ENVIRONMENTAL EFFECTS ON THE EVOLUTION OF HERBICIDE RESISTANCE IN THE GRASS WEED *ALOPECURUS MYOSUROIDES*

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In dedication to the most precious soul and wonderful Mom a person could ever wish for

Utterly beloved

Always missed

This is for you

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SUMMARY

Understanding the adaptive responses of weeds to abiotic factors and xenobiotics and the way this interact is a major unresolved issue in agricultural science. Plant defence mechanisms that prevent or minimize the number of damaged or dead plants can be defined as resistance. Of particular interest in the context of agricultural weeds, is the idea that shared pathways (e.g. physiological pathways in plants) of resistance to abiotic factors and xenobiotics might promote resistance to both drought and herbicides. The grass weed *Alopecurus myosuroides* (black-grass), has evolved resistance to several herbicides, making it an ideal species to investigate whether exposure to environmental factors promotes the evolution of resistance in grass weeds.

By conducting four linked pot experiments in a green house, evidence was obtained that drought stress accelerates the evolution of herbicide resistance in the subsequent generation of grass weed populations. Through the first two experiments, the exposure of grass weed populations with no previous history of herbicide applications to drought stress was found to confer herbicide resistance in the next generation in comparison to controlled plants. As the highest survivor plants was recorded for the plants under high droughted treatment and high dose of herbicide treatment. The second two experiments provided evidence that the mechanisms may be underlying this evolution of herbicide resistance were epigenetic. Results show that exposure of maternal plants to high drought stress may confer heritable herbicide resistance through epigenetic inheritance in the first generation.

In a final study, I investigated how several agronomically important characteristics including seed germination and emergence characteristics respond to environmental conditions. Seeds produced at high density were significantly higher in weight, size, viability and germination compared to the seeds that were produced at low density. Furthermore, a significant relationship was found between density and previous herbicide applications in parental plants. The majority of herbicides in interaction with high density caused an increase in seed germination, viability, seed weight and seed size across populations. The results also suggest an increase in seed germination, viability, seed weight and seed size in response to density in interaction with soil type. Overall, these results suggest that the environmental conditions in which the parental plants develop can be characterized as a stress-force shaping adaptation and evolution in the characteristics of weed seeds. This could occur through the phenotypic changes in the offspring that enable them to adapt in changing environments, specifically developing adaptation traits towards herbicide application. Generally, this research has shown that environmental factors can have an important role in the evolution of herbicide resistance in grass weeds.

These findings provide strong evidence that the interaction between environmental factors in which the weedy plants grow in interaction with xenobiotics, can affect the ability of grass weeds to adapt to changes in their environment.

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STATEMENT OF CONTRIBUTIONS

The introduction was written by the candidate with editorial advice from Prof. Colin Osborne and Prof. Robert P Freckleton.

1. Introduction

1.1. Weeds and global food demand

It has been proposed that, as human began cultivating wild plants, other organisms interacted with this process, such as domestic animals, pests, and other plants, which have become known as weeds and became competitors for the main crops (Walker, 1983). Weeds are the most significant biotic factors impacting the production of agriculture causing a 34% reduction of crop production throughout the globe (Oerke, 2006). Currently, crop output grown for human consumption is at risk at a global scale due to the occurrence of pests, particularly weeds, pathogens and animal pests (Oerke, 2006). Human population rise requires an increase in food and farm output for at least the next 40 years to meet demands (Godfray et al., 2010). It is expected that the world's population will reach 9.7 billion people in 2050 (UN, DESA, 2015), thus human food consumption and food security is a concern of great importance. The harshness of this problem is documented in the United Nations' sustainable development goals, SDGs (UN, 2017). These not only include no hunger, but also good health and well-being, maintainable cities and communities, responsible consumption and production. Any of the SDGs, in order to be achieved, need progress in the field of food security. In the universal efforts to understand the SDGs, not only does food production need to rise, but increasing yield must be achieved via what's known as sustainable intensification (Godfray et al., 2010). From the perspective of increasing inhabitants and food consumption, agricultural output has to be enhanced significantly. While average crop losses frequently rise with obtainable productivity, in order to achieve a high output there should be efficient crop management protection (Oerke and Dehne, 1997). Additionally, crop protection improvements can participate in agriculture sustainability and ecological resource protection. Despite the fact that effective management methods for most biotic yield restrains have been developed, but crop protection products usage is regulated by economic considerations rather than by food demand (Oerke and Dehne, 1997).

1.2. Economic effects of weedy plants

Arable weeds are the most important biotic threats to managed and natural ecosystems, and a specific problem in arable systems as they can damage and/or reduce both quality and quantity of farming output. For instance, weeds can result in the smaller size of a vegetable or the contamination of cereal grain with weed seeds. Thus, weedy plants act as a major constraint to farming production, causing significant agronomic and economic damage. Therefore, they can have an enormous effect on environmental sustainability (Powles and Yu, 2010, Javadzadeh and Fallah, 2011, Freckleton et al., 2008). Undoubtedly, the presence of weed and their management are associated with a loss of financial income, costing plant growers billions of dollars annually (Freckleton et al., 2008). The competitive abilities of weed species differ and are frequently proportional to the potential hazard they create. Weeds affect crop productivity through competition for resources, especially water, light, and inorganic nutrients. Thus they are the most important pest group in wheat production, causing potential losses of 23%, compared to only 16, 3, and 9% for pathogens, viruses, and animal pests respectively (Oerke, 2006). The hazard posed by weeds in agriculture land is correlated to weed density, the opportunity to reappear in the following season, and the ability of a certain species to regrow profusely, or to disperse in that specific milieu. The hazard is also correlated to the harshness and period of the competition with the main crop and all the related financial implications of these, together in terms of loss of crop productivity and of loss of time and money spent on management (Cousens & Mortimer 1995). Crop production loss due to weed plagues remains major concern, particularly with the rise of herbicide resistant strains of particular species. For instance, cereal crops in north-east Spain frequently suffer from the incidence of herbicide resistant annual ryegrass weeds and occasionally farm output losses up to 80% occur (Gonzalez-Andujar and Fernandez-Quintanilla, 2004). In the USA the cost of crop losses yearly due to weeds exceeded $26 billion (Pimentel et al., 2000).

A weed can be defined “with all due respect” as a plant growing out of place (Radosevich et al., 2007). In agriculture, this translates as plants other than the crop being grown for commercial production. This can include both wild plants that establish within the agricultural landscape as well as volunteers from previous crops. Weeds often exhibit rapid vegetative growth and are able to germinate, grow and reproduce in a wide range of environments (Baker, 1974). They are also very quick to adapt to changing selection pressures (Neve et al., 2009). This makes them very difficult to manage in an agricultural situation as they can quickly adapt to changing management practices. It is for these economic reasons that weeds have been studied in great detail, and methods to predict the numbers of weeds in the future have been developed. Herbicide resistance counts as one of the most important problems associated with weeds because it has developed in a number of species and weed families (Figure 1), most notably in *A. myosuroides* (Moss, 1990).

