on *Alopecurus myosuroides* genetic diversity, as it is impossible to differentiate between herbicide resistant and susceptible populations using allelic diversity, heterozygosity, or the percentage of polymorphic loci (Chauvel and Gasquez, 1994, Menchari *et al*. 2007).

There are 13 countries with herbicide resistant *Alopecurus myosuroides* populations, including France, Germany, the Netherlands, and the UK. In the UK there are populations resistant to ALS inhibitors, ACCase inhibitors, dinitroanilines, and urea and amide herbicides (Heap, 2015). It is estimated that more than 80% of farms, in the UK, where herbicides are used to control *Alopecurus myosuroides* have some form of herbicide resistance in the species (Moss *et al*. 2011). Some resistant populations in France have exhibited polygenic cross-resistance to ACCase and ALS herbicides through an accumulation of NTSR genes (Petit *et al*. 2010). In total, there are populations of *Alopecurus myosuroides* resistant to six herbicide modes of action, making it joint 5th with *L. multiflorum* in the top 15 herbicide resistant species worldwide (Heap, 2015).

Alopecurus myosuroides seed germination corresponds to the sowing times of winter cereals, with most germination occurring between October and November, with some seeds emerging prior to sowing enabling them to be removed in the stale seed bed, for example with glyphosate, and some emerging within the crop (Swain *et al*. 2006). As in these situations only a small proportion of the population is exposed to glyphosate, resistance selection pressure is low (Powles, 2008).

There are currently no glyphosate resistant *Alopecurus myosuroides* populations (Heap, 2015). However, as *Alopecurus myosuroides* is so prone to resistance evolution and is such a large problem in the UK it is possible that it could evolve glyphosate resistance here. It is very important that this does not happen, as the consequences could be extremely damaging due to the prevalence of herbicide resistant *Alopecurus myosuroides* populations in the UK.

1.5.2 *Anisantha sterilis*

Another important UK cereal weed is *Anisantha sterilis* (L. Nevski) (sterile or barren brome, syn *Bromus sterilis*), which is a grass weed found in cereal and oilseed rape crops. There are biotypes in Germany resistant to ACCase inhibitors and France resistant ALS inhibitors (Heap, 2015). *Anisantha sterilis* is widespread across UK wheat cropping regions, and is usually found in field margins, but infestations of fields are also common (Cussans *et al*. 1994). It is a mainly inbreeding species, with genetically different lines maintained through this process, however, outcrossing between lines does occasionally occur (Green *et al*. 2001).

Like *Alopecurus myosuroides*, *Anisantha sterilis* individuals produce many seeds. These seeds have low dormancy germinating almost straight after shedding. Due to the low dormancy and high seed rate, herbicides need to have a high efficacy to obtain sufficient control of *Anisantha sterilis* populations in minimum tillage situations (Lintell-Smith *et al*. 1999).

There are currently no *Anisantha sterilis* populations resistant to glyphosate (Heap, 2015), however, there are glyphosate resistant populations of *Bromus diandrus* (ripgut

brome, syn *Anisantha diandra* (Roth)) Tutin ex Tzvelev and *Bromus rubens* L. (red brome, syn *Anisantha ruben*) (L.) Nevski that infest wheat crops and fallow land respectively in Australia (Heap, 2015), where glyphosate is used in a similar way to the UK (Owen *et al*. 2014). Furthermore, as a result of minimum tillage systems and the reduced availability of other herbicide modes of action, glyphosate is often used to control *Anisantha sterilis* populations before crop sowing (Dow AgroSciences, 2014; HGCA, 2014), increasing selection pressure for resistance evolution. It is therefore possible that glyphosate resistance may evolve in this species in the UK.

1.5.3 *Arabidopsis thaliana*

As a model species with many different ecotypes genotyped, *Arabidopsis thaliana* is an ideal species to investigate the genetic variation related to variation in herbicide response. Naturally herbicide resistant *Arabidopsis thaliana* accessions have previously been discovered. El-Lithy *et al*. (2005) found that the accession Ely was atrazine resistant due to a point mutation in the chloroplast *psbA* gene as a result of high selection pressure from the use of triazine herbicides at the site of collection. Furthermore, Brotherton *et al*. (2007) found variation in glyphosate response in 72 *Arabidopsis thaliana* accessions, but no glyphosate resistance was observed. Being a model plant *Arabidopsis thaliana* accessions are an ideal tool to use in the process of understanding genetic variation to glyphosate susceptibility.

1.6 Aims

1. Investigate the extent of phenotypic variability in susceptibility to glyphosate in UK populations of:

a. *Alopecurus myosuroides*

b. *Anisantha sterilis*

2. Investigate experimental evolution of glyphosate resistance in *Alopecurus myosuroides*
3. Test the hypothesis that minor gene variation for glyphosate susceptibility (standing genetic variation) in *Alopecurus myosuroides* can be enriched under selection, resulting in phenotypes that are resistant to commercial application rates
4. Test the hypothesis that populations of *Alopecurus myosuroides* with the highest degree of variation in glyphosate susceptibility are the most prone to resistance evolution
5. Investigate possible fitness costs and trade offs related to glyphosate resistance or increased glyphosate tolerance in *Alopecurus myosuroides* populations after experimental glyphosate selection
6. Test for variation in glyphosate sensitivity in global ecotypes of *Arabidopsis thaliana* and multi-parent advanced generation inter-cross (MAGIC) lines

Chapter 2 : *Alopecurus myosuroides* (Blackgrass) Glyphosate Sensitivity Screening

2.1 Introduction

2.1.1 Variation in herbicide susceptibility

For the evolution of herbicide resistance to occur genetic variation in herbicide susceptibility must be present within a population. This variation may pre-exist as standing genetic variation, or it may be introduced via new mutations and/or gene flow (Jasieniuk *et al*. 1996). It can be assumed that even in naïve weed populations additive genetic and phenotypic variation for herbicide susceptibility will be present (Neve *et al*. 2014). Phenotypic variation is extremely important in determining how an organism responds to selection pressures, such as herbicide application (Hendry *et al*. 2011). Where this variation is heritable under selection pressure it can lead to a reduction in herbicide susceptibility in weed populations (Busi *et al*. 2013b). Therefore, understanding initial variation in pesticide susceptibility is important in investigating the early stages of resistance evolution, but has arguably been overlooked in the evolution of herbicide resistance (Neve *et al*. 2009).

Adaptation from standing genetic variation is predicted to occur over fewer generations than adaptation from new mutations, as the beneficial alleles are immediately available within the population (Barrett and Schluter, 2008). The initial frequency at which these alleles are present within the population will greatly affect the time taken for adaptation, with a high frequency leading to resistance evolution over fewer generations (Neve *et al*. 2003a). The frequency of alleles related to resistance will vary between populations both before and after herbicide exposure

(Ulber *et al*. 2013). In exposed populations where this variation in susceptibility is significant without the presence of resistance is it is possible that there is creeping resistance, where over a number of generations recombination of minor resistance alleles leads to a gradual shift towards resistance that goes unnoticed in the field (Gressel, 2009; Espeby *et al*. 2011, Chapter *1.3.5*). If creeping resistance is occurring it can be predicted that the variability in susceptibility between weed populations will increase (Espeby *et al*. 2011). Collecting herbicide sensitivity data in weed populations can be extremely useful for detecting creeping resistance, as it can highlight its occurrence in exposed populations, or be used to detect changes in populations when exposed to new herbicides.

2.1.2 Sensitivity data

Establishing the sensitivity of weed populations to herbicides can be useful in detecting a shift towards resistance, as in the short term most adaptation is likely to arise from standing genetic variation and understanding this variation can provide an excellent indication of evolutionary potential (Hendry *et al*. 2011; Ulber *et al*. 2013). This initial variation has been reported in some species, for example, Espeby *et al*. (2011) found variable response to flupysulfuron in *Alopecurus myosuroides* populations, and to sulfsulfuron in *Apera spica-venti* populations, none of which had previous exposure to the herbicides. This baseline information can only be gained from populations that have not previously been exposed to the herbicide mode of action, of which there are now few.

However, the lack of untreated populations does not mean that there is no value in comparing sensitivity amongst populations, as this provides insight into the current

level of herbicide susceptibility and resistance in weed species (Ulber *et al*. 2013). For example, when testing populations that had previously been exposed to the herbicides, Espeby *et al*. (2011) also reported variable response and previously unreported resistance in populations of *A*. *myosuroides* to fenoxaprop-P-ethyl, which conferred cross resistance to flupysulfuron, and variable response in populations of *Apera spica-venti* to isoproturon, which did not confer cross resistance to sulfsulfuron.

2.1.2.1 Glyphosate sensitivity data

In contrast to baseline variability studies of unexposed populations and those where there is variation in exposed resistance populations variation in response to glyphosate has been reported in exposed populations of different species without the presence of resistance. Escorial *et al*. (2011) found variation and decreased glyphosate susceptibility in *Bromus diandrus* between different Spanish regions of 5.9% and 13.8%. Variation in the same populations to the herbicides chlortoluron, diclofop-methyl and chlorsulfuron was also reported. Boutin *et al*. (2010) reported variation in response to glyphosate in eight plant species collected from three to seven locations around the world, with GR_{25} values ranging from 60 to 98 g ha^{-1} for *Bellis perennis* L. (English daisy), and 104 to 228 g ha^{-1} for *Digitalis purpurea* L. (common foxglove).

There is also much variation in glyphosate sensitivity in species where resistant populations have already been reported. Loureiro *et al*. (2010) found 6.9% of Spanish *Lolium rigidum* populations tested for glyphosate resistance displayed intermediate or full resistance. Kniss *et al* (2007) reported a range of glyphosate susceptibility and resistance in USA *Chenopodium album* populations ranging between complete resistance to complete susceptibility at 840-glyphosate g ha^{-1}, with a strong

relationship between past glyphosate use and reduced sensitivity. When investigating two glyphosate resistant *L. rigidum* populations from perennial crops, Collavo and Sattin (2012) found ED_{50} values of 340 and 5319 g ha^{-1}, with the latter population having high levels of resistance conferred by multiple mechanisms.

There are only a few target site glyphosate resistance alleles know and it is likely that most mechanisms are non-target site (Yuan *et al*. 2007), which can be polygenic and under diverse control (Délye *et al*. 2013a). Therefore it is possible that variation in glyphosate susceptibility will have a major impact on non-target site resistance evolution. To date no glyphosate susceptibility studies have been reported in the UK. With variation in susceptibility to glyphosate and other herbicides reported in various countries and weed species and this variation in exposed populations being indicative of creeping resistance, variation in susceptibility is something that needs to be investigated and quantified to determine the state of glyphosate susceptibility/ tolerance in the UK.

2.1.3 Objectives

The main objective of this chapter is to investigate the variation in susceptibility of UK populations of *A. myosuroides* to the herbicide glyphosate. This is accomplished through dose-response analysis to determine variation in glyphosate susceptibility amongst a set of 55 *A. myosuroides* populations collected from around the UK (16 collected in 2010, and 39 collected in 2012).

2.2 Materials and Methods

Glyphosate sensitivity was assessed in 55 *A. myosuroides* populations collected from farmer's fields in 2010 (16 populations) and 2012 (39 populations). For all experiments a collection from the Broadbalk long-term experiment at Rothamsted Research was used as an unexposed population, as this population has no previous exposure to glyphosate or any other herbicide mode of action (Moss *et al*. 2004). However, this population is not isolated and therefore cannot be classed as a standard susceptible population, as the population may have been enriched through pollen flow from other exposed populations.

2.2.1 Plant material

2.2.1.1 2010 Alopecurus myosuroides collections

Seventeen populations of *A. myosuroides* seeds were collected in 2010 (Figure 2-1, also see Appendix 1). Seeds were collected from across each field in a W shape with random plants sampled throughout the field and a minimum of 150 seed heads collected for each population.

2.2.1.2 2012 Alopecurus myosuroides collections

In July and August 2012 (16.07.12-3.08.12) 40 *A. myosuroides* populations were collected from across England (Figure 2-1, also see Appendix 2). Prior to collection, farmers were contacted using a network of contacts provided by Dow AgroScience, Rothamsted Research, ADAS, and farm advisers. Collection sites were chosen for the presence of *A. myosuroides* only, and not based on previous glyphosate use. Seven populations were provided directly by ADAS from their collections.

A population was defined as all *A. myosuroides* plants growing in a single field and seeds were only collected from one site per farm. Seeds were taken from between 130 and 531 seed heads (mean: 375) per population, with seed heads stripped of mature seed by rubbing the seed heads over a paper bag. To ensure a representative sample of the population in the collection field, seeds were collected by traversing the whole field in a W shape, collecting plants at 0.5m intervals in heavily infested fields or from every plant on the W shape in less heavily infested fields. After collection, seeds were dried and stored in paper bags at 15% RH, 15^{o}C. After drying, seed lots were cleaned to remove unfilled seeds and debris.

To enable glyphosate sensitivity to be related to previous glyphosate use and field management, a field history was requested from participating farmers (Appendix 3). Where glyphosate use history was known, populations were given a glyphosate use score between 0-10, with 0 being no glyphosate exposure and 10 being regular use (>2 uses per year, for >10 years) at high doses ($\geq$1080 g ha^{-1}) (Table 2-1, Appendix 2)

Figure 2-1: Collection sites of *A. myosuroides* populations
17 collected in 2010 (△) and 39 populations collected in 2012 (•)

Table 2-1: Glyphosate use score assigned to UK *A. myosuroides* populations Collected in 2012, based on information provided by farmers at time of collection

Score	Glyphosate Use
0	Never exposed to glyphosate
1	No recorded exposure, but possible in the past
2	Glyphosate not used on stale seed bed, but used for crop desiccation
3	Very occasional glyphosate use on seed bed at low doses, <10 years use
4	Very occasional glyphosate use on seed bed and used for crop desiccation, <10 year use
5	Use 1-2L ha^{-1} on stale seed bed once a year, >10 years use
6	Use 2L ha^{-1} on stale seed bed once a year, >10 year use
7	Use 3L ha^{-1} on stale seed bed once a year, >10 year use
8	Use >3L ha^{-1} on stale seed bed once a year, >10 years use
9	Use 3L ha^{-1} on stale seed bed twice a year, >10 years use
10	Use >2 times a year on stale seed bed, >10 years use

2.2.1 Standard procedures

2.2.1.1 Dormancy breaking

At maturity, *A. myosuroides* usually exhibit some degree of seed dormancy. Treatment under warm dry conditions can break this dormancy and increase subsequent germination (Swain *et al*. 2006). Prior to experiments, seeds were treated in paper bags at 30^{o}C in an incubator for 6 weeks.

2.2.1.2 Seed sowing directly into soil

Ungerminated seeds were sown into 90x90x100mm pots or 150 well hassey trays containing a mix of topsoil, sand and M2 compost in a 2:1:1 ratio. Pots were placed in a glasshouse compartment and covered with polythene for 9-10 days to protect against pests and promote growth. Seeds were sown into pots in order of replicate, with replicate 1 sown first.

2.2.1.3 Glasshouse conditions

Supplementary lighting was provided in the glasshouse compartment with a 17-hour day length. Supplementary lighting was provided by 6 400W high-pressure sodium luminaries, with a light threshold of turning on at 10klx and turning off at 30klx. Temperature was set at 20ºC + venting at 22ºC between 05:00 and 22:00, and 12ºC + venting at 15ºC between 22:00 and 05:00.

2.2.1.4 Thinning plants

To ensure that herbicide treated plants were a similar size, all seedlings smaller than two leaves and larger than four leaves in size (growth stage 12-13 (Hess *et al*. 1997)) were removed from pots prior to herbicide application.

2.2.1.5 Glyphosate application

Glyphosate (Roundup ProBiactive, 360g/L glyphosate present as isopropylamine salt at 480g/L (41.1% w/w) (Monsanto) (recommended field rate 540 g ha^{-1})) was applied to plants using either a Berthoud knapsack sprayer or a track sprayer (generation III research sprayer, DeVries). For both sprayers a flat fan, even spray nozzle (FE80/0.8/3) at a pressure of 3bar, and a speed of 3kph, giving an output of 200L water ha^{-1} was used.

2.2.1.6 Assessment

Around 21 days after treatment with glyphosate, plant survival was assessed with plants assigned to one of four classes (Figure 2-2). Following assessment of survival, plants were harvested 5mm above soil level and individual fresh weight was

determined. All experiments were assessed in blocks of replicates, with replicate 1 assessed first.

Figure 2-2: Example of *A. myosuroides* survival scores for glyphosate dose-response assay autumn/ winter 2011
A – alive: no observable effect compared to control (unsprayed) plants; B – alive: some observable effect e.g. yellowed leaf tips, stunted growth; C – dead: large observable effect e.g. necrosis of majority of leaf tissue; D – dead: necrosis of all leaf tissue

2.2.2 Experiment one: Glyphosate dose-response of 17 UK *Alopecurus myosuroides* populations collected in 2010

Seventeen UK *A. myosuroides* populations collected in 2010 were tested for their response to varying concentrations of glyphosate in a dose-response assay. Six glyphosate doses (0, 54, 108, 162, 270, and 405 g ha^{-1} (540 g ha^{-1} recommended rate)) were used.

Seeds were sown into 90mm Petri dishes containing three 85mm filter papers and 5ml of deionized water with 100 seeds per Petri dish. Seeds were germinated in an incubator with an alternating 23/9ºC temperature regime and a 12hr light /12hr dark photoperiod (high temperature corresponding to light phase). Fourteen days after sowing, germinated seedlings were transplanted into 70x70x80mm pots containing

topsoil, with 8 seedlings per pot, one pot per population/dose/replicate, and glasshouse compartment settings were as in *2.2.1.3*. Seedlings were transplanted into pots over 3 days in order of replicate, with replicate 1 sown first. A total of 17 populations, 6 glyphosate doses, and 3 replicates were used. Pots were placed in a split plot design, with pots containing each population randomised within dose tray, and dose trays randomised within replicate. Replicates were placed in rows in the glasshouse compartment.

Two days prior to herbicide application, plants were thinned as in *2.2.1.4*. After thinning most pots contained 6 or more individuals, however for some pots in some populations there were fewer than 6 individuals. The dose series of glyphosate was applied to the plants using a Berthould Knapsack sprayer (see section *2.2.1.5*), 19-21 days after transplanting (replicate 1 21 days after transplanting, replicate 2 20 days, replicate 3 19 days). Twenty-one to twenty-three days after treatment (DAT) plants were assessed (see section *2.2.1.6*). Replicate 1 was assessed 21 DAT, replicate 2 22 DAT, and replicate 3 23 DAT.

2.2.3 Experiment two: Glyphosate dose-response of 17 UK *A. myosuroides* populations collected in 2010

In experiment 1 there was higher than expected survival at the highest glyphosate dose used, meaning that the ED_{50} values of some populations and ED_{90} values of all populations were unable to be calculated, and standard errors were large. Therefore, to confirm the results from the experiment 1 and extend the number of doses used to improve accuracy, a second glyphosate dose-response experiment was undertaken in autumn 2013 on the 17 *A. myosuroides* populations collected in 2010. Eight

glyphosate doses were used: 0, 81, 162, 270, 405, 540, 810 and 1080 g ha^{-1} (540 g ha^{-1} recommended rate).

Seeds were treated under warm conditions (see section *2.2.1.1*), then sown into 90x90x90mm pots (see section *2.2.1.2*). Seedlings were transplanted into pots over 3 days in order of replicate, with replicate 1 sown first, replicates 2 and 3 sown on the second day, and replicate 4 sown on the third day. A total of 17 populations, 8 glyphosate doses, and 4 replicates were used. Pots were placed in a randomised split plot design, with pots containing each population randomised within dose tray, and dose trays randomised within replicate. Replicates were placed in rows in the glasshouse compartment.

Glasshouse settings were those in *2.2.1.3*. Twenty-one days after sowing plants were thinned to 8 plants per pot (see section *2.2.1.4*) (except where there was poor germination) and glyphosate doses were applied 22-24 days after sowing (replicate 1 24 days after sowing, replicates 2 and 3 23 days, replicate 4 22 days) using a Berthoud knapsack sprayer (see section *2.2.1.5*). Plants were assessed 19-22 DAT (see section *2.2.1.6*). Replicate 1 was assessed 19 DAT, replicate 2 20 DAT, replicate 3 21 DAT, and replicate 4 22 DAT.

2.2.4 Experiment three: Glyphosate dose-response of 40 UK *A. myosuroides* populations collected in 2012

In October to December 2012, a glyphosate dose-response assay was performed to assess glyphosate susceptibility of the 40 *A. myosuroides* populations. Eight doses (0,

81, 162, 270, 405, 540, 810 and 1080 g ha^{-1} (540 g ha^{-1} recommended rate)) were used.

Seeds were treated under warm conditions (see section *2.2.1.1*) and sown directly into 90x90x90mm pots (see section *2.2.1.2*), with glasshouse conditions set as *2.2.1.3*. Seedlings were transplanted into pots over 8 days in order of replicate, with replicate 1 sown first, replicate 2 sown on day 2, replicate 3 day 3, replicate 4 day 4, and due to one of the replicates being destroyed by mice, replicate 5 days 7-8. A total of 40 populations, 8 glyphosate doses, and 5 replicates were used. Pots were placed in a split plot design, with pots containing each population randomised within dose tray, and dose trays randomised within replicate. Replicates were placed in rows in the glasshouse compartment.

Plants were thinned 18 days after sowing to eight plants per pot (see section *2.2.1.4*) (varying depending on germination) and a track sprayer was used to treat plants with one of the eight glyphosate doses 24 to 28 days after sowing (see section *2.2.1.5*). Spraying took place twice, with replicates 1 and 2 sprayed together 28 (replicate 1) and 27 (replicate 2) days after sowing, and replicates 3, 4, and 5 sprayed together, 28 (replicate 3), 27 (replicate 4), and 24 (replicate 5) days after sowing.

Plants were assessed a minimum of 20 days and a maximum of 28 DAT with glyphosate (*2.2.1.6*).Each replicate was assessed over two days, replicate 1 was assessed 20-21 DAT, replicate 2 22-23 DAT, replicate 3 22-23 DAT, replicate 4 25-26 DAT, and replicate 5 27-28 DAT.

2.2.6 Data analysis

2.2.6.1 Dose-response curve analysis

Dose-response curves can be used to assess the sensitivity of a plant population to a particular herbicide and to compare sensitivities between populations (Seefeldt *et al*. 1995). A range of different models, for example, log-logistic (symmetrical distribution) and Weibull models (1 & 2) (asymmetrical distribution) (Figure 2-3) can be used to assess dose-response, further discussed in Knezevic *et al*. (2007). Models can also be binomial or continuous. Binomial models use binomial data, such as survival data, where proportions are calculated and vary between 1 and 0. Continuous models are used for continuous data, such as fresh weight, and has no constraint on values used.

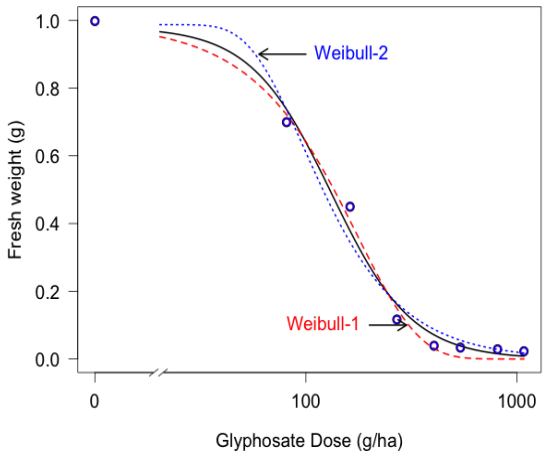

Figure 2-3: Example of three types of dose-response curves
Log-logistic (black) a symmetrical distribution, and asymmetrical distribution Weibull-1 (red) that has a steep decrease in slope towards the lower limit, and Weibull-2 (blue) that has a steep decrease in slope towards the upper limit, adapted from Ritz and Streibig (2012)

There are four parameters used to calculate dose-response curve, upper limit (b), lower limit (c), slope (d), and ED_{50} (the estimated dose at which 50% of the population is affected) (e). Each of the four parameters can be constrained or unconstrained in different models. In 2-parameter binomial models both the upper and lower limits are

constrained to 1 and 0 respectively for all populations. In 3-parameter binomial and continuous models only the lower limit is constrained to 0. Both the slope and ED_{50} can also be constrained to the same value for all populations. Models with constrained slopes and ED_{50} can be compared to unconstrained models using ANOVA analysis to determine whether there is significant variation in the two parameters. The ED_{50} and ED_{90} (the dose at which 90% of the population is affected) can be calculated from dose-response analysis and are useful parameters to assess variability in sensitivity between different populations (Seefeldt *et al*. 1995). T-tests can be used within the model to calculate selectivity indexes and the estimated ratio of effective dose, the ratio of the difference between the doses needed for ED_{50} of the unsusceptible population, compared to the susceptible population. If the estimated ratio of effective dose is >2 it can be inferred that the unsusceptible population is resistant (Collavo and Sattin, 2014).

2.2.6.2 Dose-response data analysis

Results were analysed using the R statistical package (version 2.15.3) and dose response curve (DRC) analysis. For survival data binomial 2-parameter models were fitted, and for fresh weight data 3-parameter models were fitted. To determine the best model to use, log-logistic, Weibull-1, and Weibull-2 models were fitted separately to each data set. Once models were fitted a lack of fit test was performed on each model to determine whether they were significantly different from the saturated model (an ANOVA model). As a lack of fit test was performed, the p-value needs to be >0.05, as no significant difference between the saturated ANOVA model and the dose-response model is needed to ensure that the dose-response model fitted represents the data. The model with the best fit and no significant difference from the saturated model (p>0.05)

was used. Where all models were significantly different to the saturated model the model that was least significantly different was chosen, and each population was run individually to determine which did not fit the model.

For fresh weight data, once the model was selected, boxcox transformation was used to determine whether fresh weight data needed to be transformed. However, for all experiments in this chapter boxcox transformation compromised model fit for fresh weight data, and was therefore not used.

To determine whether ED_{50} and/or slope needed to be constrained, once the model was chosen it was run with both a constrained ED_{50} and slope together, and a constrained ED_{50} and a constrained slope separately. Constrained models were then compared to the unconstrained model using ANOVAs. Where there was no significant difference between the models the constrained model was used, where there was significant difference between the models the unconstrained model was used, as there was significant variance in the ED_{50} and/or slope.

Once the final model was chosen, ED_{50} and ED_{90} values for survival data, and GR_{50} (growth rate) and GR_{90} values for fresh weight data of the populations were calculated. T-tests were then performed to determine whether any populations had significantly different ED_{50}, ED_{90}, GR_{50}, and GR_{90} values compared to the unexposed AHE110 (Broadbalk 2010 collection) for 2010 populations and AHE112 (Broadbalk 2012 collection) for 2012 populations.

2.2.6.3 Experimental data analysis

For experiment 1 survival data, a binomial log-logistic 2-parameter model with an unconstrained ED_{50} and constrained slope was used (model fit: $p<0.001$). There were 7 populations that did not fit to the log-logistic 2-parameter model. However, when these populations were removed from the model the model fit was still significant ($p=0.0135$). Despite the poor fitting models, to enable comparison of glyphosate susceptibility between these populations the log logistic 2-parameter model with a constrained slope is presented.

For experiment 1 fresh weight data, an unconstrained binomial log-logistic 2-parameter model was used (model fit: $p=0.0011$). When the model was individually fitted to each population three of the seventeen populations had models that did not fit (Table 2-2), without these populations model fit was $p=0.2299$, indicating that the model fit well to the remaining fourteen populations.

Table 2-2: Three of seventeen UK *A. myosuroides* populations tested in a glyphosate dose-response assay that did not fit to a Weibull-2 3-parameter model, with p-values

Population	p-value
ARU110	0.0301
ASC110	0.0152
AWA210	0.0324

For experiment 2 survival data, a binomial log-logistic 2-parameter model with an unconstrained ED_{50} and constrained slope was used (model fit: $p=0.5623$). For fresh weight data a fully constrained Weibull-2 3-parameter model was used (model fit: $p=0.9929$).

ED_{50} and GR_{50} values and rank of the two dose-response assays of experiments 1 and 2 were compared using correlation analysis. ED_{50} and GR_{50} values of the same population were also compared between experiments 1 & 2 using at T-test set to the 95% confidence level.

For experiment 3 survival data, a binomial log-logistic 2-parameter model with a constrained ED_{50} and unconstrained slope was used (model fit: <0.001). When the model was individually fitted to each population eight of the forty populations had models that did not fit (Table 2-3), without these populations model fit was p=1, indicating that the model fit well to the remaining thirty-two populations.

Table 2-3: Eight of forty UK *A. myosuroides* populations tested in a glyphosate dose-response assay that did not fit to a log logistic 2-parameter model, with p-values

Population	p-value
ACA212	0.0437
ACA812	0.0011
ALE112	<0.001
ALI312	<0.001
ANR112	0.0091
ASF312	<0.001
ASO212	<0.001
AWA612	0.0087

For experiment 3 fresh weight data an unconstrained Weibull-2 3-parameter model was used (model fit: p=0.9589). For experiment 3, correlation analysis was also used to determine any relationship between ED_{50}, ED_{90}, GR_{50} and GR_{90} values and previous glyphosate exposure score.

2.3 Results

2.3.1 Experiment 1: Dose-response of 17 2010 *A. myosuroides* populations

2.3.1.1 Experiment 1: Log logistic 2-parameter survival dose-response analysis

There was no significant difference between the unconstrained model and the model with unconstrained ED_{50} and constrained slope (LR-value = 23.15, p-value = 0.081), but there was significant difference between the unconstrained model and the model with constrained ED_{50} and unconstrained slope (LR=27.233, p=0.0269), meaning that there was significant variance between the populations in ED_{50}, but not slope.

For this model, ED_{50} ranged from 253 to 395-glyphosate g ha^{-1} (Table 2-4) with population AYO110 having the lowest ED_{50} and AWA110 the highest. No populations where ED_{50} could be calculated had significantly different ED_{50} values from the unexposed AHE110 (Broadbalk), showing that although ED_{50} varied significantly among the populations, there was no significant variance compared to the unexposed population, which was not the most susceptible population (Table 2-4).

For ANO110, ASC110 and ASX110 survival did not fall below 50% at the highest dose used (405 g ha^{-1}) and therefore ED_{50} was not calculated for these populations. However, these populations were included in the ANOVA analysis comparing the models with constrained and unconstrained ED_{50}, therefore, these populations contributed to the significant variation in ED_{50}. The high survival also meant that ED_{90} values could not be calculated, as survival did not fall below 10% for any population (Figure 2-4).

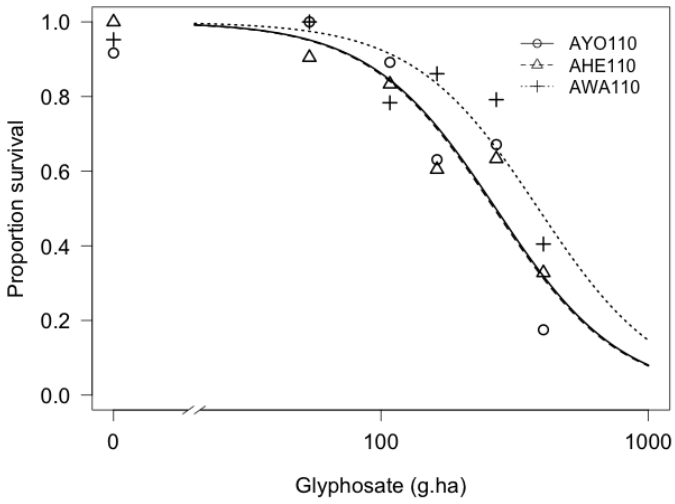

Figure 2-4: Dose response curves for log-logistic 2-parameter model for glyphosate survival
The least sensitive (AWA110), most sensitive (AYO110) and for the unexposed Rothamsted population (AHE110)

Table 2-4: ED_{50} values for glyphosate dose-responses of 17 UK *Alopecurus myosuroides* populations collected in 2010

Population	ED_{50} (g ha^{-1})	Standard error
AYO110	253.6	27.03
AHE110	283.7	55.35
AES110	300.5	59.82
ASU110	301.7	42.08
AKE110	317.2	62.28
ALI110	337.5	36.98
AWA210	346.0	47.16
ABE110	355.3	44.78
ACA110	360.9	37.92
ABE210	361.6	47.36
ALE110	374.4	69.04
AKE210	386.7	71.77
AWA110	394.6	78.30
ANO110	NA	NA
ASC110	NA	NA
ASX110	NA	NA

2.3.1.2 Experiment one: dose response analysis for plant fresh weight data

There was a significant difference between the unconstrained model and model with constrained GR_{50} and slope (F=1.5088, p=0.0339), showing that there is significant

variation in the GR_{50} and slope of the populations. GR_{50} values ranged from 62-glyphosate g ha^{-1} to 260 g ha^{-1} (Table 2-5). GR_{90} values were larger than the highest dose used and could not be estimated. Three populations had significantly different GR_{50} values compared to the unexposed AHE110 (Broadbalk) (Table 2-5).

Table 2-5: GR_{50} of glyphosate dose-response of 17 UK *Alopecurus myosuroides* populations collected in 2010
GR_{50} *p-value compared to AHE110 (unexposed) **<0.01, ***<0.001

Population	GR_{50}	Standard error
ARU110	62.1	44.51
AES110	93.9	25.61
ASC110	112.5	18.73
AHE110	118.7	27.94
ANO110	137	30.2
AKE110	143.1	32.33
AWA210	168.9	27.4
ACA110	172	23.34
AWA110	173.8	31.57
ALE110	176.1	32.93
ABE210	182	20.92
ASU110	185	30.89
ALI110	185.3	36.44
ABE110	207	25.77
ASX110	209.7**	31.58
AYO110	214.0**	26.79
AKE210	260.8***	36.45

The ranking of ED_{50} and GR_{50} of the populations is similar, for example the unexposed, AHE110, is one of the most susceptible populations for both (Table 2-4, Table 2-5). However, there is some discrepancy with AYO110, which was the most sensitive population for survival, but one of the least sensitive populations for fresh weight, with a significantly higher GR_{50} compared to AHE110 (Table 2-5). This may be due to there being little difference between the fresh weight of dead and alive plants, resulting in a high GR_{50}.

2.3.2 Experiment 2: Repeat glyphosate dose-response of 17 UK *A. myosuroides* populations collected in 2010

2.3.2.1 Experiment 2: Log logistic 2-parameter survival dose-response analysis

Again there was variation in glyphosate susceptibility of the 17 UK *A. myosuroides* populations collected in 2010 (Figure 2-5). In this experiment ARU110 conformed to dose-response analysis. There was no significant difference between the unconstrained model and model with unconstrained ED_{50} and constrained slope (LR value = 15.537, p-value = 0.4857), but there was significant difference between the unconstrained model and the model with constrained ED_{50} and unconstrained slope (LR=44.125, $p<0.001$) showing that there is significant variation in the ED_{50} but not slope of the populations.

ED_{50} values ranged from 244-glyphosate g ha^{-1} to 344 g ha^{-1} (Figure 2-6). The unexposed AHE110 had the lowest ED_{50} values (Figure 2-6). Six populations had significantly higher ED_{50} values than AHE110 (Figure 2-6). The higher doses used in the dose-response enabled ED_{90} values to be calculated with values ranging from 406-glyphosate g ha^{-1} to 573 g ha^{-1} (Figure 2-6). Due to the constrained slope, ED_{90} values are directly proportional to ED_{50} values, therefore, the 6 populations with significantly higher ED_{50} values compared to AHE110, also had significantly higher ED_{90} values.

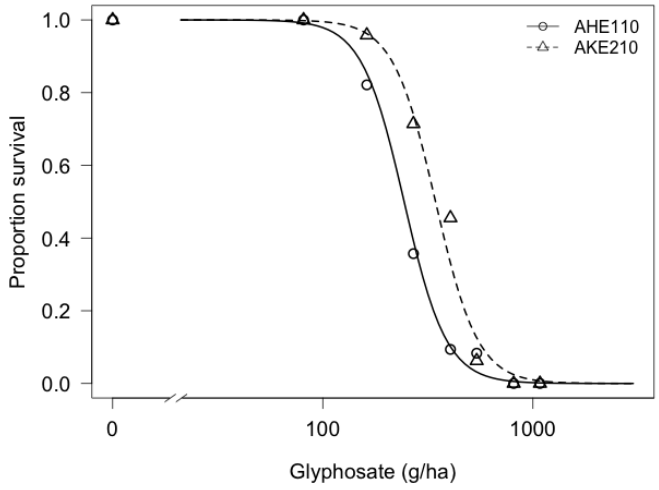

Figure 2-5: Log logistic 2-parameter model with constrained slope glyphosate dose-response curve analysis of survival
Of 2 of 17 UK *Alopecurus myosuroides* populations collected in 2010 carried out in 2012, with the highest ED_{50} value (AKE210) and lowest values (AHE110)

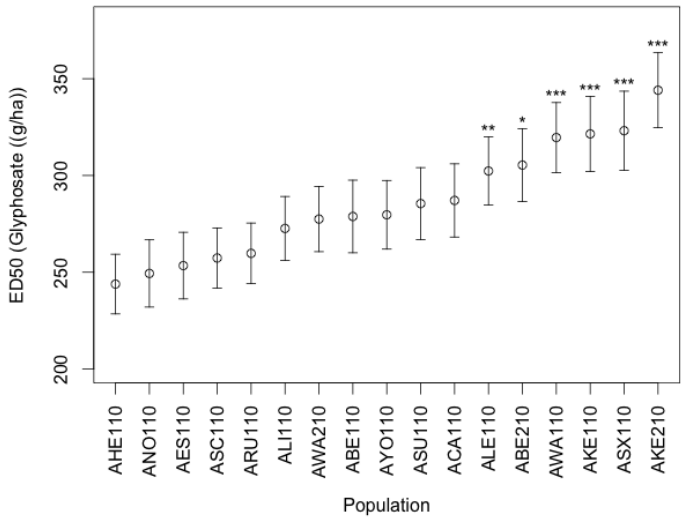

Figure 2-6: Plot of ED_{50} values and standard error of glyphosate log-logistic 2-parameter dose-response assay with a constrained slope of 17 *Alopecurus myosuroides* populations
ED_{50} and ED_{90} *p-value <0.05 compared to AHE110 (unexposed), **<0.01, ***<0.001

2.3.2.2 Experiment 2: Weibull-2 3-parameter plant fresh weight dose-response analysis

There was no significant difference when the unconstrained model was compared to the model with a constrained GR_{50} and slope (LR value = 0.985, p-value = 0.493).

Meaning that there was no significant variation in glyphosate susceptibility between the fresh weight of the populations. GR_{50} and GR_{90} values for all populations were 177 (±4.79) g ha^{-1} and 405 (±22.23) g ha^{-1}, respectively.

2.3.3 Comparison of experiment 1 and experiment 2 dose-response assays of 17 UK *A. myosuroides* populations collected in 2010

The patterns in variance between the ED_{50} and ED_{90} values between experiments 1 and 2 are similar (Figure 2-7). The standard error for the repeat experiment 2 dose-response was much smaller than that of the experiments 1 dose-response (Figure 2-7).

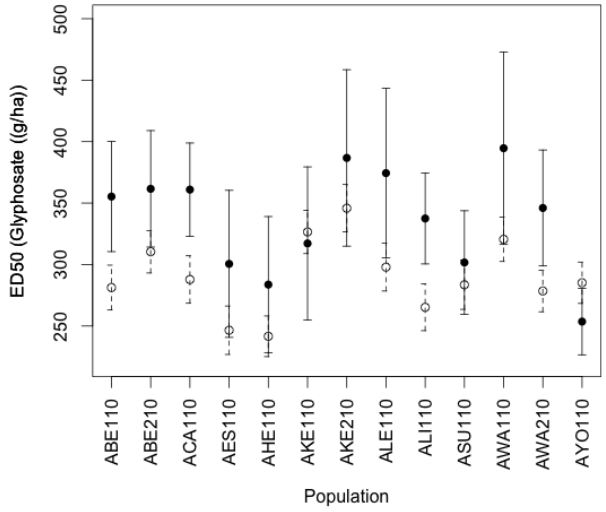

Figure 2-7: ED_{50} values and standard error of 2 glyphosate dose-response assays of 17 *Alopecurus myosuroides* populations collected in 2010
ED_{50} with standard error of experiment 1 (•) and repeat experiment 2 (ₒ) dose response of 13 of the 17 *Alopecurus myosuroides* populations collected in 2010, 4 of the populations are not included as there was no ED_{50} from the experiment 1 dose-response assay analysis

The ED_{50} values and ranking of susceptibility between the two dose-response assays was similar with a significant positive correlation between the ED_{50} values (0.59, p=0.0339, R^2=0.2886) and the rank of the ED_{50} values (0.65, p=0.0165, R^2=0.3677) of

the two dose-response assays (Figure 2-8). Furthermore, T-test analysis showed no significant difference between the ED_{50} values of the populations between the two dose-response experiments, as all the lower and upper limits passed through 0 (Table 2-6). The similarity in response of the populations between the two dose-responses suggests that the survival results from the first dose-response are consistent with those of the repeat experiment 2.

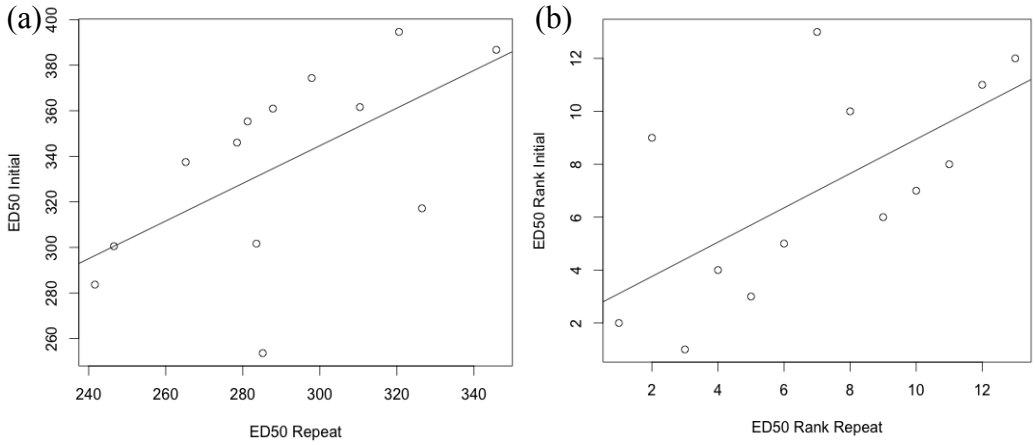

Figure 2-8: Correlation of parameters of 2 dose-response assay analyses of 17 UK *Alopecurus myosuroides* populations
(a) ED_{50} values (0.59, p=0.0339, R^2=0.2886) and (b) rank of ED_{50} values (0.65, p=0.0165, R^2=0.3677) of two glyphosate dose-response assays of 13 of the 17 UK *A. myosuroides* populations, 4 of the populations are not included as there was no ED_{50} from the experiment 1 dose-response assay analysis

Table 2-6: T-test analysis of ED_{50} values of 2 dose-response assay analyses of 17 UK *Alopecurus myosuroides* populations
13 of the 17 UK *A. myosuroides* populations, 4 of the populations are not included as there was no ED_{50} from the experiment 1 dose-response assay analysis

Population	Estimate	Standard error	Lower limit	Upper limit
ABE110	74.1	48.4	-20.7	168.8
ABE210	51.2	50.4	-47.6	150.0
ACA110	73.1	42.6	-10.3	156.6
AES110	54.0	42.7	-29.8	137.7
AHE110	42.1	57.8	-71.2	155.3
AKE110	-8.9	64.7	-135.8	118.0
AKE210	40.9	74.3	-104.7	186.6
ALE110	76.5	71.7	-64.1	217.1
ALI110	72.3	41.6	-9.2	153.8
ASU110	18.1	46.6	-73.2	109.4
AWA110	74.1	80.4	-83.4	231.6
AWA210	67.5	50.1	-30.7	165.7
AYO110	-31.6	31.8	-94.0	30.8

2.3.4 Experiment 3: 40 2012 *A. myosuroides* populations glyphosate dose-response assays

2.3.4.1 Experiment 3: Log-logistic 2-parameter survival dose-response curve analysis

There was no significant difference between the unconstrained model and the model with a constrained ED_{50} and unconstrained slope (LR value = 48.1, p-value = 0.1514), but there was significant difference between the unconstrained model and the model with unconstrained ED_{50} and constrained slope (LR=68.792, p=0.0023), meaning that there is significant variance in slope, but not ED_{50}.

For this model ED_{50} was constrained to 280 (±2.34) g ha^{-1}. As there was significant difference between the slopes of the populations there was variation in ED_{90} values, with 5 populations having significantly higher values than AHE112 (Figure 2-9). ED_{90}

values varied between 354 and 610 g ha^{-1}, with AYO112 having the lowest value and AES112 the highest (Figure 2-9). The significant variation in slope shows that there are differences in the variation of glyphosate susceptibility within the populations, with populations with shallow slopes having higher variation (e.g. AES112), and those with steeper slopes less variation (e.g. AYO112) (Figure 2-10).

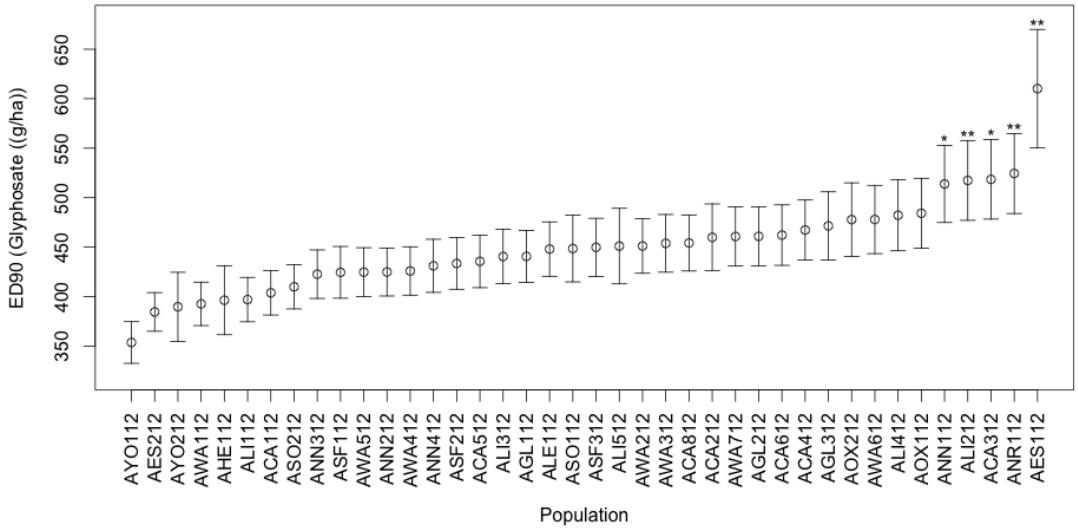

Figure 2-9: ED_{90} values and standard error of glyphosate dose-response assay of 40 UK *Alopecurus myosuroides* populations
Log-logistic 2-parameter model with constrained ED_{50} and unconstrained slope of 40 *Alopecurus myosuroides* populations from the UK, ED_{90} *p-value <0.05 compared to AHE112 (unexposed), **<0.01

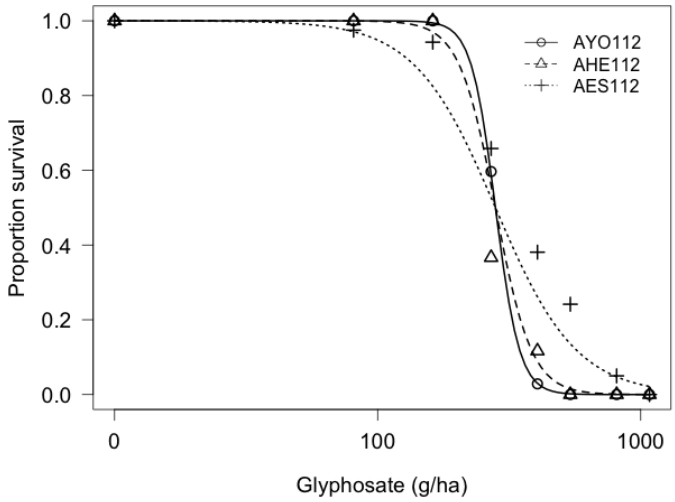

Figure 2-10: Log logistic 2-parameter model with constrained ED_{50} glyphosate dose-response curve analysis of survival
3 of 40 UK *Alopecurus myosuroides* populations collected in 2012, with the populations the highest (AES110) and lowest ED_{90} values (AYO112), and the unexposed (AHE112)

2.3.4.2 Experiment 3: Weibull-2 3-parameter plant fresh weight dose-response curve analysis

There was significant difference between the unconstrained model and model with constrained GR_{50} and slope (F-value = 1.9858, $p<0.001$), meaning that there was significant variation between populations in both the GR_{50} and the slope of dose response curves.

GR_{50} values ranged between 122-glyphosate g ha^{-1} and 199 g ha^{-1} (Figure 2-11a), with surviving plants still being affected by glyphosate application. Thirty populations had significantly higher GR_{50} values than AHE112. GR_{90} values ranged from 275 to 466 g ha^{-1}. ASO112 and AWA212 had shallow slopes, which can explain why they have low GR_{50} values but high GR_{90} values. There were no populations with a significantly different GR_{90} to the unexposed AHE112 due to high standard errors (Figure 2-11b).

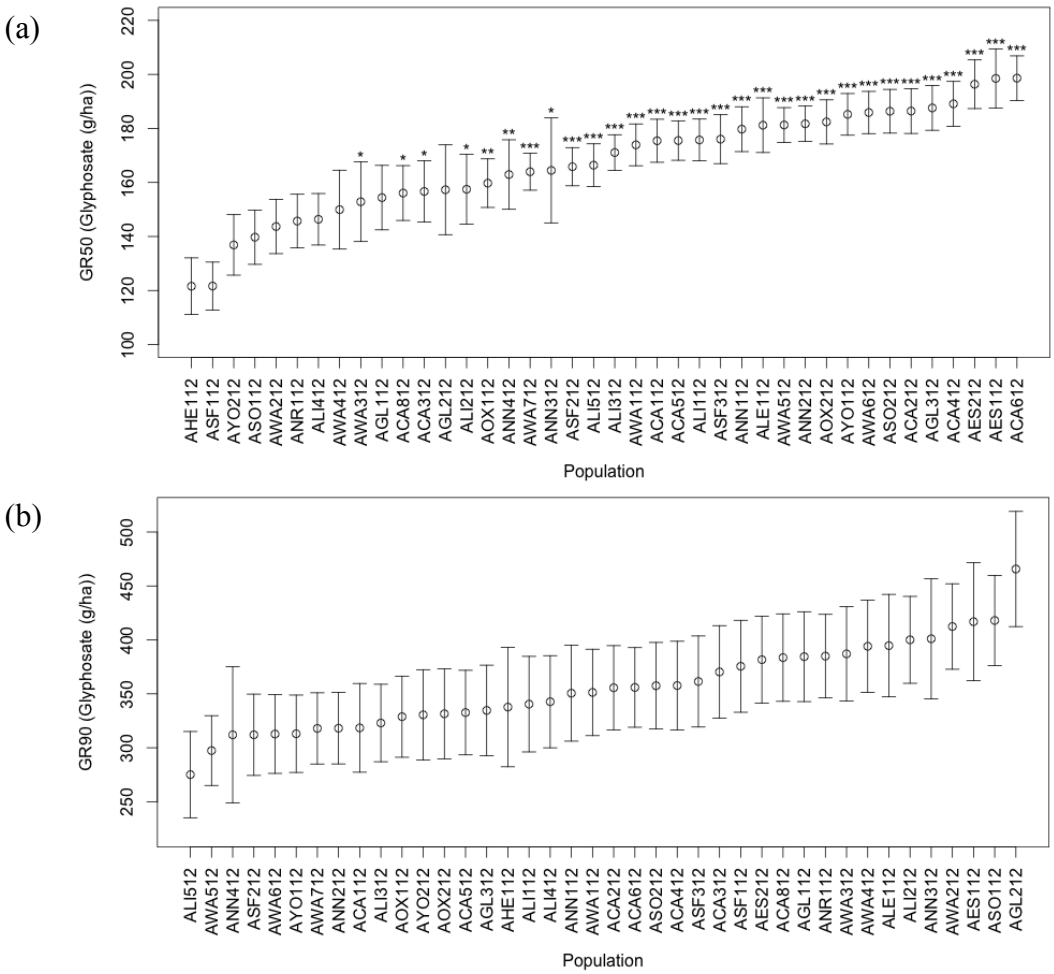

Figure 2-11: GR_{50} and GR_{90} with standard error of Weibull-2 3-parameter glyphosate dose-response assay
(a) GR_{50} and (b) GR_{90} values of glyphosate dose-response assay of 40 *Alopecurus myosuroides* populations from the glyphosate dose-response assay of 40 UK *Alopecurus myosuroides* populations collected in 2012

2.3.4.3 Relationships between past glyphosate exposure and glyphosate susceptibility

There was no significant correlation between ED_{90} of the 25 UK *A. myosuroides* populations collected in 2012 where glyphosate exposure data was provided and their glyphosate exposure score (p=0.432). Considering this lack of correlation and that the population with the highest glyphosate exposure, AES112 (Peldon), had the highest ED_{90}, and that the unexposed broadbalk population (AHE112) had one of the lowest ED_{90} values the relationship between glyphosate exposure and glyphosate

susceptibility in populations is not clear cut and may involve other factors, such as the standing genetic variation present within a population.

There was a significant (p=0.002, R^2=0.3151) positive correlation (0.586) between GR_{50} and previous glyphosate exposure score (Figure 2-12), but no correlation between GR_{90} and previous glyphosate exposure score (p=0.890). However, the R^2 value is low, suggesting that there are other factors influencing the GR_{50} value. This suggests that exposure to glyphosate selection pressure can effect the variation in glyphosate susceptibility between populations, but that this variation is also dependent on other factors.

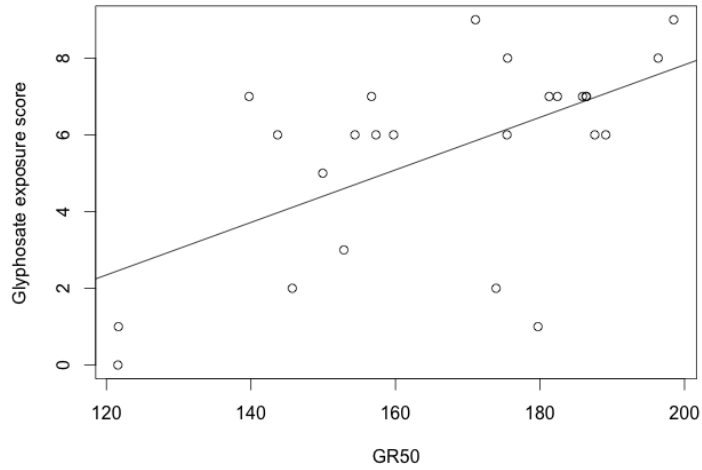

Figure 2-12: Correlation of GR_{50} of UK *Alopecurus myosuroides* populations and glyphosate exposure score
Correlation (0.586, p<0.01, R^2=0.3151) between GR_{50} and previous glyphosate exposure score of glyphosate dose-response assay of 25 UK *Alopecurus myosuroides* population

2.4 Discussion

No glyphosate resistance was found in the 55 UK *A. myosuroides* populations tested, with good control at field rate (540-glyphosate g ha^{-1}) for most populations. Individuals surviving at field rate were greatly affected by glyphosate having stunted growth, shown by the much lower GR_{50} and GR_{90} values, when compared to ED_{50} and ED_{90} values. There was, however, variation in glyphosate susceptibility between these populations, although this variation was not large. For the 17 populations collected in 2010, ED_{50} varied between 254-395 g ha^{-1} for experiment 1, and 242-346 g ha^{-1} for experiment 2. Of the 40 populations collected in 2012, there was no significant variance in ED_{50} values, but there was significant variance in slope between the populations, and ED_{90} values varied between 354 and 610 g ha^{-1}. GR_{50} values ranged between 122-199 g ha^{-1} and GR_{90} values ranged from 275 to 466 g ha^{-1}.

2.4.1 Variation in glyphosate susceptibility in *A. myosuroides*

The ED values and variance in glyphosate susceptibility of these *A. myosuroides* populations is much greater than that of baseline studies that have assessed glyphosate susceptibility in unexposed populations of *L. rigidum* and *B. diandrus* where ED_{50} values ranged between 85-117 g ha^{-1} and 17-46 g ha^{-1}, respectively (Barroso *et al*. 2010). Although most of the *A. myosuroides* populations in this study have previously been exposed to glyphosate, the unexposed Broadbalk population (AHE110/12) had a much higher range of ED_{50} values when tested (242-284 g ha^{-1}), suggesting *A. myosuroides* may have lower initial susceptibility to glyphosate compared with other grass species. Although, in exposed populations of the grass *Chloris polydactyla* (L.) Sw. GR_{50} values ranged between 64-254 g ha^{-1} (Barroso *et al*. 2014), similar to the

range in GR_{50} values found in *A. myosuroides* in this study, suggesting that *A. myosuroides* populations may respond in a similar way to glyphosate selection pressure as some other grass species.

Although no studies have previously investigated the variability in glyphosate susceptibility in *A. myosuroides*, variation in susceptibility to other herbicide modes of action has previously been reported, with LD_{50} values ranging from 3-87 g ha^{-1} for Swedish populations exposed to flupysulfuron (Espeby *et al*. 2011) and percentage control biomass for German populations exposed to the field rates of flupysulfuron (9.3 g ha^{-1}) and clodinafop (53.5 g ha^{-1}) ranging from 17-120% (Ulber *et al*. 2013). This variation is much greater than the response found in the 56 *A. myosuroides* populations tested in this study and could be due to glyphosate having a relatively low resistance risk, as well as its use in combination with other herbicide modes of action, meaning that rare glyphosate resistance traits are controlled (Neve *et al*. 2003a). This is in contrast to the use of clodinafop and flupyrsulfuron, both ALS herbicides that can be used within wheat crops allowing time for a larger proportion of the population to germinate and be exposed (Llewellyn and Powles, 2001). The lower variation in response found in this study suggests that although there is variation in glyphosate susceptibility in *A. myosuroides* populations, the risk of resistance evolution is lower than that of other commercial herbicides. This may also explain why there was no significant variation in ED_{50} for the 40 populations collected in 2012.

2.4.2 Reduced glyphosate susceptibility in populations if *A. myosuroides*

A total of 11 of the 55 *A. myosuroides* tested in this study had significantly lower glyphosate susceptibility compared to the unexposed Broadbalk (AHE110/12),

suggesting that these populations, which have been exposed to glyphosate, have significantly decreased susceptibility. Similar variability and decreases in glyphosate susceptibility have been reported in Spain and Australia, where glyphosate use for weed removal before crop sowing in cereal producing areas is similar to that of the UK (Loureiro *et al*. 2010; Owen *et al*. 2014). For example, populations of *L*. *rigidum* and *B*. *diandrus* collected from Spain have shown similar variable responses to glyphosate as the *A*. *myosuroides* populations tested in this study, with 4.6% of 45 Spanish populations of *L*. *rigidum* tested showing early stages of resistance evolution (Loureiro *et al*. 2010), and levels of intermediate resistance of 79 *B*. *diandrus* populations ranging from 5.9-13.8% (Escorial *et al*. 2011). In Western Australia the early stages of glyphosate resistance evolution may also be under way in *L*. *rigidum* populations. In 2003 only 3 (<1%) of 500 populations were found to have intermediate resistance and no populations had full resistance (Owen *et al*. 2007), however in a 2010 study in the same region of 359 populations found 3 (6%) to have intermediate resistance and 1 (<1%) with full resistance (Owen *et al*. 2014). The variation in glyphosate susceptibility in grass species in both Spain and Australia, and the potential evolution of resistance in these areas, suggests that the variation to glyphosate susceptibility in *A*. *myosuroides*, exposed to similar glyphosate use, found in this study may also potentially lead to glyphosate resistance evolution over time in the UK. It would therefore be interesting to investigate the potential resistance risk that this variation poses through further experiments, such as glyphosate selection experiments.

The Peldon (AES112) population, which is already highly resistant to a number of herbicide modes of action, including phenyl-urea, dinitroaniline, and ACCase (Hall *et al*. 1997; Moss *et al*. 2007), was the population that had the lowest glyphosate

susceptibility in the dose-response of the 40 populations collected in 2012. From the herbicide use history collected from this site, glyphosate is now used 2-3 times a year on the stale seedbed (Appendix 2), as this is one of the few herbicide modes of action remaining to the farmer. This exposes the population to a selection pressure higher than those on other farms where glyphosate is used once a year. Past glyphosate exposure and higher selection pressure has previously resulted in some species having reduced glyphosate susceptibility (Kinss *et al*. 2009), which may be the case for Peldon, as exposure and selection pressure is high.

Considering the Peldon population and the prevalence of herbicide resistance to other modes of action in the UK it would be interesting to investigate any possible relationship between existing herbicide resistance and the potential for glyphosate resistance in *A*. *myosuroides*. It would also be interesting to resample Peldon on a regular basis to track any shifts in glyphosate susceptibility over time and compare these to the shifts of other populations where glyphosate use it lower.

2.4.3 Previous glyphosate exposure and susceptibility

The positive correlation between GR_{50} and glyphosate use score, suggests that there is some relationship between increased glyphosate exposure and reduced glyphosate sensitivity. Relationships between glyphosate exposure and response to glyphosate, including resistance, has been reported in populations of *Conyza canadensis* (Okada *et al*. 2013), and *Chenopodium album* with low, intermediate and high glyphosate mortality related to rotating and low glyphosate exposure for *Chenopodium album* (Kniss *et al*. 2007). The relationship between glyphosate exposure and increased GR_{50} and evidence that other species have positively responded to glyphosate exposure

suggests that glyphosate exposure in these populations has lead to some shift towards resistance, but due to a lack of relationship between glyphosate exposure and survival this relationship may be weak and other factors, such as initial allele frequency from standing genetic variation may be more important (Neve *et al*. 2003a). Conversely, as discussed above, the high ED_{90} of the glyphosate exposed AES112 population and the low ED_{90} of the unexposed AHE112 population the amount of glyphosate exposure may influence the susceptibility of some populations over time. Considering this and the lack of relationship between ED_{90} and past glyphosate exposure, and the low R^2 value for the relationship between past glyphosate exposure and GR_{50}, it would be interesting to investigate the other factors influencing the differences in ED and GR values in *A*. *myosuroides* populations, and further investigate how much of an influence glyphosate exposure has on susceptibility.

2.4.4 Conclusion

This is the first study to investigate variation in glyphosate susceptibility in *A*. *myosuroides* populations. No glyphosate resistance was found, but there is significant variation in susceptibility, with this variation being confirmed in both the 2010 collected populations and those collected in 2012 and a total of 11 populations having significantly lower susceptibility compared to the unexposed Broadbalk (AHE110/12). Variation in glyphosate susceptibility was less than that found in other *A*. *myosuroides* populations treated with different herbicide modes of action, possibly due to glyphosate having a lower resistance risk. Although this variation is not large, it is similar to that found in other grass species exposed to glyphosate in comparable cropping systems where it is leading to the early stages of glyphosate resistance evolution. AES112 (Peldon) was a population of concern in both the dose-response

assay and glyphosate screen and it would be interesting to investigate the likelihood of glyphosate resistance evolution in this and other populations. There is some relationship between past glyphosate exposure and GR_{50}, however this relationship was not found for any of the other ED or GR values, suggesting that there may be a relationship between glyphosate exposure and reduced sensitivity, but that it is weak. The processes of glyphosate resistance evolution will be explored in chapter 3 using low dose glyphosate selection experiments.

Chapter 3 : Low-dose glyphosate selection of 10 UK *Alopecurus myosuroides* populations

3.1 Introduction

3.1.1 Accumulation of quantitative traits

Adaptation in a population to rapid environmental changes, such as herbicide application, can either occur through new advantageous mutations or by utilizing alleles already present within the population from standing genetic variation (Hermisson and Pennings, 2005). Weed species generally have high standing genetic variation and can therefore easily adapt to environmental change (Murphy and Lemerle, 2006). As discussed in Chapters 1 (*1.4.5*) and 2 (*2.1.1*), standing genetic variation can contribute to creeping resistance. It is important to understand the evolutionary processes, such as selection at low doses, that can lead to creeping resistance, as understanding these processes will shed light on the impact of dose rates on resistance evolution and on strategies that can be implemented to slow resistance evolution (Neve *et al*. 2014).

Assuming variation in herbicide susceptibility in weed populations is a normally distributed quantitative trait (Paran and Zamir, 2003), where applied herbicide rates act within this range of standing quantitative genetic variation, rare survivors with reduced herbicide sensitivity may survive and reproduce (Figure 3-1). If the trait is also heritable, in outcrossing species there can be an accumulation of minor alleles from standing genetic variation related to reduced susceptibility. Over time and generations

this can lead to a gradual reduction in herbicide susceptibility that may ultimately result in creeping resistance, with an increasing dose needed to control the population (Figure 3-2; Ulber *et al*. 2013; Neve *et al*. 2014; Chapter *1.3.5*). This has been shown to work in theory in glasshouse experiments, with low dose-selection leading to an accumulation of quantitative alleles resulting in herbicide resistance over a number of generations (Neve and Powles, 2005b; Busi *et al*. 2013b). However, if there is a lack of genetic variation or the herbicide selection pressure acts outside of this variation resistance will only evolve from novel mutation (Neve *et al*. 2014).

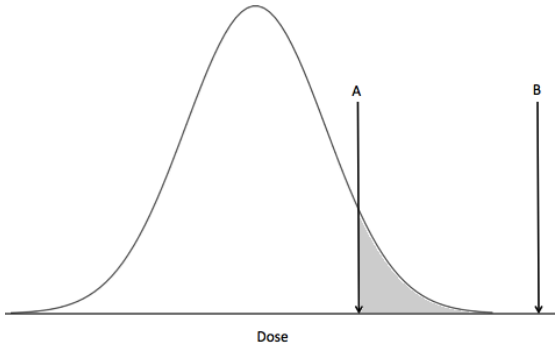

Figure 3-1: A hypothetical weed population where herbicide susceptibility is a normally distributed quantitative trait
(A) Where dose of herbicide applied acts within the standing quantitative genetic variation of a population and does not control the whole population, enabling less susceptible individuals (grey area) to survive and produce progeny. (B) Where dose of herbicide applied controls the whole population, leaving no survivors

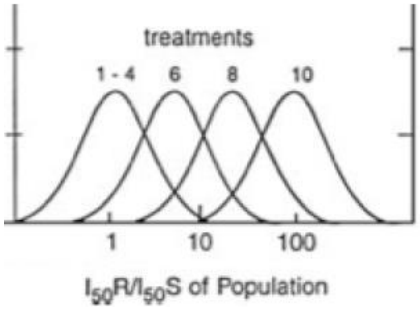

Figure 3-2: Population distribution of number of treatments of diclofop-methyl needed to control a *Lolium rigidum* population after yearly treatment with 375 g ha^{-1}, from Gressel (2009)

3.1.2 Low dose selection

As discussed in Chapter 1 (*1.3.5*), herbicide resistance evolution through low dose selection has already been investigated in different species using multiple modes of action, including *Lolium rigidum* using pyroxasulfone (Busi *et al*. 2012), glyphosate (Busi and Powles, 2009), and diclofop-methyl in glasshouse selection experiments (Neve and Powles, 2005a; Busi *et al*. 2013b) and in the field (Manalil *et al*. 2011), in *Alopecurus myosuroides* using fenoxaprop-p-ethyl (Lynch, 2014), and in *Amaranthus tuberculatus* using glyphosate (Zelaya and Owen, 2005).

Target site and possibly non target site glyphosate resistance has already been detected in arable farm populations of *Lolium* spp. exposed to repeated applications of low doses (360 g ha^{-1}) in Italy (Collavo and Sattin, 2014), showing that herbicide resistance selection from using low doses is not only possible in experimental situations, but also under use by farmers. Furthermore, polygenic resistance has been found to confer glyphosate resistance in field populations of *L. rigidum* (Simarmata *et al*. 2005), and ALS and ACCase resistance in field populations of *Alopecurus myosuroides* (Petit *et al*. 2010). There is also a possibility that reduced glyphosate susceptibility endowed by multiple alleles may have increased the chance of a single gene mutation for resistance in a *L. rigidum* population, which has full glyphosate resistance conferred by a single gene at high doses, but polygenic resistance at low glyphosate doses, although this study was conducted after the evolution of resistance, so this cannot be confirmed (Lorraine-Colwill *et al*. 2001).

Low herbicide doses can be applied to weed populations in the field in a number of ways, either through deliberately using below field rate doses to treat weeds within the

crop, as shown by Collavo and Sattin (2014), or to reduce costs, like in Australia (Neve *et al*. 2003a). Lower herbicide rates can also be applied to weeds through poor spray application where part of the field receives a lower than recommended rate of herbicide due to human error, or through spray drift, where a lower rate reaches crops and weeds due to smaller herbicide droplets drifting onto areas where the herbicide has not been applied (Baylis, 2000). As a result of either using reduced rates or through poor application, the dose of herbicide applied to weeds may act within the standing genetic variation, resulting in a buildup of minor resistance alleles over time.

Resistance evolution can vary between populations under low dose selection for polygenic resistance, and depends on the initial frequency of minor resistance alleles within the population (Renton *et al*. 2011). Therefore, populations that are initially less susceptible to a herbicide may evolve resistance over fewer generations than more susceptible populations, when exposed to low herbicide dose selection. To date there have been no studies investigating the potential for different *A*. *myosuroides* populations to evolve glyphosate resistance under low-dose selection, despite the risk of resistance evolution in this species. As there are already populations of *A*. *myosuroides* resistant to 6 herbicide modes of action (Heap, 2015) there may be major implications for possible glyphosate resistance evolution in this species, as due to resistance there are few post-emergent chemical control options left, the main one of which is glyphosate.

3.1.1 Objectives

The main objectives of this chapter are to

- Investigate potential evolution of glyphosate resistance in *A*. *myosuroides* by testing the hypothesis that minor gene variation for glyphosate susceptibility

(standing genetic variation) can be enriched under glyphosate selection, resulting in phenotypes with reduced sensitivity to commercial application rates

- Test the hypothesis that populations with the highest degree of variation in glyphosate susceptibility are the most prone to resistance evolution

To accomplish this an experimental evolutionary approach was used, where, under glasshouse conditions, a total of ten *A. myosuroides* populations were recurrently exposed to glyphosate doses that selected within the range of standing genetic variation. Response to glyphosate selection was observed after two to three generations of selection using dose-response assays.

3.2 Materials and Methods

3.2.1 Plant material

3.2.1.1 2010 Alopecurus myosuroides field collections

Based on the differential dose-responses of the 17 *A. myosuroides* populations collected in 2010 (see section *2.2.2*), and seed availability, four populations were chosen for glyphosate selection experiments. AHE110 (Broadbalk) and AES110 were chosen as more susceptible populations (AHE110 has had no previous exposure to glyphosate). ASC110 and ARU110 were chosen as they demonstrated lower sensitivity to glyphosate.

3.2.1.2 2012 Alopecurus myosuroides field collections

To identify populations from amongst the 2012 field collection with contrasting levels of sensitivity to glyphosate, a resistance index was calculated for each population based on initial dose response results (see section *2.2.4*). This index was calculated as the product of percentage survival and relative mean fresh weight at two glyphosate doses (162 and 270 g glyphosate ha^{-1}, data not shown). Based on this index, six populations were chosen for further selection experiments. ACA212, ACA412, AES112, and AES212 were less susceptible populations. AWA712 exhibited 'intermediate' sensitivity and ASF112 represented a sensitive population.

3.2.2 Standard procedures

3.2.2.1 Dormancy breaking

To break dormancy seeds were placed in an incubator at 30ºC for six weeks before sowing.

3.2.2.2 Growing and thinning

For selection experiments, plants were grown in 300 well hassey trays, two trays per population, one for glyphosate treated and one for untreated control lines. Trays were placed in a glasshouse compartment in a completely randomised design. Glasshouse settings were: supplementary lighting provided between 05:00 and 22:00. Temperature was set at 20ºC + venting at 22ºC between 05:00 and 22:00, and 12ºC + venting at 15ºC between 22:00 and 05:00. Seedlings were left to germinate and grow to the 2-4 leaf stage, at which point the number of seedlings per tray was thinned to between 150 and 175 (with one plant per well of the hassey tray), removing excess plants and any plants with <1 leaf and >4.

3.2.2.3 Glyphosate treatment and assessment

Once thinned, plants were treated with glyphosate at the 2-4 leaf stage, either using a Berthoud knapsack sprayer or a track sprayer (see section *2.2.1.5*). Unselected control lines were not treated with glyphosate. After glyphosate treatment plants were left for 3-5 weeks before being assessed. Plants from both treated and untreated lines were assessed as dead or alive, and were cut to 10mm above the soil surface. To confirm survival, plants were left to grow for a further 8-11 days and survivors were assessed for regrowth. Only individuals that had regrown were assessed as alive. Where

survival was too high (>50% in the majority of populations) plants were re-treated with the same glyphosate dose and assessed as before. Environmental conditions can greatly affect the efficacy of glyphosate (Boutin *et al*. 2010; Owen and Powles, 2010). This caused large variation in survival of the *A*. *myosuroides* selected lines between years, resulting in the need for to glyphosate treatments in some years of selection.

3.2.2.4 Bulk crossing

The survivors from glyphosate treated lines were removed from the hassey trays and repotted. In order to provide a corresponding unselected line for each population, an identical number of untreated plants were randomly selected and repotted in the same way. Pots for each glyphosate-selected and untreated line were moved to polythene tunnels and placed in pollen cages (Figure 3-3) with one line per cage. Plants were grown to maturity in ambient conditions so that all plants for each line could bulk cross, producing a single seed population for that line for subsequent characterization by dose response. Seeds were collected as they matured and stored at 15% RH 15ºC until they were needed for further use.

Figure 3-3: Pollen cage compartment
Mesh netting used for the walls of the compartment allows air movement to facilitate pollination, whilst minimizing pollen movement between cages. Pollen flow between cages was further limited by ensuring that reproducing populations were not placed in adjacent cages

3.2.3 Glyphosate selection of 2010 populations

Populations collected in 2010 were selected over 3 generations and were treated with varying below field rate doses of glyphosate. Doses were selected based on the variable response to glyphosate in *A. myosuroides* populations in chapter 2 (see section *2.3.1*), with doses chosen where there was the maximum variation in susceptibility/ survival between the populations.

3.2.3.1 First generation of 2010 selection

For the first generation of selection of 2010 populations in February 2012, seeds were pre-germinated before being transplanted. Seeds were sown into 90mm Petri dishes containing three 85mm filter paper and 5ml of deionized water. Petri dishes were sealed with parafilm and seeds were left to germinate in an incubator set at 23/9^{o}C with 12/12hr lighting for seven days. Seedlings were then transplanted into hassey trays containing topsoil, one seedling per well. Trays were placed in a glasshouse compartment (see section *3.2.2.2*) and plants were left to grow for 16 days before being thinned to 175 plants per tray (see section *3.2.2.2*). Seventeen days after transplanting 324-glyphosate g ha^{-1} was applied to selection lines using a Berthoud knapsack sprayer (see section *2.2.1.5*). Control lines were not treated. Twenty-three days after glyphosate treatment, survival was assessed and plants were cut 10mm above soil height (see section *3.2.2.3*). Survivors were assessed for regrowth 11days after cutting. Surviving plants were repotted into 90x90x90mm pots containing topsoil and M2 compost in a 2:1 ratio, one plant per pot. Eighteen days after repotting treated lines were again sprayed with 324-glyphosate g ha^{-1}, using a track sprayer (seed section *2.2.1.5*), as survival for three of the four lines was >50%. Thirteen days after

treatment (DAT) plants were assessed for survival and moved to pollen cages to bulk cross and produce seeds (see section *3.2.2.4*). This process is shown in Figure 3-4.

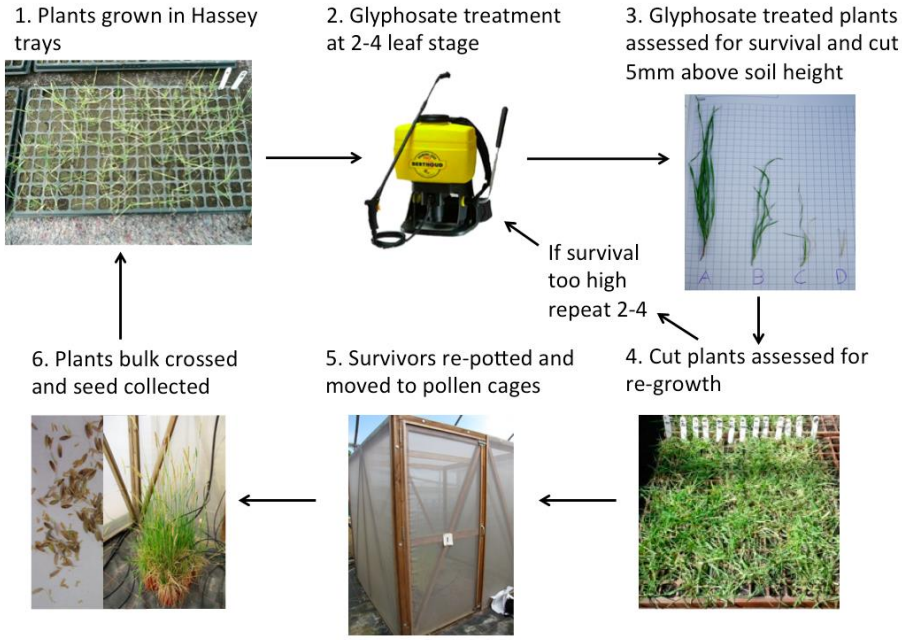

Figure 3-4: Glyphosate selection experiment procedure
For UK *Alopecurus myosuroides* populations selected for glyphosate survival at below field rate doses

3.2.3.2 Second generation of 2010 selection

For the second generation, in December 2012 seeds were treated under warm conditions (see section *3.2.2.1*), before two seeds were placed into each well of a hassey tray containing a mix of topsoil, M2 compost, and sand in a 2:1:1 ratio, one hassey tray per treated and untreated line per population. Trays were placed in a randomised design in a glasshouse compartment (see section *3.2.2.2*) and covered in polythene for 7 days to protect against pests and promote germination. Seeds were allowed to germinate and grow for 25 days before being thinned (see section *3.2.2.2*). Four days after thinning plants were treated with 360-glyphosate g ha^{-1} using a Berthoud knapsack sprayer (see section *2.2.1.5*). Twenty-one DAT plants were assessed for survival and cut (see section *3.2.2.3*). Plants were reassessed for survival

10 days after cutting. Again survival was too high, with all glyphosate treated lines having >50% survival, and plants were retreated with the same dose using a Berthoud knapsack sprayer twelve days after cutting. Survival was reassessed 21 days after the second glyphosate treatment and survivors were repotted into 6-inch pots containing a mix of topsoil, M2 compost, and sand in a 2:1:1 ration, 5-7 plants per pot. Pots were then moved to pollen cages to allow bulk crossing (see section *3.2.2.4*).