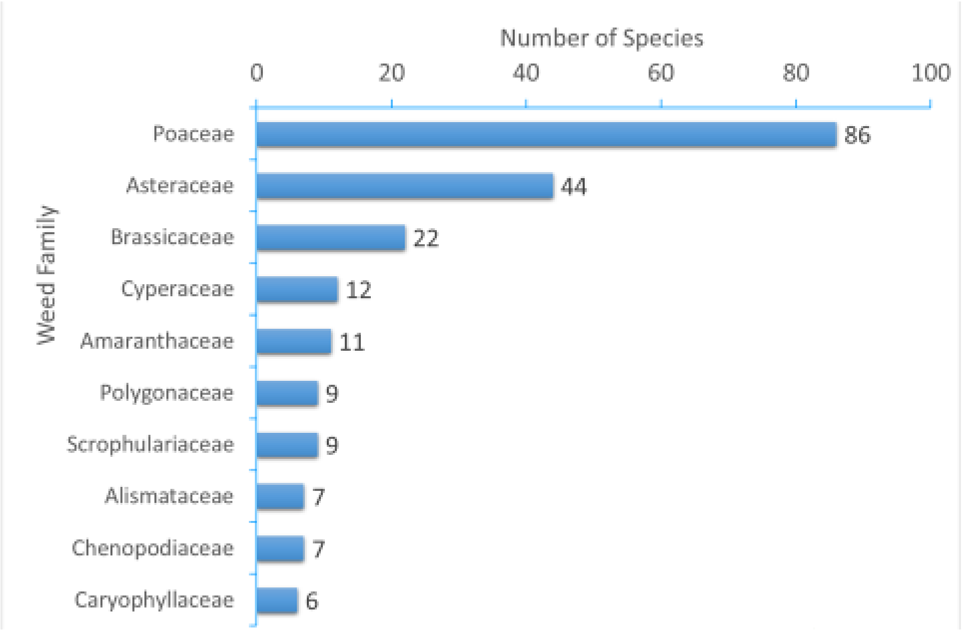

Figure 1. The number of herbicide resistant species for the plant families containing the most weed species. The graph adapted from (Heap, 2021) The International Survey for Herbicide Resistance database (http://www.weedscience.org).

1.3. Evolution of herbicide resistance as a global threat

The evolution of herbicide resistance in arable weeds is increasing rapidly on a global scale, threatening food security throughout the world (Delye et al., 2013b, Heap, 2014, Matzrafi et al., 2014, Powles and Yu, 2010, Hicks et al., 2018, Moss et al., 2019). Herbicide resistance refers to the ability of a plant to withstand and regrow after exposure to a certain dose of herbicide that would usually be lethal to the wild species (Katerova and Miteva, 2010, Yuan et al., 2007). Since the late 1940s, synthetic herbicides have been used on arable farms (i.e. crop fields) to control weeds (Oerke, 2006, Busi et al., 2013). They have been the most effective method to control weeds, killing approximately 99% of the weeds targeted (Foster et al., 1993), and replacing labour-intensive, animal and mechanical control of weeds (Oerke, 2006, Powles and Yu, 2010). After herbicide development, the arable surface area treated and variety of weed species targeted by herbicides increased rapidly and globally (Oerke, 2006). However, this efficacy was rapidly interrupted by the occurrence of herbicide resistance. Resistance to herbicide in weeds appeared for the first time in 1968, when failure to control common groundsel (*Seneca vulgaris* L.) was observed (Ryan, 1970). From 1968 to the 1970s, the most important instances of weed resistance to herbicide were to triazines. Since then, the development of herbicide resistance in weeds has increased worryingly

(Neve et al., 2009). By September 2021, resistance had been confirmed in 263 weed species in 95 different crop types in 71 countries, affecting the efficacy of 164 different herbicides from 21 of the 31 known herbicide sites of action (Heap, 2021).

Currently, herbicide resistance is extensively recognized as the result of the adaptive evolution of weed populations to the extreme selection pressure exerted by herbicides (Jasieniuk et al., 1996, Christoffers, 1999, Neve et al., 2009). Herbicide use has resulted in the evolution of plants and adaptation through the selection of genetic features that endowed resistance and allowed weedy plant populations to persist despite the herbicide application. Larger numbers and different modes of resistance have evolved in weeds than in other pests because of the widespread use of herbicides in comparison to pesticides (Gressel, 2009, Busi et al., 2013). Thus, the evolution of herbicide resistance in weeds is a significant issue in agriculture systems because of its high level of development, and since the ineffectiveness of herbicidal weed control is a major constraint to farming worldwide (Busi et al., 2013).

Herbicide resistance can be endowed in weeds through two mechanisms (Figure 2) monogenic target-site resistance (TSR), which is commonly inherited as a dominant allele (Delye, 2005, Powles and Yu, 2010, Delye et al., 2011). It is based on single point mutations which change the amino acid structure and inhibit herbicides from efficiently binding to the target enzyme. Plants with these types of mutations demonstrate high resistance to the corresponding herbicide. Delye et al., (2013a) concluded that resistance to herbicides will be conferred by unlinked point mutations. These can exist in weed populations within their standing genetic variation, and in their highest frequencies have detrimental pleiotropic impacts, so that their subsequent selection will be facilitated by applications of herbicides. Non-target-site resistance (NTSR) is the second set of the herbicide resistance mechanisms, which are currently the most problematic mechanisms in arable weeds. NTSR mechanisms are a subset of the physiological responses of weeds to abiotic stress, which is mimicked by herbicide application. Physiological stress-response mechanisms cause a decrease in the amount of herbicide molecules reaching their target sites, thereby preventing lethal action. NTSR mechanisms can endow resistance to a number of herbicides with different target proteins (Cummins et al., 1997, Letouze and Gasquez, 2001, Délye, 2013, Delye et al., 2013a); they arise from a quantitative set of characters that is determined by the expression of several genes. Furthermore, the presence of pre-existing alleles in the resistant weed plant genome is probable (Delye et al., 2013a). Consequently, as a result of repeatedly using certain types of herbicide on the same land, many different species of weeds have developed NTSR to these chemicals. Previously it has been documented that changed metabolism or translocation may be NTSR based (Beckie and Tardif, 2012). In herbicide-resistant biotypes, both enhanced metabolism and reduced translocation inhibit phytotoxic levels of herbicide from reaching the site of action. In general, enhanced metabolism is responsible for cross-resistance through the herbicide site of action (Beckie and Tardif, 2012). In a herbicide-resistant biotype, multiple resistance is generally defined with more than two mechanisms, either as a result of selection of sequential herbicide sites of action or resistance alleles accumulating in offspring as a result of pollen flow in self-incompatible species like "black-grass" *A. myosuroides* Huds, "rigid ryegrass" *Lolium rigidum* Gaudin, and *Kochia scoparia* (L.). Consequently, in weed populations, herbicides can select for every pre-existing mechanism endowing resistance (Beckie and Tardif, 2012). The evolution of NTSR has thus become a common example of weed adaptation. In future, weed control will benefit from a superior combination of ecological and evolutionary principles to evaluate the long-term reactions of weed populations to changing weed control, farming environments and worldwide climate (Neve et al., 2009).