3.2.3.3 Third generation of 2010 selection

For the third generation of selection, in February 2014 seeds were treated under warm conditions (see section *3.2.2.1*) before two seeds were placed into each well of a hassey tray containing a mix of topsoil, M2 compost and sand in a 2:1:1 ratio, one hassey tray per treated and untreated line per population. Trays were placed in a randomised design in a polythene tunnel and covered in polythene for 7 days to protect against pests and promote germination. Twenty-eight days after sowing plants were thinned to 150 plants per tray (see section *3.2.2.2*). Four days after thinning, treated lines were treated with glyphosate at 360 g ha^{-1} using a track sprayer (see section *2.2.1.5*). Survival was assessed 23 days after treatment and plants were cut 10mm above soil height (see section *3.2.2.3*). Eight days after assessment plants that had regrown were repotted into 6-inch pots containing a mix of topsoil, M2 compost, and sand in a 2:1:1 ration, 5-7 plants per pot, and moved to pollen cages to bulk cross (*3.2.2.4*).

3.2.4 Glyphosate selection of 2012 populations

Populations collected in 2012 were selected over 2 generations, and were treated with varying below field rate doses of glyphosate. Doses were selected based on the

variable response to glyphosate in *A. myosuroides* populations in chapter 2 (see section *2.3.4*). Doses were chosen where there were the largest differences in survival between the *A. myosuroides* populations tested. The aim of this was to have a range in survival between the glyphosate selected populations.

3.2.4.1 First generation of 2012 selection

The procedure for the first generation of selection was the same to that of the second generation of 2010 selected lines in *3.2.3.2* with the exception of the glyphosate dose used. Treated lines were treated with a dose of 405 g ha^{-1}. In this first selected generation, to investigate whether increasing genetic diversity increases the likelihood of resistance evolution, a sub set of 5 survivors from each of the four less susceptible populations collected in 2012 (ACA212, ACA412, AES112, and AES212) were put in a pollen cage and allowed to bulk cross and create a new line – MIX.

3.2.4.1 Second generation of 2012 selection

For the second generation of selection of 2012 lines procedure was the same at that of the third generation of selected lines in *3.2.3.1*. For this generation a lower dose of 360 g ha^{-1} was used, as glyphosate efficacy in 2014 was high and selection at a higher dose would not have provided enough survivors to allow for bulk crossing.

3.2.4 Glyphosate dose-response experiments to assess responses to selection.

Following completion of selection experiments, two dose response experiments were used to determine the response of all seed populations to recurrent glyphosate selection. One dose response was used for each of the sets of selected populations,

those collected in 2010 and those in 2012. A total of seven doses were used for each dose-response (0, 81, 162, 270, 405, 540, and 810-glyphosate g ha^{-1}). For each population, dose response experiments were performed on every selected generation as well as the final generation for unselected (control) lines (Table 3-1).

Table 3-1: Glyphosate selected lines of *Alopecurus myosuroides* populations used in a dose-response assay
To assess variance in glyphosate susceptibility between treated and untreated lines

Line name	Treated/ untreated line	Generation of selection
Population C3	Untreated 2010	3
Population C2	Untreated 2012	2
Population T1	Treated 2010/ 2012	1
Population T2	Treated 2010/ 2012	2
Population T3	Treated 2010	3

Although dose-response experiments were set up 2 weeks apart, protocol was exactly the same for each experiment, with the exception that there were 5 replicates for the 2010 lines, and due to poor germination 4 replicates for the 2012 lines (3 for AWA112 C3).

Seeds were treated under warm conditions (see section *3.2.2.1*) before being sown into 90mm Petri dishes containing three 85mm filter paper and 5ml of deionized water over a period of 5 days, one replicate per day starting with replicate 1. Petri dishes were sealed with parafilm and seeds were left to germinate in an incubator set at 23/9^{o}C with 12/12hr lighting for seven days. Once seeds had germinated, over a period of 5 days seedlings were sown into 90x90x90mm pots containing a mix of topsoil, M2 compost and sand in a 2:1:1 ratio, 6 plants per pot, one pot per line, dose, and replicate. One replicate was sown per day, starting with replicate 1. Pots were placed in a glasshouse compartment (see section *3.2.2.2*) in a split plot design, with pots containing each line randomised within dose tray, and dose trays randomised within

replicate. Replicates were placed in rows in the glasshouse compartment. Pots were covered in polythene for 4 days to protect against pests and promote growth.

Seedlings were grown for 10 days before being thinned to remove plants smaller than 2 leaves and larger than 4 leaves (growth stage 12-13). The dose series of glyphosate was applied to the plants 13 to 15 days after transplanting using a track sprayer (see section *2.2.1.5*). Replicates 1 and 2 were treated together 13-14 days after transplanting (14 days replicate 1, 13 days replicate 2) and replicates 3, 4, and 5 were treated together 13-15 days after transplanting (15 days replicate 3, 14 days replicate 4, 13 days replicate 5). Twenty to twenty-two DAT seedlings were assessed for survival (see section *2.2.1.6*) and plants were cut 5mm above soil height. Replicate 1 was assessed 20 DAT, replicate 2 21 DAT, replicate 3 20 DAT, replicate 4 21 DAT, and replicate 5 22 DAT. After cutting, the above ground plant biomass was placed in paper bags, one bag per pot, and dried in a drying oven at 70^{o}C for 72 hours, before pot dry weight was measured.

3.2.5 Statistical analysis

Results were analysed using the R statistical package (version 2.15.3) and dose response curve (DRC) analysis. Dose-response results were analysed as described in chapter 2 (see sections *2.2.6.1* and *2.2.6.2*).

For the dose-response of the 2010 selected lines a Weibull-1 2-parameter model with constrained slope and unconstrained ED_{50} was used to assess survival (model fit =0.9981), and a log-logistic 3-parameter model with constrained slope and ED_{50} was

used to assess dry weight (model fit =1). The estimated ratios of effective dose were also calculated using T-test analysis.

For the dose-response of 2012 selected lines a Weibull-1 2-parameter model with an unconstrained ED_{50} and constrained slope was used to assess survival (model fit =0.1214), and a Weibull-2 3-parameter model with a constrained slope and unconstrained ED_{50} was used to assess dry weight (model fit = 0.3279). The estimated ratios of effective dose were also calculated using T-test analysis.

3.3 Results

3.3.1 Survival of treated lines per generation

Herbicide efficacy varied between years, resulting in much variation in survival of the selected individuals and the need for more than one treatment in some years. After a first glyphosate treatment, survival for the first and second generation of selection for the 2010 lines and the first generation for the 2012 lines was high (Table 3-2, Table 3-3). After a second treatment with the same glyphosate dose survival for these generations was lower (Table 3-2, Table 3-3). For the third generation of selection for 2010 lines and second for 2012 line, glyphosate efficacy was high and survival low and no second glyphosate treatment was needed (Table 3-2, Table 3-3). Number of survivors varied each year, as did the survival ranking of each population.

Table 3-2: Number of *Alopecurus myosuroides* plants alive and dead after glyphosate selection treatment 2010 lines
At 324 g ha^{-1} (T0) and 360 g ha^{-1} (T1&T2). Some plants classed as alive at first treatment did not survive to the second treatment.

Survivors used to create line	First treatment		Second treatment	
	Alive	Dead	Alive	Dead
AES110 T0	97	78	56	27
AHE110 T0	105	70	73	21
ARU110 T0	157	18	94	56
ASC110 T0	72	103	44	32
AES110 T1	94	51	34	60
AHE110 T1	78	74	20	58
ARU110 T1	80	70	24	56
ASC110 T1	108	42	78	30
AES110 T2	22	128		
AHE110 T2	52	98		
ARU110 T2	27	123		
ASC110 T2	22	128		

Table 3-3: Number of *Alopecurus myosuroides* plants alive and dead after glyphosate selection treatment 2012 lines
At 405 g ha^{-1} (T0) and 360 g ha^{-1} (T1). Some plants classed as alive at first treatment did not survive to the second treatment.

Survivors used to create line	First treatment		Second treatment	
	Alive	Dead	Alive	Dead
ACA212 T0	101	49	32	69
ACA412 T0	89	61	49	40
AES112 T0	105	45	69	36
AES212 T0	108	33	52	56
ASF112 T0	76	62	35	41
AWA712 T0	118	32	56	62
MIX T0			40	
ACA212 T1	31	119		
ACA412 T1	24	126		
AES112 T1	40	110		
AES212 T1	50	100		
ASF112 T1	45	105		
AWA712 T1	20	130		
MIX T1	20	130		

3.3.1 2010 lines dose-response assay

3.3.1.1 Dose-response survival of 2010 lines

There was no significant difference between the unconstrained model and the model with unconstrained ED_{50} and constrained slope (LR=10.64, p=0.9406), but there was a significant difference between the unconstrained model and the model with constrained ED_{50} and unconstrained slope (LR=105.29, p<0.001), meaning that there was significant variation in ED_{50} between the lines, but not in slope, and that treated lines responded positively to glyphosate selection.

All populations responded to glyphosate selection, with significantly higher ED_{50} values in the third generation treated lines (T3) compared to the untreated lines (C3) (Figure 3-5, Figure 3-6), confirming that variation in glyphosate susceptibility is heritable and can be selected for. The constant slope between the selected lines shows

that although selection has decrease glyphosate susceptibility in *A. myosuroides* populations, it has not increased variance for glyphosate response within the selected lines.

AES110, initially chosen as a more glyphosate susceptible population, had the largest increase in ED_{50}, with T3 having an estimated ratio of effective dose of 1.51 when compared to the untreated line (Figure 3-6). Agronomic levels of glyphosate resistance did not evolve over three generations of selection, as high mortality was observed at glyphosate field rate (540 g ha^{-1}) and above. However, in line AES110 T3 there were survivors at field rate that survived to reproductive maturity and produced viable seeds.

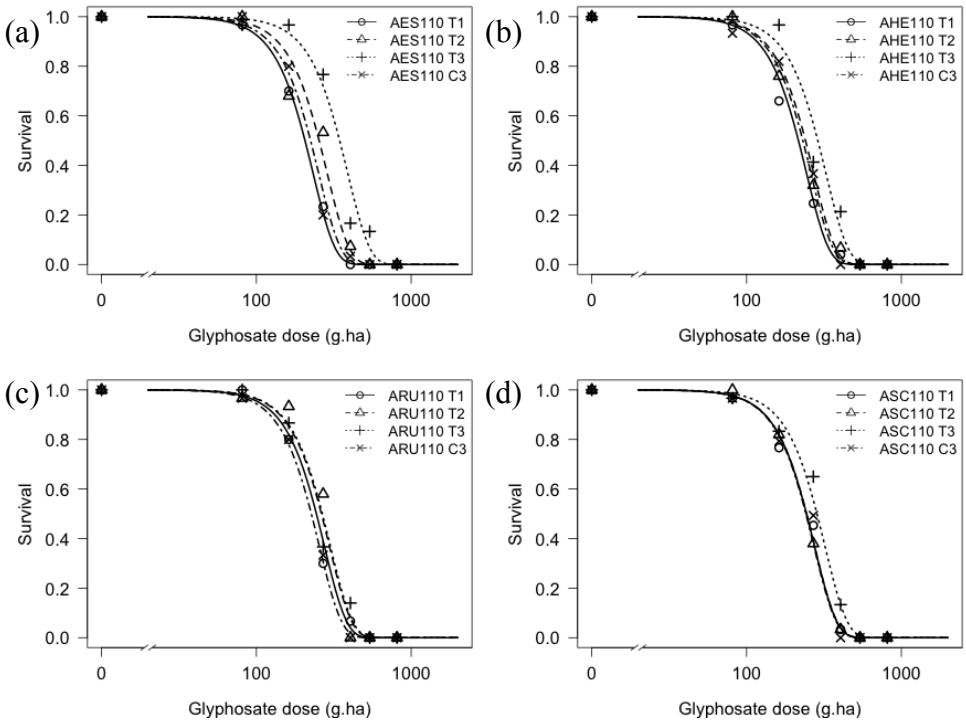

Figure 3-5 a, b, c, & d: Weibull-1 2-parameter model of glyphosate dose-response curve analysis of survival of 2010 selected lines
Slope set to 3.04, three generations of *Alopecurus myosuroides* selected with below field rate doses of glyphosate and the third untreated generation (C3) of four UK populations of *A. myosuroides* collected in 2010

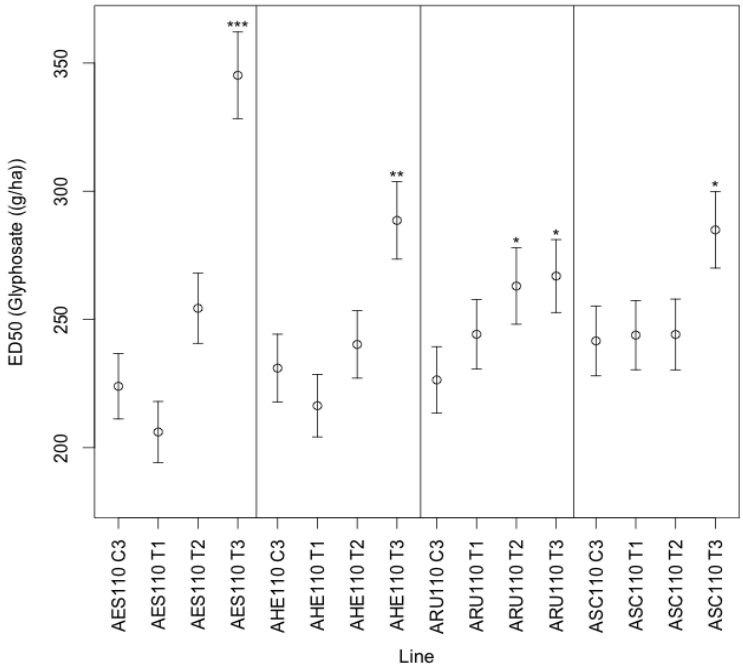

Figure 3-6 a&b: ED_{50} values and standard error of glyphosate dose-response curve analysis of survival of 2010 selected lines
Selected with below field rate doses of glyphosate and the third untreated generation (C3) of four UK populations of *A. myosuroides* collected in 2010, *compared to third untreated generation $p<0.05$, **<0.01, ***<0.001

3.3.1.2 Dry weight dose-response 2010 lines

There was no significant difference between the unconstrained model and the model with constrained ED_{50} and slope (F-value = 0.6423, p=0.954), meaning that there was no significant variation to glyphosate response in dry weight between the lines. GR_{50} was 141 (±3.99) g ha^{-1}, and GR_{90} was 339 (±17.63) g ha^{-1}. This shows that although the 2010 populations responded to three generations of selection in survival, there was no to little response in fresh weight, meaning that survivors were still strongly affected by glyphosate.

3.3.3 Dose-response of 2012 lines

3.3.3.1 Dose-response survival of 2012 lines

There was no significant difference between the unconstrained model and the model with unconstrained ED_{50} and constrained slope (LR=27.422, p=0.1238), but there was a significant difference between the unconstrained model and model with constrained ED_{50} and unconstrained slope (LR=133.22, p<0.001), meaning that there was significant variance in ED_{50} between the lines, but not slope.

No glyphosate treated lines became resistant over two generations of selection, with good control at field rate and higher. However, five of the six treated populations responded to glyphosate selection, with significantly higher ED_{50} values in treated lines compared to the untreated lines (Figure 3-7, Figure 3-8), again confirming that variation in glyphosate susceptibility is heritable and can be selected for. Furthermore, the constant slope between the selected lines, again, shows that although selection has decrease glyphosate susceptibility in *A. myosuroides* populations, it has not increased variance for glyphosate response in the selected lines.

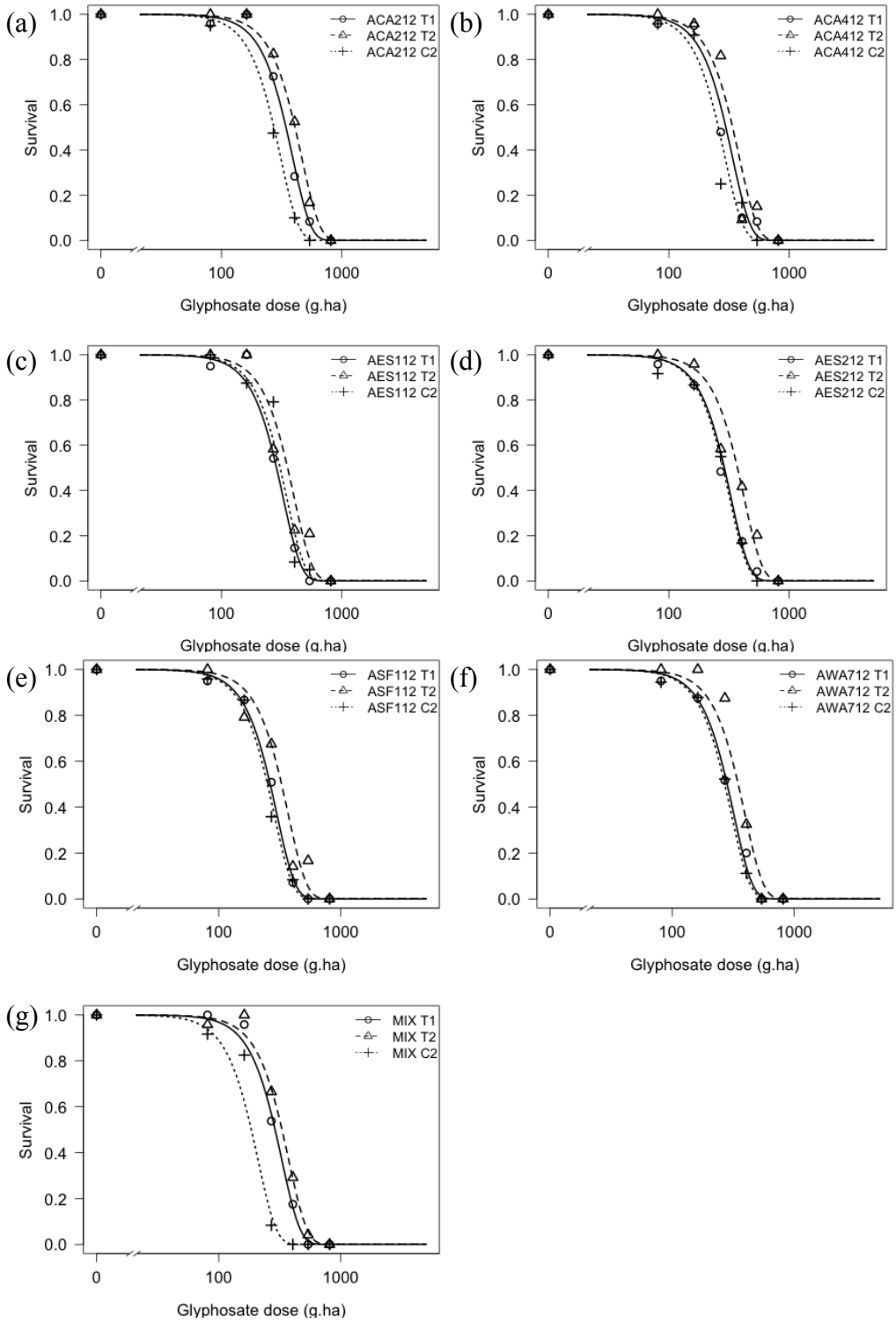

Figure 3-7 a, b, c, d, e, f, & g: Weibull-1 2-parameter model of glyphosate dose-response curve analysis of survival of 2012 selected lines
Slope set to 2.89, survival of two generations of *Alopecurus myosuroides* selected with below field rate doses of glyphosate and the second untreated generation (C2) of six UK populations of *Alopecurus myosuroides* collected in 2012 and one mixed population of ACA212, ACA412, AES112, and AES212

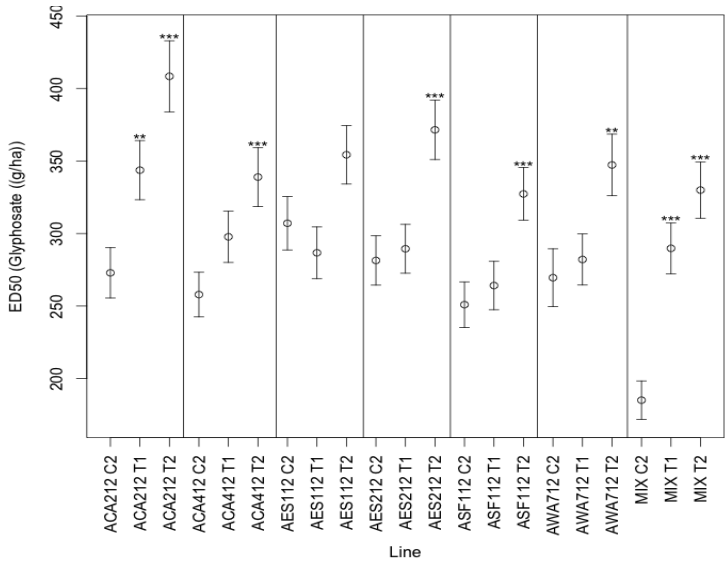

Figure 3-8 a&b: ED_{50} values and standard error of glyphosate dose-response curve analysis of survival of 2012 selected lines
Selected with below field rate doses of glyphosate and the second untreated generation (C2) of six UK populations of *Alopecurus myosuroides* collected in 2012 and one mixed population of ACA212, ACA412, AES112, and AES212, **compared to second generation untreated control line $p<0.01$, ***<0.001

AES112 had the smallest change in glyphosate susceptibility with no significant shift in glyphosate susceptibility of either generation of the treated line compared to the untreated line (Figure 3-8) and an estimated ratio of effective dose of 1.15 between the untreated control line C2 and the second generation of the treated line T2. This is despite AES112 having the highest ED_{50} and ED_{90} values for the untreated control line. The largest change between ED_{50} values of the treated and untreated lines was for MIX, with the untreated line having and ED_{50} of 185 (±13.3)-glyphosate g ha^{-1} and the second generation of the treated line having an ED_{50} of 330 (±19.3) g ha^{-1} (Figure 3-8). The estimated ratio of effective dose for MIX compared to the untreated control line was 1.57 for the first generation of treatment T1, and 1.78 for the second generation of treatment T2.

3.3.3.2 Dose response dry weight 2012 lines

There was no significant difference between the unconstrained model and model with unconstrained GR_{50} and constrained slope ($F=0.9607$, $p=0.5094$), but there was a significant difference between the unconstrained model and model with constrained GR_{50} and constrained slope ($F=2.3766$, $p<0.001$), meaning that GR_{50} varied significantly between the populations, but slope did not. AWA712 C2 had large standard errors, probably due to the reduced number of replicates. Three lines had significantly greater GR_{50} values for the second generation of the treated lines compared to the untreated control lines (Figure 3-9, Figure 3-10), showing that in these populations variation in glyphosate susceptibility is also heritable for growth as well as survival, and that survivors of selected lines were less affected by glyphosate than unselected lines.

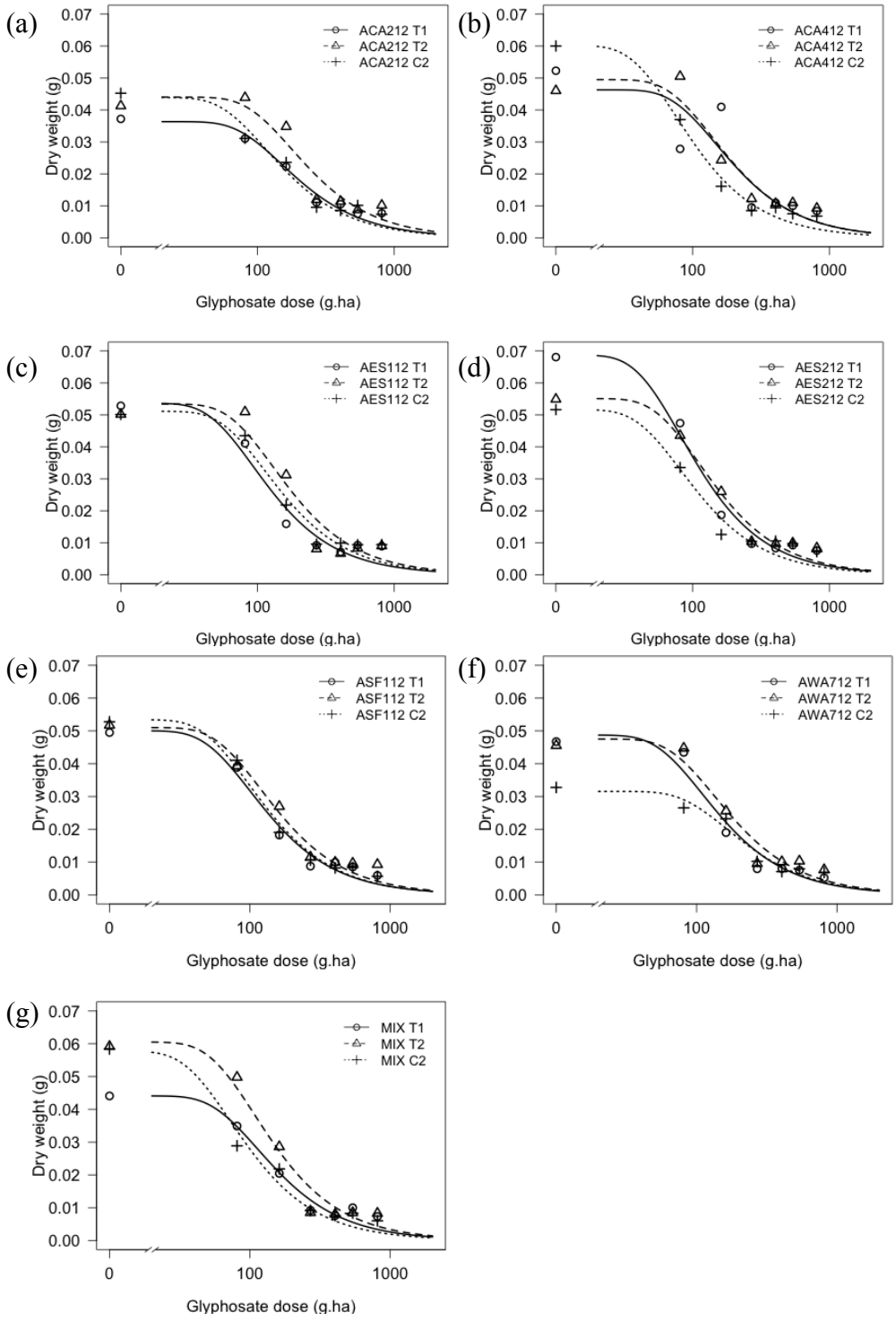

Figure 3-9 a, b, c, d, e, f, & g: Weibull-2 3-parameter model of glyphosate dose-response curve analysis of dry weight of 2012 selected lines
Two generations of *A*. *myosuroides* selected with below field rate doses of glyphosate and the second untreated generation (C2) of six UK populations of *A*. *myosuroides* collected in 2012 and one line of four populations crossed together (Mix – ACA212, ACA412, AES112, and AES212)

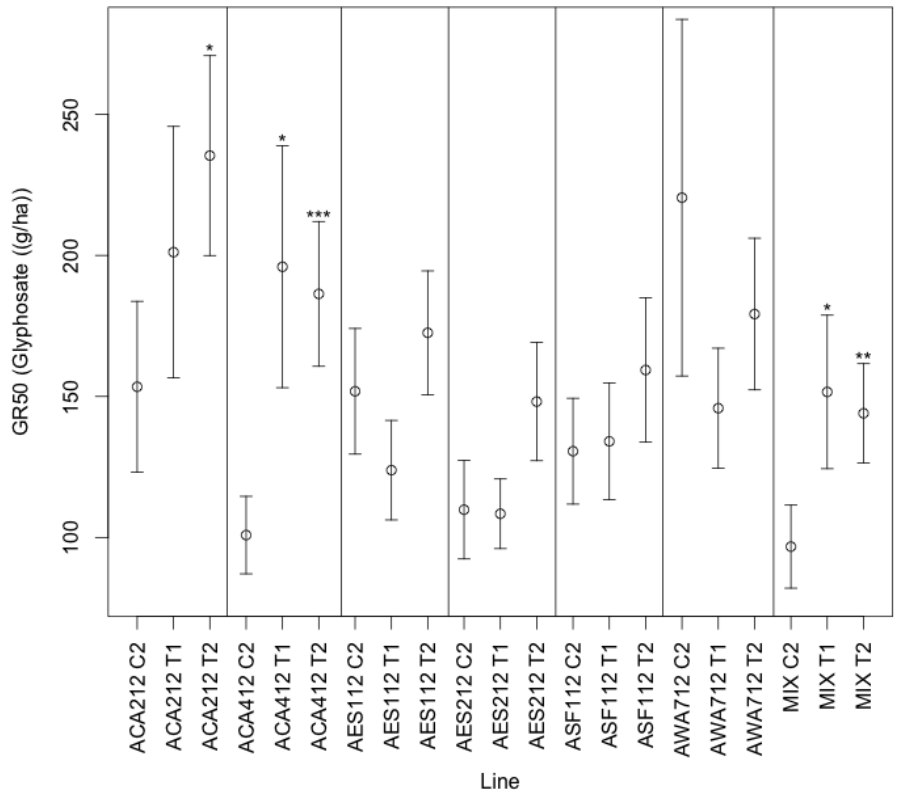

Figure 3-10 a & b: GR_{50} values and standard error of glyphosate dose-response curve analysis of dry weight of 2012 selected lines
A. myosuroides selected with below field rate doses of glyphosate and the second untreated generation (C2) of six UK populations of *A. myosuroides* collected in 2012 and one line of four populations crossed together (Mix – ACA212, ACA412, AES112, and AES212), *compared to second untreated generation $p<0.05$, ***<0.001

3.4 Discussion

3.4.1 Change in susceptibility of glyphosate treated lines

No selected lines became resistant over the period of glyphosate selection, with the estimated ratio of effective dose <2 for all treated lines compared to untreated control lines (Collavo and Sattin, 2014). However, there was a significant shift towards reduced susceptibility in nine of the ten blackgrass populations after two to three generations of glyphosate selection. The largest differences between selected and unselected lines were with survival, rather than dry weight, suggesting that although individuals in selected lines were surviving and able to reproduce at higher doses, these individuals were still greatly affected by glyphosate application and were not resistant. The gradual decrease in glyphosate susceptibility over the generations suggests that there was a build up of minor alleles related to this trait (Neve *et al*. 2005a). However, to confirm whether the cause of the shift towards lower susceptibility is monogenic or polygenic further experiments are needed to produce F_1 and F_2 generations, and backcrosses (Busi *et al*. 2013b).

The shift towards lower glyphosate tolerance in the selected lines in this study was not as much as might have been expected when compared to other herbicide selection studies. After 3 rounds of selection with diclofop-methyl a population of *L. rigidum* had a 56-fold increase in LD_{50} compared to the untreated controls (Neve and Powles, 2005b). Lynch (2014) found that after one generation of selection with below field rate doses of fenoxaprop-p-ethyl, *A. myosuroides* populations had large shifts towards resistance, although population size had a significant impact on the shift towards resistance. It is probable that the shift towards lower glyphosate susceptibility in this

study is much lower than that to other herbicides due to less additive genetic variation for glyphosate susceptibility, as mutation rates for alleles related to glyphosate resistance are much lower than that for other herbicide modes of action (Jander *et al*. 2003).

However, the shift was similar to that of other glyphosate selection studies, the largest shift in ED_{50} between untreated control lines and the third generation of treated selected lines was for the AES110 population, where the estimated ration of effective dose was 1.59 for the treated line compared to the untreated line. With a larger estimated ratio of effective dose for the treated MIX line of 1.78, compared to the untreated control line. In comparison, after three generations of glyphosate selection LD_{50} values in a selected *L. rigidum* population doubled (Busi and Powles, 2009). Like *A. myosuroides*, *L. rigidum* is prone to herbicide resistance evolution. Currently, there are multiple glyphosate resistant *L. rigidum* biotypes in seven countries (Heap, 2015). It is therefore concerning that the decrease in glyphosate susceptibility in *A. myosuroides* populations in this study is similar to the decrease found in a glyphosate resistance prone weed, suggesting that there is also the potential for *A. myosuroides* to evolve glyphosate resistance.

Conversely, there was a 3.1-fold decrease in susceptibility in selected *Amaranthus tuberculatus* populations (Zelaya and Owen, 2005). *Amaranthus* spp. are also prone to glyphosate resistance evolution, with multiple glyphosate resistant *L. rigidum* biotypes in seven countries, and many glyphosate resistant *Amaranthus tuberculatus* biotypes across the USA (Heap, 2015). It is therefore probable that *A. myosuroides* populations are less susceptible to glyphosate resistance evolution than *Amaranthus tuberculatus*

populations, and may not evolve high levels of glyphosate resistance, unlike *Amaranthus tuberculatus* populations.

It would be interesting to continue the selection experiments on these lines to determine whether it can result in individuals resistant to field rate glyphosate doses, and how many generations would be needed for this. It would also be interesting to discover whether the change in glyphosate susceptibility has a limit, as selection for plant traits can continue to increase over multiple generations (Moose *et al*. 2004), but glyphosate selection at low doses can have a limit of 3-4 generations (Busi and Powles, 2009).

3.4.2 Relationship between initial glyphosate susceptibility and change in susceptibility of treated lines

In this study, there is no significant correlation between the ED_{50} and ED_{90} values of the final treated generation and the final untreated generation (data not shown). The lack of relationship between treated and untreated lines is similar to that found by Neve and Powles (2005b), who found no relationship between initial susceptibility and shift towards resistance in *L. rigidum* populations selected using diclofop-methyl. This is also similar to Okada *et al*. (2013), who found that genetic diversity had no effect on the presence of glyphosate resistance in Californian populations of *Conyza canadensis* when under glyphosate selection pressure. It may also be that there are genetic differences in the mechanisms of variation in glyphosate susceptibility within each of the populations, accounting for the differences in response to glyphosate selection (Neve and Powles, 2005b).

It is interesting to note that AES112, the least susceptible population in the glyphosate screen of 40 populations (Chapter 2) responded the least to glyphosate selection. It may be that AES112 has already undergone similar selection in the field and has reached the limit of glyphosate susceptibility shifts under low dose selection and therefore no longer responds under these selection pressures (Busi and Powles, 2009). Considering the lack of correlation between the ED_{50} of untreated lines and the change in ED_{50} in treated lines and the lack of response in AES112 to selection, it appears that populations with the highest degree of pre-selective variation in glyphosate susceptibility are not the most prone to resistance evolution, and initial variation to glyphosate susceptibility does not effect the change in glyphosate susceptibility in populations selected using below field rate doses.

3.4.3 Conclusions

A. *myosuroides* populations have the potential to respond to below field rate doses of glyphosate selection, with minor gene variation for glyphosate susceptibility (standing genetic variation) enriched under glyphosate selection, resulting in decreased susceptibility. However, this response is not as strong as the response to selection using other herbicide modes of action in *A*. *myosuroides* but is similar to the response to glyphosate selection in different grass species. This suggests that although *A*. *myosuroides* does respond to glyphosate selection pressure at below field rate doses, the risk of resistance evolution to glyphosate in *A*. *myosuroides* is less than that to other herbicide modes of action within the species but has a similar risk of glyphosate resistance evolution compared to other grass species. Further experiments are needed to determine whether the change in susceptibility is polygenic or monogenic. It would

also be interesting to continue the selection experiments to determine whether the shifts in reduced susceptibility will eventually result in resistance at field rate.

Populations with the highest initial degree of variation in glyphosate susceptibility are not the most prone to resistance evolution, with no relationship found between initial glyphosate susceptibility and decrease in glyphosate susceptibility after selection with below field rate doses.

Chapter 4 : Fitness cost of glyphosate susceptibility variation in selected lines of *Alopecurus myosuroides*

4.1 Introduction

4.1.1 Fitness and fitness costs

Fitness is a central concept in evolutionary genetics (Vila-Aiub *et al*. 2011) being the relative contribution of a genotype or phenotype to subsequent generations in a given environment. Fitness is usually expressed as a measure of the reproductive success of the genotype/phenotype of interest in comparison to others (Lawrence, 2005; Vila-Aiub *et al*. 2009a). Fitness costs are expressed when individual genotypes or phenotypes that confer increased fitness in response to a selection pressure (e.g. presence of herbicides resulting in herbicide resistance) result in reduced fitness due to negative pleiotropic effects (or trade-offs) in environments where the selection pressure is not present (e.g. absence of herbicides) (Vila-Aiub *et al*. 2009a; Vila-Aiub *et al*. 2015a). Trade-offs are evident where one trait improves (e.g. resistance) at the cost of another (e.g. growth) (Garland, 2014). It has been hypothesized that trade-offs can act within a population to maintain intermediate traits, such as defense and tolerance (Baucom and Mauricio, 2008). Trade-offs can be investigated on an evolutionary level through selection experiments (Garland, 2014).

Plant resistance and/or defense from attack, for example from herbivory, pathogens, or herbicides, can lead to fitness costs or trade-offs in the absence of the stress, due to the allocation of limited resources to the resistance mechanism and away from other processes, such as growth and reproduction (Bergelson and Purrington, 1996;

Purrington, 2000; Heil, 2001; Garland, 2014). Fitness costs of resistance can also occur if the resistance mechanism compromises the effectiveness of normal metabolic processes, for example, compromised enzyme kinetics (Yu *et al*. 2015), or alters ecological interactions, for example with pollinators (Purrington, 2000; Vila-Aiub *et al*. 2009a). Costs of resistance can also appear to be caused by linkage effects, where alleles not related to resistance, but are linked to the resistance loci, confer a resistance cost. However, this is not a true cost of resistance, as it is not conferred by the resistance mechanism itself (Purrington, 2000).