In the 1980s, herbicide metabolism was first suspected as a resistance mechanism, when numerous weed populations, especially of (*L. rigidum*) and (*A. myosuroides*), were recognised with resistances to herbicides spanning multiple sites of action (Yu and Powles, 2014). In the 1990s, frequent reports started of enhanced herbicide metabolism involving enzyme systems including cytochrome P450s mono-oxygenases (P450s) and glutathione S-transferases (GSTs) (Devine and Preston 2000). GSTs are a multigene family of isozymes, recognised to catalyze the conjugation of glutathione (GSH) to various electrophilic and hydrophobic substrates (Kumar and Trivedi, 2018). GSTs have been linked with plant developmental processes and are responsive to a multitude of stressors.

Grass weeds included approximately in all primary cases of herbicide metabolism as a resistance mechanism, with GST-based metabolism of atrazine herbicide in velvetleaf (*Abutilon theophrasti*) and P450-based metabolism of mecoprop (methylchlorophenoxypropionic acid) in common chickweed (*Stellaria media*) being remarkable exclusions (Coupland et al., 1990, Anderson and Gronwald, 1991). Today, enhanced herbicide metabolism is more common in grass weeds, however a number of cases of broadleaf weeds evolving enhanced herbicide metabolism have been described (Yu and Powles, 2014).

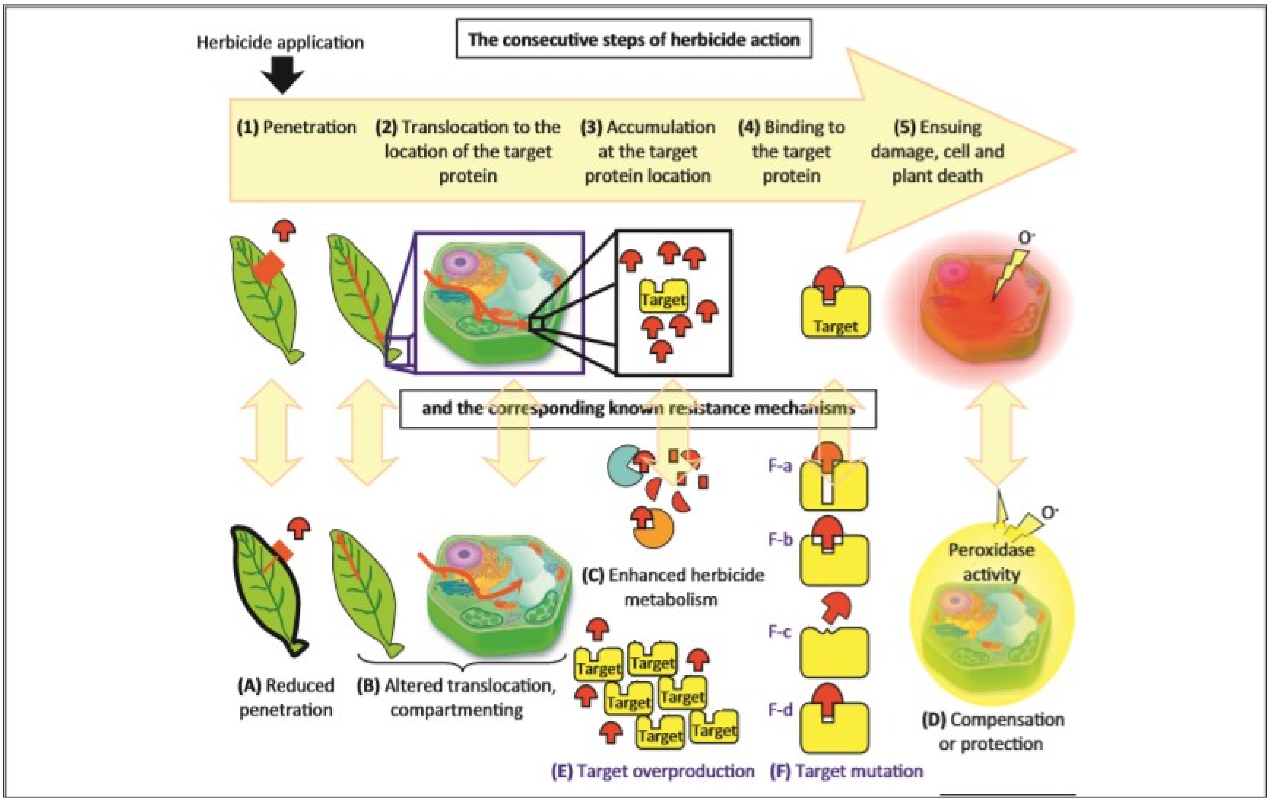

Figure 2. The modes of herbicide action (top) and the resistance mechanisms that have evolved in weeds in response to each action (bottom). After application, (1) the herbicide molecules penetrate, (2) are translocated to the location of the target protein (here, the chloroplast of meristem cells), (3) accumulate at the location of the target protein, and (4) bind to the target protein, thus (5) disrupting biosynthesis pathways or vital cell structures, and/or generating cytotoxic molecules active oxygen that damage cells and ultimately causes plant death). Multiple mechanisms of resistance interfering with the herbicide action steps have evolved in weeds. Non-target-site resistance (NTSR) mechanisms include (A) decrease in herbicide penetration due to changes in cuticle properties and/or plant habit, (B) changed translocation of the herbicide away from the target protein, (C) stimulated degradation (metabolism) of the herbicide, or (D) stimulated neutralization of cytotoxic molecules produced by herbicide action (illustrated: neutralization of active oxygen by peroxidases). Target-site resistance mechanisms consist of (E) regulatory mutations causing target protein overproduction that compensates for the herbicide inhibitory action, and/or (F) structural mutations that adjust the 3D structure and electrochemical properties of the target protein. Structural mutations can have no, moderate, or strong negative effects on the stability of herbicide binding to the target protein, which result in (F-a) no, (F-b) moderate or (F-c) marked decrease in herbicide sensitivity at the protein level, respectively; or can (F-d) riase the stability of herbicide binding to the target protein, which results in an increase in herbicide sensitivity (i.e., hypersensitivity) at the protein level. Adapted from (Delye et al., 2013b).

1.4. The response of grass weed species to different abiotic stresses (stress-interaction)

Under both natural and agricultural farm conditions, plants are exposed to environmental conditions called stresses (i. e. abiotic and biotic) through their life (Zhu, 2002, Vaahtera and Brosche, 2011, Vila-Aiub et al., 2011), and in general, an individual plant has the ability to adapt its metabolism, eliciting different physiological and biochemical changes under environmental stress conditions (Smirnoff and Cumbes, 1989, Arnholdt-Schmitt, 2004). Plants adaptation and/or responsiveness to these stresses particularly result from the modification of physiology and metabolism through the life cycle within the framework of the genetic background (Pastori and Foyer, 2002). Through the evolution of defence strategies plants have evolved responses to both biotic and abiotic stresses, by extensively controlling and modifying their metabolic systems (Pastori and Foyer, 2002). Various factors limit and participate in preventing mortality, including stress harshness, plants' genetic background and life history of each plant. Thus the survival of each single plant is dictated by these factors. Therefore, the interaction between the genome and environment must be an important focus, the nature of differences in phenotype directing plants to environmental signals via successful responses (Pastori and Foyer, 2002). In their stress responses, plants make the use of common pathways and components. In particular, the phenomenon known as cross tolerance allows plants to adapt to a variety of stresses following exposure to a particular stress (Pastori and Foyer, 2002). However, gene expression regulates the main part of plant adaptation with abiotic stresses, and the great readjusting of gene expression through regulation of transcription counts as one of the characteristic traits of plant defences against these stresses (Vaahtera and Brosche, 2011). Consequently, the major key to survival is the capability of plants to respond and/or adapt to stresses rapidly and in an appropriate way (Vaahtera and Brosche, 2011).