4.1.2 Effects of fitness costs

The initial frequency of resistance alleles (pre-selective frequency) can be affected by fitness costs, with alleles with low or no fitness costs occurring at higher initial frequencies in a population compared to alleles with a high fitness cost (Murphy and Lemerle, 2006). This has an effect on the rate of selection and fixation needed for these alleles in resistance evolution (post-selective frequency). For example, glyphosate resistance was initially slow to evolve, and it has been suggested that the fitness costs associated with glyphosate resistance are high, slowing the evolution of resistance (Preston *et al*. 2009). If fitness costs are large enough in the absence of the herbicide selection pressure, the frequency of resistant individuals will decline (Preston and Wakelin, 2008; Vila-Aiub *et al*. 2009a). Furthermore, understanding fitness costs can have implications on resistance management, with fitness costs exploited in resistance management by using control methods, such as crop competition and herbicide rotation (Preston *et al*. 2009, Vila-Aiub *et al*. 2011).

Many plant life history traits may contribute to fitness costs that manifest as reduced reproductive success, such as reduced seed production (Pedersen *et al*. 2007), decreased biomass (Jordan, 1996), lowered reproductive biomass (Soltani *et al*. 2008), and reduced competitiveness (Vila-Aiub *et al*. 2009b). The timing of onset of these traits can be important, early traits, for example reduced early vigour, may have a higher fitness cost in an environment with high competition and can be selected against (Paris *et at*. 2008). It is therefore important to measure a number of life history traits, as this can give rise to a better understanding of the mechanistic basis of the fitness cost, leading to better designed management practices to maximize the expression of the cost.

4.1.3 Fitness costs related to herbicide resistance

The mechanism of resistance and the resistant species can cause differences in fitness cost, for example, in a review of 88 studies investigating the cost of resistance, 50% found fitness costs, 5% found fitness benefits, and 45% were inconclusive (Bergelson and Purrington, 1996). Menchari *et* al. (2008) found no fitness cost conferred by the Leu-1781 or Asn-2041 ACCase mutations in ACCase resistant *Alopecurus myosuroides* populations, but, when homozygous, the Gly-2078 mutation conferred reduced plant height, biomass, and seed production. Giacomini *et al*. (2014) and Vila-Aiub *et al*. (2014), both found no fitness cost in glyphosate resistant *Amaranthus palmeri*, when resistance was endowed by EPSPS gene amplification, but Cockerton (2013) found a cost in glyphosate resistant *Amaranthus tuberculatus* with the same resistance mechanism. Additionally, in some species tolerance rather than resistance to glyphosate can cause trade-offs, with tolerance to glyphosate resulting in a lack of resistance to leaf damage (Baucom and Mauricio, 2008).

Alternatively, in some cases resistance can increase the fitness in the absence of the herbicide, as well as the presence of it. Wang *et al*. (2014) found that glyphosate resistant crop-weed hybrids of *Oryza sativa f*. *spontanea* (wild rice) and GM-glyphosate resistant *Oryza sativa*, endowing an overexpression of the EPSPS gene, had increased seed production of 48-125% when compared to non-transgenic control hybrids. However, the validity of these claims has been disputed (Gressel *et al*. 2014; Grunewald and Bury, 2014).

4.1.4 Fitness costs in the presence of competition

Where fitness costs are a result of allocation of resources to the resistance mechanism the cost may only become apparent when resources are limited, this will not be apparent under optimal laboratory conditions (Heil, 2001), but may be observed in the presence of resource competition. There are two key factors when assessing fitness costs associated with herbicide resistance, crop competition and competition between resistant and susceptible individuals. This is because fitness costs can vary in the presence and absence of competition, and fitness can vary with different levels of competition (Pedersen *et al*. 2007; Vila-Aiub *et al*. 2009b). Therefore, assessing fitness in the presence of crop competition allows a more realistic assessment of what may happen in the field, which can enable the better application of resistance management practices. For example, in the presence of crop competition *Lolium rigidum* individuals with a P450 enzymatic complex that conferred resistance to multiple modes of action, had significantly reduced biomass production and mean competitive response for reproductive traits than susceptible individuals (Vila-Aiub *et al*. 2009b). Fitness costs in the presence of competition could lead to selection against

herbicide resistant phenotypes and add to the maintenance of genetic polymorphism related to herbicide resistance by preventing the fixation of new resistance alleles (Vila-Aiub *et al*. 2009a).

4.1.5 Genetic background

One major component that needs to be controlled in fitness cost experiments is genetic background, ensuring that costs measured are a result of the resistance alleles (Vila-Aiub *et al*, 2015a). Many fitness studies have compared resistant and susceptible populations without controlling for genetic background (Zeleya *et al*. 2004; Soltani *et al*. 2008; Davis *et al*. 2009; Shrestha *et al*. 2010; Brabham *et al*. 2011; Lehnhoff *et al*. 2013), with it concluded that 75% are flawed for this reason (Délye *et al*. 2013a). This means that if genetic background is not controlled it cannot be concluded that resistant and susceptible alleles are the cause of the observed fitness cost, as these traits may actually be due to population differences (Neve, 2007). For example, Giacomini *et al*. (2014) found that the fitness differences in glyphosate resistant and susceptible *Amaranthus palmeri* populations was due to differences between the populations at different fitness-related loci and not due to the glyphosate resistance mechanism.

Furthermore, it is possible that genetic background can have an effect on resistance costs related to the resistance mechanism. Paris *et al*. (2008) found that genetic background either enhanced or reduced the fitness cost of the *axr1-3* 2,4-D resistance allele in differing *Arabidopsis thaliana* crosses, with the suggestion that there may have been compensatory alleles in the different genetic backgrounds for the different fitness traits. It is therefore important to assess fitness costs in susceptible and resistant plants with the same or similar genetic background and to assess a number of different

genetic backgrounds (Menchari *et al*. 2008, Vila-Aiub *et al*. 2009a). One way to control for differences in genetic background is to compare resistant and susceptible individuals from the same population. This can be done by cloning individuals in the same population, enabling individual plants to be phenotyped by treating one set of clones, whilst fitness cost experiments can be completed on the remaining set of clones (Pedersen *et al*. 2007).

4.1.1 Objectives

The objective of this chapter is to assess whether there are fitness trade-offs associated with reduced glyphosate susceptibility in *Alopecurus myosuroides*. The presence of costs will be assessed by measuring variation in key fitness related life history traits amongst more and less glyphosate sensitive individuals within two populations of *A. myosuroides* that have undergone glyphosate selection (chapter 3). Life history traits are measured in the presence and absence of competition with wheat.

4.2 Materials and Methods

4.2.1 Plant material

Glyphosate screening of the second-generation of 2010 glyphosate selected lines (Chapter 3) showed that the selected populations with the largest difference in susceptibility between the treated (T2) and untreated (C2) lines at that time were AHE110 and ARU110 (data not shown). Therefore these lines were chosen to undertake a fitness cost experiment to determine if there was a significant difference in fitness in the absence of glyphosate between susceptible and less susceptible individuals.

4.2.2 Experimental design

4.2.2.1 Plant cloning

In October 2013, seeds from glyphosate selected lines, AHE110 T2 and ARU110 T2, and unselected lines, AHE110 C2 and ARU110 C2, were sown into 84 well hassey trays containing a mix of topsoil, sand, and M2 compost in a 2:1:1 ratio, one hassey tray for each line. Trays were placed in a glasshouse with a 17-hour day length with supplementary lighting, with temperature set at 20ºC + venting at 22ºC between 05:00 and 22:00, and 12ºC + venting at 15ºC between 22:00 and 05:00. Once seeds had germinated, plants were left to grow for 10 weeks to the four-tiller stage.

When at the four-tiller stage, plants were removed from the hassey trays and clones were produced by separating individual tillers. Fifteen plants were selected from the untreated lines (C2) and 35 from the treated lines (T2), to make a total of 50 plants per population. Plants were cloned using a scalpel to section the plants at the base of each

tiller to produce four clones, each with roots and leaves (Figure 4-1). Once separated, root and shoot material was trimmed to provide four clones of uniform size from each parent plant. For each cloned individual, one clone was transplanted into a hassey tray to be treated with 270-glyphosate g ha^{-1}, one into a hassey tray to be treated with 405 g ha^{-1}, one into the centre of a 6-inch pot (area 182.4 cm^2, effective plant density 55 plants m^{-2}) with no other plants, and one into the centre of a 6-inch pot into which four wheat seeds were sown (var. ribband). Pots and trays were filled with standard potting mix (see above). Wheat seeds were placed 5cm away from the *A. myosuroides* clone and 7cm from the other wheat plants to provide an effective density of 222 plants m^{-2} (Figure 4-2). Wheat seeds were sown on the same day as clones were transplanted. One day after sowing, wheat seeds in pots sown with population ARU110 were predated by mice, necessitating resowing of wheat seeds four days after initial transplanting of clones. After repotting, hassey trays and plant pots were returned to the glasshouse to enable cloned blackgrass plants to establish.

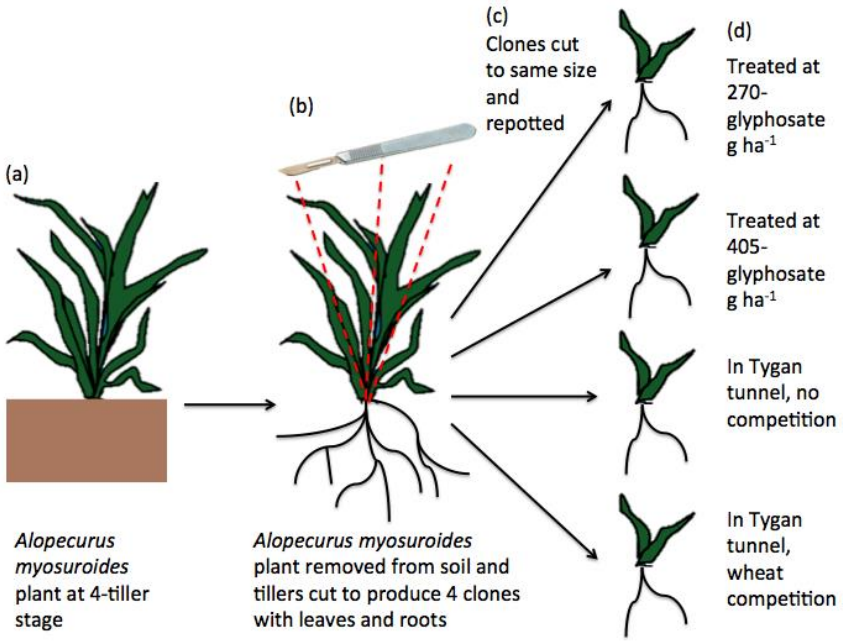

Figure 4-1: Cloning of *Alopecurus myosuroides* plants
(a) at the four-tiller stage, (b) plants were removed from soil and sectioned at the base of each tiller using a scalpel to produce four clones with roots and leaves, (c) which were cut to the same size, (d) clones were then repotted and treated in one of four ways

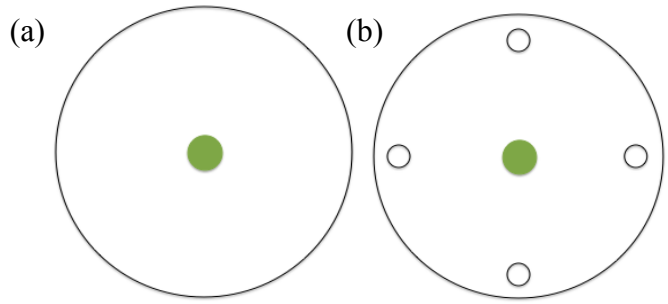

Figure 4-2: Layout of *Alopecurus myosuroides* clones
Clones in green, in 6-inch pots (a) without competition and (b) in competition with four wheat plants (white), wheat spaced 5cm from clone and 7cm from other wheat

4.2.2.2 Glyphosate susceptibility phenotyping

Glyphosate treated clones were left to grow for 8 days before being treated with one of two glyphosate doses, 270 g ha^{-1} or 405 g ha^{-1}, using a Berthoud knapsack sprayer (*2.2.1.5*). Twenty-eight days after glyphosate treatment, clones were assessed for glyphosate susceptibility (Table 4-1). Plants were grouped into one of three phenotypes, tolerant, intermediate, and susceptible, depending on the glyphosate response of the clones at the two glyphosate doses (Table 4-2).

For population AHE110 there were 11 susceptible, 8 intermediate, and 31 tolerant plants. For ARU110 there were 18 susceptible, 14 intermediate, and 17 tolerant plants. One ARU110 clone died without the application of glyphosate, therefore, the total number of plants included in the fitness cost experiment was 49 for ARU110 and for 50 for AHE110.

Table 4-1: Glyphosate effect score for glyphosate treated *Alopecurus myosuroides* clones

Score	Criteria
A	No observable glyphosate affect
B_1	Some observable glyphosate affect, but plant still healthy
B_2	Observable glyphosate effect, plants not healthy, but still alive
C	Plants dead, but not fully desiccated
D	Plants dead and desiccated

Table 4-2: Glyphosate susceptibility scores of clones
Treated at 270 g ha^{-1} and 405 g ha^{-1}, spit into glyphosate susceptibility phenotypes, tolerant (blue), intermediate (yellow), and susceptible (red). For example, plants whose clones exhibited phenotype B_1 at both applied glyphosate doses were designated as B_1B_1. Plants with the B_1B_1 phenotype were classified as being glyphosate 'tolerant'

		Clone treated at 405 g ha^{-1}				
		A	B_1	B_2	C	D
Clone treated at 270 g ha^{-1}	A	AA	AB_1	AB_2	AC	AD
	B_1	B_1A	B_1B_1	B_1B_2	B_1C	B_1D
	B_2	B_2A	B_2B_1	B_2B_2	B_2C	B_2D
	C	CA	CB_1	CB_2	CC	CD
	D	DA	DB_1	DB_2	DC	DD

4.2.2.3 Fitness cost experiment

After cloning, plants in 6-inch pots were left to grow in the glasshouse for 28 days, before being moved to a Tygan tunnel. Plants were arranged in three blocks within the Tygan tunnel to account for environmental gradients from the front to the back of the tunnel. For each population, one third of the plants of each phenotype (S, I and T) were randomly selected and placed in each block. Within a block, the two identical clones from each parent plant (with and without competition) were placed next to each other Pots containing only wheat were placed around the edge of each block to reduce edge effect, and pots were re-randomised within blocks every 14 days.

4.2.2.4 Plant assessment

The parameters for *A. myosuroides* individual clones were estimated seed production, above soil dry weight, and 100 seed weight. Where *A. myosuroides* clones were in competition with wheat, dry weight of wheat seed heads, tiller number, and dry weight of wheat plants above soil height were measured. Wheat plants were assessed by pot, not individually, with results of mean total pot dry weight presented.

A. myosuroides seeds are shed from parent plants over an extended period during maturation. Therefore, total seed production per plant was assessed by means of an allometric relationship between flower head length and seed number. In order to determine this relationship, in April 2014 after anthesis but before maturity, one seed head from each clone was placed in a pollen bag to collect seeds at maturity. At harvest, pollen bags containing the shed seeds and seed head were removed from each plant and stored for subsequent seed counting. Where possible, any unshed *A. myosuroides* seeds from the remaining un-bagged seed heads were collected. All other seed heads were then removed and bagged for subsequent measurement of total seed head length for each plant. Once seed heads were removed, *A. myosuroides* biomass was cut at soil height and placed in a paper bag for subsequent drying at 70^{o}C for 72 hours. The number of tillers produced by each wheat plant was counted, total wheat seed heads per pot were removed and bagged and wheat above ground biomass was harvested and placed in paper bags. Wheat seeds and biomass were dried at 70^{o}C for 72 hours.

To estimate the relationship between seed head length and seed production, seed head length was measured and number of seeds counted for each seed head. Seed head lengths were also measured for all seed heads from which seed was not collected. Seed weight was measured by weighing 100 seeds from each plant that un-bagged seeds were collected from.

4.2.3 Statistical analysis

4.2.3.2 Estimation of seed production

A linear regression in R (version 2.15.3) was used to estimate the relationship between seed head length and seed number (Equation 4.1). Linear regressions were separately fitted for each population with and without competition and models were compared using ANOVA analysis to determine if there significant effects of population identity and/or competition on model parameters.

$$Seed\ number = a * seed\ head\ length + b \quad \text{(Equation 4.1)}$$

Where a is the slope and b the intercept

4.2.3.2 Assessment of life history trade-offs

To account for the unbalanced experimental design, life history data were modelled using linear mixed effect restricted maximum likelihood (REML) in GenStat (version 17.1). For *A. myosuroides* traits (seed production, biomass, and 100 seed weight), plant (clone) was modeled as a random factor with competition, population and phenotype as fixed factors. Model output was interpreted to determine if there were significant effects of fixed factors (and interactions) on life history traits with a significant effect of phenotype interpreted as evidence of life history trade-offs. For wheat data, the response variables (wheat plant biomass, tiller number, and seed head biomass) were modeled with plant (clone) as a random factor and populations and phenotype as fixed factors. Based on the diagnostic plots of the linear mixed effect restricted maximum likelihood models the data is normally distributed and did not need transforming.

4.3 Results

4.3.1 *Alopecurus myosuroides*

4.3.1.1 Alopecurus myosuroides estimated seed number

ANOVA analysis showed that there was a significant difference between linear regression models for population and competition ($p<0.001$), therefore, separate linear regression models were fitted to each population with and without competition (Figure 4-3, equation 4.2a-d). Test statistics for each regression model were: AHE: F=27.6, R^2=0.3658, $p<0.001$, AHE with wheat competition (AHE W): F=29.28, R^2=0.3789, $p<0.001$, ARU: F=46.2, R^2=0.4963, $p<0.001$, ARU with wheat competition (ARU W): F=51.28, R^2=0.5218, $p<0.001$.

$$AHE: 2.278 * seed\ head\ length - 50.213 \text{ (Equation 4.2a)}$$

$$AHE\ W: 1.9794 * seed\ head\ length - 13.6175 \text{ (Equation 4.2b)}$$

$$ARU: 1.9 * seed\ head\ length - 8.6401 \text{ (Equation 4.2c)}$$

$$ARU\ W: 2.2439 * seed\ head\ length - 32.6927 \text{ (Equation 4.2d)}$$

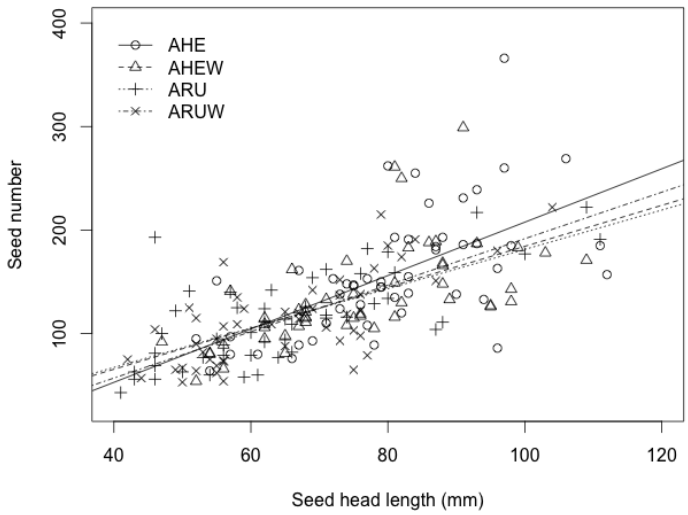

Figure 4-3: Relationship between seed head length (mm) and number of seeds produced per seed head
With linear regression models showing the relationship between the two for two populations (AHE and ARU) of *Alopecurus myosuroides* in a fitness cost experiment with (W) and without wheat competition

Phenotype did not have an affect on estimated seed production of the clones (F=0.92, p=0.403). Competition had a significant effect on decreasing seed production in the clones (F=764.53, p<0.001) and there was a significant interaction between competition and population (F=16.51, p=<0.001), with competition significantly reducing the mean number of seeds produced by individuals from AHE in competition, compared to individuals in competition from ARU (Figure 4-4). There was no interaction between population and phenotype (F=2.33, p=0.103) (Figure 4-4). There was also no interaction between competition, population, and phenotype (F=0.29, p=0.751) (Figure 4-4).

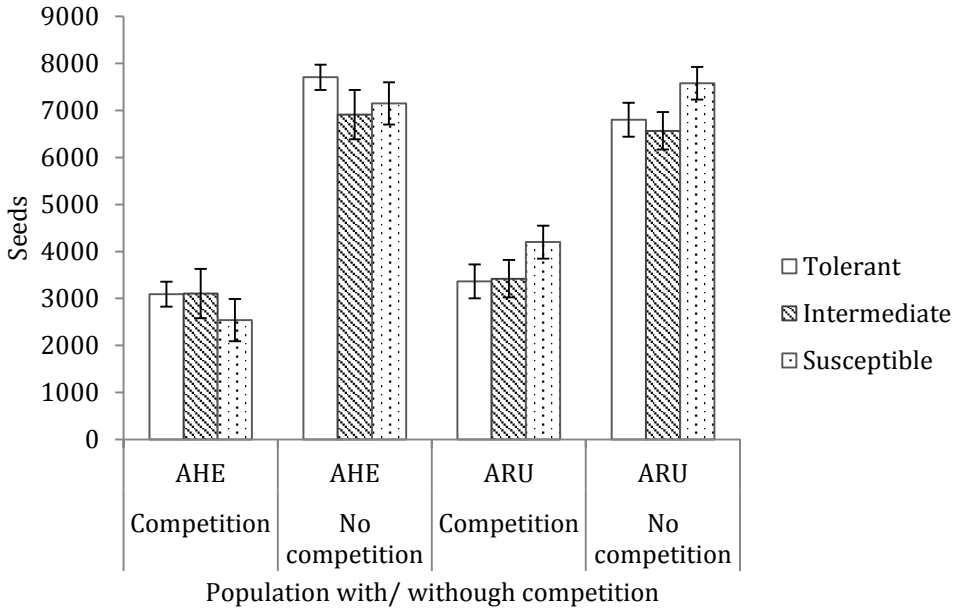

Figure 4-4: Estimated mean seed production per plant and standard error of clones of *Alopecurus myosuroides* plants
from two populations (AHE and ARU) with and without wheat competition

4.3.1.2 Alopecurus myosuroides plant biomass

Phenotype did not have a significant effect on *A. myosuroides* biomass (F=0.59, p=0.555, Figure 4-5). Competition with wheat significantly affected *A. myosuroides*

biomass (F=1026, p<0.001), with clones in competition having significantly lower biomass than clones without competition (Figure 4-5). There was also a significant interaction between competition and population (F=12.69, p<0.001), with individuals in competition with wheat from ARU significantly less affected than individuals in competition with wheat from AHE (Figure 4-5). The interaction between competition and phenotype was not significant (F=2.58, p=0.081), suggesting there is no interaction between the two factors (Figure 4-5). There was no interaction between competition, population and phenotype (F=0.29, p=0.752).

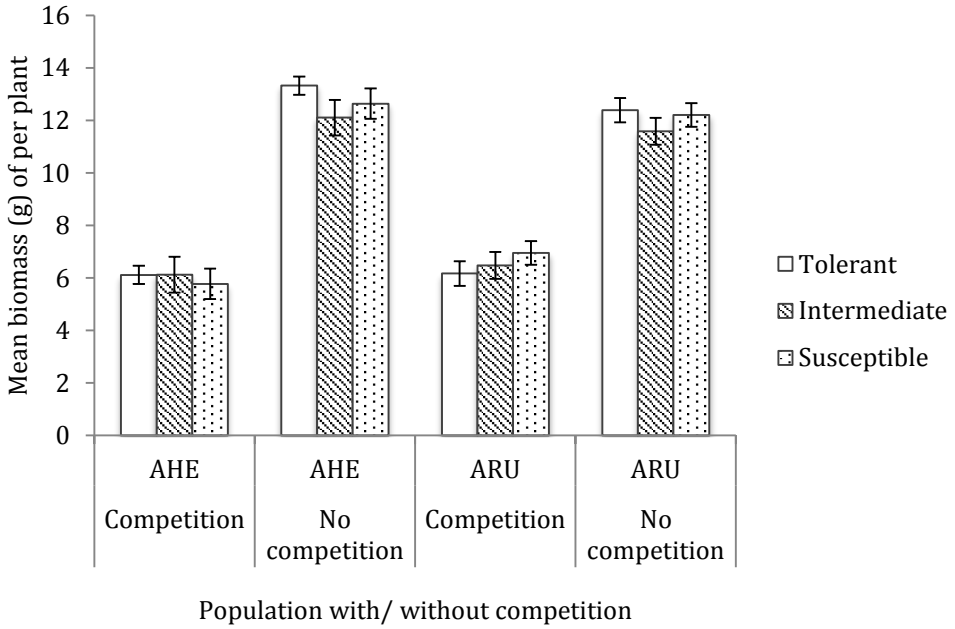

Figure 4-5: Mean biomass (g) and standard error of clones of *Alopecurus myosuroides* plants
from two populations (AHE and ARU) with and without wheat competition

4.3.1.3 Alopecurus myosuroides 100 seed weight

Phenotype did not have a significant effect on *A. myosuroides* seed weight (F=1.15, p=0.321) (Figure 4-6). Competition with wheat (F=13.76, p<0.001) significantly affected the 100 seed weight, with individuals in competition having significantly lower 100 seed weight compared to individuals not in competition, for both

populations. However, the effect of competition on 100 seed weight was not as great as that on seed production and biomass (Figure 4-4, Figure 4-5, Figure 4-6). Population was also a significant factor (F=4.11, p=0.046), with ARU having significantly higher 100 seed weight than AHE, for individuals both with and without competition (Figure 4-6). There was no interaction between competition, population, and phenotype (F=0.68, p=0.51).

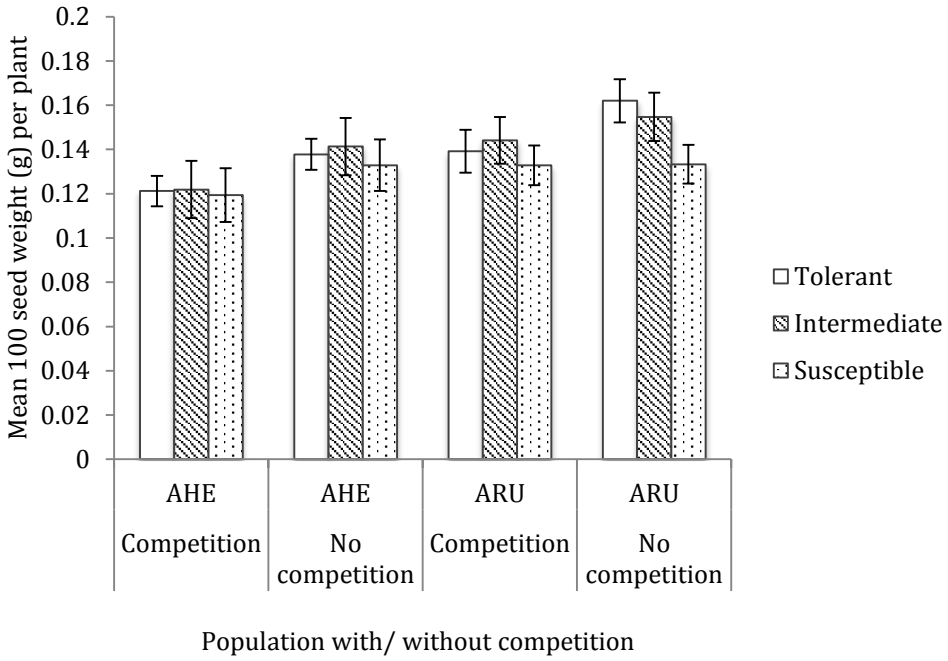

Figure 4-6: Mean weight (g) and standard error of 100 seed weight of clones of *Alopecurus myosuroides* plants
from two populations (AHE and ARU) with and without wheat competition

4.3.2 Wheat

4.3.2.1 Wheat biomass

A. myosuroides population had a significant effect on wheat dry weight (F=12.27, p<0.001), with ARU having a greater competitive effect than AHE (Figure 4-7).

Phenotype was not significant (F=0.13, p=0.881) and there was no interaction between population and phenotype (F=0.78, p=0.463) (Figure 4-7).

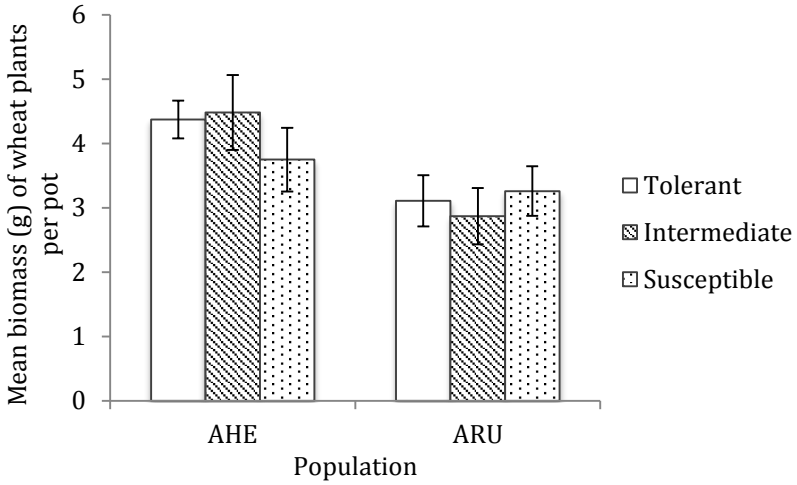

Figure 4-7: Mean biomass and standard error of pot wheat dry weight (4 plants per pot) when in competition with two populations (AHE and ARU) of *Alopecurus myosuroides* with different glyphosate susceptibility phenotypes

4.3.2.2 Wheat tiller number

A. myosuroides population had a significant effect on wheat tiller number (F=4.59, p=0.035), with ARU significantly decreasing number of wheat tillers per pot compared to AHE (Figure 4-8). Phenotype was not significant (F=0.57, p=0.57). There was no interaction between phenotype and population (F=2.83, p=0.064) (Figure 4-8).

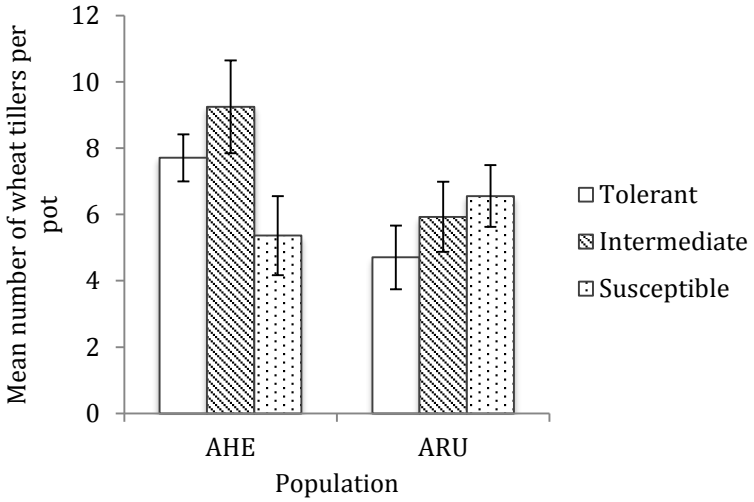

Figure 4-8: Mean number of tillers and standard error of pot wheat dry weight (4 plants per pot) when in competition with two populations (AHE and ARU) of *Alopecurus myosuroides* with different glyphosate susceptibility phenotypes

4.3.2.3 Wheat head biomass

A. myosuroides population had a significant effect on wheat head biomass (F=4.95, p=0.028), with individuals from ARU significantly decreasing wheat head biomass compared to AHE individuals (Figure 4-9). Phenotype was not significant (F=0.64, p=0.53) and there was no interaction between population and phenotype (F=0.89, p=0.414) (Figure 4-9).

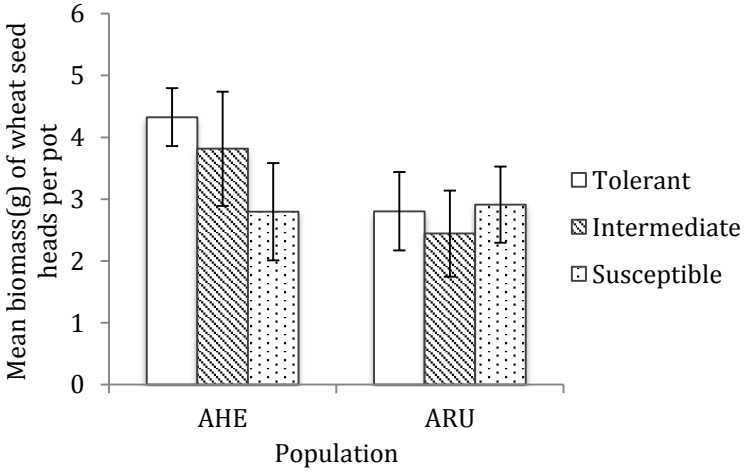

Figure 4-9: Mean dry seed head weight and standard error of pot wheat dry weight (4 plants per pot) when in competition with two populations (AHE and ARU) of *Alopecurus myosuroides* with different glyphosate susceptibility phenotypes

4.4 Discussion

4.4.1 No major fitness costs related to reduced glyphosate susceptibility

There appear to be no major fitness costs or trade-offs related to decreased glyphosate susceptibility in either population, as glyphosate susceptibility phenotype was not a significant factor for any of the traits assessed. This finding counters the theory that glyphosate resistance has been slow to evolve due to major fitness costs associated with resistance (Preston *et al*. 2009), and it would appear that the trait of reduced glyphosate susceptibility in the glyphosate selected *A*. *myosuroides* populations would be able to spread through a population unhindered by fitness costs.

There are a number of studies that have also found no fitness costs related to various herbicide resistance mechanisms. For example, EPSPS gene amplification in *Amaranthus palmeri* conferring resistance to glyphosate (Giacomini *et al*. 2014; Vila-Aiub *et al*. 2014), the absence when in competition of a fitness cost and trade-offs of glyphosate resistant *Lolium rigidum* (Pedersen *et al*. 2007), and the Ile-1781-Leu mutation in *Lolium rigidum* (Vila-Aiub *et al*. 2015b) and *A*. *myosuroides* (Menchari *et al*. 2008) to ACCase inhibitors. However, this study did not use glyphosate resistant individuals, but ones with variation in glyphosate susceptibility, and therefore, there may be larger fitness costs in further glyphosate-selected generations if glyphosate resistance does evolve.

There may also be a lack of major fitness costs, as the decreased glyphosate susceptibility in the populations assessed in this study originates from standing genetic variation rather than new mutations. Adaptation from standing genetic variation can

result in different distributions of fitness effect size than adaptation from new mutation, with high amounts of polymorphisms at genetic loci within populations having neutral or only slightly deleterious fitness (Brcic-Kostic, 2005; Barrett and Schluter, 2008). Furthermore, it is predicted that under relatively weak selection, such as the low herbicide dose selection in this study, only less costly mechanisms of resistance will be maintained (Bergelson and Purrington, 1996).

4.4.2 Minor fitness costs

Although there were no major fitness costs associated with decreased glyphosate susceptibility, there is the possibility that there are minor fitness costs associated with the trait that were not detected, as there were some interactions, for example between competition and phenotype for *A. myosuroides* biomass, that were not significant, but showed a trend towards a change in fitness. It is possible that any minor fitness costs associated with reduced glyphosate susceptibility are being masked by the genetic variation within the *A. myosuroides* populations. This may be because smaller fitness costs are hard to detect due to the inherent variation in fitness between individuals within a population (Reed and Frankham, 2003). Furthermore, *A. myosuroides* is a genetically diverse species and evolution of herbicide resistance appears to have little effect on the genetic diversity between susceptible and resistant populations (Chauvel and Gasquez, 1994, Menchari *et al*. 2007). This study, also, only assessed fitness at only one density of competition and one growth stage, which again may mask minor fitness costs, as the extent of fitness costs can be density dependent and vary with life history stage (Pedersen *et al*. 2007).

For any further experiments it would be advisable to assess fitness costs at different life history stages and at different levels of competition to determine if there are any minor fitness costs related to reduced glyphosate susceptibility. Additionally, using glyphosate-selected lines that have undergone more generations of glyphosate selection in further experiments should increase the difference in glyphosate susceptibility in individuals and the likelihood that any differences in the fitness of more and less susceptible individuals would be clearer. It may also be worthwhile to use multigenerational studies, as these can highlight minor fitness costs more easily than single-generational studies (Roux *et al*. 2005a). However, when using multigenerational studies it is necessary to mitigate genetic drift by having large population sizes, as well as, needing large numbers of replicates and generations to detect small fitness costs (Vila-Aiub *et al*. 2015a). Introducing interspecific competition may also enable the further assessment of fitness costs between more and less glyphosate susceptible individuals (Vila-Aiub *et al*. 2009b).

4.4.3 Restoring genetic factors

It has also been suggested that herbicide resistance mechanisms may confer fitness costs in the early stages of resistance evolution and spread through a population, but that these fitness costs are compensated for by indirect selection of fitness restoring genetic factors from standing genetic variation, making fitness costs undetectable by the time the resistance is noticed at a field level (Darmency *et al*. 2015). This however, has been disputed, with the suggestion that any increase in fitness in subsequent resistant generations is a result of breaking of linkage effects, rather than compensation (Purrington, 2000). Therefore, if full resistance were to evolve conferring a fitness cost in further glyphosate selection studies, it would provide an

excellent opportunity to study the effects of fitness restoring genetic factors on a resistance mechanism and investigate whether it is a result of compensatory alleles, or breaking of linkage effects.

4.4.4 Exploitation of fitness costs

It has previously been suggested that fitness costs related to increased glyphosate tolerance could be exploited in management practices to slow evolution of the trait, or reduce the frequency of resistant alleles once resistance has evolved (Baucom and Mauicio, 2004; Preston *et al*. 2009). However, there is growing evidence that resistant frequencies do not always decline in the absence of herbicide selection pressure (Andrews and Morrison, 1997; Zeleya *et al*. 2004; Roux *et al*. 2005a&b; Chauvel *et al*. 2009; Brabham *et al*. 2011; Darmency *et al*. 2011), making management practices that exploit fitness costs redundant in these situations. The lack of fitness costs between glyphosate susceptible and glyphosate tolerant *A*. *myosuroides* individuals in this study means that it will not be possible to use management practices to exploit fitness costs related to decreased glyphosate susceptibility in *A*. *myosuroides* populations that have undergone evolution under low glyphosate dose exposure to prevent glyphosate resistance evolution in these situations.

4.4.5 Genetic background

Population was a significant factor affecting 100 seed weight of *A*. *myosuroides* individuals, and biomass, tiller number, and seed head biomass of wheat plants. There was also a significant interaction between competition and population for estimated seed number for *A*. *myosuroides*. Therefore, it is possible that if one of these populations was treated as resistant and the other susceptible in this experiment, it may

have been wrongly concluded that the differences between the populations was a result of a fitness cost of reduced glyphosate susceptibility, which is not the case. This supports the notion that genetic background needs to be controlled in fitness cost experiments to enable the proper analysis of the fitness of resistance alleles (Neve, 2007; Menchari *et al*. 2008, Vila-Aiub *et al*. 2015a).

4.4.6 Conclusions

There are no major fitness costs related to reduced glyphosate susceptibility in *A. myosuroides* populations that have undergone glyphosate selection at low doses. This may be due to the trait originating from standing genetic variation, and therefore the alleles already had little or no fitness cost before selection, or the trait, like some glyphosate resistance mechanisms, having no major fitness cost. There may be minor fitness costs associated with variation in glyphosate susceptibility, but these need to be further investigated. It does not appear that major fitness costs related to reduced glyphosate susceptibility mechanisms selected for under low glyphosate doses prevent the evolution of glyphosate resistance, making management practices that exploit fitness costs in these situations redundant.

Chapter 5 : *Anisantha sterilis* (sterile brome) glyphosate sensitivity screening

5.1.1 *Anisantha sterilis*

Over the past 40 years *Anisantha sterilis* (L.) Nevski (sterile or barren brome, syn *Bromus sterilis* L.) has emerged as a problematic UK weed, with a significant increase in the presence of weedy populations in cereal crops since 1978 (Smart *et al*. 2005). This is a consequence of the introduction of minimum tillage techniques, an increase in winter cereal production, and a lack of available herbicides for control of Brome (Clarke *et al*. 2000; Escorial *et al*. 2011). Consequently, glyphosate is often used to control *A*. *sterilis* populations before crop sowing (Dow AgroSciences, 2014; HGCA, 2014).

As discussed in Chapter 1 (see section *1.5.2*), *A*. *sterilis* is species that usually infests field margins, but also infests fields. Individual plants produce many seeds with low dormancy, which germinate quickly after shedding and have short viability (<1 year) (Lintell-Smith *et al*, 1999; Green *et al*. 2001; Steinmann and Klingebiel, 2004). Due to this, infestations can be effectively controlled through ploughing, however in low tillage situations high herbicide efficacy is needed to control *A*. *sterilis* infestations, as seed production and germination is high (Lintell-Smith *et al*, 1999).

In contrast to *Alopecurus myosuroides*, *A*. *sterilis* is a predominantly selfing species and this may impact the rate of, and potential for, evolution of herbicide resistance. Selfing species tend to have lower genetic diversity and recombination rates compared

to outcrossing species and this may impact rates of adaptation to novel environments and the potential for spread of novel traits (such as herbicide resistance) via gene flow (Campbell and Kessler, 2013). However, UK *A*. *sterilis* populations have relatively high genetic diversity, despite selfing, which may in fact be maintained through occasional outcrossing (Green *et al*. 2001). Therefore, it may be that the probability of resistance evolution in outcrossing vs. selfing may vary in evolutionary processes, but not likelihood, and it would interesting to contrast resistance evolution in both outcrossing and selfing species.

5.1.2 Herbicide resistance in *Anistantha*/ *Bromus* species

Presently, there are *A*. *sterilis* biotypes resistant to ALS inhibitors in France and ACCase inhibitors in Germany, but there are no current reports of herbicide resistant biotypes in the UK, including to glyphosate (Heap, 2015). There are, however, glyphosate resistant populations of *Bromus diandrus* (ripgut brome, syn *Anisantha diandra* (Roth) Tutin ex Tzvelev) and *Bromus rubens* L. (red brome, syn *Anisantha ruben* (L.) Nevski) that infest wheat crops and fallow land, respectively, in Australia (Heap, 2015), where glyphosate is used in a similar way to the UK (Owen *et al*. 2014). Some Australian *B*. *diandrus* populations have been found to have increased EPSPS gene copy number, which can cause resistant individuals to survive five times the dose of wild type individuals (Malone *et al*. 2015).

There are Brome species also resistant to other herbicide modes of action, *B*. *diandrus* and *Bromus rigidus* Roth. (stiff brome, syn *Anisantha rigida* (Roth) Tzvelev) have populations resistant to ACCase inhibitors (Boutsalis and Preston, 2006) and ALS-inhibitors (Heap, 2015), with ALS resistance in *B*. *rigidus* conferred by enhanced

metabolism (Owen *et al*. 2012). There are populations of *Bromus tectorum* L. (downy brome, syn *Anisantha tectorum* (L.) Nevski) resistant to photosystem II inhibitors, conferred by enhanced metabolism (Menendez *et al*. 2006), and to ACCase-inhibitors possibly conferred by an ACCase target site mutation (Ball *et al*. 2007). Additionally, one population of *B*. *tectorum* is reported to be resistant to ACCase-inhibitors and ALS-inhibitors through enhanced metabolism, photosystem II inhibitors through a target site mutation, and ethofumesate possibly through enhanced metabolism (Park and Mallory-Smith, 2005). Finally, there are populations of *Bromus japonicus* Thunb. (Japenese brome) and *Bromus secalinus* L. (rye brome) resistant to ALS-inhibitors only (Heap, 2015).

5.1.3 Variation in glyphosate response in *Anisantha*/ *Bromus* species

Even without the presence of resistance, glyphosate response in Brome species varies amongst populations with and without a history of glyphosate exposure. In baseline glyphosate variability testing of *B*. *diandrus*, I_{50} (the herbicide dose resulting in 50% reduction of biomass or survival) ranged from 85-glyphosate g ha^{-1} to 117 g ha^{-1}, for six previously unexposed populations collected in Spain (Barroso *et al*. 2010). For 77 Spanish populations of *B*. *diandrus* with varying histories of exposure to glyphosate, percent undamaged plants at 400-glyphosate g ha^{-1} ranged from 0-100% (Escorial *et al*. 2011). Therefore, there may also be variation in response to glyphosate in other *Bromus*/ *Anisantha* populations.

Previous work has indicated that there may be some UK populations of *A*. *sterilis* with reduced sensitivity to glyphosate at doses that may compromise efficacy in the field. There were reports from an agrochemical company of glyphosate application failure,

with the population sent to Rothamsted Research for testing. When this and a further 12 UK populations of *A. sterilis* were tested for glyphosate susceptibility at 360 and 540 g ha^{-1}, reduction in fresh weight varied from 9-59% and 27-94% respectively. Furthermore, two of the populations tested (OXON and SEL 11) were shown to have greatly reduced glyphosate susceptibility with only a 27% and 40% reduction in fresh weight at 540 g ha^{-1} (Moss and Hull, unpublished).

5.1.4 Objectives

The objectives of this chapter are to:

- Assess variability in sensitivity to glyphosate amongst a random collection of 44 *A. sterilis* populations from England through a glasshouse screening experiment.
- Perform dose response experiments on the least sensitive populations to determine if reduced glyphosate sensitivity is evolving in UK populations.

5.2 Materials and methods

5.2.1 Plant material

A. sterilis seeds were collected from fields in the UK in the years between 2007 and 2011 (Table 5-1). All populations other than OXON, ROAD, PATH, and SEL 11, were provided by ADAS from collections made by farmers. The OXON and SEL 11 populations have previously been found to have reduced glyphosate susceptibility (Moss and Hull, unpublished). The SEL 11 population was derived from 6 surviving individuals treated with 270 g ha^{-1} glyphosate from a field with suspected glyphosate resistance, reported by a farmer for glyphosate control failure. The ROAD population was collected 500m from the field where the SEL 11 population originated. In 2012 a population from Oxfordshire (OXON) was also reported by a farmer for glyphosate control failure found to have reduced glyphosate susceptibility and a population (PATH) was collected 20m from the field where the OXON population was sampled, to be tested as a standard against OXON. Both ROAD and PATH populations were collected from areas with no previous glyphosate exposure adjacent to the suspected resistant populations.

Table 5-1: 44 *A. sterilis* populations collected in UK used in glyphosate susceptibility screening

Population	County	Field	Date sampled
08D106	Shropshire	Green Graves	21/07/2008
08D115	West Sussex	Perrydown	22/07/2008
08D125	Gloucs	Warren Field	25/07/2008
08D129	Lincs	Croake Hill	24/07/2008
08D137	West Midlands	Dairy Field	24/07/2008
08D153	Hampshire	Borough SU29469173	30/07/2008
08D21	Cambs	Fruitcage	15/07/2008
08D34	Gloucs	Home Field	14/07/2008
08D42	Hampshire	Stakes	15/07/2008
08D59	Norfolk	Milehans	16/07/2008
08D73	Cambs	Extra Close	21/07/2008
08D86	Cambs	Pamplins North	22/07/2008
09D118	Leics	The Drift	13/08/2009
09D34	Shropshire	Ten Acre Top	10/07/2009
09D37	Glous	Gayton Left	10/07/2009
09D59	Cambs	Sykes	18/07/2009
09D65	Herefordshire	Ammonds Meadow - Hall	14/07/2009
09D87	Oxford	Devils pool hill	23/07/2009
09D89	Edinburgh	Dow trials	23/07/2009
09D92	Darlington	Dow trials	2009
09D94	Norfolk	Dightles	25/07/2009
10D2	Norfolk	Middle Common	11/07/2010
10D48	Oxfordshire	Home Ground	16/07/2010
10D66	Hereford	Williamson Heath	22/07/2010
10D75	Humberside	#1	21/07/2010
10D80	Co Durham	72 acre	02/08/2010
10D82	Co Durham	86 acre	02/08/2010
11D032	Oxfordshire	Pieces	15/07/2011
ADAS	CAMBS	Extra Close	19/07/2010
BBBARN07	Gloucestershire	Barn Ground	29/07/2007
BBBUI07	Co Durham	Buildings	28/07/2007
BBFIRS07	Shropshire	Firs	02/08/2007
BBFLI07	E Sussex	Flinty	03/08/2007
BBFUL07	Oxon	Fulwell Lodge	01/08/2007
BBHM07	N Yorks	Tommy Ireland	31/07/2007
BBORC07	Gloucestershire	Orchard Field	28/07/2007
BBRED07	Hants	Redenham	17/07/2007
BBWAD07	Herefordshire	Redman the riddings	13/07/2007
BBWES07	Herts	Paul Cherry	27/07/2007
BBWP207	Cambs	Whitepits	26/07/2007
OXON	Oxfordshire	Hopyard Bank	20/07/2008
PATH	Oxfordshire	Foot path 20m from Hopyard bank	11/07/2013
ROAD	Rutland	Roadside 500m from original plants of SEL 11	09/06/2011
SEL 11	Rutland	Bulk crossed from 6 surviving plants treated with 270 g ha glyphosate	06/10

5.2.2 Standard procedures

5.2.2.1 Germination and Sowing

Seeds were sown into 90mm Petri dishes containing 3 Whatman filter papers and 5.5 ml distilled water. Petri dishes were placed in a Sanyo MLR-350 environmental test chamber for 5 days with a 14 hours light (17ºC) and 10 hours dark (11ºC) photoperiod. After five days, five germinated seeds were sown into VDT 90mm pots containing sterilised Kettering loam and lime free grit (3-6mm) in a 4:1 ratio, with the addition of 2kg m^{-3} Osmocote fertilisers. Pots were placed in a glasshouse compartment and grown to the 3-leaf stage (16 days). Plants were watered as required.

5.2.2.2 Glyphosate application

At the 3-leaf stage, plants were thinned to four plants per pot, with individuals smaller or larger than 3-leaves removed (growth stage 13). Five days after thinning plants were treated with glyphosate (NuFarm Clinic Ace: aqueous solution of the isopropylammonium salt Glyphosate, 360g/L, recommended field rate 540 g ha^{-1}) using a laboratory track sprayer, with a Teejet 110015VK flat fan ceramic nozzle, at a pressure of 210 kPa. Plants were not watered for 24 hours after spraying, to prevent washing off of the glyphosate.

5.2.2.3 Assessment

Twenty-one to twenty-two days after glyphosate treatment, plants were assessed for survival (Figure 5-1) and cut at soil height for dry weight measurements.

Figure 5-1: Example of *Anisantha sterilis* survival scores for glyphosate dose-response assay
A – alive: no observable effect compared to control (unsprayed) plants; B – alive: some observable effect e.g. yellowed leaf tips, stunted growth; C – dead: large observable effect e.g. necrosis of majority of leaf tissue; D – dead: necrosis of all leaf tissue

5.2.3 Experiment 1: Glyphosate screen

A glyphosate screen was used to assess the glyphosate susceptibility of 44 *A. sterilis* populations in winter 2014. Four replicates and three glyphosate doses (0, 360, and 540 g ha^{-1} – recommended field rate) across a total of 528 pots were used, one pot per dose, replicate and population. Seeds were germinated in incubators and grown for 5 days before 5 seedlings were transplanted into each pot (*5.2.2.1*). All replicates were sown on the same day (19/02/14), and all replicates were transplanted on the same day (24/02/14). Pots were placed in a glasshouse compartment in a split plot design, with pots randomised within dose in trays, one tray per dose, and dose trays randomised within replicate. Replicates were ordered in rows across the glasshouse compartment. Glasshouse conditions were set at 14 hours light 16ºC, 10 hours dark 12ºC for the first 14 days of the experiment, then changed to frost free ambient settings for the

remainder of the experiment. Plants were thinned 5 days before glyphosate doses were applied. Control pots were treated with tap water and treated pots were treated with either 360 or 540-glyphosate g ha^{-1}. Glyphosate was applied using a laboratory track sprayer (*5.2.2.2*), with a water volume of 208 l ha^{-1}. After spraying pots were moved back to the glasshouse and left for a further 21 days after treatment before being assessed for survival (*5.2.2.3*).

5.2.4 Experiment 2: Further glyphosate screen

As mortality was high in experiment 1, a further glyphosate screen was carried out on 42 of the 44 *A. sterilis* populations from experiment 1, in January to March 2015 (not 08D21 and BBFIR07, due to poor germination). Four replicates and two glyphosate doses were used (0 and 270 g ha^{-1}) across a total of 336 pots, one pot per dose, replicate, and population. Seeds were germinated in incubators and grown for 5-6 days before 5 seedlings were transplanted into each pot (*5.2.2.1*). All replicates were sown on the same day (14/01/15), replicates 1 and 2 were transplanted 5 days after sowing on 19/1/15 and replicates 3 and 4 6 days after sowing on 20/1/15. Pots were placed in a glasshouse compartment in a split plot design, with pots randomised within dose in trays, one tray per dose, and dose trays randomised within replicate. Replicates were ordered in rows across the glasshouse compartment. Glasshouse conditions were set at 14 hours light with supplementary lighting, 16ºC, 10 hours dark, 8ºC.

Five days before glyphosate treatment plants were thinned. Glyphosate was applied on 9/2/15, 20-21 days after transplanting (21 days replicates 1&2, 20 days replicates 3&4) using a laboratory track sprayer with a water volume of 194 l ha^{-1} (*5.2.2.2*). Control pots were treated with tap water and treated pots were treated with 270 g ha^{-1}

glyphosate (*5.2.2.2*). After glyphosate treatment plants were left for a further 21 days before being assessed for survival, all replicates were assessed on the same day (*5.2.2.3*). After assessment of survival, total aboveground plant biomass per pot was harvested, placed in paper bags and dried at 70ºC for 72 hours (*5.2.2.3*).

5.2.5 Experiment 3: Glyphosate dose-response assay

A dose-response assay was performed to further investigate the variability in glyphosate susceptibility in eleven *A. sterilis* populations. PATH, ADAS, and ROAD were used as susceptible controls, and 09D118, SEL 11, OXON, 10D82, 08D59, 09D34, 09D87, and BBRED07 were included as less susceptible populations, based on the results of the earlier glyphosate screen (Experiment 1). Eight doses (0, 81, 121.5, 162, 270, 405, 540, and 810 g ha^{-1}) and four replicates were used, across a total of 352 pots. Experiment 3 was set up concurrently with experiment 2, and the principals of experimental set up were the same, with seeds sown, seedlings transplanted, plants thinned, glyphosate treatments applied, and assessment taking place on the same days as experiment 2. The same glasshouse settings, and water volume for spraying were also used (*5.2.4*). At treatment, control pots were treated with tap water, and treated pots were treated with one of the seven glyphosate doses.

5.3.6 Statistical analysis

Results were analysed using the R statistical package (version 2.15.3).

For survival in experiments 1 and 2 linear mixed effect models were used to assess survival and compare the proportion survival, transformed using empirical logit, against the unexposed control populations ADAS, PATH, and ROAD, and determine

the restricted maximum likelihood parameters. Linear mixed effect models enable the comparison of both fixed and randomised terms in linear models, and are further discussed in Bates et al. (2012). Proportion survival was the response, population the operator, and dose the grouping factor.

For survival in experiment 2 Fisher's test analysis was also used to compare the survival of each population against the susceptibles ADAS, PATH, and ROAD, individually at 270 g ha^{-1}. This analysis was not used for experiment 1, as survival was too low.

For experiment 2 the proportion dry weight of the control of treated plants was calculated and analysed using two-way ANOVA analysis to assess the interaction between population and replicate at the glyphosate dose of 270 g ha^{-1}. Tukey's HSD test analysis was the used on the ANOVA analysis to determine which populations were significantly different compared to the unexposed population ROAD. ROAD was used in this analysis, as there was no significant difference between the three susceptible populations ADAS, PATH, and ROAD, and ROAD was the population with the lowest proportional dry weight of the three.

The DRC package was used for experiment 3 and analysis was performed as described in chapter 2 (*2.2.6.1* and *2.2.6.2*). A Weibull-2 2-parameter model with an unconstrained slope and ED_{50} was used to assess survival for ten of the eleven populations in experiments 3 (model fit = 0.1953). For the population 09D118 a Weibull-2 3-paramter model was used, as the data did not biologically fit a 2-parameter model due to high survival (50% at the highest dose used) (model fit =

0.3082). Also, as survival was too high for 09D118 (50% at 810 g ha^{-1}) ED_{90} could not be estimated. For dry weight data a log-logistic 4-parameter model with a constrained slope was used. As the residuals were not normally distributed the data was transformed using a boxcox transformation (model fit = 0.9226). For both survival and dry weight data in experiment 3 the estimated ratios of effective dose were calculated using T-test analysis for ED_{50} and GR_{50} values of the treated populations OXON and SEL 11 and compared to their corresponding untreated populations PATH and ROAD. The less sensitive 09D118 population was also compared against PATH and ROAD, for this comparison the Weibull-2 3-parameter model used for 09D118 was also fitted to PATH and ROAD to enable comparison, this data is only presented in relation to 09D118, for all other population comparisons to PATH and ROAD a Weibull-2 2-parameter model was used.

5.3 Results

5.3.1 Experiment 1: Glyphosate screen

5.3.1.1 Survival

There was no survival at both 360 and 540-glyphosate g ha^{-1} for 34 of the populations, and low survival for eight of the remaining 10 populations at 360 g ha^{-1}, with the exceptions being OXON and 09D118 (Figure 5-2). Only 2 populations had any survival at 540 g ha^{-1} and this survival was low (Figure 5-2). Linear mixed effect model analysis showed that OXON ($p<0.001$) and 09D118 ($p<0.05$) were the only populations with significantly different survival compared to the susceptible control populations ADAS, PATH and ROAD, across all three doses used.

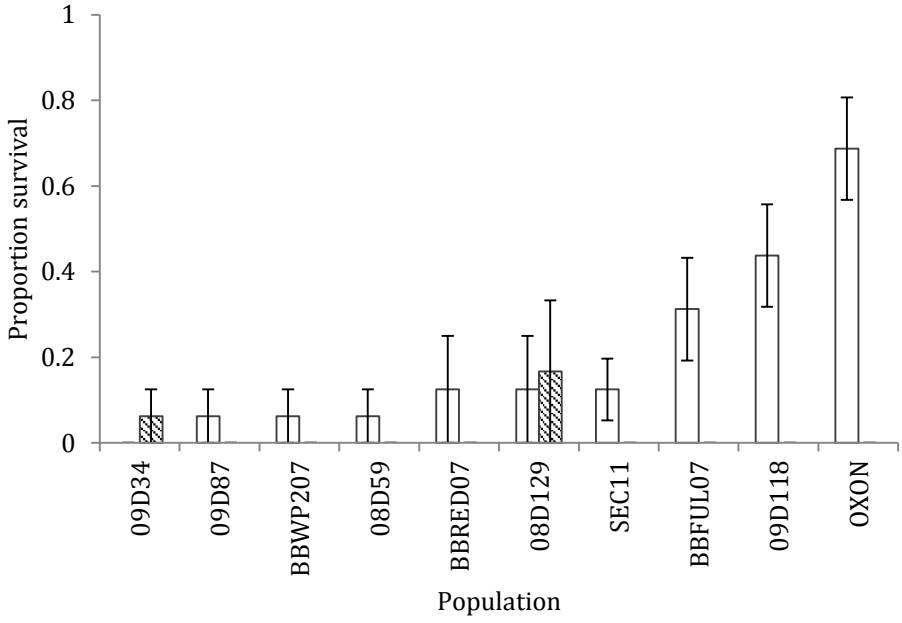

Figure 5-2: Mean proportion survival and standard error for 10 UK populations of *Anisantha sterilis* treated with glyphosate
At 360 g ha-1 (white) and 540 g ha-1 (hatched). For all other populations, there was no survival

5.3.2 Experiment 2: Further glyphosate screen

5.3.2.1 Survival

Survival varied between a proportion of 0.125 and 1 at 270-glyphosate g ha^{-1} (Figure 5-3). Linear mixed effect model analysis showed that OXON and SEL 11 were the only populations with significantly different survival compared to ADAS across both doses ($p=0.016$). The linear mixed effect model showed no populations with significantly different survival when compared to the PATH and ROAD populations across both doses.

At 270-glyphosate g ha^{-1}, Fisher test analysis showed that SEL 11 ($p=0.002$), OXON ($p=0.002$), 09D118 ($p=0.003$), 10D66 ($p=0.027$), and 09D37 ($p=0.033$) had significantly higher survival compared to ADAS, suggesting that these populations may have reduced glyphosate sensitivity. OXON had significantly higher survival at 270 g ha^{-1} when compared to the unexposed population PATH, which was collected in conjunction with OXON ($p=0.013$). SEL 11 also had significantly higher survival compared to ROAD the unexposed population collected in conjunction with SEL 11 ($p=0.03$), suggesting that both the glyphosate exposed OXON and SEL 11 populations have decreased glyphosate susceptibility compared to the unexposed PATH and ROAD populations, despite the probability of these populations having similar genetic backgrounds.

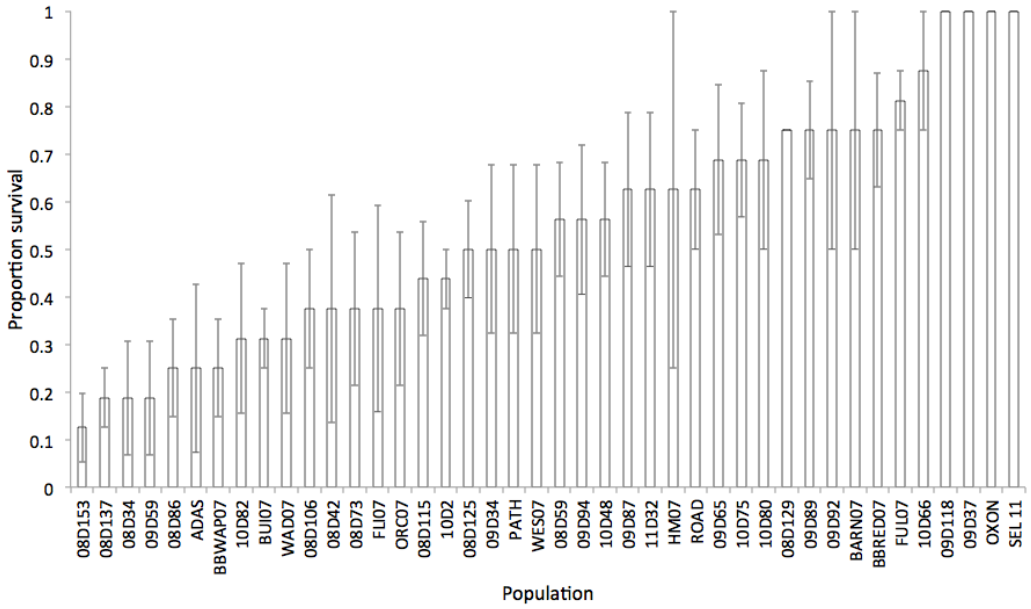

Figure 5-3: Proportion survival and standard error for 42 UK populations of *Anisantha sterilis* treated with glyphosate at 270 g ha^{-1}

5.3.2.2 Dry weight

At 270 g ha^{-1} mean proportion dry weight of the control dose varied between 0.178 and 0.852 at 270 g ha^{-1} (Figure 5-4). Two-way ANOVA analysis and Tukey's HSD test showed that replicate was not a significant factor and that there was no significant difference in proportion dry weight between the three susceptible populations, so ROAD was used for comparison. Three (SEL 11, OXON, 09D118) of the 42 populations tested had significantly different dry weight at 270-glyphosate g ha^{-1} when compared to ROAD (Figure 5-4). OXON ($p<0.001$) and SEL 11 ($p=<0.001$) also had significantly higher dry weight compared to PATH and ROAD respectively, showing again that the exposed populations OXON and SEL 11 are significantly less susceptible to glyphosate compared to unexposed populations collected from the same area.

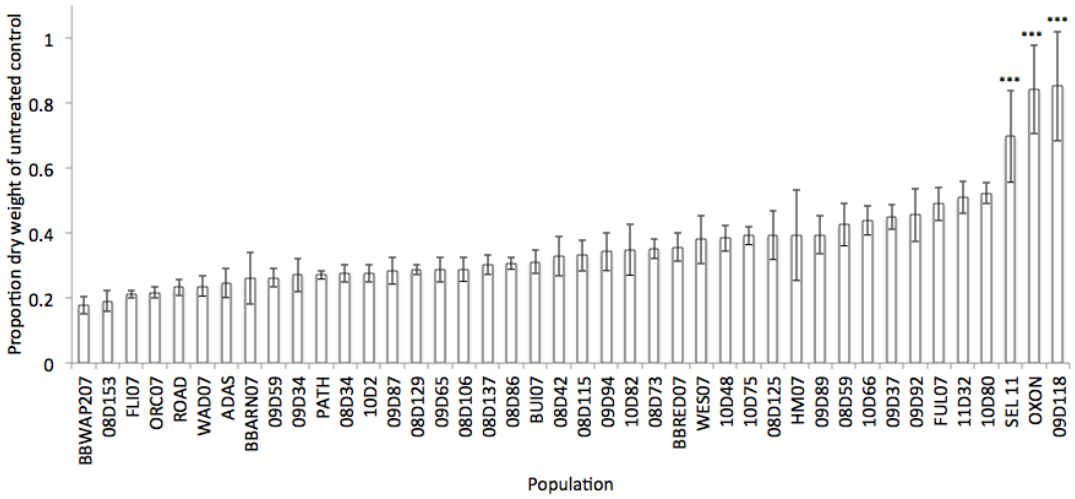

Figure 5-4: Mean proportion dry weight of untreated control and standard error of 42 UK populations of *Anisantha sterilis* treated with glyphosate
At 270 g ha^{-1}, *p<0.05 when compared to ROAD

5.3.3 Experiment 3: Glyphosate dose-response

5.3.3.1 Survival

There were significant differences between the unconstrained model and model with constrained ED_{50} values (LR value = 35.85, p<0.001) and the unconstrained model and model with constrained slope (LR value = 69.79, p<0.001), meaning that both ED_{50} and slope varied significantly between the populations. ED_{50} values ranged from 241 to 821-glyphosate g ha^{-1} and ED_{90} values ranged from 283 g ha^{-1} to 1081 g ha^{-1} (Figure 5-5). Survival was high for both OXON and 09D118 at field rate, with individuals affected but still relatively healthy (Figure 5-6).

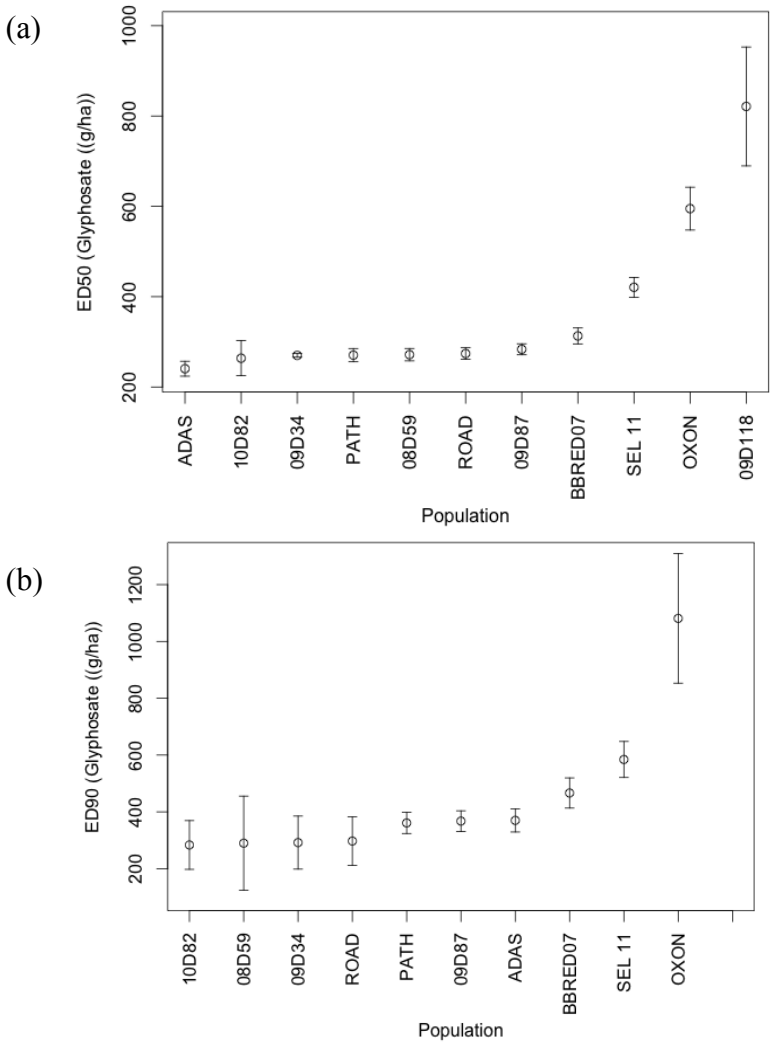

Figure 5-5: Plot of ED_{50} and ED_{90} values and standard error of glyphosate Weibull-2 2-parameter dose-response assay of 11 *Anisantha sterilis* populations
(a) ED_{50} and (b) ED_{90} values

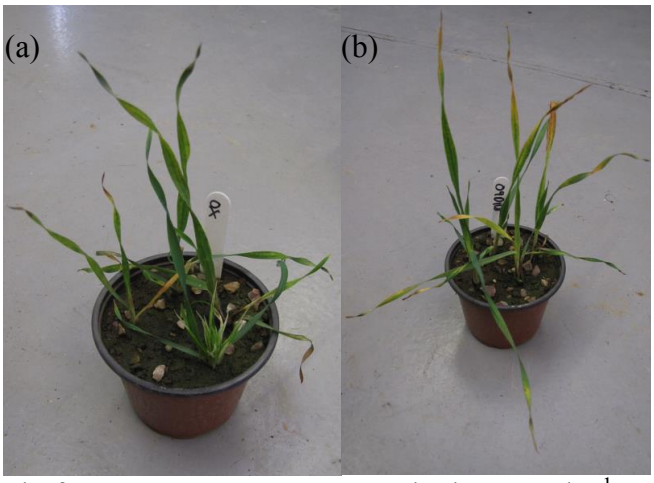

Figure 5-6: Survival of *Anisantha sterilis* at 540-glyphosate g ha^{-1}
(a) OXON and (b) 09D118 individuals at field rate (540-glyphosate g ha^{-1}) Individuals are affected by glyphosate, but were still reasonably healthy and would be able to grow and produce progeny

T-test analysis showed that both OXON and SEL 11 had significantly higher ED_{50} ($p<0.001$) and ED_{90} ($p<0.01$) values when compared to PATH and ROAD, despite being collected from adjacent areas. For ED_{50} the estimated ratio of effective dose between OXON and PATH is 2.2, meaning that a 2.2 times higher dose is needed to control 50% of the OXON population compared to PATH (Figure 5-7a). However, although SEL 11 and ROAD are significantly different, the estimate ratio of effective dose is much lower at 1.55 for these populations (Figure 5-7b). This shows that there has been a significant reduction in glyphosate susceptibility of both the OXON and SEL 11 populations, with OXON having an ED_{50} value more than 2 times higher than the unexposed PATH population. This doubling of ED_{50} and healthy survivors at field rate (Figure 5-6) suggests that OXON has evolved practical glyphosate resistance, where the weed population is not controlled at usual field rates (In Kuk *et al*. 2008).

No unexposed adjacent population was assessed for the population with the highest ED_{50}, 09D118. However, the estimated ratio of effective doses for 09D118 compared to the unexposed PATH and ROAD is 3.05 and 3.02 respectively (Figure 5-7c). Along with the healthy survivors at field rate (Figure 5-6), this also suggests that this population has significantly decreased susceptibility to glyphosate and has evolved practical glyphosate resistance, however, without a corresponding unexposed population this cannot be confirmed in this experiment.

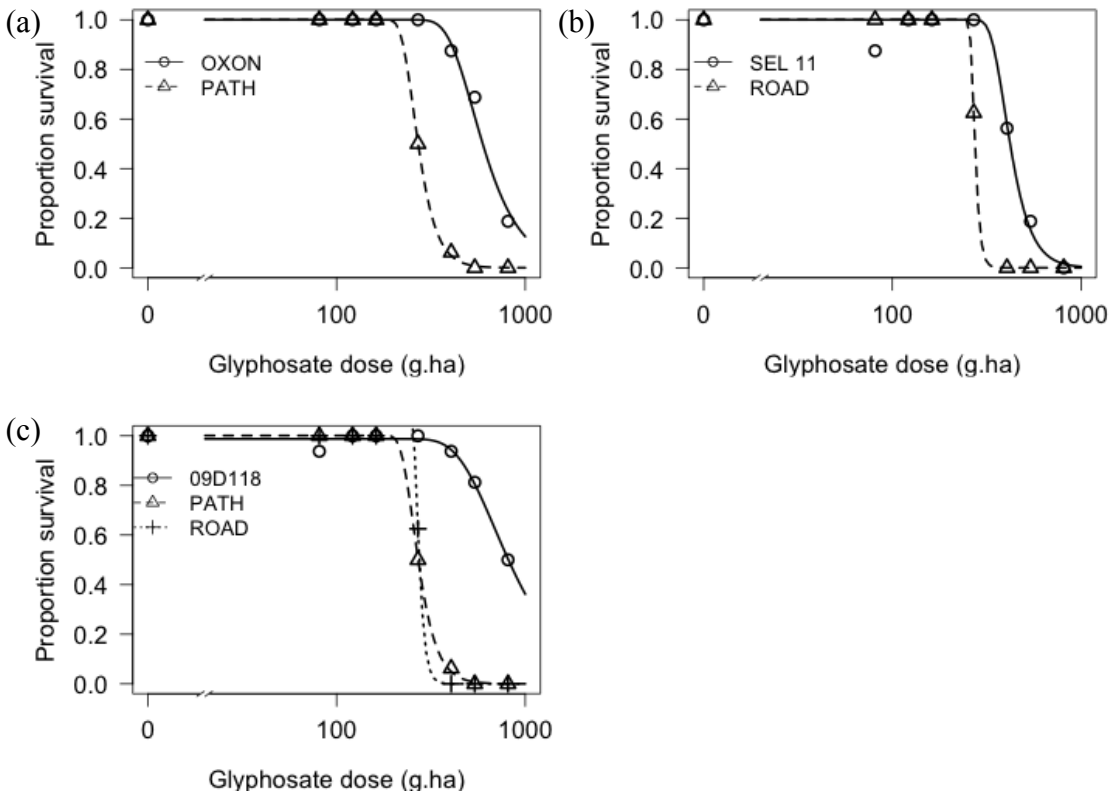

Figure 5-7: Weibull-2 2-parameter model of glyphosate survival of *Anisantha sterilis* populations
Exposed and unexposed paired populations collected from the same area (a) OXON (exposed) and PATH (unexposed), and (b) SEL 11 (exposed) and ROAD (unexposed) of *Anisantha sterilis* and (c) the least susceptible population tested (09D118) compared to the unexposed populations PATH and ROAD

5.3.3.2 Dry weight

There was a significant difference between the unconstrained model and model with constrained GR_{50} (F value = 6.78, $p<0.001$), but no significant difference between the unconstrained model and model with constrained slope (F value = 0.2217, $p=0.9226$), meaning that there was significant variation in GR_{50} between the populations, but not in slope. GR_{50} values ranged from 143 to 307-glyphosate g ha^{-1} (Figure 5-8).

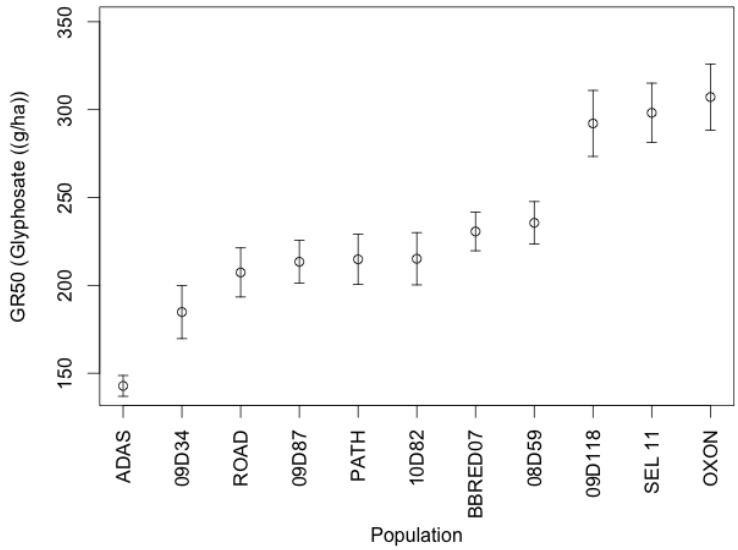

Figure 5-8: Plot of GR_{50} values and standard error of glyphosate Weibull-2 2-parameter dose-response assay of 11 *Anisantha sterilis* populations

T-test analysis showed that both OXON (p=0.0363) and SEL 11 (p=0.0316) had significantly higher GR_{50} values when compared to PATH and ROAD, respectively (Figure 5-8, Figure 5-9a&b). The estimated ratio of effective dose for OXON and PATH was 1.44, and for SEL 11 and ROAD 1.45. The significant difference in dry weight between OXON and PATH, and SEL 11 and ROAD supports that there has been a significant decrease in glyphosate susceptibility in both OXON and PATH, however, the estimated ratio of effective dose is less than 2 for these populations suggesting that for dry weight these populations are not resistant. There was no significant difference between the GR_{50} value of 09D118 compared to PATH and ROAD (Figure 5-9), which is in contrast to the survival data, but also highlights the need for an unexposed population collected from the same area as 09D118 to use for comparison, as the lack of difference may be due to genetic factors.