Multiple stress interactions can play effective roles in plant stress physiology (Beck et al., 2007). Stress tolerance in plant may also be adjusted by previous exposure to stress. This "stress memory" in plants is a common theme underlying responses to a range of stresses (i.e. biotic or abiotic), and may be due to the accumulation of self-protective compounds and transcription factors that adjust genes endowing stress tolerance (Bruce et al., 2007, Walter et al., 2013). Thus prior exposure to an abiotic stress makes a plant more tolerant and/or resistant to future stress exposure. (Walter et al., 2013) describes the ecological stress memory as any response of an individual plant following a stress exposure that adjusts the plant's response towards forthcoming stress occurrence, including the interaction with other environmental factors. Environmental stress memory may appear when plants make modifications to their structure or physiology upon exposure to stress that may continue after the stress stops. Consequently, with increasing stress frequency, plants may not have returned to their prior reference condition in the time interval between two stress events, thereby influencing the plant response to recurrent stress. This stress memory maintained by the plant after a stress occurrence may enable a more rapid stress response and increased stress tolerance (Bruce et al., 2007, Walter et al., 2011). At the cellular level, Tanou et al., (2012) have demonstrated the "priming phenomenon" where prior exposure to a catalyst postpones the adverse impacts of abiotic stress factors in plants, and consequently leads to a greater survival. There are many examples of this phenomenon. (Walter et al., 2011) established that an increase in photo-protection occurs in individual grass plants under repeated drought when compared to plants that were not exposed to drought previously. (Onate et al., 2011) also showed that Common Nettle (*Urtica dioica*) exposed to combined drought and nutrient deficiency in its juvenile phase shows enhanced drought stress tolerance in mature leaves, particularly in reproductive shoots. In a study of the combined effect of drought stress and repeated selection with sublethal dose of herbicide on adaptive gene expression and herbicide effectiveness on Junglerice (*Echinochloa colona*), Lariza et al., (2020) showed that under drought stress, repeated exposure to sub-lethal dose of herbicide decreases the species sensitivity to herbicide thus reduces the efficacy of herbicide. Under *in vitro* conditions, (Goh et al., 2003) found that *Arabidopsis thaliana* repeatedly exposed to high levels of abscisic acid (ABA), or subjected to drought stress signalling and response, led to the formation of an ecological stress memory, where gene expression was changed in response to subsequent stress events compared to untreated plants. Additionally, (Cuk et al., 2010) showed that the activity of antioxidative enzymes such as catalase and ascorbate peroxidase, which are frequently upregulated under drought, is inherited to the offspring of *A. thaliana*. As a result, evidence shows that an ecological stress memory may be inherited after exposure of maternal plants to a stress treatment (Walter et al., 2013).

However, studies investigating the relationship between environmental stresses (example: drought stress) and its effect on the evolution of herbicide resistance have never been tested especially in (*A. myosuroides*) populations, even though it is an important aspect to focus on for understanding the mechanism of the evolution of herbicide resistance. Continuously, plants are exposed to a large number of stresses, both biotic and abiotic, that adversely impact their growth, productivity and reproductive success. In order to survive stressful conditions, plants have evolved complex and sophisticated strategies. For example, they can deal with xenobiotics such as herbicides by using defences that originally evolved for other functions. There is growing evidence that resistance due to increased herbicide metabolism is commonly associated with enzymes that have major roles in plant responses to stress. Hence herbicide resistance is a trait that evolves quickly through the co-option of a suite of physiological mechanisms originally evolved to allow plants to survive environmental stress. Consequently, it might be expected that stress tolerance and herbicide resistance are functionally linked. A series of mechanisms including epigenetic modifications assumed to be involved in such responses to previous exposure, in addition to physiological, metabolic and morphological changes.

1.5. Aims and Objective

The aim of this thesis was to investigate the effect of drought as an abiotic stress on the evolution of herbicide resistance in grass weeds, to identify how rapid selection for herbicide resistance occurs in different species, by examining populations which differ in their previous exposure to herbicides and environmental conditions. The work also aimed to investigate the correlations between exposure to different levels of herbicide and other aspects of biology, particularly germination behaviour. This understanding can be used as the basis for thinking about management as well as future research, and to determine whether the inheritance of herbicide resistance occurs through genetic or epigenetic mechanisms. I use (*A. myosuroides* Huds) as a study system because it is a particularly problematic weed of winter sown crop in the UK and other Western European countries such as Germany and France, where its control is of concern. *A. myosuroides* is a major annual grass weed which is well adapted to recent agricultural production systems, such as non-ploughing cultivations, early sowing dates, and crop rotations dominated by winter sown crops. Due to its well adapted growth behaviour to winter crops, *A. myosuroides* can be very competitive and as a result needs to be managed on a regular basis. In the following chapters I use comparative methods to test the effect of drought stress on the evolution of herbicide resistance and further investigate the adaptation of the species to changes in environmental conditions such as density, soil type and exposure to different herbicide via seed characteristics changes and germination behaviour.

Chapter 2. DROUGHT EXPOSURE LEADS TO RAPID ACQUISITION AND INHERITANCE OF HERBICIDE RESISTANCE IN THE WEED *ALOPECURUS MYOSUROIDES*

Plants can deal with xenobiotics such as herbicides by using defences that originally evolved for other functions. There is growing evidence that resistance due to increased herbicide metabolism is commonly associated with enzymes that have major roles in plant responses to stress. I tested the hypothesis that stress tolerance and herbicide resistance are indirectly link through common physiological mechanisms. Results reveal that exposure of grass weed populations to drought stress can confer herbicide resistance in the subsequent generation. This provides insights into the possible mechanisms that drive resistance, as well as the types of ecological conditions that pre-adapt species to evolve resistance to herbicides.

Chapter 3. MECHANISMS THAT UNDERPIN THE EVOLUTION OF HERBICIDE RESISTANCE IN THE WEED *ALOPECURUS MYOSUROIDES*: EPIGENETICS AND THE ROLE OF ENVIRONMENTAL STRESS RESPONSES.