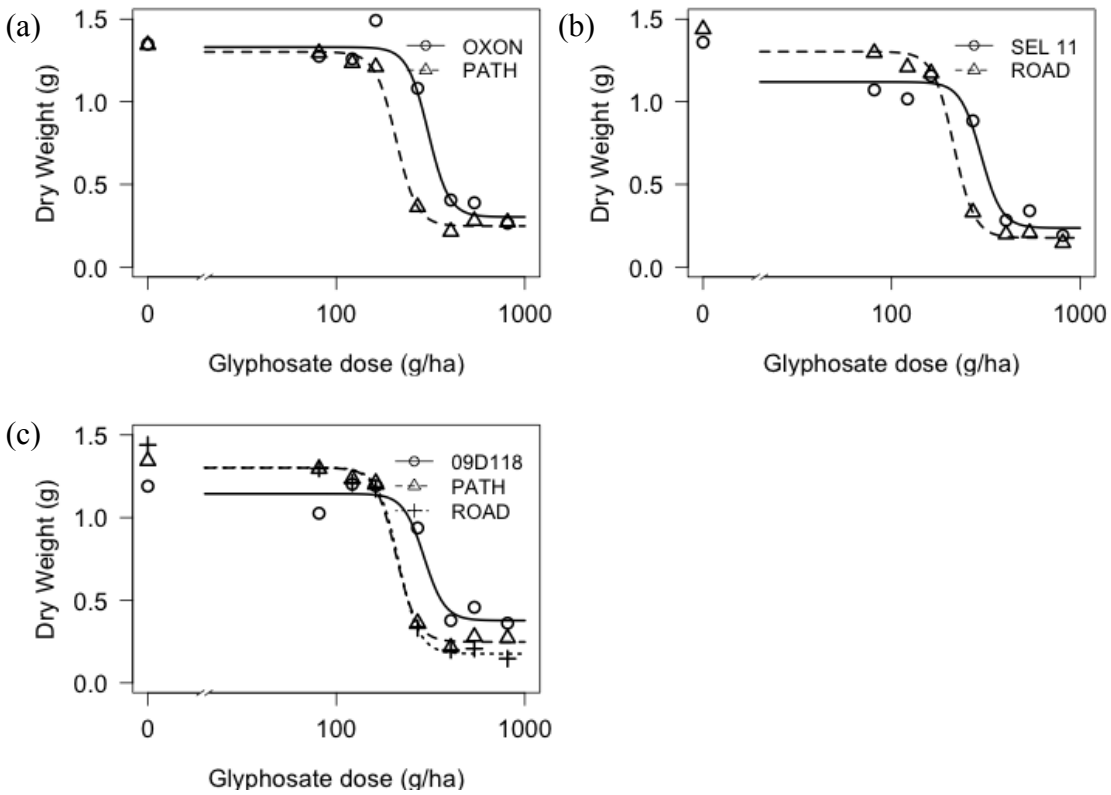

Figure 5-9: Log-logistic 4-parameter model of glyphosate dry weight of *Anisantha sterilis* populations

Exposed and unexposed paired populations collected from the same area (a) OXON (exposed) and PATH (unexposed), and (b) SEL 11 (exposed) and ROAD (unexposed) of *Anisantha sterilis* and (c) the least susceptible population tested (09D118) compared to the unexposed populations PATH and ROAD

5.4 Discussion

5.4.1 Variation in glyphosate susceptibility

There is significant variation in glyphosate susceptibility of the UK *A*. *sterilis* populations tested, with significant differences in both glyphosate screens of 44 UK populations and dose-response analysis of 11 populations. This variation to glyphosate in all populations tested is similar to other selfing species that have had previous glyphosate exposure. For example, the variation in dry weight in experiments 2 and 3 is similar to that found by Escorial *et al*. (2011) where reduction in fresh weight at 400 g ha^{-1} ranged from 2-79% in glyphosate exposed populations of *B*. *diandrus*, some of which were resistant. The range of the variation in glyphosate susceptibility is also similar to that of other selfing weed species exposed to glyphosate selection pressure, for example, in *Oryza sativa* L. (red rice), where percent injury range at 400 g ha^{-1} was between 41 and 100% (Burgos *et al*. 2011). This suggests that the *A*. *sterilis* populations tested in this study have responded to glyphosate selection, as the response is similar to populations where resistance has evolved.

Although the variation in response to glyphosate in the *A*. *sterilis* populations tested in this study is similar to that of other previously glyphosate exposed populations of other species, it is much greater than that of unexposed populations. For example, the dry weight of the unexposed populations in experiment 3 (ADAS, PATH, and ROAD) ranged from 142 to 214 g ha^{-1}, whereas the I_{50} values of unexposed *B*. *diandrus* populations ranged from 85 to 117 g ha^{-1} (Barroso *et al*. 2010). Furthermore, the ED_{50} values of ADAS (241 g ha^{-1}), PATH (271 g ha^{-1}) and ROAD (274 g ha^{-1}) were also

much greater than the LD_{50} of the unexposed *B*. *diandrus* (59 g ha^{-1}) population in Malone *et al*. (2015).

5.4.2 Glyphosate dose acting within standing genetic variation

These higher GR_{50} and ED_{50} values in unexposed *A*. *sterilis* populations compared to unexposed *B*. *diandrus* populations suggests that glyphosate susceptibility in *A*. *sterilis* unexposed populations in the UK may be lower than that of other unexposed *Anisantha*/ *Bromus* species. This may have major implications in glyphosate resistance evolution of UK *A*. *sterilis* populations, as it is possible that the glyphosate doses used on *A*. *sterilis* populations in the UK act within the standing genetic variation of *A*. *sterilis* populations, which can lead to resistance evolution (discussed in chapters 1 (*1.4.2*) and 2). Furthermore, the field rate of glyphosate used for brome control in other countries (a rate of 686 g ha^{-1} is recommended to control brome species in Canada (Monsanto, 2010)) is higher than that used in the UK (540 g ha^{-1}), which may be due to some *Anisantha*/ *Bromus* species being naturally less susceptible to glyphosate than other grass species. The source of the standing genetic variation in glyphosate susceptibility may be a result of high levels of polymorphism in *A*. *sterilis* populations, providing the alleles needed for a change in glyphosate susceptibility (Green *et al*. 2001). There is therefore a need for baseline data of *A*. *sterilis* populations to determine how much the populations in this study may have responded, and whether *A*. *sterilis* is initially less susceptible to glyphosate than other species in the genus, meaning that a higher glyphosate field rate is needed to prevent resistance evolution as a result of the dose acting within standing genetic variation.

5.4.3 Significant change in glyphosate susceptibility in exposed and unexposed populations

Both the glyphosate exposed OXON and SEL 11 populations had significantly decreased glyphosate susceptibility compared to the glyphosate unexposed populations PATH and ROAD collected from adjacent areas to OXON and SEL 11, respectively. It can be assumed that these paired populations have similar genetic backgrounds (Escorial *et al*. 2011), and therefore, the significant differences in glyphosate susceptibility in the exposed populations is due to an evolved reduction in susceptibility. This contrasts to the findings of Escorial *et al*. (2011), where no difference in response to glyphosate was found between collections of *B*. *diandrus* made within the field and at field edges where selection pressure of glyphosate should have varied, with the field individuals not being self-sustaining and originating for the field margins. *A*. *sterilis* also has limited primary and secondary seed dispersal, with median dispersal distances of 2.3 – 4.8m (Rew *et al*. 1996, Steinmann and Klingebiel, 2004). This suggests that although PATH and OXON, and SEL 11 and ROAD, may have similar genetic backgrounds sustained through outcrossing and pollen flow (Green *et al*. 2001), both OXON and SEL 11 are self sustaining in the field, and do not come from PATH and ROAD each year even though they are from the same geographical location.

5.4.4 Practical glyphosate resistance

OXON was initially collected, as there was a reported failure of glyphosate in the field for this population (Moss and Hull, unpublished). There were healthy survivors of OXON at field rate in experiment 3, and there has been a significant reduction in glyphosate susceptibility in this population with the estimated ratio of effective dose

compared to the unexposed PATH population of >2, with these results confirming those found by Moss and Hull (unpublished). Herbicide resistance can be said to have evolved when a population has the inherited ability to survive and reproduce following exposure to a herbicide dose lethal to the wild type (Weed Science Society of America, 1998). Furthermore, where the estimated ratio of effective dose it >2 between susceptible and unsusceptible populations it can be inferred that the unsusceptible population is resistant (Collavo and Sattin, 2014). One drawback of comparing ED and GR values of possible resistant populations to unexposed, susceptible populations is the possibility of identifying populations with higher ED and GR values as resistant, when in fact they are still susceptible at herbicide rates used in the field and the cause of the high difference in values is a result of low initial susceptibility. Therefore, when comparing possible resistant populations to unexposed, susceptible populations, it is important to take into account the survival of the resistant populations at field rate, populations that have higher ED and GR values compared to unexposed, susceptible population and are also not controlled at field rate can be classified as being practically resistant (In Kuk *et al*. 2008). As OXON has an estimated ratio of effective dose compared to the unexposed PATH population of >2 and was not controlled at field rate of 1.5x the field rate of glyphosate, it would appear that the OXON population has evolved practical glyphosate resistance. As a result of this practical resistance the farmer that farms the field where OXON was collected may find it much harder to control *A*. *sterilis* in the field, as there will be many more survivors after glyphosate application, resulting in a larger population size. It may also mean that the farmer will need to use other management strategies, such as ploughing to control this *A*. *sterilis* population.

The only other population to have healthy survivors at field rate is 09D118. This population also has ED_{50} estimate ratio of effective dose >2 compared to the unexposed populations, ROAD and PATH. Therefore, it also appears that 09D118 is practically glyphosate resistant. However, further experiments with an unexposed population near the collection site of 09D118 are needed to confirm that this population is practically glyphosate resistant and determine whether the population has responded to glyphosate selection pressure or if it is naturally less susceptible.

The ED_{50} values for OXON (594 g ha^{-1}) and 09D118 (821 g ha^{-1}) are much higher than LD_{50} values reported for Australian glyphosate resistant *B*. *diandrus* populations, which ranged from 288 and 275 g ha^{-1} (Malone *et al*. 2015) and are more similar to those of the first reported case of glyphosate resistance evolution, where LD_{50} values of the resistant *Lolium rigidum* population ranged from 600 – 1800 g ha^{-1} (Powles *et al*. 1998). The estimated ratio of effective dose for OXON and 09D118 is also similar to that found by Collavo and Sattin (2014), in the first reported cases of glyphosate resistance in European annual crops. Therefore, it appears that OXON and 09D118 have a similar or higher levels of glyphosate resistance compared to other grass weed species in countries where glyphosate is used in a similar way to the UK.

Interestingly, in experiment 1 there was good control for all populations at field rate, calling into question the practical resistance of OXON and 09D118. The difference in survival between experiment 1 and experiments 2 and 3 is probably due to differences in the environmental conditions of the experiments, which can greatly affect the efficacy of glyphosate (Boutin *et al*. 2010; Owen and Powles, 2010), rather than the populations not being practically resistant. Therefore, to confirm resistance a repeat

glyphosate dose-response is needed to confirm the results of the 09D118 population in experiment 3. Experiments investigating the heritability of the resistance trait in both 09D118 and OXON are also required, as for a population to be resistant the trait must be heritable (Weed Science Society of America, 1998; Heap, 2015).

5.4.5 Mechanism of glyphosate resistance

This study has not investigated the mechanism of the practical glyphosate resistance of OXON and 09D118. However, as hypothesised above the cause for the change in glyphosate susceptibility and resistance evolution may be a result of the glyphosate doses used acting within the standing genetic variation of the *A. sterilis* populations. It is also possible that the resistance in OXON and 09D118, and variation in glyphosate susceptibility in the remaining populations is conferred by an increased EPSPS gene copy number (Malone *et al*. 2015) or from a single gene. It would, therefore, be interesting to determine if the reduced susceptibly and practical resistance in the glyphosate exposed populations is the result of a build up of minor alleles related to reduced susceptibility, or whether it is conferred by increased EPSPS copy number, or a single allele.

Genetic analysis could be used to further investigate the difference in glyphosate susceptibility in these populations, as genetic differentiation in resistant and susceptible individuals in selfing species can vary significantly within the same population (Aper *et al*. 2010), with results possibly exposing the mechanism for future glyphosate resistance evolution in these populations. It would also be interesting to determine if these populations are resistant to any other herbicide modes of action, as

losing the use of in-crop post-emergent herbicides can greatly increase the risk of glyphosate resistance evolution (Neve *et al*. 2003b).

5.4.1 Conclusions

There is significant variation in glyphosate susceptibility in UK *A*. *sterilis* populations, with two populations tested having practical glyphosate resistance. This variation is greater than the variation in baseline data for a closely related species, but is similar to that of exposed populations in the same genus. Some of the variation in glyphosate susceptibility can be attributed to a reduction in susceptibility in glyphosate-exposed populations when compared to unexposed populations collected from the same location.

Chapter 6 : Variation in glyphosate susceptibility in global and UK accessions of *Arabidopsis thaliana*

6.1 Introduction

6.1.1 Herbicide resistant *Arabidopsis thaliana*

Atrazine resistance has been reported in *Arabidopsis thaliana* accessions collected from UK railway lines, with the *Ely* accession collected from Ely railway station in 1988, found to have evolved triazine resistance (El-Lithy *et al*. 2005). After its ban in 1993, glyphosate replaced the use of atrazine for weed control on railway lines in the UK and is now regularly used in these situations (Ramwell *et al*. 2004; El-lithy *et al*. 2005), thus exposing weeds, such as *A*. *thaliana*, to glyphosate selection pressure. The precedent for evolution of herbicide resistance in *A*. *thaliana* on railway lines and a history of exposure to glyphosate, mean that the potential for evolution of glyphosate resistance in this species is worthy of further study.

Furthermore, using a model plant species, such as *Arabidopsis thaliana*, in herbicide resistance studies can help to enhance understanding of the phenotypic and genotypic basis of reduced and variable herbicide susceptibility within diverse, characterized global accessions of a weedy plant species (Brotherton *et al*. 2007; Vila-Aiub *et al*. 2009b). There are many herbicide resistant *A*. *thaliana* accessions, however a number of these have been derived from ethyl methanesulfonate mutagenized lines or have had the resistance gene transgenically inserted (Vila-Aiub *et al*. 2009b). As the resistance mechanisms in these accessions have not resulted from random mutation and/or

standing genetic variation, and subsequent selection, they are less useful when investigating the evolution of herbicide resistance.

6.1.2 Variation in herbicide susceptibility in *Arabidopsis thaliana*

However, variation in susceptibility to different herbicides in other accessions of *A. thaliana* has been reported, with Roux *et al*. (2005c) finding large differences in susceptibility to twenty-two different ALS herbicides between Col and Ler accessions, with ED_{50} values for mesosulfuron of 376 mg ha^{-1} and 1507 mg ha^{-1}, respectively. Brotherton *et al*. (2007) found some variation in glyphosate susceptibility in *A. thaliana* accessions, with responses ranging from very susceptible to less sensitive (Brotherton *et al*. 2007). However, these accessions had not previously been exposed to glyphosate selection pressure, and it would be interesting to compare variation in glyphosate sensitivity in accessions with previous exposure to glyphosate to infer if responses to glyphosate selection are possible and detectable.

6.1.3 Quantitative trait loci

It is likely that any variation in susceptibility to glyphosate in *A. thaliana* accessions is underpinned by genetic variation at a number of genes (a quantitative trait). Quantitative trait loci (QTL) are the areas of the genome that contain genes related to quantitative traits (Collard *et al*. 2005). QTLs can be used to identify areas of the genome that contain genetic variation in the form of single nucleotide polymorphisms (SNPs), which can cause a change in amino acid sequence and the function of the protein coded (Collard *et al*. 2005). As few as 56 *A. thaliana* accessions can be used to detect 98% of SNPs shared between geographic regions, and 67 accessions can be used to detect 98% of all common SNPs (Cao *et al*. 2011).

6.1.4 Multi-parent advanced generation inter-cross *Arabidopsis thaliana* lines

Multi-parent advanced generation inter-cross (MAGIC) lines can also be used to investigate QTLs related to phenotypic traits (Kover *et al*. 2009). To create MAGIC lines multiple parental accessions were crossed for four generations, before lines were inbred for six generations to create recombinant inbred lines (RILs). This inbreeding means that there is little to no variation within the lines and they therefore do not need to be re-genotyped each time new phenotypic traits are investigated (Kover *et al*. 2009).

MAGIC lines occupy a place between naturally occurring *A*. *thaliana* accessions and existing synthetic populations (Kover *et al*. 2009). Naturally occurring accessions and backcrosses are easily produced, but have high levels of heterozygosity, causing variation in genotype and phenotype meaning that both need to be assessed in each generation (Seymour *et al*. 2012). Synthetic populations, such as traditional RILs, are the result of crosses between two parent lines, meaning that there can only be a maximum two alleles at a locus (Kover *et al*. 2009; Gnan *et al*. 2014). This reduces genetic variation and means that pleiotropic effects cannot be studied, as the presence of an allele can randomly increase the value of any two traits 50% of the time (Gnan *et al* 2014). Using MAGIC lines overcomes these problems, as mapping accuracy is increased due to the large number of accumulated recombinant events. They also have high allelic and phenotypic diversity, due to the number of parent lines (19), which increases the number of QTLs that segregate in crosses and provides more evidence of a shared genetic mechanism (Kover *et al*. 2009; Gnan *et al*. 2014). MAGIC lines are already available for *A*. *thaliana*, meaning that investigating QTLs related to

differences in glyphosate susceptibility in *A. thaliana* is made possible, unlike in other weed species, such as *Alopecurus myosuroides*, where there are no such lines available and which would take years to create.

6.1.1 Objectives

The objectives of this chapter are to:

- Investigate the variability in glyphosate susceptibility in a random collection of 30 global accessions of *A. thaliana* (baseline variability)
- Compare glyphosate susceptibility of accessions collected from UK railways (and previously characterised for atrazine resistance) to the baseline data
- Use MAGIC lines to explore the genetic basis of reduced glyphosate sensitivity in natural accessions

6.2 Materials and Methods

6.2.1 Standard procedures

6.2.1.1 Germination, transplanting and spraying

A. thaliana seeds were scattered onto trays containing standard *Arabidopsis* potting mix (Levington's F2 compost, sand and vermiculite in a 6:1:1 ratio). Seed trays were covered in foil and placed in a 2°C incubator for 4-6 days for stratification. After stratification the seeds were allowed to germinate and grow (differing conditions for each experiment) before transplanting. When at the cotyledon stage, seedlings were carefully removed from the seed trays and transplanted into 70x70x70mm pots containing standard *Arabidopsis* potting mix, with one plant in the centre of each pot.

Plants were treated with glyphosate at the rosette stage before bolting, unless otherwise stated. Glyphosate was applied using the same methods as in Chapter 2 (*2.2.1.5*), with either a Berthoud knapsack sprayer or a track sprayer.

6.2.1.2 Assessment

At assessment, plants were given a score of between 0 and 100% depending on glyphosate injury level (Figure 6-1, Appendix 4), with 0% being no observable effect and 100% complete mortality. Plants with a score of >75% were classified as dead.

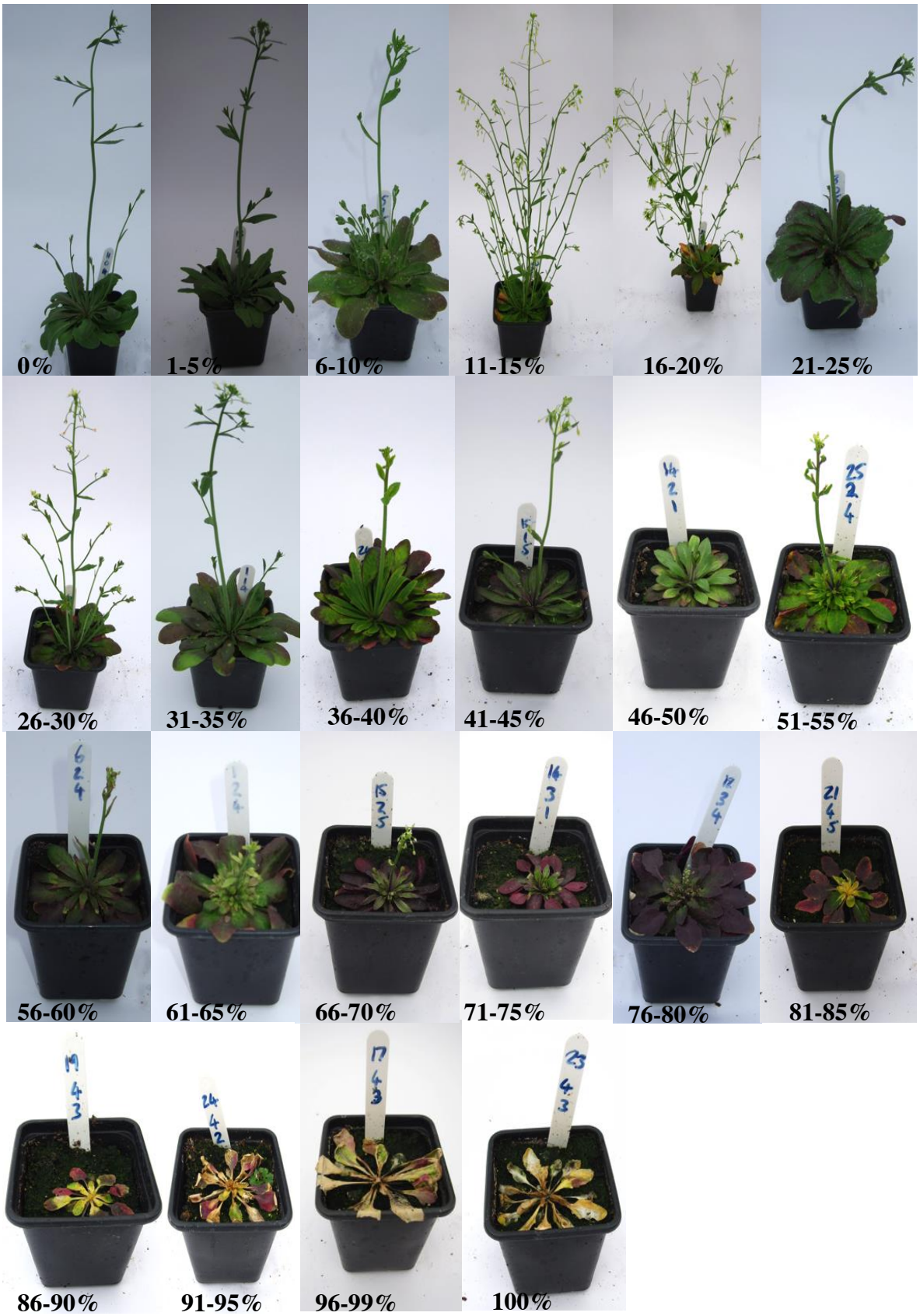

Figure 6-1: Examples of *A. thaliana* scoring criteria
Each individual plant was scored in relation to the other individuals within the accession and not between accessions

6.2.2 Experiment 1: Assessing glyphosate sensitivity amongst a global collection of *Arabidopsis thaliana* accessions.

Thirty global accessions of *Arabidopsis thaliana* (Table 6-1) were used to carry out a dose-response assay to assess differences in glyphosate susceptibility. Five glyphosate doses (0, 54, 108, 180, 360 g ha^{-1}) were used with five replicates.

Table 6-1: *Arabidopsis thaliana* accessions used in glyphosate dose-response assay

Accession	Origin	Region
Bra-1	UK	Cumbria
Col-0	USA	Columbia (South Carolina)
Ler-0	Poland	Landsberg
C24	Portugal	Coimbra
Can-0	Canary Isles	Las Palmas/ Mirador
Hi-0	Netherlands	Hilversum
Kn-0	Lithuania	Kaunas
Po-0	Germany	Poppelsdorf
Sf-2	Spain	San Feliu
Wil-2	Russia	Wilna
Ws-0	Ukraine	(Wassilewskija) Djnepr
Zu-0	Switzerland	Zürich
Rrs-7	USA	Indiana
Ull-2-5	Sweden	Ullstorp
Pu2-23	Czech Republic (Croatia)	Prudka
Ren-1	France	Rennes
Br-0	Czech Republic	Brno (Brunn)
Gy-0	France	La Miniere
Mrk-0	Germany	Märkt/Baden
Kas-1	India	Kashmir
Nok-3	Netherlands	Noordwijk
Ms-0	Russia	Moscow
Dog-4	Turkey	Dogruyol
Del-10	Serbia	Deliblato sands
Vie-0	Spain	North, Pyrenees
Yeg-1	Armenia	Yeghegis
Mer-6	Spain	Merida
Lov-1	Sweden	Lovvik
Uod-1	Austria	Ottenhof
Est-1	Estonia	Estland

Seeds were sown in seedling trays and stratified for four days (*6.2.1.1*). After stratification, seed trays were moved to a Conviron room for 17 days to promote seed germination (16h day at 150 μmol m^{-2} sec^{-1} photons, 8h night). After 17 days, seed trays were moved to a polythene tunnel at +3.5ºC ambient and left to grow for six to seven days before being transplanted into pots (replicates 1 and 2 six days, replicates 3, 4, and 5, seven days) (*6.2.1.1*). For each of the 30 accessions there were 5 replicate pots per glyphosate dose, there were 5 doses, making a total of 750 pots.

Once transplanted, pots were arranged in a randomised split plot design, with accessions randomised within dose tray and dose trays randomised within replicate. Replicates were placed in the polythene tunnel in rows. The plants were left to grow in the thermogradient tunnel for 69-70 days before glyphosate treatment (replicates 1 and 2 70 days, replicates 3, 4, and 5 69 days). Spraying was conducted using a Berthoud knapsack sprayer (*2.2.1.5*). Glyphosate treatments were applied to accessions at the rosette stage, apart for C24, Sf-2, Br-0 and Mer-6, where all (C24, Sf-2) or some (Br-0, Mer-6) plants had begun to bolt.

Individual plants were assigned a glyphosate injury score 41-42 days after spraying (Replicates 1 and 2 41 days, replicates 3, 4, and 5 42 days). To prevent shading, once plants had been assessed the space between pots was increased from 0cm to 7cm. Plants were assessed again for survival 74 days (03.04.12) after spraying.

6.2.3 Experiment 2: 8 UK railway line accessions of *Arabidopsis thaliana* dose-response assay

6.2.3.1 Plant material

Seeds from eight populations of *A. thaliana* individuals were collected by Padraic Flood from UK railway lines in Essex in 2012 and tested for triazine resistance (Table 6-2) (Heap 2015; Flood, unpublished). Seed collections were made in pairs, one from a population that was triazine resistant and one triazine susceptible population from an adjacent area not treated with herbicides (Table 6-2) (Flood, unpublished). Collections from these *A. thaliana* populations were deposited in a genebank and given accession numbers (Table 6-2). Two of the more glyphosate sensitive (Gy-0 and Mer-6) and two of the less sensitive (Kas-1 and Ms-0) accessions from experiment 1 were used as a comparison for the eight UK railway line accessions.

Table 6-2: *Arabidopsis thaliana* UK railway accessions included in the glyphosate dose-response assay
Triazine resistant (R) and susceptible (S) populations were collected from UK railway lines. Triazine R and S populations are paired being collected from adjacent triazine treated and untreated areas (for example, B-4-b and B-0-a). Control accessions represent more and less susceptible wild (previously unexposed accessions)

Triazine resistant	Triazine susceptible	Reference populations with high/low susceptibility
B-4-b	B-0-a	Gy-0 (High)
Cam-2	Cam-0	Mer-6 (High)
M-1-a	M-1-e	Kas-1 (Low)
P-4-a	P-3-a	Ms-0 (Low)

6.2.3.2 Dose-response assay, spring 2014

Seeds were sown on 11/2/14 and stratified for 6 days (*6.2.1.1*). After stratification, seed trays were placed in a polythene tunnel for 21 days before transplanting (*6.2.1.1*). Seedlings were transplanted 21-22 days after being moved to the polythene tunnel (reps 1-5: 21 days, reps 6-8: 22 days), one plant per pot (*6.2.1.1*), and accessions were

randomised within dose trays and doses trays randomised within replicate in a randomised split plot design. Replicates were placed in rows in the polythene tunnel. A total of 8 replicates, 7 doses, and 12 accessions were used, totaling 672 pots. Dose trays were placed in plastic containers and pots were watered from below as required, to prevent seedlings being washed away.

Fourteen to fifteen days after transplanting, plants were treated with one of 7 glyphosate doses using a track sprayer (replicates 1-5 15 days, replicates 6-8 14 days) (*2.2.1.5*). Twenty-eight days after glyphosate treatment plants were assessed for survival and glyphosate injury score (*6.2.1.2*).

6.2.4 Experiment 3: *Arabidopsis thaliana* MAGIC line screen

To assess the response to glyphosate of MAGIC lines a screen of the 19 parent lines and 100 MAGIC lines (Table 6-3) was conducted using three glyphosate doses (0, 135 and 216 g ha^{-1} (recommended rate 540 g ha^{-1})), with 5 replications. These doses were chosen, as they were the doses at which there was the largest variation in response between populations in experiment 1. The MAGIC lines used were based on a varied selection across the set of lines to give the maximum amount of recombination across the genome and represented crosses between all 19-parent lines.

Table 6-3: *Arabidopsis thaliana* parent and MAGIC lines used for glyphosate screen

Parent lines	MAGIC lines				
Bur-0	5	66	143	247	376
Can-0	10	75	144	250	378
Col-0	11	76	146	251	379
Ct-1	12	77	149	255	391
Edi-0	18	79	150	261	397
Hi-0	24	81	151	282	409
Kn-0	26	83	167	285	413
Ler-0	28	88	176	291	416
Mt-0	30	89	177	295	421
No-0	31	94	182	313	437
Oy-0	32	96	183	314	446
Po-0	38	98	185	328	447
Rsch-4	42	103	189	330	453
Sf-2	45	104	192	338	455
Tsu-0	51	112	193	339	483
Wil-2	52	125	195	342	485
Ws-0	60	138	209	366	492
Wu-0	61	139	226	367	499
Zu-0	62	141	234	370	511
	63	142	239	373	523

In February 2014, MAGIC lines seeds were sown in seed trays and stratified for 5 days (*6.2.1.1*). After stratification, seed trays were kept in a polythene tunnel at +4^{o}C ambient for 12 days for seeds to germinate and establish. After 11-14 days, germinated seedlings were transplanted into pots, one plant per pot (*6.2.1.1*) (replicate 1 11 days, 2 12 days, 3 13 days, and 4 and 5 14 days). Pots were moved into a glasshouse compartment with a 10 hour light period with supplementary lighting as required (20^{o}C +venting at 22^{o}C) and a 14 hour dark period (12^{o}C +venting at 14^{o}C), darkness being achieved with blackout blinds. Day length was shortened to help prevent bolting, as it is stimulated by long days. Pots were placed in a radomised split plot design in the glasshouse compartment with accessions randomised within dose tray and dose trays randomised within replicate. Replicates were placed in rows in the glasshouse compartment. Plants were left to grow for 20-23 days before treatment

with glyphosate using a Berthoud knapsack sprayer (*2.2.1.5*) (replicate 1 23 days, 2 12 days, 3 22 days, and 21 and 5 20 days). 21-24 days after spraying, plants were assessed for survival, glyphosate injury, and aboveground fresh weight (*6.2.1.2*) (replicate 1 21 days, 2 22 days, 3 23 days, and 4 and 5 24 days). Above ground fresh weight was measured by cutting the root at soil height underneath the *A. thaliana* rosette, each rosette was then placed on the scale for weight measurement.

6.2.4 Statistical analysis

Results were analysed using the R statistical package (version 2.15.3). The DRC package was used for experiments 1 & 2. Dose-response results were analysed as in chapter 2 (see sections *2.2.6.1* and *2.2.6.2*).

For experiment 1 survival data, a log-logistic 2-parameter model with constrained slope and unconstrained ED_{50} was used (model fit = 1). For score data a Weibull-1 4-parameter model with constrained slope and unconstrained ED_{50} was used (model fit = 0.9933).

For experiment 2 survival data, a Weibull-1 2-parameter model with constrained ED_{50} and constrained slope was used (model fit = 0.6445). For glyphosate injury score a log logistic 3-parameter model with the upper limit constrained at 100, a constrained slope and unconstrained ED_{50} was used (model fit = 0.1799).

For experiment 3, parent lines were classified as susceptible, intermediate, or tolerant to glyphosate by multiplying proportion survival, mean injury score, and proportion fresh weight of control at each of the treated doses (135 and 216 g ha^{-1}), the

populations were then ranked in order of susceptibility and classified. MAGIC line results were analysed using the happy.hbrem package in the R statistical package (version 2.15.3) to give an output of QTLs associated with the variation in phenotypic assessment of survival and glyphosate injury score. This was accomplished by the package performing a genome scan to determine the threshold for statistical significance, finding all QTLs with significant genome wide logP values, and determining the founder parent accessions for each QTL (Mott and Kover, 2015). Out put also includes the estimated phenotypic contribution of each parent line to the QTL locus (Tair, 2015). The genes in the area of the QTL peaks associated with glyphosate response were identified and the parent lines that contributed the most and least to the QTL peak were investigated for the number of single nucleotide polymorphisms in these regions compared to the standard accession Col-0 using Tair (2015) and 1001genome project (Ossowski *et al*. 2008; Cao *et al*. 2011). The number of SNPs between the identified parent lines were compared, and where there were different SNPs in gene encoding regions, the genes were identified using Tair (2015) and the 1001 genome project.

6.3 Results

6.3.1 Experiment 1: *Arabidopsis thaliana* dose-response of 30 global accessions

6.3.1.1 Survival dose-response

There was a significant difference between the unconstrained model and model with constrained ED_{50} (LR value=74.9, $p<0.001$), but no significant difference between the unconstrained model and model with constrained slope (LR value = 27.7, p=0.543), meaning that there is significant variation in the ED_{50} of the 30 accessions, but not in the slope.

There were large differences in survival between populations at the 180 and 360 g ha^{-1} doses. ED_{50} values ranged between 125-glyphosate g ha^{-1} and 342 g ha^{-1} (Figure 6-2), ED_{90} values ranged between 189 and 463 g ha^{-1}. There were many populations with equal ED_{50} values, possibly due to the constrained slope, and low number of replicates meaning that responses were similar for some populations.

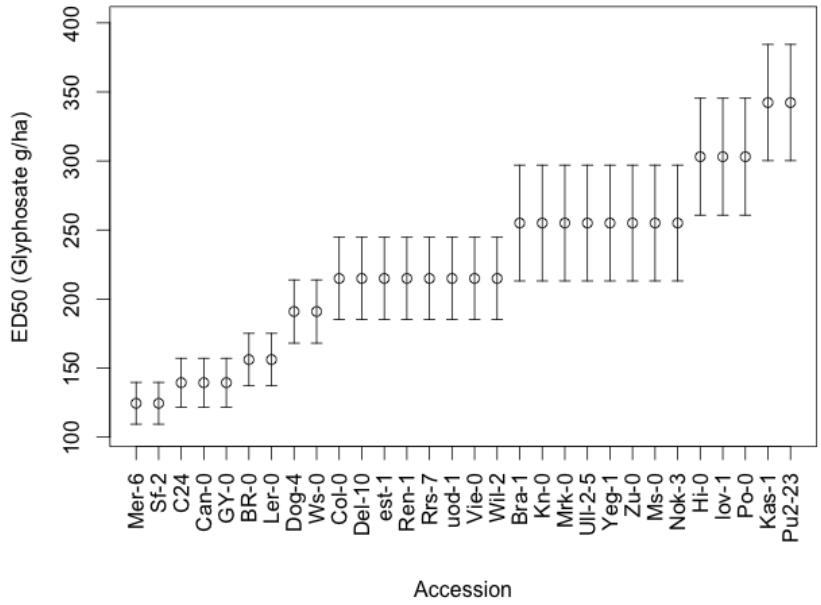

Figure 6-2: ED_{50} and standard error of 2-parameter log-logistic glyphosate dose response assay of survival for 30 global accessions of *Arabidopsis thaliana*

6.3.1.2. Glyphosate injury score assessment dose-response curve

There was a significant difference between the unconstrained model and model with constrained ED_{50} (F value=3.435, p<0.001), but no significant difference between the unconstrained model and model with constrained slope (F value = 0.7015, p=0.8785), meaning that there is significant variation in the ED_{50} of the 30 accessions, but not in the slope. ED_{50} values ranged from 61-glyphosate g ha^{-1} to 149 g ha^{-1} (Figure 6-3). ED_{90} values ranged from 116-glyphosate g ha^{-1} to 284 g ha^{-1}.

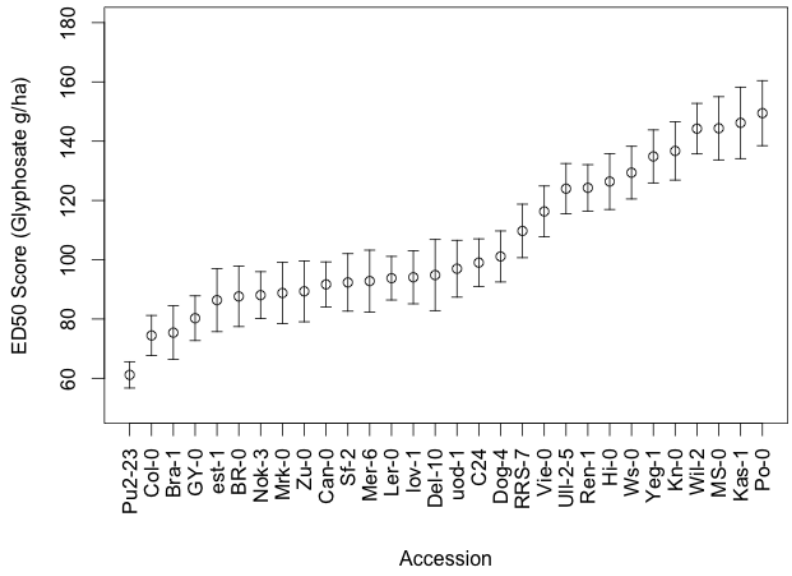

Figure 6-3: ED_{50} and standard error of Weibull-1 4-parameter glyphosate dose-response assay of glyphosate injury score for 30 global accessions of *Arabidopsis thaliana*

6.3.2 Experiment 2: Glyphosate dose-response assay of eight UK railway line *A. thaliana* accessions

6.3.2.1. Survival analysis

There was no significant difference between the unconstrained model and the model with constrained ED_{50} and slope (LR value = 30.87, p=0.0988), meaning that there was no significant variation in ED_{50} of the accessions. For this constrained model ED_{50}

was 186 g ha^{-1}, and the slope was 1.99. This suggests that none of the accessions exposed to glyphosate have responded to the selection pressure.

6.3.2.2 Glyphosate injury score analysis

There was a significant difference between the unconstrained model and model with constrained ED_{50} (F value=3.839, $p<0.001$), but no significant difference between the unconstrained model and model with constrained slope (F value = 1.467, p=0.1396), meaning that there is significant variation in the ED_{50} of the accessions, but not in the slope.