I carried out a further experiment to investigate the mechanism by which plants had acquired heritable herbicide resistance on exposure to drought. I tested the hypothesis that exposure to stresses, via an epigenetic mechanism, such as drought in a cloned parental generation tend to promote the evolution of herbicide-resistance in the subsequent generation, and that such evolution maybe mediated through inherited epigenetic mechanisms. Results show that exposure of maternal plants to high drought stress may confer heritable herbicide resistance through epigenetic inheritance. Knowledge that this herbicide resistance may arise epigenetically promotes better understanding of the evolution of the mechanism of herbicide resistance in grass weeds.

Chapter 4. THE IMPACT OF PLANT DENSITY, SOIL TYPE AND EXPOSURE TO HERBICIDE ON GERMINATION AND EMERGENCE CHARACTERISTICS OF POPULATIONS OF *ALOPECURUS MYOSUROIDES*.

Seed germination is a key process in weed population dynamics, and is predicted to be under strong selection pressure to ensure germination under circumstances that increase plant survival. The effects of previous management, different soil types and two levels of plant density (high and low) of 43 populations of plant (*A. myosuroides*) on germination and emergence characteristics of seeds were studied. Seeds produced at high density were significantly higher in weight, size, viability and germination rate compared to the seeds that were produced at low density. Furthermore, a significant relationship was found between density and previous herbicide applications in parental plants. The majority of herbicides in interaction with high density caused an increase in seed germination, viability, seed weight and seed size across populations. The results also suggest an increase in seed germination, viability, seed weight and seed size in response to density in interaction with soil type. Overall, these results suggest that the environmental conditions in which the parental plants develop can be characterized as a stress-force shaping adaptation and evolution in the characteristics of weed seeds. This could occur through the phenotypic changes in the offspring that enable them to adapt in changing environments, specifically developing adaptation traits towards herbicide application.

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CHAPTER TWO

Figure 1. Sample of *A. myosuroides* plants that used in dose response study (herbicide exposure) following parental exposure to drought stress.

STATEMENT OF CONTRIBUTIONS

The initial ides of this chapter were developed by Prof. Robert Freckleton. The method which was used to conduct the drought stress treatment was constructed by the candidate. All the data collection and writing were carried out by the candidate. Data analysis was carried out by the candidate with editorial advice from Prof. Robert Freckleton and Prof. Colin Osborne. General editorial advice was provided by Prof. Robert Freckleton and Prof. Colin Osborne.

CHAPTER TWO

DROUGHT EXPOSURE LEADS TO RAPID ACQUISITION AND INHERITANCE OF HERBICIDE RESISTANCE IN THE WEED *ALOPECURUS MYOSUROIDES*

Abstract

Globally, the evolution of herbicide resistance in arable weeds is increasing rapidly and poses a growing threat to global food security. Plants can deal with xenobiotics such as herbicides by using defences that originally evolved for other functions. There is growing evidence that resistance due to increased herbicide metabolism is commonly associated with enzymes that have major roles in plant responses to stress. Consequently, it might be expected that stress tolerance and herbicide resistance are functionally linked. An experiment was set up to test this hypothesis and whether resistance is mediated through a heritable mechanism. Experimentally we exposed a parental generation of two species of grass weeds "black-grass" (*Alopecurus myosuroides*) and "annual meadow grass" (*Poa annua*) to three levels of drought stress and observed whether there were consequent effects on herbicide resistance in the subsequent generation (F1). Plants from the F1 were exposed to lethal or sub-lethal doses of fenoxaprop-p-ethyl herbicide at the 2-3 tiller stage. In terms of both survival and dry mass we observed enhanced resistance to lethal and sub-lethal herbicide doses in the F1 plants of both species when the parents had been exposed to high and medium drought levels compared with controls. Overall, the results suggest that exposure of grass weed populations to drought stress can confer herbicide resistance in subsequent generations. Knowledge of whether this herbicide resistance arises genetically or epigenetically will promote better understanding of the evolution of the mechanism of herbicide resistance in grass weeds.

Keywords: "Black-grass", Stress response, Drought tolerance, Drought and herbicide interaction, Resistance development, Multiple stress interactions, Selection pressure.

2.1. Introduction

Since the origins of agriculture, arable weeds have been among the most important biotic factors limiting crop production (Oerke, 2006, Matzrafi et al., 2014). Weeds cause yield reductions of up to 34% across the globe and are thus a major threat to food security (Oerke, 2006, Matzrafi et al., 2014, Delye et al., 2013b). Herbicide application is considered by many as the most effective weed control method, and has made great contributions to food production throughout the world (Powles and Yu, 2010, Busi et al., 2013, Matzrafi et al., 2021). However, because of the repeated use of these xenobiotic chemicals, herbicide efficacy rapidly has been interrupted by the evolution of herbicide resistance in arable weeds threatening health and food security throughout the world (Hicks et al., 2018, Varah et al., 2020). The greatest challenge in weed control is the identification of selective herbicides for use in crop fields (Rubin, 1996), with grass weeds particularly difficult to control selectively in cereals because of their close relatedness to the crops (Rubin, 1996). Herbicide resistance can be defined as the ability of weed plants to survive following a herbicide treatment that would usually be expected to be lethal to a wild type (Reade et al., 2004, Katerova and Miteva, 2010) and plants can develop resistance through natural selection (Katerova and Miteva, 2010). By September 2021, resistance had been confirmed in 264 weed species in 95 different crop types in 71 countries, affecting the efficacy of 164 different herbicides from 21 of the 31 known herbicide sites of action (Heap, 2021).

Herbicide resistance in weeds is conferred by one of two broad mechanisms: monogenic target-site resistance (TSR) or non-target-site resistance (NTSR). Target-site resistance is commonly inherited as a dominant allele (Delye, 2005, Matzrafi et al., 2021). This kind of resistance is based on single point mutations in herbicide-binding proteins, which lead to changes in the amino acid structure and may inhibit herbicides from efficiently binding to the target enzyme (Delye et al., 2002, Matzrafi et al., 2021). Plants with these types of mutations can demonstrate high resistance to the corresponding herbicide (Delye et al., 2002).

Non-target-site resistance is controlled by a subset of physiological pathways responsible for the response to abiotic stress, many of which are induced in weeds by herbicide application (Cummins et al., 1997, Letouze and Gasquez, 2001, Délye, 2013, Matzrafi et al., 2021). Plants have evolved complex physiological systems of stress detection, response and signalling that activate both specific and general responses (Vaahtera and Brosche, 2011). The physiological basis of NTSR mechanism is usually the stimulation of herbicide metabolism or detoxification mediated by cytochrome P450 monooxygenases (CYPS) (Vila-Aiub et al., 2009a), glutathione S-transferases (GSTs) (Reade et al., 2004) and other Phase II metabolism enzymes (Powles and Yu, 2010). As the ability to metabolize (degrade or detoxify) sufficient of the herbicide for the plant to survive can arise naturally in tolerant species, or can be enriched under environmental stress conditions (Yu and Powles, 2014, Nandula et al., 2019)

Among these various routes, pathways that remove reactive oxygen species (ROS) are particularly important. Reactive oxygen species, including H_2O_2, are produced in response to a range of stresses, and are important in stress signalling, but can cause cell damage unless eliminated. In plants, GSTs are assumed to play major roles in oxidative stress metabolism, although their regulation is not well understood (Chen et al., 2012). These mechanisms cause a decrease in the amount of herbicide that reaches its target sites, thereby preventing herbicide's action (Cummins et al., 1997, Letouze and Gasquez, 2001, Yuan et al., 2007). Moreover, Glutathione S-transferases have been linked with responses to biotic and abiotic stress, hormone signals and evolutionary change (Moons, 2005, Frova, 2006). Better understanding of the environmental factors that impacts the evolution of herbicide resistance in plants is essential for understanding the mechanisms of resistance (Busi et al., 2013).