ED_{50} values ranged between 61-glyphosate g ha^{-1} and 123 g ha^{-1} (Figure 6-4, Table 6-4). There were 5 accessions with significantly different ED_{50} values than the more glyphosate susceptible Gy-0 accession, and three with significantly different values from the other more susceptible accession Mer-6. There were two triazine resistant/ susceptible pairs with significantly different ED_{50} values (Table 6-4). Although there were accessions collected from railway lines with significantly different ED_{50} values compared to the four global accessions used as a comparison, there were no railway line accessions that were significantly outside of the variation in response of the four global accessions.

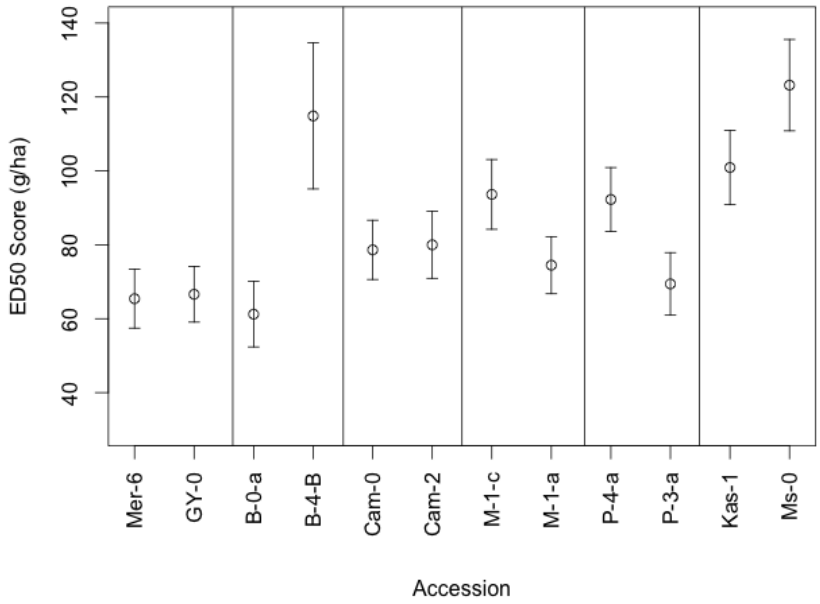

Figure 6-4: ED_{50}, and standard error of log-logistic 3-parameter glyphosate dose-response assay of glyphosate injury score of 12 *Arabidopsis thaliana* accessions
More glyphosate susceptible accessions (Gy-0 and Mer-6) more glyphosate tolerant accessions (Kas-1 and MS-0) and 8 remaining accessions collected from UK railway lines. Accessions are split into pairs collected from adjacent glyphosate unexposed (left of pair) and exposed (right of pair) sites

Table 6-4: ED_{50}, and standard error of log-logistic 3-parameter glyphosate dose-response assay of glyphosate injury score of 12 *Arabidopsis thaliana* accessions
More glyphosate susceptible accessions (GY-0 and Mer-6), more glyphosate tolerant accessions (Kas-1 and Ms-0), and 8 remaining accessions collected from UK railway lines

Accession	ED_{50}	Standard error	Significantly different to Mer-6	Significantly different to Ms-0	Traizine resistant and susceptible pair significantly different
GY-0	66.6	7.54		***	
Kas-1	100.9	10.06	*		
Mer-6	65.4	7.95		***	
Ms-0	123.2	12.37	***		
B-0-a	61.2	8.86			
B-4-B	114.9	19.75	*		***
Cam-0	78.6	8.02		**	
Cam-2	80.0	9.06		**	
M-1-a	74.5	7.72		***	
M-1-c	93.6	9.45			
P-3-a	69.4	8.46		*	
P-4-a	92.3	8.63	***		*

6.3.3 MAGIC line glyphosate screen

The response to glyphosate of the 19 MAGIC line parents varied for each of the responses measured. Mean glyphosate injury score varied from 34.6% to 79.8% at 135-glyphosate g ha^{-1}, and 70.8% to 96.8% at 216 g ha^{-1} (Table 6-5). Proportion survival varied from 0 to 1 at 135 g ha^{-1}, and 0 to 0.75 at 216 g ha^{-1} (Table 6-5). Mean fresh weight varied from 0.27g to 2.30g at 135 g ha^{-1}, and 0.14g and 1.16g at 216 g ha^{-1} (Table 6-5). There were 6 more susceptible parent line accessions, 6 intermediate accessions, and 7 more tolerant accessions (Table 6-5).

At 135-glyphosate g ha^{-1}, MAGIC line analysis of phenotype for glyphosate injury score showed two peaks on chromosome 2, with the first between 12428271 base pairs (b.p.) and 12612808 b.p., (distance: 184537 b.p.) with a peak at 12612808 b.p. The second peak was between 12757304 b.p. and 13083366 b.p. (distance: 326062 b.p.), with a peak at 12974734 b.p. (Figure 6-5). There was also a peak on Chromosome 2 for related to phenotype of fresh weight analysis between 12180334 b.p. and 12757304 b.p. (distance: 576970 b.p.), with a peak at 12612808 b.p., the same as for glyphosate injury score (Figure 6-5). There were no peaks at 216 g ha^{-1}, and no peaks associated with survival (Figure 6-5).

Table 6-5: 19 *Arabidopsis thaliana* MAGIC parent lines response to glyphosate at 3 doses
Response measured as: mean glyphosate injury score, proportion survival, and mean fresh weight, at 3 glyphosate doses (0, 135 and 216 g ha^{-1}), and phenotype in glyphosate response, S- susceptible, I- intermediate, T- tolerant

Parent lines	Mean injury score			Proportion survival			Mean fresh weight			Prop. fresh weight		Survival*Score*prop fresh weight		Rank		Phenotype
	0 g ha^{-1}	135 g ha^{-1}	216 g ha^{-1}	0 g ha^{-1}	135 g ha^{-1}	216 g ha^{-1}	0 g ha^{-1}	135g ha^{-1}	216 g ha^{-1}	135 g ha^{-1}	216 g ha^{-1}	135 g ha^{-1}	216 g ha^{-1}	135 g ha^{-1}	216 g ha^{-1}	
Bur-0	0	34.6	77.2	1	1	0.2	5.68	2.30	0.52	0.405	0.091	0.01171	0.00024	1	7	T
Can-0	0	60	83	1	0.6	0.2	7.25	1.52	0.69	0.209	0.095	0.00209	0.00023	9	8	I
Col-0	0	68.2	86.6	1	0.8	0.2	6.12	0.93	0.41	0.152	0.066	0.00178	0.00015	11	10	I
Ct-1	0	59.6	75	1	1	0	7.14	0.89	0.57	0.125	0.079	0.00210	0.00000	8	12	I
Edi-0	0	51.6	70.8	1	0.8	0.4	7.20	2.04	0.75	0.283	0.105	0.00439	0.00059	3	3	T
Hi-0	0	73.8	89.4	1	0.4	0	6.36	0.51	0.26	0.080	0.041	0.00044	0.00000	17	17	S
Kn-0	0	56	81.4	1	1	0.2	4.64	0.98	0.46	0.212	0.099	0.00378	0.00024	5	6	T
Ler-0	0	73.6	90.4	1	0.6	0	3.26	0.46	0.22	0.142	0.068	0.00116	0.00000	14	15	S
Mt-0	0	53.5	70.8	1	1	0.75	7.42	1.24	1.16	0.167	0.156	0.00311	0.00166	6	1	T
No-0	0	53.8	85.2	1	1	0.2	5.82	1.22	0.29	0.210	0.051	0.00390	0.00012	4	11	I
Oy-0	0	51.4	77.4	1	0.6	0.2	3.95	1.55	0.63	0.391	0.160	0.00457	0.00041	2	4	T
Po-0	0	68.8	77.8	1	0.6	0.4	5.46	0.98	0.73	0.179	0.133	0.00157	0.00068	13	2	T
Rsch-4	0	53.8	83.8	1	0.6	0.2	5.01	1.00	0.53	0.200	0.106	0.00223	0.00025	7	5	T
Sf-2	0	76.2	96.8	1	0.2	0	5.12	0.27	0.14	0.053	0.028	0.00014	0.00000	18	18	S
Tsu-0	0	79.8	91.8	1	0	0	8.95	0.91	0.36	0.102	0.041	0.00000	0.00000	19	19	S
Wil-2	0	73.8	93.6	1	0.4	0	3.93	0.41	0.24	0.103	0.062	0.00056	0.00000	16	16	S
Ws-0	0	64.4	85.4	1	0.6	0	4.94	0.96	0.33	0.194	0.066	0.00181	0.00000	10	13	I
Wu-0	0	63.2	82.4	1	1	0	5.55	0.57	0.37	0.102	0.066	0.00162	0.00000	12	14	S
Zu-0	0	71	81.2	1	0.2	0.2	4.61	1.08	0.41	0.234	0.090	0.00066	0.00022	15	9	I

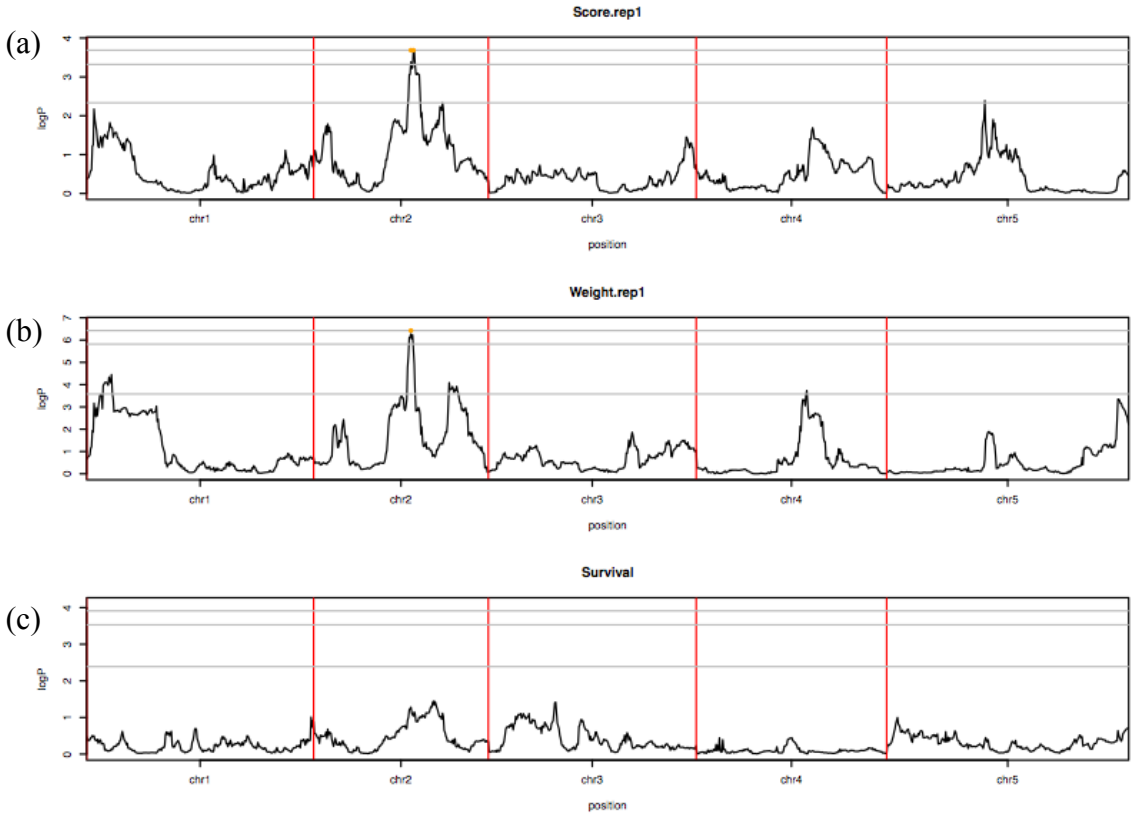

Figure 6-5: QTL peaks associated with variation in glyphosate response in 100 *Arabidopsis thaliana* MAGIC lines
Arabidopsis thaliana MAGIC line analysis of 100 MAGIC lines and 19 parent lines treated with 135-glyphosate g ha^{-1}, assessment of glyphosate (a) injury score, (b) fresh weight, and (b) survival, showing two QTL peaks on chromosome 2 associated with glyphosate injury score, and QTL one peak on chromosome 2 associated with fresh weight. Red lines represent chromosome length

The Rsch-4 parent line was the line that contributed most to lower glyphosate injury score to the peaks between 12428271 b.p. and 12612808 b.p. and 12757304 b.p. and 13083366 b.p. associated with glyphosate injury score, and Sf-2 the parent line that contributed the most to high glyphosate injury score (Figure 6-6). The Rsch-4 parent line is a more glyphosate tolerant line and Sf-2 a more susceptible line (Table 6-5). For the peak associated with fresh weight between 12180334 b.p. and 12757304 b.p. the parent line most associated with higher fresh weight at the peak was Rsch-4 with most of the other parent lines being associated with lower fresh weight (Figure 6-6).

Therefore, the parent line that contributes most to MAGIC lines having reduced glyphosate susceptibility is Rsch-4, and the parent line that contributes most to increased glyphosate susceptibility is Sf-2. Analysis of the area of chromosome 2 between 12428271 b.p. and 13083366 b.p. for Rsch-4 and Sf-2 showed that there are 6074 different SNPs, of these 2471 are associated with 193 genes, the remaining SNPs are within introns (Tair, 2015) (Appendix 5).

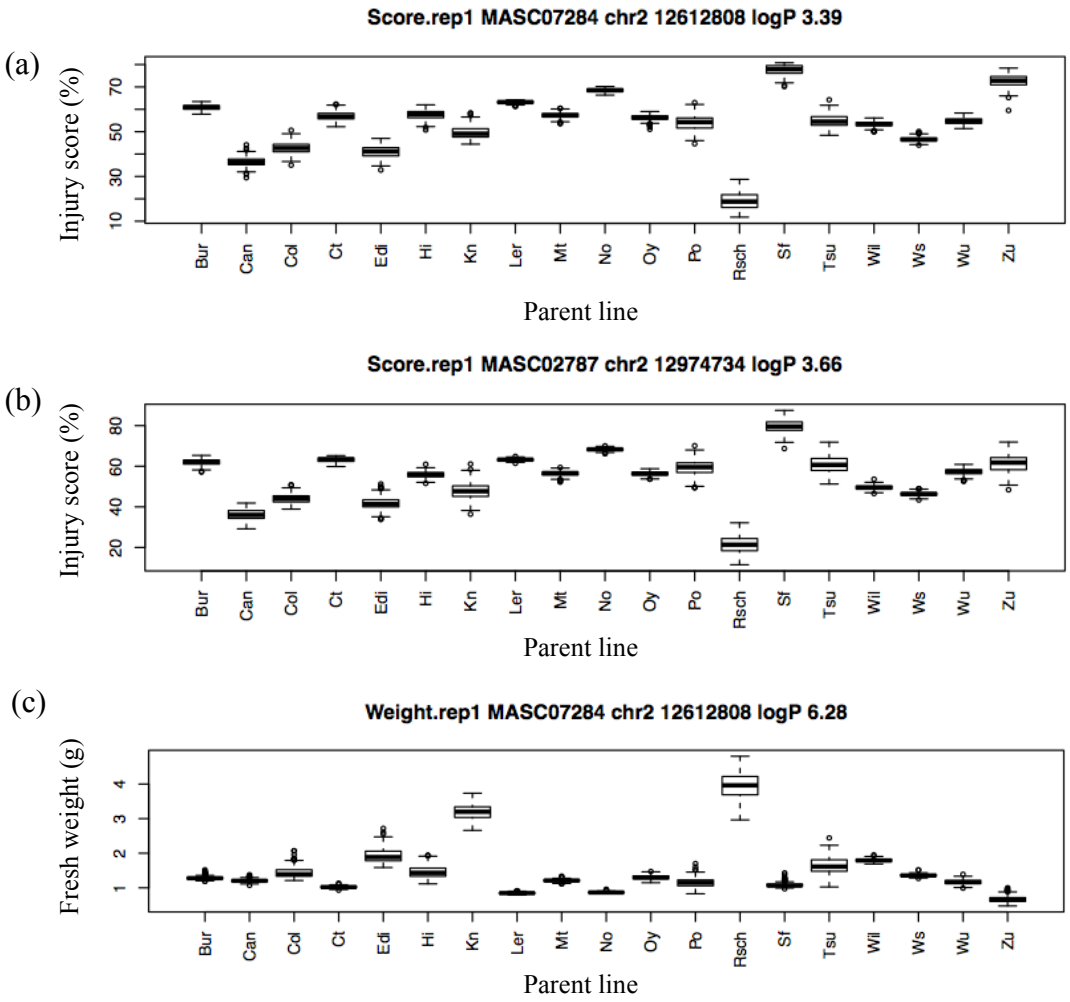

Figure 6-6: Estimate phenotypic contribution of each MAGIC parent line to the QTL locus for glyphosate injury score and fresh weight at 135-glyphosate g ha^{-1}

The gene locus at the 12612808 b.p. peak on chromosome 2 associated with glyphosate injury score and fresh weight is AT2G29390, where there is a protein

coding gene that encodes a sterol 4-alpha-methyl-oxidase, involved in acetyl-CoA metabolic processes, amongst others (Tair, 2015). There is a single nucleotide polymorphism (SNP) and an amino acid substitution at 12612808 b.p. for Sf-2 compared to Col-0, there are also further SNPs and amino acid substitutions for this accession on the AT2G29390 gene (Appendix 5).

The gene locus at the 12974734 b.p. peak on chromosome 2 associated with glyphosate injury score is AT2G30440, a protein coding gene that encodes a thylakoid processing peptidase, which is involved in proteolysis and signal peptide processing, and is expressed in guard cells (Tair, 2015). There is an SNP at 12974734 b.p. for the parent line Sf-2, but not Rsch-4 when compared to Col-0 (Tair, 2015).

In the region of chromosome 2 between 12180334 b.p. and 13083366 there are 26 transporter genes, 15 genes associated with metabolism and oxidation reduction, 12 genes associated with phosphatase activity, and 6 glycosyltransferase genes (Appendix 5). There are also 23 genes of unknown function and 7 pseudogenes (Appendix 5). The genes with the most SNPs are AT2G29000 with 223 SNPs, AT2G28990 with 154 SNPs, and AT2G28970 with 104 SNPs. These genes all encode proteins with kinase activity, involved in ATP binding, and protein amino acid phosphorylation (Tair 2015) (Appendix 5). 130 of the genes have 10 or fewer SNPs (Tair, 2015) (Appendix 5).

6.4 Discussion

6.4.1 Variation in glyphosate response in *Arabidopsis thaliana*

There were significant differences in glyphosate susceptibility based on measures of survival and glyphosate injury score, for the 30 global accessions of *A. thaliana*. This variation in glyphosate response was much greater than that found in the dose-response assay of 6 *A. thaliana* accessions assessed by Brotherton *et al*. (2007). However, this may be due to the much greater number of accessions assessed in this study and the use of dose-response analysis enabling the comparison of ED_{50} values. There were a number of more (GY-0 and Mer-6) and less susceptible (Kas-1 and Ms-0) accessions, which responded similarly in both the dose-response assays of the 30 global accessions and the triazine resistant UK railway accessions.

It is clear that the UK railway accessions have not responded to glyphosate selection pressure, and it is possible that *A. thaliana* accessions do not respond to glyphosate selection pressure. Despite the range in glyphosate susceptibility in the trazine resistant accessions, for all of the assessment criteria, none of the accessions collected were significantly outside the range of the more and less glyphosate susceptible accessions tested in the first dose-response assay. Brotherton *et al*. (2007) exposed the accession Col-0 to seven generations of glyphosate selection, finding no change in susceptibility between the generations. However, in Brotherton *et al*. (2007) only one accession was selected and glyphosate doses were low. It therefore may be interesting to subject more triazine susceptible UK railway accessions to glyphosate selection to determine whether or not they respond.

There was some significant difference in glyphosate susceptibility between triazine resistant accessions and their paired triazine susceptible UK railway accessions. The triazine resistant glyphosate exposed UK railway accession P-3-a was significantly more susceptible to glyphosate than the triazine susceptible UK railway accession P-4-a, collected from an adjacent glyphosate unexposed location. Triazine resistance can cause negative cross-resistance, where the resistance causes the plants to be more susceptible to other herbicide modes of action (Gadamski *et al*. 2000). This phenomenon has been reported in triazine resistant biotypes of *Conyza canadensis* when treated with glyphosate (Gadamski *et al*. 2000) and in *Amaranthus hybridus* L. (smooth pig weed) when treated with bentazon (Jordan *et al*. 1999), as well as in other triazine resistant species. Fitness costs associated with resistance can also contribute towards negative cross-resistance (Gadamski *et al*. 2000). The *Ely A. thaliana* accession first found to be atrazine resistant had a fitness cost associated with the resistance phenotype (El-Lithy *et al*. 2005). Therefore, negative cross-resistance and fitness cost may explain why there appears to have been little to no response to glyphosate selection pressure and why some of the triazine resistant UK railway accessions appear to be more glyphosate susceptible than the triazine susceptible UK railway accessions, despite exposure to and selection pressure from glyphosate.

6.4.2 *Arabidopsis thaliana* MAGIC line analysis

There were two peaks associated with the MAGIC line screen, one for glyphosate injury score and fresh weight, and one for glyphosate injury score only. The AT2G29390 gene at the peak at 12612808 b.p. on chromosome 2 associated with glyphosate injury score and fresh weight, encodes a sterol 4-alpha-methyl-oxidase

involved in acetyl-CoA metabolic processes, amongst others (Tair, 2015). There is no evidence that the processes this gene is involved in are related to herbicide resistance.

The gene at the second peak on Chromosome 2 at 12974734 b.p. associated with glyphosate injury score is AT2G30440, which encodes a thylakoid processing peptidase, involved in proteolysis and signal peptide processing (Tair, 2015). During formation of EPSPS, the pre-EPSPS molecule is cleaved in the chloroplast through proteolysis, with a transit peptide crucial in the transport of the pre-EPSPS molecule into the chloroplast (Della-Cioppa *et al*. 1986). It is possible that the AT2G30440 gene is involved in this process and may have an effect on glyphosate susceptibility, which requires further investigation. However, proteolytic pathways are involved in protein degradation upon herbicide treatment and glyphosate treatment can alter the activity of these pathways in plants (Zulet *et al*. 2013), so it is possible that this is the reason for the peak associated with AT2G30440.

Analysis of differing SNPs between the parent line that contributed most to decrease glyphosate susceptibility, Rsch-4, and the parent line that contribute most to increased susceptibility, Sf-2, showed that there were 15 genes with SNPs in the region of chromosome 2 related to metabolism and oxidation-reduction. There is no evidence of any glyphosate resistance mechanisms related to metabolism (Duke, 2011; Ribeiro *et al*. 2015), but glyphosate can have differing effects on redox metabolism in resistant and susceptible individuals (Vivancos *et al*. 2011), suggesting that variation in these genes are other potential candidates for further investigation. There were also 12 genes in the region of chromosome 2 associated with phosphatase activity. Glyphosate can reduce phosphatase activity from 5% to 98% in some situations, possibly as a result of

the presence of the phosphoric acid group acting as a competitive inhibitor (Gianfreda *et al*. 1993; Sannino and Gianfreda, 2001). It is therefore possible that the variation in the genes with phosphatase activity is resulting in variation in the reduction of phosphatase activity.

It is interesting that there were many transport protein genes with SNPs at the peaks on Chromosome 2. Glyphosate resistance endowed by reduced translocation has been reported in many plant species, including, *Lolium multiflorum* (Perez-Jones *et al*, 2007), *Lolium rigidum* (Wakelin *et al*. 2004), and *Digitaria insularis* L. Fedde (sourgrass) (de Carvalho and da Costa Aguiar Alves, 2012). The transport genes highlighted in this study may be possible candidates for further investigation into the genetic basis of reduced glyphosate translocation. There are also six glycosyltransferase genes with SNPs in the region of the two peaks on chromosome 2 (Tair, 2015). Glycosyltransferases can detoxify herbicides by the addition of sugars and have been shown to play a role in non-target site resistance (NTSR) in *Alopecurus myosuroides* biotypes resistant to multiple herbicides (Yuan *et al*. 2007). The addition of sugars onto glyphosate by glycosyltransferases may change the structure of the molecule and reduce its ability to be transported around the plant (Shaner, 2009). It would therefore also be interesting to further investigate these genes.

Rsch-4 and Sf-2 where the parent lines that were most and least associated with the peak on chromosome 2 between 12757304 b.p. and 13083366 b.p. For further experiments to investigate any role the genes highlighted in this region have, it would be interesting to use crosses from the Rsch-4 and Sf-2 parent lines to assess the glyphosate susceptibility of the progeny. If there is a single gene response, it would be

expected for there to be a 50/50 split in segregation in the back cross and F_2 generation, however, if it is a polygenic response the split would not be 50/50 in the progeny (Okada and Jasieniuk, 2014). It would also be interesting to repeat the MAGIC line screen using lines derived from the Rsch-4 and Sf-2 parent lines, with the hypothesis that lines from Rsch-4 would be less susceptible than those from Sf-2.

6.4.3 Conclusions

There is variation in glyphosate susceptibility in *A. thaliana* accessions, which is greater than previously found. UK railway triazine resistant accessions exposed to glyphosate in amenity use on railway lines have not responded to glyphosate selection pressure. Moreover, triazine resistance may have caused negative cross-resistance, as some of the resistant accessions were more susceptible to glyphosate than the susceptible accessions. Further glyphosate selection experiments of triazine susceptible UK railway accessions are needed to determine whether *A. thaliana* responds to glyphosate selection pressure.

The MAGIC line analysis showed a region of chromosome 2 that has QTLs related to variation in glyphosate response in *A. thaliana*. Many genes between the 2 peaks on chromosome 2 were identified to have SNPs between the parent lines that contributed most, Rsch-4, and least, Sf-2, to decreased glyphosate susceptibility. These genes include a thylakoid processing peptidase gene, genes associated with oxidation-reduction and metabolism, and genes associated with translocation. Further experiments using more MAGIC lines, and crosses of the Rsch-4 and Sf-2 parent lines, are needed investigate the possible role of these genes in glyphosate susceptibility.

Chapter 7 : General Discussion

There is currently a lack of information regarding levels of variation in glyphosate susceptibility in UK weed species. This thesis provides a starting point for detecting decreases in glyphosate susceptibility in *Alopecurus myosuroides*, *Anisantha sterilis*, and *Arabidopsis thaliana* populations from the UK and investigating the evolutionary processes that may lead to resistance.

7.1 Research in context

It is important to understand the phenotypic variation within a species when investigating how that species may respond to environmental change, such as herbicide application, as phenotype determines how organisms react with their environment (Hendry *et al*. 2011). It is also important to understand the genetic architecture that underlies these phenotypic traits, as this will show whether the variation in phenotype is due to plasticity or is the result of additive genetic variation in quantitative traits (Sultan, 2000; Conner *et al*. 2003). Furthermore, understanding the genetic architecture will also show if the trait is heritable (Conner *et al*. 2003), for example, by responding to selection (Neve and Powles, 2005b), and whether there are fitness costs and/or trade-offs related to the trait (Merilä and Sheldon, 1999). Chapters 2, 3, 4, 5, and 6 have begun to address these questions for glyphosate resistance evolution in the UK.

7.1.1 Variation in glyphosate susceptibility in populations of *Alopecurus myosuroides*, *Anisantha sterilis*, and *Arabidopsis thaliana*

This study has determined the extent of phenotypic variation in glyphosate susceptibility of populations of three UK weed species, *Alopecurus myosuroides*, *Anisantha sterilis*, and *Arabidopsis thaliana*, showing significant variation in susceptibility for all three species studied (Chapters 2, 5 & 6). The largest variation seen between populations was found within *Anisantha sterilis*, where two populations have evolved practical glyphosate resistance (Chapter 5). The variation for both *Alopecurus myosuroides* and *Arabidopsis thaliana*, although significant, is much lower than that of *Anisantha sterilis*, and no resistance was found, despite populations having been exposed to glyphosate selection pressure (Chapters 2 & 6).

The low variation in glyphosate susceptibility in *Alopecurus myosuroides* (Chapter 2) and *Arabidopsis thaliana* (Chapter 6) is comparable to the variation in glyphosate susceptibility seen in other species where there is currently no glyphosate resistance (Boutin *et al*. 2011; Brotherton *et al*. 2007; Espeby *et al*. 2014). Furthermore, the variation in glyphosate susceptibility in *Alopecurus myosuroides* populations is much less than the variation to other herbicides previously reported in this species (Espeby *et al*. 2011; Ulber *et al*. 2013). However, the significant correlation between GR_{50} and glyphosate use score for *Alopecurus myosuroides* populations collected in 2012, suggests that some of the variation in susceptibility in the populations is due to glyphosate exposure and selection pressure (Chapter 2). Conversely, it appears that *Arabidopsis thaliana* accessions, which have previously evolved resistance to triazine herbicides, have not responded to glyphosate selection pressure following its use on railway lines, as the variation in susceptibility in these populations is still low and

within the range observed for unexposed populations (Chapter 6). This is similar to the findings of Brotherton *et al*. (2007), where there was no change in glyphosate susceptibility in progeny of Col-0 individuals exposed to glyphosate selection in the laboratory. The low variation in both *Alopecurus myosuroides*, one of the most herbicide resistant prone weeds, and *Arabidopsis thaliana* populations where triazine resistance has evolved, supports the notion that glyphosate is a herbicide with a relatively low risk of resistance evolution (Neve *et al*. 2003a).

However, the variation in glyphosate susceptibility was much greater in the *Anisantha sterilis* populations and more similar to closely related species where glyphosate resistance has evolved (Escorial *et al*. 2011). Two *Anisantha sterilis* populations were found to have practical glyphosate resistance, with one population having significantly higher ED_{50} and GR_{50} values than a glyphosate-unexposed population collected from an adjacent area (Chapter 5). This significant result shows that some UK *Anisantha sterilis* populations have responded to glyphosate selection pressure. The ED_{50} values of the practically resistant populations were higher than those found for glyphosate resistant populations of *Bromus diandrus* (Malone *et al*. 2015), and more similar to those of the first case of glyphosate resistance in *Lolium rigidum* (Powles *et al*. 1998). This suggests that *Anisantha sterilis* populations are at more risk of further glyphosate resistance evolution in the UK under current use, than *Alopecurus myosuroides* and *Arabidopsis thaliana* populations.

Interestingly, both *Alopecurus myosuroides* and *Anisantha sterilis* glyphosate-unexposed populations have ED_{50} values much higher than those of other unexposed populations of other grass species such as *Bromus diandrus* and *Lolium rigidum*, and

are more similar to unexposed populations of broad leaf weedy species (Figure 7-1). This initial lower susceptibility compared to other grass species may have implications for glyphosate resistance evolution, decreasing the number of generations needed for resistance to evolve. This may be a result of having initial lower susceptibility making it more likely that doses lower than field rate applied in the field, for example from miss application or drift, can act within the standing genetic variation of *Alopecurus myosuroides* and *Anisantha sterilis* populations, leading to a build up of minor alleles related to resistance (Busi *et al*. 2013b). This lower initial susceptibility may mean that higher glyphosate dose rates are needed for *Alopecurus myosuroides* and *Anisantha sterilis* compared to other grass species to prevent further resistance evolution through the build up of minor alleles.

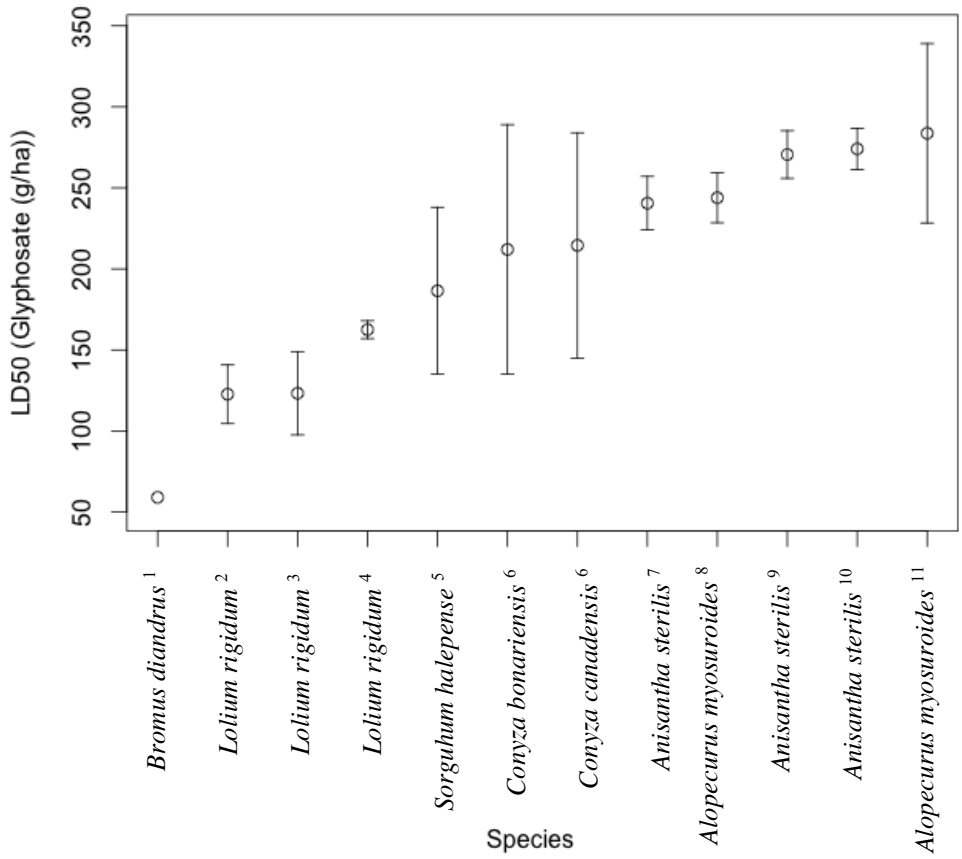

Figure 7-1: Glyphosate LD_{50} values for known susceptible populations of seven different plant species

LD_{50} values of known susceptible populations of *Alopecurus myosuroides* and *Anisantha sterilis* tested in glyphosate dose-response assays have lower values compared to known susceptible populations of different species from a range of glyphosate susceptibility studies. LD_{50} and standard error bars are reported values from studies where single values are given (1, 6), or are mean LD_{50} and standard error where more than 1 value was given (2, 3, 4, 5). *Alopecurus myosuroides* – LD_{50} values and standard error for the unexposed population Broadbalk tested in two separate dose-response assays. *Anisantha sterilis* – LD_{50} and standard error for three susceptible populations tested in one dose-response assay

[1]Malone *et al*. (2015), [2]Busi *et al*. (2009), [3]Powles *et al*. (1998), [4]Collavo and Sattin (2014), [5]Vila-Aiub *et al*. (2007), [6]Vila-Aiub *et al*. (2008), [7]ADAS susceptible population, [8]Broadbalk susceptible population 2013 dose-response,
[9]PATH susceptible population, [10]ROAD susceptible population, [11]Broadbalk susceptible population 2011 dose-response

7.1.2 Response of *Alopecurus myosuroides* populations to glyphosate selection

Even though the variation in glyphosate susceptibility in *Alopecurus myosuroides* populations was not large (Chapter 2), these populations can still respond to glyphosate selection pressure at doses below field rate that act within the standing

genetic variation of the populations and shift towards lower glyphosate susceptibility, showing that the variation in glyphosate susceptibility in these populations is heritable (Chapter 3). Additionally, the gradual decrease in glyphosate susceptibility between the selected generations suggests that the mechanism for this decrease is polygenic and has resulted from an increased frequency of alleles related to reduced herbicide susceptibility within the populations (Neve and Powles, 2005b, Busi and Powles, 2009). This may have major implications on the future control of *Alopecurus myosuroides* using glyphosate, as it is possible that populations in the field may evolve quantitative resistance from standing genetic variation as a result of poor glyphosate application, exposing them to low dose selection (Owen and Zeleya, 2005; Neve and Powles, 2005a). This is especially worrying, as reducing herbicide rates to 'the necessary amount' became EU law in 2014, meaning that lower herbicide use rates, for example, through applying herbicides in a field at variable rates within one application, may become more prevalent (Weis *et al*. 2012). The results in chapter 3 demonstrate that understanding the evolutionary process of the potential for quantitative glyphosate resistance could affect the management practices used to control *Alopecurus myosuroides* (Neve *et al*. 2014). For example, encouraging higher rather than lower use rates and good application practices.

After 2-3 generations of selection no glyphosate resistance evolved in *Alopecurus myosuroides* and it is possible that recombination over more generations may lead to resistance (Neve and Powles, 2005b), especially as it is likely that most glyphosate resistance mechanisms are non-target site (Yuan *et al*. 2007), for which the genetic basis of resistance is unknown (Shaner, 2009), but the mechanisms may be polygenic. On the other hand the populations may reach the maximum shift possible under low

dose selection before becoming resistant (Busi and Powles, 2009). If this is the case, it may be that a decrease in glyphosate susceptibility is possible under low dose exposure, but any field evolved glyphosate resistance in UK *Alopecurus myosuroides* populations will be a result of rare major monogenetic traits. Even if rare monogenetic traits are required for glyphosate resistance evolution in UK *Alopecurus myosuroides* populations, a build up of minor genes related to reduced susceptibility may lead to larger populations sizes and therefore increase the chance of a single gene mutation being present in the population (Blackshaw, 2006; Gressel, 2009, Neve *et al*. 2009).

7.1.3 Fitness costs of glyphosate selected *Alopecurus myosuroides* populations

There appears to be no major fitness costs associated with decreased glyphosate susceptibility in populations exposed to low dose glyphosate selection (Chapter 4). This is possibly a result of the variation in glyphosate susceptibility originating from standing genetic variation (Brcic-Kostic, 2005; Barrett and Schluter, 2008), or of the resistance mechanism conferring no fitness cost, as has been found in other studies (Menchari *et al*. 2008; Pedersen *et al*. 2009; Giacomini *et al*. 2014; Vila-Aiub *et al*. 2014; Vila-Aiub *et al*. 2015b). This lack of major fitness cost suggests that if glyphosate resistance were to evolve from standing genetic variation this evolution will not be slowed due to fitness costs. It is also more likely that there will be a higher frequency of alleles present within populations related to reduced susceptibility, even when selection pressure is low, resulting in fewer generations of selection needed for resistance evolution (Vila-Aiub *et al*. 2011). Furthermore, if glyphosate application were to be stopped due to the lack of fitness cost it is likely that the resistance alleles would persist in the population (Vila-Aiub *et al*. 2015b).

7.1.4 *Arabidopsis thaliana* MAGIC lines

Arabidopsis thaliana MAGIC line analysis highlighted an area on chromosome 2 that may be related to variability in glyphosate susceptibility in the species (Chapter 6). There are many genes in this peak that may be related to herbicide resistance, for example, glycotransferases, which have been found to confer herbicide resistance in some *Alopecurus myosuroides* populations through detoxification (Yu *et al*. 2007). This is the first study to use *Arabidopsis thaliana* MAGIC lines to investigate the natural allelic variation underlying quantitative traits related to variation in herbicide susceptibility, and has provided some promising results for further investigation into the genes involved. Considering the prevalence of transporter genes and glycotransferase genes within the area of chromosome 2, investigation of this region could highlight candidate genes related to NTSR mechanisms, such as glyphosate translocation, for which the genetic mechanisms are currently unknown (Shaner, 2009). Identification of these ecologically important genes will help develop the prediction of the evolutionary trajectory of non-target site glyphosate resistance evolution (Bergelson and Roux, 2010).

7.2 Risk of glyphosate resistance evolution in the UK

7.2.1 Risk in species tested

It is clear that there is variation in glyphosate response in the species investigated in this project, and that *Alopecurus myosuroides* and *Anisantha sterilis* populations can respond to glyphosate selection, at low doses in the glasshouse (*Alopecurus myosuroides* (Chapter 3)), and under farm management practices in the field (*Anisantha sterilis* (Chapter 5)). This shows that despite the resistance risk for

glyphosate being lower than that of most other herbicide modes of action, there is still the potential for quantitative resistance evolution from standing genetic variation in UK weedy species. This indicates that there is a need for continued monitoring of UK weed populations for changes in glyphosate susceptibility, particularly as herbicide resistance to other modes of action is so prevalent in the UK (Moss *et al*. 2011; Heap, 2015) and glyphosate resistance has evolved in grass species under similar use in Italy (Collavo and Sattin, 2014) and Australia (Owen and Powles, 2010).

One of the main threats of glyphosate resistance evolution in the UK is with its use in arable crops to control grass weeds that are already resistant to many herbicide modes of action (WRAG, 2015). With resistance to multiple herbicide modes of action in >80% of farms that use herbicides to control *Alopecurus myosuroides* in the UK (Moss *et al*. 2011), *Alopecurus myosuroides* herbicide resistant populations are in a similar position to that of Australian *Lolium rigidum* populations were in 15 years ago, before glyphosate resistance evolved in wheat cropping systems (Neve *et al*. 2004). *L. rigidum* populations in Australia resistant to multiple mode of action, including glyphosate, have not only target site resistance, but also reduced translocation (Yu *et al*. 2007), which is possibly under polygenic control. Similarly, it is probable that the mechanism for the reduced glyphosate susceptibility in the selected lines of *Alopecurus myosuroides* is polygenic (Chapter 3). Furthermore, it is predicted that the loss of in-crop post emergent herbicides, such as those *Alopecurus myosuroides* populations are currently resistant to greatly increases the risk of glyphosate resistance evolution, as there is an increased reliance on other modes of action increasing selection pressure, and there are fewer modes of action available to remove survivors of other applications (Neve *et al*. 2003b). It is therefore likely that with the significant

variation in glyphosate susceptibility and a positive response to glyphosate selection, under current farming methods quantitative glyphosate resistance will evolve in UK *Alopecurus myosuroides* populations. However, due to the low variation in susceptibility between the populations tested in this study, and the small incremental decrease in glyphosate susceptibility in selected populations, glyphosate resistance evolution in *Alopecurus myosuroides* is likely to be slower than that of other herbicide modes of action in the species and to glyphosate in other species.

On the other hand, due to a lack of apparent fitness cost associated with variation in glyphosate susceptibility in *Alopecurus myosuroides* populations it is probable that the previous hypothesis that glyphosate resistance is slow to evolve due to high fitness penalties (Preston *et al*. 2009) is incorrect. Therefore, it is likely that fitness costs will not slow the rate of quantitative glyphosate resistance evolution from standing genetic variation in UK *Alopecurus myosuroides* populations.

However, as no fitness costs were found this begs the question as to why glyphosate resistance has been so slow to evolve. One reason for this could be because the frequency of glyphosate resistance mutations is much lower than that of other herbicide modes of action (Jander *et al*. 2003). Glyphosate also has a lower resistance risk than other herbicide modes of action, particularly when used traditionally for weed removal before crop sowing, as selection pressure is low due to only a proportion of the population being exposed, and in combination with other herbicide modes of action, which can remove survivors of glyphosate application (Neve, 2008; Cook *et al*. 2010). These factors will impact the rate at which *Alopecurus myosuroides* and *Anisantha sterilis* populations evolved quantitative glyphosate resistance from

standing genetic variation, meaning that the process will be slower than that of resistance evolution to other herbicide modes of action.

Despite the prevalence of herbicide resistance being lower in UK *Anisantha sterilis* populations compared to *Alopecurus myosuroides* populations (Heap, 2015), the results of this study suggest that the risk of resistance evolution in some populations of *Anisantha sterilis* are high, as two populations have already evolved practical resistance. The high ED_{50} value of these populations, as well as, the significant difference between paired glyphosate exposed and unexposed populations, and survival at field rate and 1.5x field rate of glyphosate (Chapter 5) shows that there has already been a shift towards lower glyphosate susceptibility in the field. Therefore, not only do the practically resistant populations need to be monitored and managed, the remaining populations need to be monitored and may benefit from proactive management strategies to prevent glyphosate resistance evolution.

7.2.2 Risk with the introduction of genetically modified glyphosate resistant crops

The risk of glyphosate resistance evolution is further increased with the possibility of the introduction of glyphosate resistant crops (GRCs) in the UK, either through genetic modification or plant breeding. Where GRCs have been introduced, the reliance on glyphosate as the sole chemical control method has increased. This, along with a decreased use of integrated weed management strategies, such as soil cultivation, herbicide rotation, and the use of residual herbicides, has increased glyphosate resistance selection pressure (Johnson *et al*. 2009). The use of GRCs can lead to shifts in weed species composition, with weeds less susceptible to the herbicide becoming more prevalent in the field (Owen, 2008). All this combined can lead to

rapid glyphosate resistance evolution, for example, glyphosate resistant *Conyza canadensis* evolved only three years after the introduction of GRCs (Owen and Zeleya, 2005). In the UK wheat is the most widely grown crop (UK Government, 2015) and there are already many problems with herbicide resistance grasses in these crops (Moss *et al*. 2011), therefore if glyphosate resistant wheat were to be introduced it is likely uptake would be high. However, considering the prevalence of glyphosate resistant grass species in other countries with more than one resistance mechanism, glyphosate resistant weeds are likely to evolve in glyphosate resistant wheat crops (Lyon *et al*. 2002).

From the results of this study, if GRCs were to be introduced into the UK it could result in weed species shift, with an increased prevalence of less susceptible weed populations, such as those found for *Anisantha sterilis*, and the AES112 (Peldon) population of *Alopecurus myosuroides*, and this combined with the increased glyphosate resistance selection pressure, may in turn accelerate the evolution of glyphosate resistance in these species.

7.3 Management strategies to reduce the risk of glyphosate resistance evolution

There are many different management strategies that can be used to reduce the risk of herbicide resistance. These include using a range of herbicide modes of action (Beckie and Tardiff, 2012), using herbicides with soil residual activity (Beckie, 2011), crop rotation so other modes of action can be used (Neve, 2008), crop competition (Blackshaw *et al*. 2006), and weed seed collection at harvest to prevent weed seed input into the seedbank (Walsh and Powles, 2007). It has also been proposed that

using rotation of high and low doses of a herbicide could prevent both recessive monogenic resistance and polygenic resistance, whilst also reducing herbicide usage (Gardner *et al*. 1998), although this has not been tested.

It has already been suggested that to reduce the risk of glyphosate resistance evolution in the UK, farmers should prevent individuals that have survived the herbicide application from growing and reproducing by removing them through cultivation, using the right dose to prevent low dose selection, use alternative weed control methods, such as using other modes of action and non-chemical control, avoid dependence on glyphosate, and monitor fields so any problems can be detected early (WRAG, 2015). Non-tillage situations greatly increase the risk of glyphosate resistance evolution, whereas minimum tillage with a high degree of soil disturbance after glyphosate appliction decreases the risk of resistance evolution almost to zero (Neve *et al*. 2003b). Therefore, as well as the management practices suggested by WRAG (2015), tillage should be used after glyphosate application on stale seedbeds to remove any survivors, before crop sowing. In the case of *Anisantha sterilis* populations where there is reduced glyphosate sensitivity and practical resistance, ploughing to bury the seed should be an effective control method, as this will prevent germination (Clarke *et al*. 2000), and reduce the soil seedbank, as *Anisantha sterilis* has very short seed viability of <1 year (Steinmann and Klingebiel, 2004). Reducing the density of weeds within fields will also reduce the probability of glyphosate resistance evolution, which can be achieved in *Alopecurus myosuroides* through integration of cultural practices, such as delayed sowing and spring cropping (Chauvel *et al*. 2009).

However, the use of glyphosate resistance genetically modified crops (GMCs) leads to reduced tillage and non-tillage in many instances, increasing the risk of resistance evolution (Johnson *et al*. 2009) and many of the cases of glyphosate resistance worldwide have evolved in conjunction with these crops, therefore it is important to consider management strategies in these situations. If GRCs were to be introduced in the UK, to prevent weed species shifts and the evolution of glyphosate resistant weeds, rotation with conventional crops and other herbicide modes of action should also be used (Gustafson, 2008; Lutman *et al*. 2008; Neve, 2008). This is the case in Canada, where use of genetically modified glyphosate resistant oilseed rape is used in rotation with conventional crops, and glyphosate resistance has not evolved in these situations (Harker *et al*. 2012). Stacking herbicide resistance genes in GMCs may enable the use of more in-crop herbicide use, with knowledge of resistant weeds present in the field contributing to the use of stacked GMCs (Beckie and Tardiff, 2012). If GRCs were to be introduced into the UK it would be recommended that they be used in rotation with conventional herbicide susceptible crops and with a range of different herbicide modes of action, as well as with non-chemical control methods, such as tillage.

As it appears that there are no fitness costs related to the mechanism of glyphosate susceptibility variation in the *Alopecurus myosuroides* populations tested (Chapter 4), this suggests that it would not be practical to use management practices that may exploit fitness costs to try to prevent resistance evolution (Preston *et al*. 2009), as there is a high chance that they would not be successful.

Most importantly, farmers need to be educated on the use of glyphosate and strategies to reduce the risk of resistance evolution, as well as being incentivised to use these methods (Beckie, 2011).

7.4 Conclusions

- UK populations of both *Alopecurus myosuroides* and *Anisantha sterilis* are at risk of evolving quantitative glyphosate resistance, with some populations of *Anisantha sterilis* already practically resistant
- The variation in glyphosate susceptibility in *Alopecurus myosuroides* is heritable and can be selected for leading to populations with reduced susceptibility
- Due to the low glyphosate exposure of *Alopecurus myosuroides* populations in the UK, and the small decreases in susceptibility in selected populations, glyphosate resistance *Alopecurus myosuroides* may be slower than that of other grass species
- UK populations of *Anisantha sterilis* need to be monitored for glyphosate resistance and management strategies implemented to prevent further resistance evolution
- There is an area of chromosome 2 of *Arabidopsis thaliana* which may be related to variation in glyphosate susceptibility and further investigation may highlight genes associated with NTSR mechanisms
- Management strategies, such as tillage, need to be implemented to slow or prevent the evolution of glyphosate resistance in the UK, however, management strategies exploiting fitness costs related to quantitative

glyphosate resistance can not be used, as there are no fitness costs related to this

7.5 Future research

This thesis has established the glyphosate sensitivity of 55 *Alopecurus myosuroides* and 44 *Anisantha sterilis* populations. This data can now be used to monitor any changes in glyphosate susceptibility in these populations, enabling the processes of resistance evolution in these populations to be investigated in the field.

Investigating other UK weedy species for variability in glyphosate susceptibility should be a future research area, particularly investigating species that are prone to glyphosate resistance evolution, for example *Lolium* species. However, as *Alopecurus myosuroides* is such a problematic weed species in the UK, and there is significant variation in glyphosate susceptibility between populations, and a response to glyphosate selection, there should be a strong focus on the potential for glyphosate resistance evolution in this species, as well as management strategies that can be used to prevent or slow resistance. There should also be a strong focus on *Anisantha sterilis,* as populations of this species have already shown shifts towards lower susceptibility and have evolved practical resistance in the field. The implications of this practical resistance also needs to be investigated, for example whether the size of the population has increased in the field since the collection was made, whether the susceptibility/ resistance in the population has increased or decreased over time, and if there have been any management implications for the farmer, such as having to introduce ploughing to control the *Anisantha sterilis* population due to the failure of glyphosate applications in the field.

Investigating the baseline variability in UK weed populations, particularly in *Alopecurus myosuroides* and *Anisantha sterilis* populations, is also an area that needs further research, as in this study only one unexposed population of *Alopecurus myosuroides* and two (probable) unexposed populations of *Anisantha sterilis* were used. By finding more glyphosate unexposed weed populations to use in sensitivity screening the baseline variability to glyphosate in UK weed species can be established, and it can be determined how much of the variability found in exposed population is due to baseline varibability and how much is a result of exposure to glyphosate.

Continuation of glyphosate selection experiments on *Alopecurus myosuroides* is needed to determine whether the selective adaptation can continue (Moose *et al*. 2004) and the lines can become resistant, or whether the shift in decreased susceptibility will reach a limit (Busi and Powles, 2009). Furthermore, the focus of this research should be on non-target site resistance, as the glyphosate selection experiments have shown a gradual decrease in glyphosate susceptibility, which is possibly under polygenic control (Délye *et al*. 2011). Continuation of the glyphosate selection experiments, may also produce resistant individuals allowing for further fitness cost experiments to investigate the possibility of minor fitness costs relating to glyphosate resistance. These experiments should be multigenerational, as these can highlight minor fitness costs more easily than single-generational studies (Roux *et al*. 2005a).

The heritability of the variation in glyphosate resistance in *Alopecurus myosuroides* and the practical resistance in *Anisantha sterilis* needs to be studied in more detailed genetic experiments, as the heritability will affect the rate of resistance evolution

(Jasieniuk *et al*. 1996) and one of the criteria for confirming herbicide resistance is heritability of the trait (Weed Science Society of America, 1998; Heap, 2015). The segregation of the trait in progeny in heritability experiments of *Alopecurus myosuroides* and *Anisantha sterilis* may also determine whether the resistance mechanism is monogenic or polygenic (Busi *et al*. 2013b).

Further investigation into the area of chromosome 2 highlighted by the *Arabidopsis thaliana* MAGIC line experiment is needed to determine which, if any, of the genes in the region have a role in the variation of glyphosate susceptibility, and whether they are involved resistance mechanisms, such as reduced translocation. There should be a focus on the Rsch-4 and Sf-2 parent lines, which contributed most and least to reduced glyphosate susceptibility.

Models, such as HERMES by Monsanto, have already been developed to try to predict glyphosate resistance evolution and management strategies to slow this evolution (Gustafson, 2008). It would therefore be beneficial to utilize these existing models and integrate data from this study, to help predict glyphosate resistance evolution in the UK and management strategies that can help prevent or slow glyphosate resistance evolution in the UK.

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Appendix 1: Population information of 17 UK *A. myosuroides* populations collected from across England in summer 2010

Population	Region	Location	Crop
ABE110	Bedfordshire	Barton le Clay	Wheat
ABE210	Bedfordshire	Bedford	Wheat
ACA110	Cambridgeshire	ADAS, Extra close	Wheat
AES110	Essex	Manningtree	Wheat
AHE110	Hertfordshire	Broadbalk, Rothamstead	Wheat
AKE110	Kent	Hothfield, Ashford	Wheat
AKE210	Kent	Ruckinge, Ashford	Wheat
ALE110	Leicestershire	Loddington	Wheat
ALI110	Lincolnshire	Walcott	Wheat
ANO110	Nottinghamshire	Radcliffe on Trent	90xOSR, 62xwheat
ARU110	Rutland	Rockingham	Wheat
ASC110	Scotland	Westfield, Bathgate	Wheat
ASU110	Suffolk	Cavendish, Sudbury	Wheat
ASX110	Sussex	Haywards Heath	No crop at collection
AWA110	Warwickshire	Warwick	Wheat
AWA210	Warwickshire	Leamington	Wheat
AYO110	Yorkshire	Moffat	NA

Appendix 2: Population information of 40 UK *A. myosuroides* populations collected from across England in summer 2012

Population name	Collection date	Address/ site	Crop at collection	Known/ suspected resistance	Seed heads collected	G use Score
ACA112	30.7.12	Morborne, Peterborough	1st winter wheat	Suspected Atlantis	503	6
ACA212	30.7.12	Stilton	1st winter wheat	Suspected	436	7
ACA312	30.7.12	Connington, Peterborough	1st winter wheat	Suspected Atlantis	311	7
ACA412	31.7.12	Chesterton, Peterborough	1st winter wheat	Fops and dims known, suspect Atlantis	405	6
ACA512	2.8.12	Haddon, Cambridshire,	Winter wheat	None	453	8
ACA612		Elsworth, Cambridgeshire				
ACA812		Boxworth, Cambridgeshire				
AES112	19.7.12	Peldon, Essex	Winter wheat	Known ALS, ACCase	368	9
AES212	19.7.12	Wallraven, Peldon, Essex	Winter wheat		184	8
AGL112	17.7.12	Bledington, Oxfodshire	2nd winter wheat	None	277	6
AGL212	17.7.12	Bledington, Oxfordshire	2nd winter wheat	Suspected Atlantis	312	6
AGL312	26.7.12	Poulton, Cirencester	Winter wheat		218	6
AHE112	26.7.12	Rothamsted Research	Winter wheat			0
ALE112		Leicestershire				
ALI112	1.8.12	Bourne, Lincs	Winter wheat		314	
ALI212	1.8.12	Sutton St James, Lincolnshire	Winter wheat		413	
ALI312	1.8.12	Sutton St James, Lincolnshire	4th winter wheat	Suspected ALS and ACCase	335	9
ALI412	3.8.12	North Owersty, Lincolnshire	Winter wheat		446	
ALI512		Culverthorpe, Lincolnshire	Winter Wheat	Suspected ALS		
ANN112	24.7.12	Chipping Warden, Banbury	Winter wheat	Suspect everything but glyphosate	531	1
ANN212	24.7.12	Byfield, Daventry	Winter wheat		288	
ANN312	24.7.12	Lower Boddington, Daventry	Winter wheat		225	
ANN412	30.7.12	Nassington, Northamptonshire	Winter wheat	Atlantis	499	
ANR112	2.8.12	Marshland St James, Wisbech	2nd winter wheat	None	443	2
AOX112	20.7.12	Deddington	2nd Winter wheat		442	6
AOX212	20.712	Duns Tew, Deddington	Winter barley	Suspected Atlantis and Chlotoylon	414	7
ASF112	19.7.12	Hintlesham, Suffolk	Winter wheat	None	348	1
ASF212		Halesworth, Suffolk				
ASF312		Lowestoft, Suffolk				
ASO112	16.7.12	Somerset	Winter wheat	Suspected fop and dim	457	7
ASO212	16.7.13	Somerset	Winter wheat		316	7
AWA112	17.7.12	Barton on the heath	2nd winter wheat	Suspected Atlantis	493	2
AWA212	23.7.12	Oxhill	Winter wheat	Suspected Atlantis	526	6
AWA312	23.7.12	Stratford-upon-Avon	Winter wheat	Suspected Atlantis and Chlotoylon	328	3
AWA412	24.7.12	Fenny Compton, Leamington	Winter wheat	Suspected fops and dims	360	5
AWA512	26.7.12	Coventry	2nd winter wheat	Suspected cycloxydim	209	7
AWA612	26.7.12	Idlicote	Winter wheat	Suspected Atlantis	500	7
AWA712	26.7.12	Shotteswell, Banbury	Winter wheat		502	
AYO112		East Yorkshire				
AYO212		Northallerton, North Yorkshire				

Appendix 3: Glyphosate use questionnaire given to farmers where seed *A. myosuroides* seed was collected in 2012

Name and address:_____

_____

_____

Name / location of field_____

When was glyphosate first used in the collection field?_____

What is the field size?_____

What is the soil type in the field?_____

What is the current crop in the field?_____

What crop rotation is used in the field?_____

_____

Do you know of any existing herbicide resistance in the blackgrass on your farm and in the collection field? Yes: No:

If yes, please give details:_____

_____

_____

Have you had any problems controlling blackgrass with glyphosate in this field?
Yes: No:
If yes, please give details:_____

_____

Do you use a stale seedbed?

Yes: No:

If yes, please give details:_____

_____

Year	Time of application	Product	Rate	Crop	Reason for application	Success treating blackgrass	Additional information

Appendix 4: *Arabidopsis thaliana* scoring assessment

0% -	Alive no observable adverse effect
1-5% -	Very slight observable effect, some purpling to tip of 1-2 leaves
6-10% -	Very slight observable effect, some purpling to tip of 3-5 leaves
11-15% -	Slight observable effect, some purpling to edges of 1-2 leaves and tips of 3-5, some stunted growth
16-20% -	Observable effect, more so than 11-15%,
21-25% -	Observable effect, purpling to edges of 3-5 leaves, some stunted growth
26-30% -	Observable effect, purpling to edges of >5 leaves, some stunted growth
31-35% -	Observable effect, purpling to edge of leaves and majority of 1 leaf purple, stunted growth
36-40% -	Observable effect, purpling to edge of leaves and majority of 2 leaves purple, stunted growth
41-45% -	Observable effect, purpling to edge of leaves and majority of >2 leaves purple, stunted growth
46-50% -	Observable effect, 1 leaf purple, majority of other leaves purple, stunted growth
51-55% -	Observable effect, 1 leaf purple, other leaves mainly purple, some yellowing to leaf edges stunted growth
56-60% -	Observable effect, 2 leaves purple, other leaves mainly purple, yellowing to leaf edges, stunted growth
61-65% -	Observable effect, whole leaves purple, stunted growth
66-70% -	Observable effect, whole leaves purple, yellowing to one leaf, stunted growth
71-75% -	Observable effect, whole leaves purple, yellowing 2 leaves, leaves mostly purple, some green in some leaves, stunted growth
76-80% -	Observable effect, whole leaves purple, slight yellowing >2 leaves, more than 71-75%, some green in some leaves, stunted growth
81-85% -	Observable effect, majority all leaves completely purple, at least one whole leaf yellow, still some green in some leaves, stunted growth
86-90% -	Dead, all leaves purple or yellow, some browning to leaves
91-95% -	Dead, more so than 86-90% whole leaves brown
96-99% -	Dead, majority of leaves brown and shriveled, one or two completely yellow
100% -	Completely dead, all leaves brown and shriveled

Appendix 5: Genes between 12180334 base pairs (b.p.) and 13083366 b.p with different single nucleotide polymorphisms (SNPs) between Rsch-4 and Sf-2 accessions, position of gene, number of SNPs, and function of gene

Gene	Position on Chromosome 2 (B.P.)	Number of SNPs	Function
AT2G28507	12196406 - 12196552	3	Unknown
AT2G28510	12199098 - 12200765	2	DNA binding, involved in regulation of transcription
AT2G28520	12209896 - 12215895	5	ATPase activity, involved in ATPase synthesis coupled proton transport
AT2G28540	12218045 - 12223828	3	Nucleotide binding, zinc ion binding
AT2G28560	12237052 - 12239086	1	Double stranded DNA repair
AT2G28600	12251784 - 12254823	3	Nucleotide acid binding
AT2G28610	12262013 - 12263415	1	involved in regulation of lateral axis-dependent flower development
AT2G28620	12265167 - 12270020	1	Microtubule motor activity, involved in microtubule-based movement
AT2G28640	12284625 - 12286645	1	Involved in exocytosis
AT2G28650	12289152 - 12291045	23	Involved in exocytosis
AT2G28670	12300259 - 12301790	21	Disease resistance response
AT2G28680	12302963 - 12304799	61	Nutrient reservoir activity
AT2G28690	12306890 - 12308628	22	Unknown
AT2G28700	12317166 - 12318746	6	DNA binding, involved in regulation of transcription
AT2G28710	12322386 - 12323584	3	Sequence specific DNA binding activity, involved in regulation of transcription
AT2G28725	12328605 - 12328943	1	Unknown
AT2G28750	12331627 - 12332774	18	Pseudogene
AT2G28780	12340034 - 12343421	8	Unknown
AT2G28800	12356364 - 12359252	3	Chloroplast membrane protein ALB3, involved in may play a role in plant senescence

AT2G28830	12366748 - 12370684	39	Ubiquitin protein ligase activity, involved in response to chitin
AT2G28840	12378337 - 12380742	2	Zinc ion binding
AT2G28850	12383480 - 12384961	24	Electron carrier activity, involved in oxidation reduction
AT2G28860	12388201 - 12389872	7	Electron carrier activity, involved in oxidation reduction
AT2G28890	12405596 - 12408235	1	Protein phosphatase, involved in serine/theronine phosphatase activity
AT2G28900	12414114 - 12415578	17	Protein transmembrane transporter activity,
AT2G28910	12415780 - 12417385	10	Zinc ion binding, involved in positive regulation of calcium ion transport
AT2G28920	12418017 - 12418454	4	Zinc ion binding
AT2G28930	12424775 - 12426674	40	Protein serine/threonine kinase activity, involved in protein amino acid phosphorylation
AT2G28940	12426710 - 12428730	61	Protein amino acid phosphorylation, function protein kinase activity
AT2G28950	12431341 - 12433595	13	Encodes and expansin, involved in the formation of nematode-induced syncytia in roots of AT
AT2G28960	12437914 - 12442347	16	Process protein amino acid phosphorylation, protein serine/threonine kinase activity
AT2G28970	12443919 - 12448163	104	Protein seronine/threonine kinase activity, ATP binding, protein amino acid phosphorylation
AT2G28980	12449336 - 12454356	1	Pseudogene, transposable element gene
AT2G28990	12455055 - 12459541	154	Kinase activity, protein amino acid phosphorylation
AT2G29000	12460781 - 12465037	223	Protein seronine/threonine kinase activity, ATP binding, protein amino acid phosphorylation
AT2G29010	12465764 - 12469464	41	Pseudogene
AT2G29020	12469724 - 12471700	4	Produces Rab5interacting family protein
AT2G29030	12471773 - 12471844	5	Triplet codon amino acid adaptor activity
AT2G29040	12472425 - 12474962	12	Catalytic activity, unknown process
AT2G29045	12476372 - 12477107	5	Encodes a member of a family of small, secreted, cysteine rich protein
AT2G29050	12478146 - 12480372	1	Serine type endopepsidase activity
AT2G29060	12481744 - 12484087	1	Sequence specific DNA binding transcription factor activity
AT2G29065	12484843 - 12486983	2	Transcription factor
AT2G29070	12487274 - 12489518	1	Ubiquitin dependent protein catabolic process,
AT2G29080	12489627 - 12493285	5	ATP-dependent peptidase activity, proteolysis, protein catabolic processes
AT2G29090	12494851 - 12499723	14	ABA hydroxylase activity, involved in ABA catabolism

AT2G29100	12501092 - 12504912	4	Intracellular ligand gated ion channel activity, cellular calcium ion homeostasis
AT2G29110	12506880 - 12510552	10	Intracellular ligand gated ion channel activity, cellular calcium ion homeostasis
AT2G29120	12511292 - 12515895	13	Intracellular ligand gated ion channel activity, cellular calcium ion homeostasis
AT2G29125	12523342 - 12524109	11	Involved in shoot development
AT2G29130	12524889 - 12527747	16	Lactase activity, response to water deprivation
AT2G29140	12530838 - 12535399	81	Encodes Arabidopsis Pumilio proteins, regulated mRNA stability and translation
AT2G29150	12535715 - 12536964	26	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29160	12537251 - 12539334	41	Pseudogene
AT2G29170	12541864 - 12542282	17	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29180	12543023 - 12543835	24	Unknown function and process
AT2G29190	12543812 - 12548494	16	Encodes Arabidopsis Pumilio proteins, regulated mRNA stability and translation
AT2G29200	12548931 - 12553433	13	Encodes Arabidopsis Pumilio proteins, regulated mRNA stability and translation
AT2G29210	12558051 - 12562348	4	RNA splicing
AT2G29220	12562781 - 12564664	7	Kinase activity, protein amino acid phosphorylation
AT2G29250	12578909 - 12580780	5	Kinase activity, protein amino acid phosphorylation
AT2G29260	12582519 - 12584101	1	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29263	12584243 - 12584428	1	Unknown function and process
AT2G29270	12584502 - 12585096	1	Pseudogene
AT2G29280	12585889 - 12586330	1	Pseudogene
AT2G29290	12585856 - 12587742	7	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29300	12588191 - 12589759	4	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29310	12590059 - 12591363	4	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29320	12592148 - 12593686	4	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29330	12594590 - 12596330	21	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29340	12597115 - 12599203	9	Oxidoreductase activity, involved in oxidation reduction and metabolic processes
AT2G29350	12600914 - 12602556	10	Scenscence associated gene, SAG13, encodes a short chain alcohol dehydrogenase
AT2G29370	12606059 - 12607618	1	Oxidoreductase activity, involved in oxidation reduction and metabolic processes

AT2G29390	12610543 - 12612894	7	Encodes a sterol 4-alpha-methyl oxidase, involved in acetyl-CoA metabolic processes
AT2G29400	12613081 - 12615399	5	Protein phosphatase, serine/threionine phosphatase activity
AT2G29410	12616564 - 12617987	9	Metal tolerance protein, involved in anion transmembrane transport
AT2G29430	12619554 - 12620027	1	Unknown
AT2G29440	12620060 - 12621148	1	Glutathione S-transferase
AT2G29450	12624586 - 12625637	1	Glutathione S-transferase, involved in oxidative stress response and toxin catabolic processes
AT2G29510	12634514 - 12638018	1	Unknown
AT2G29550	12644047 - 12646037	2	Encodes a beta-tubulin, involved in response to cadmium ion, response to salt stress
AT2G29560	12646560 - 12649906	1	Phosphopyruvate hydratase activity, involved in glycolysis
AT2G29580	12651923 - 12654336	2	RNA binding, nucleotide binding
AT2G29600	12655151 - 12657296	2	Galactose oxidase
AT2G29605	12657909 - 12660087	9	Unknown
AT2G29610	12661312 - 12662417	1	Pseudogene
AT2G29620	12662741 - 12665803	1	Unknown
AT2G29630	12667034 - 12670311	14	Thiomine biosynthesis, detection of bacterium, glucosinolate metabolic process
AT2G29640	12671206 - 12673300	1	Josephin like protein, involved in proteolysis
AT2G29650	12673383 - 12676049	8	Anion transporter, Inorganic phosphate transporter
AT2G29654	12677279 - 12677461	2	Unknown
AT2G29660	12678890 - 12680667	1	Regulation of transcription, nucleic acid binding
AT2G29670	12681958 - 12685087	16	Binding in the chloroplast
AT2G29679	12689276 - 12689398	15	Unknown
AT2G29680	12689581 - 12692872	9	Cell division control protein, involved in cell cycle
AT2G29690	12693871 - 12696975	8	Functional anthranilate synthase protein, involved in aromatic amino acid biosynthetic process
AT2G29710	12698673 - 12700343	5	UDP-glycosyltransferase
AT2G29720	12700401 - 12702341	16	Monooxygenase activity
AT2G29730	12703537 - 12705181	6	Glycosyltransferase activity, involved in metabolic processes
AT2G29740	12706710 - 12708367	16	UDP-glycosyltransferase

AT2G29750	12709727 - 12711696	18	UDP-glycosyltransferase
AT2G29760	12712884 - 12715852	11	Organelle transcript protein 81, Chloroplast RNA editing factor
AT2G29770	12715411 - 12716875	4	Galactose oxidase
AT2G29780	12717786 - 12719328	6	Galactose oxidase
AT2G29790	12720884 - 12722455	8	Unknown
AT2G29800	12722939 - 12724602	4	Galactose oxidase
AT2G29810	12726102 - 12727253	4	Galactose oxidase
AT2G29820	12728362 - 12729528	6	Galactose oxidase
AT2G29830	12730411 - 12731599	8	Galactose oxidase
AT2G29840	12732387 - 12733983	6	Zinc ion binding
AT2G29860	12737666 - 12738388	13	Galactose oxidase
AT2G29890	12744150 - 12749596	14	Villin-like protein, involved in DNA methylation
AT2G29910	12751147 - 12753117	6	F-box/ RNI-like superfamily protein
AT2G29920	12754494 - 12755384	1	Involved in purine nucleobase transport
AT2G29930	12756356 - 12758558	2	F-box/ RNI-like superfamily protein
AT2G29940	12760139 - 12766623	9	ATPase activity, involved in drug transmembrane transport and pleiotropic drug resistance
AT2G29950	12767585 - 12768435	18	Involved in positive regulation of circadian rhythm
AT2G29960	12769033 - 12770579	9	Peptidyl-prolyl cis--trans isomerase, involved in protein folding, protein targeting to vacuole
AT2G29970	12776386 - 12779938	1	Double Clp-N motif-containing p-loop nucleoside triphosphate hydrolase
AT2G29990	12793340 - 12795913	5	NADH dehydrogenase activity, involved in oxidation reduction
AT2G29995	12796907 - 12799419	35	Unknown
AT2G30000	12803853 - 12804977	11	PHF5-like protein
AT2G30010	12805724 - 12809386	23	Triptochome Birefringence-like protein
AT2G30020	12814410 - 12816088	23	Encodes AP2C1, acts as a MAPK phosphatase that negatively regulates MPK4 and MPK6
AT2G30040	12821710 - 12823169	2	Protein seronine/threonine kinase activity, involved in protein amino acid phosphorylation
AT2G30050	12824451 - 12826684	2	Nucleotide binding, involved in membrane budding
AT2G30060	12826915 - 12828919	5	Ran GTPase binding, involved in intracellular transport, translocation

AT2G30070	12834993 - 12838626	15	Potassium ion transmembrane transporter
AT2G30080	12838730 - 12840112	20	Cation transmembrane transporter
AT2G30090	12843583 - 12845597	6	N-acetyltransferase activity, involved in metabolic processes
AT2G30100	12847828 - 12849669	9	Pentatricopeptide
AT2G30110	12852372 - 12857617	7	Ubiquitin-protein ligase activity, response to cadmium ion and other organisms
AT2G30115	12858462 - 12860142	7	Unknown
AT2G30120	12860541 - 12861910	6	Involved in cell differentiation, flower development
AT2G30140	12872134 - 12873820	16	UDP-glycosyltransferase activity
AT2G30150	12874706 - 12876122	2	UDP-glycosyltransferase activity, involved in metabolic processes
AT2G30160	12877838 - 12879649	2	Involved in transport, mitochondrial transport, transmembrane transport
AT2G30170	12879675 - 12881498	4	Phosphoprotein phosphotase activity
AT2G30180	12881591 - 12881661	6	Involved in process translational elongation
AT2G30190	12882734 - 12882804	4	Involved in process translational elongation
AT2G30200	12882955 - 12885534	5	Transferase activity, involved in metabolic processes
AT2G30210	12887446 - 12889874	10	Lactase activity, involved in oxidation reduction
AT2G30220	12891266 - 12892537	23	Hydrolase activity, involved in lipid metabolic processes
AT2G30230	12897071 - 12897885	5	Unknown
AT2G30240	12899907 - 12902779	54	Encodes a plasma membrane localised potassium transporter
AT2G30250	12903236 - 12905198	4	Member of WRKY transcription factor family, involved in abiotic stress response
AT2G30260	12905402 - 12907509	7	Involved in cis-assembly of pre-catalytic spliceosome
AT2G30270	12907841 - 12909261	10	Involved in purine nucleobase transport
AT2G30280	12909593 - 12912340	2	Involved in transcriptional regulation in RNA-directed DNA methylation and plant development
AT2G30290	12912519 - 12915912	11	Vacuolar sorting receptor, involved in protein targeting to vacuole
AT2G30300	12919401 - 12921222	6	Unknown
AT2G30310	12923055 - 12924380	4	Hydrolase activity, involved in lipid metabolic processes
AT2G30320	12925728 - 12927896	9	Pseudouridine synthase
AT2G30330	12928767 - 12929737	3	Biogenesis of lysosome-related organelles complex 1

AT2G30340	12931219 - 12932582	2	Unknown
AT2G30360	12936979 - 12938834	1	Protein kinase, phosphorylates AHA2, appears to regulate the activity of proton transporters
AT2G30362	12937351 - 12939638	4	Potential natural antisense gene, over laps with AT2G30360
AT2G30370	12940455 - 12942409	4	Negative regulation of stomatal complex development
AT2G30380	12948284 - 12950573	11	Unknown
AT2G30390	12951062 - 12954114	5	Ferrochelatase, involved in heme biosynthetic process
AT2G30395	12954174 - 12955089	2	Ovate family protein
AT2G30400	12956561 - 12957554	1	Ovate family protein, involved in N-terminal protein myristoylation
AT2G30410	12959289 - 12960810	21	Tubulin folding factor, involved in tubulin complex assembly
AT2G30420	12960827 - 12962162	37	Enhancer of TRY and CPC 2, involved in regulation of transcription
AT2G30424	12964506 - 12965468	4	Trichomeless 2, involved in regulation of transcription
AT2G30430	12968176 - 12968412	1	Involved in purine nucleobase transport
AT2G30432	12968615 - 12970209	12	Trichomeless 1, involved in regulation of trichome morphogenesis
AT2G30440	12972877 - 12975497	4	Thylakoid processing peptidase
AT2G30450	12975948 - 12976018	2	Involved in translational elongation
AT2G30460	12976146 - 12978868	5	Involved in purine nucleobase transport
AT2G30470	12980507 - 12985043	15	High level expression of sugar inducible gene, involved in regulation of transcription
AT2G30480	12987876 - 12993403	22	Involved in protein folding, response to heat,
AT2G30490	12993663 - 12995770	17	Transcinnamate 4-monooxygenase activity
AT2G30500	12998159 - 13000102	22	Kinase interacting family protein, involved in response to arsenic containing substances
AT2G30505	13000973 - 13002563	13	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AT2G30520	12262013 - 12263415	56	Signal transducer activity, involved in phototropism
AT2G30550	13014848 - 13017153	5	Galactolipase activity, involved in lipid metabolic processes
AT2G30560	13017347 - 13018857	10	Unknown
AT2G30570	13019028 - 13020194	1	Photosystem II reaction centre
AT2G30575	13020397 - 13024208	10	Transferase activity, involved in carbohydrate biosynthetic processes
AT2G30580	13026000 - 13030661	5	Ligase, involved in stress related transcriptional changes

AT2G30590	13033476 - 13035593	10	Sequence specific DNA binding factor, involved in regulation of transcription
AT2G30600	13036876 - 13041684	42	Involved in cell adhesion
AT2G30615	13041085 - 13042586	27	Unknown
AT2G30620	13044931 - 13046511	16	DNA binding, involved in nuleosome assembly
AT2G30630	13046672 - 13049219	22	ATP binding, involved in cell killing
AT2G30640	13049509 - 13051773	5	Encodes a member of a domesticated transposable element gene family
AT2G30650	13053777 - 13056380	8	Catalytic activity, involved in metabolic processes
AT2G30660	13058250 - 13061673	10	Catalytic activity, involved in metabolic processes
AT2G30670	13069313 - 13070904	9	Oxidase reductase activity, involved in oxidation reduction, metabolic processes
AT2G30680	13073648 - 13075396	10	Unknown
AT2G30690	13076229 - 13078595	1	Involved in plant-type cell wall modification, pollen tube development
AT2G30700	13081847 - 13085281	3	Unknown