Epigenetic mechanisms play essential roles in the regulation of stress-related gene expression (Chinnusamy and Zhu, 2009), They operate via the generation of small RNAs and modifications in chromatin, and may contribute to transcriptional and post-transcriptional controls over gene expression, which are critical for environmental stress responses. The inheritance of epigenetic changes is via mitotic cell divisions and they can, in some circumstances, be passed to the next generation. Epigenetic mechanisms may regulate many genetic functions, including replication, transcription, DNA repair, gene transposition and cell differentiation (Madlung and Comai, 2004, Angers et al., 2010). Consequently, they represent an important potential mechanism for stress memories (Kinoshita and Seki, 2014).

Recently, it has also been shown that plants have the ability to remember previous environmental exposure and can benefit from this to augment responses when these environmental conditions reappear (Kinoshita and Seki, 2014). For example, Ding et al., (2012) showed that previous exposure to drought stress conditions helped *Arabidopsis* plants to respond to subsequent stress through rapid adaptive alterations to gene expression. Similarly, *Urtica dioica* (L.) (common nettle) subjected to combined drought and nutrient stress in the juvenile phase showed enhanced drought tolerance in mature leaves (Onate et al., 2011). Furthermore, (Lariza et al., 2020) in a study on *Echinochloa colona* (junglerice) showed that repeated selection with a sublethal dose of herbicide under drought stress selected *E. colona* plants with greater adaptability to the subjected conditions, with a transgenerational impact, causing a reduced sensitivity to herbicide management. They also showed that exposure to drought stress changed the general plant physiology and decreased the efficacy of herbicide. Thus, prior experiences of biotic and abiotic stresses can alter the response of an individual plant to subsequent stresses (Tahkokorpi et al., 2007, Onate et al., 2011). This phenomenon can be described as the "priming effect" (Tanou et al., 2012) or "stress memory" (Ding et al., 2012, Walter et al., 2013). A series of mechanisms including epigenetic modifications (Robertson and Wolf, 2012), physiological, metabolic and morphological changes (Walter et al., 2013) are assumed to be involved in such behaviour.

This study reports an experiment designed to evaluate the hypothesis that exposure to stresses such as drought could promote herbicide resistance in the first generation and to test whether such resistance is heritable. Two model species of grass weeds in which a parental generation was exposed to varying levels of drought, and the offspring exposed to different levels of herbicide.

Alopecurus myosuroides Huds (annual "black-grass") and *Poa annua* L. (annual meadow-grass) were used as model species for the study of herbicide resistance in grass weeds. *A. myosuroides* is the primary weed of winter crop fields in northern Europe, which can increase crop lodging and cause crop production losses of more than 44% (Reade et al., 2004). Multiple herbicide resistance (MHR) in *A. myosuroides* is an important issue, due to the significant competition of this weed with cereal crops (Powles and Yu, 2010). Multiple herbicide resistance was reported for the first time in *A. myosuroides* in 1982 at Peldon in Essex, England, and soon after was very rapidly recorded throughout Europe (Moss, 1990, Hall et al., 1997). In common with other grass weeds, *A. myosuroides* is an obligate out-crosser with a self-incompatible reproduction system (Chauvel and Gasquez, 1994). This type of reproduction has the ability to enhance the spread of herbicide resistance in the weed population (Matzrafi et al., 2014).

P. annua is common worldwide as a weed of farmed and waste ground habitats (Warwick, 1979). *P. annua* has a self-compatible reproduction system (Ellis, 1973) and, as a winter annual weed, is a significant issue in managed turfgrass (Cross et al., 2013). The herbicides frequently used to control *P. annua* in managed turfgrass systems have been those that target Acetolactate Synthase (ALS). However the number of *P. annua* populations resistant to this herbicide is rising (Cross et al., 2015).

The purpose of this research was to identify how rapid selection for herbicide resistance occurs in grass species such as *A. myosuroides* and *P. annua*, through the interaction of abiotic stress such as drought, and a xenobiotic such as a herbicide; by examining populations which differ in their previous environmental conditions. The abiotic stress was different levels of water availability using three levels of drought stress, and the xenobiotic stress was provided by spraying the first generation of droughted parental with two herbicide doses (lethal and sublethal). The objectives were to (1) measure the impacts of drought on growth and morphological traits for *A. myosuroides* and *P. annua* to characterise their response to drought stress, and (2) characterize rapid selection to herbicide resistance in *A. myosuroides* and *P. annua* by applying different herbicide doses to the generation after the drought treatment.

2.2. Materials and methods

2.2.1. Plant material

The response to drought stress of five populations of each species was investigated in a greenhouse environment, (Arthur Willis Environment Centre at The University of Sheffield). All the populations of *P. annua* were obtained from Royal Botanical Garden, Kew, while *A. myosuroides* populations were obtained from Herbiseed, Ltd, UK, in 2015, based on their confirmation the seeds had no previous treatments of herbicides, as they had an annual production area of totally herbicide free ALOMY which was accepted by the research industry as susceptible (the information was obtained at www.herbiseed.com) however, the source is not available to any further extent. Furthermore, the locations, year of collection and source of the populations are summarized in Fig 2 and Table 1. The aim was to propagate first generation plants (F1) from parents (P) that had been exposed to different drought stress levels.

2.2.2. Drought stress treatment

In March 2015, nine seeds of each of the five populations for both species were planted in square plastic pots (200 mm), containing a standard potting mixture (50% compost + 50% vermiculite) with planting depth of 50 and 25 mm for each species, *Alopecurus* and *Poa* respectively. Saucers were used for each pot to avoid the loss of soil nutrient contents by leaching. The pots were maintained in a greenhouse with average day and night temperatures of 20 °C and 15 °C respectively, and were well-watered to ensure seed germination. Following emergence, seedlings similar in height (40 mm) and number of leaves (1 leaf) were thinned to three plants per pot to ensure sufficient plant material. All the information about planting dates and other measurements is summarized in (Table 2). Plant height, above ground biomass and seed weight were measured to estimate the influence of drought stress on the species phenotypes. In addition, surviving and dead plants were assessed to evaluate the tolerance of *A. myosuroides* and *P. annua* to drought stress.

Plant height was recorded 48 days after emergence (DAG), from May to August 2015, at the end of each drought period and before re-watering the plants for both species. The shoots of all plants were measured at the soil level to the end of the longest leaf. During the anthesis stage and before pollen emission, the plants were covered by a pollen-proof bag to ensure cross-pollination with members of the same population only (Neve and Powles, 2005a).

The experiment was conducted as a complete randomized design with four replicates and three treatments including an unstressed control group (well-watered plants), and two drought treatments. The drought stress treatments were initiated 30 DAG. The low drought treatment was applied by withholding water until the shoots of approximately 25% of plants had died back, the first period of low drought treatment was started on 10th April, 2015 until 12th May, 2015 (roughly 5 weeks); the second period was started on 18th May, 2015 until 12th June, 2015 (3 weeks) and the last period of drought treatment was applied on 18th June, 2015 until 22nd July 2015 (5 weeks). The high drought treatment was applied by withholding water until 75% of plants had died back, the first period of high drought treatment was started on 10th April, 2015 until 21st May 2015 (approximately 6 weeks); the second period was applied on 27th May, 2015 until 23rd June, 2015 (roughly 4 weeks) and the last period was initiated on 29th June, 2015 until 30th July, 2015 (4 weeks). After each period of drought treatment the mortality rates were assessed at each level of the treatment, and the plants were re-watered as normal watering (twice per week) until the appearance of drought tolerance shoots. Visual assessment by the same observer was made using consistent criteria to monitor the growth and mortality rate of each group ("low and high" drought). In addition, the soil moisture content of each pot was monitored after each period of drought by measuring the apparent dielectric constant (ThetaProbe, Delta-T-Devices, Cambridge-England). Then, the drought treatment was stopped and plants were re-watered (for two weeks) after the drought treatments, and many re-sprouted and flowered.

During harvest time and after 4 weeks of withholding irrigation the aboveground biomass of a single mature plant per pot was harvested (2nd September, 2015 for the low drought treatment and 9th September, 2015 for the high drought treatment). This allowed the impact of water stress on aboveground biomass production to be evaluated. Aboveground biomass was hand-harvested and directly weighed with a scale (EP 6102C, max 100 g, d=0.01 g, Ohaus Corporation, Parsippany, NJ, USA). After harvesting, seeds of each plant were separated and weighed using a high precision scale (GH-252-EC, max = 250 g, min= 1 mg, d=0.01/0.1 mg, A&D Instruments, Abingdon, UK). Seeds were stored in dark and dry conditions (fridge at 4 °C) until further use. The percentage reductions in plant height, biomass and seed production were calculated relative to the unstressed control plants for each drought treatment level.

2.2.3. Dose-response experiment

In January 2016, a herbicide dose–response experiment was conducted (Figure 1) to investigate (i) the effect of previous selection for drought stress on the incidence of herbicide resistance in different grass weed populations (ii) the rapid selection of herbicide resistance in different species and populations with contrasting herbicide exposure histories. Nine seeds from the F1 generation of all populations for both species were planted in circular plastic pots (100 mm in diameter, 215 mm depth and 4 L volume), pots containing standard potting mixture as previously described, and were maintained in a greenhouse, environment (Arthur Willis Environment Centre, University of

Sheffield). Following seed sowing, pots were thoroughly watered from above to ensure seed germination and through the course of the experiment, plants were watered as required. After seed emergence, seedlings similar in height and number of leaves (height: 40 mm, and 1 leaf) were thinned to three plants per pot. At the 2-3 tillers stage, these seedlings were sprayed with fenoxaprop-p-ethyl herbicide (as "Puma Super" – 69 g a.i. h^{-1}, Bayer Crop Science) using two different doses lethal dose 40 g a.i. h^{-1} and sub-lethal dose 20 g a.i. h^{-1}. From other research data it seemed that a dose around 15-25 g a.i. ha^{-1} would cause a small/moderate growth reduction, while a higher dose anywhere between 30-50 g a.i. ha^{-1} would cause a more pronounced response, at the expense of some plants dying. I was keen to avoid complete mortality for some of the plants this is why low dose of 20 g a.i. h^{-1} "sub-lethal dose", was chosen. On the other hand, I wished to observe a larger mean growth reduction with some mortality, then I went with slightly higher dose 40 g a.i. h^{-1} called lethal dose. Each dose of the herbicide was applied separately in different chambers which had the same conditions (in terms of light and temperature) to avoid any cross contamination between the plants. Subsequently, the plants were relocated back into the main chamber after they dried out. Fenoxaprop-p-ethyl herbicide was used because resistance to fenoxaprop-p-ethyl is linked with the selection for NTSR (Letouze and Gasquez, 2001, Delye et al., 2007, Delye et al., 2015) or enhanced metabolism, this is where the target plants are able to detoxify the herbicide before it reaches the target site. Additionally, the occurrence of NTSR is predicted to be derived by exposure to abiotic stress. Also the herbicide is used very widely to control *A. myosuroides*. For example, in Germany, fenoxaprop-p-ethyl is one of the most common herbicides used in cereal crop fields for selective control of *A. myosuroides* over long periods (Rosenhauer et al., 2015).

Four weeks after herbicide application dead and damaged plants were assessed. Plants were scored as dead if they had yellow/burned leaves following herbicide treatment. Scoring was carried out by the same observer for all plants. Surviving plants were categorised in two ways to account for the differential outcomes of exposure to herbicide: plants were categorised as surviving if they showed no visible effects of herbicide exposure, or damaged if they survived but with obvious effects on above ground tissues. To evaluate the impacts of herbicide exposure, we first combined surviving and damaged individuals, and calculated these as a proportion of the plants treated. Second, we calculated the proportion of plants which survived, compared with the fraction of those that died or were damaged. These two approaches measure resistance in slightly different ways. The first measures the plants that survive application, whether they are damaged or not; the second measures those plants that are unaffected by the herbicide application. One plant per pot for each species was harvested at the soil surface and dried at 50 °C for three days. The dry weight of each surviving and damaged plant of each species was measured using high precision scales (GH-252-EC, A&D Instruments). The methods that were used in this study are shown in Figure 1 for further clarification.

This experiment was conducted as a complete randomized block design: there were 20 pots per block (five blocks in total), five populations of *A. myosuroides and P. annua*, two levels of herbicide doses, and as pre-treatment, and three levels of drought stress for a total of 300 experimental units (pots). One set of F1 plants for each drought treatment from each population was sprayed with fenoxaprop-p-ethyl herbicide ("Puma Super" – 69 g a.i. L^{-1}, Bayer Crop Science) at 40 g a.i. h^{-1} (lethal dose) and the other set of F1 plants were sprayed with the herbicide at 20 g a.i. h^{-1} (sublethal dose), Herbicides were applied using a flat nozzle sprayer (3l capacity) delivering 0.79 gallons 0.20 minutes^{-1} (equivalent to 4 gallons minutes^{-1} with pressure up to 100 PSI) herbicide in Max 45 PSI, applied with a fine spray and 3BAR pressure.

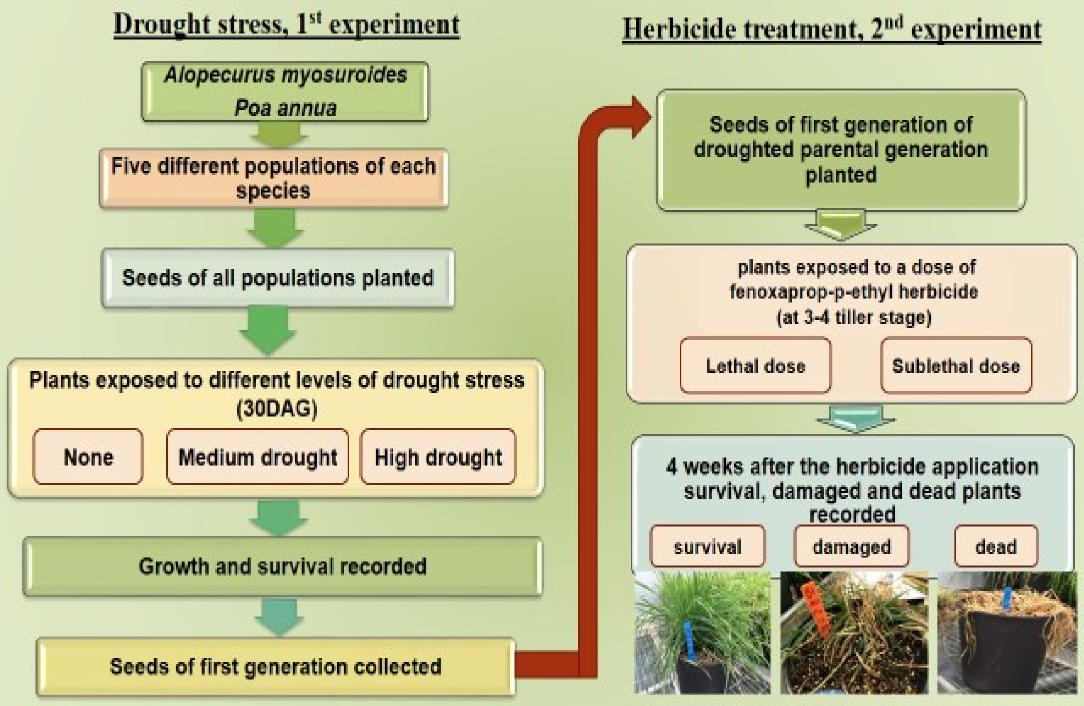

Figure 2. Shows the summary of the methods of both experiments for this study. First experiment: five different populations with no previous herbicide treatment for both species (*A. myosuroides and P. annua*) were selected. Seeds of all populations were planted, 30 days after germination plants were exposed to different drought treatments (None, Medium and high). Growth and survival of the plants were recorded for each treatment. Seeds of the first generation of droughted parental plants were planted and then exposed to two different dose of fenoxaprop-p-ethyl herbicide (lethal and sublethal). Four weeks after the herbicide application survival, damaged and dead plants were recorded. Additionally, one plant per pot for the intact and damaged plants were harvested and dried to record the plant biomass.

2.2.4. Statistical analysis

R (R Core Team, 2015) and lme4 (Douglas et al., 2015) were used to analyse the response of the P generation to drought treatment (first experiment). Population and replicates were entered into the model as factors. P-value were obtained by using two-way ANOVA to compare the mean responses in the drought stress experiment with two factors. Data on the effect of drought in P generation on plant height, biomass and seed mass were log transformed. Generalised Linear Model (glm) was also performed to analyse the survival plants of drought treatments.

For the herbicide application experiment (second experiment) Generalised Linear Mixed Model, using the 'lme4' package (Douglas et al., 2015) was fitted to explore the effects of drought stress in parental plants upon the evolution of herbicide resistance in the F1 generation for all survived populations. Binomial error structure was assumed in this experiment as the dependent variable was a binary outcome. Successive models with all variables and an interaction between drought stress and herbicide resistant were also constructed. The main hypothesis was that exposure of the P generation to drought would influence the response of the F1 generation to herbicide exposure. In the models for the F1 generation, we therefore included an interaction between the P generation exposure to drought and herbicide treatment.

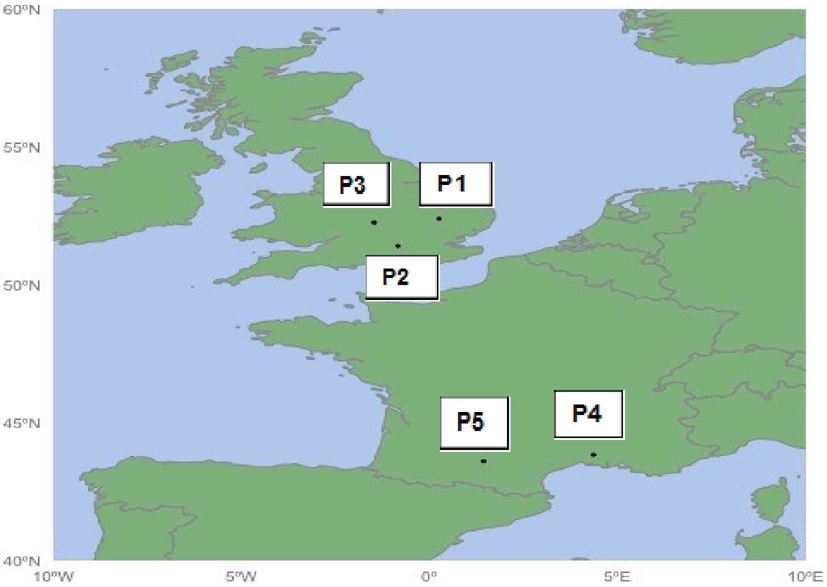

Figure 3. The black dots on the map represent the locations of sampling the *A. myosuroides* weed. "P= population", P1= Ely Cambridgeshire, UK; P2= Wokingham Berkshire, UK; P3= Warwickshire, UK; P4= Nimes Languedoc-Roussillon, France; P5= Toulouse Pyrenees.

Table 1. Origins, source and year of collection (Y.O.C) of *A. myosuroides* and *P. annua* of parental populations.

Species	Population	Location	Source	Y.O.C.
A. myosuroides	1	Ely Cambridgeshire, UK	Organic field margin (Herbiseed, Ltd, UK)	2014
	2	Wokingham Berkshire, UK	Winter wheat crop (Herbiseed, Ltd, UK)	2011
	3	Warwickshire, UK	Winter wheat crop (Herbiseed, Ltd, UK)	2012
	4	Nimes Languedoc-Roussillon, France	Spring wheat crop (Herbiseed, Ltd, UK)	2012
	5	Toulouse Pyrenees, France	Winter wheat crop (Herbiseed, Ltd, UK)	2014
P. annua	1	-	Royal Botanic Garden, UK	-
	2	-	Royal Botanic Garden, UK	-
	3	-	Royal Botanic Garden, UK	-
	4	-	Royal Botanic Garden, UK	-
	5	-	Royal Botanic Garden, UK	-

Table 2. Dates of planting, germination, drought treatment application, soil moisture content measurement and the number of days between each irrigation for both drought treatments.

Species	Date of planting	date of germination	Plants thinning (the average of height and the number of leaves)	Date of low drought treatment application	Date of high drought treatment application	Dates of soil moisture content measurement for both drought treatments and species	Number of days between each irrigation
Alopecurus myosuroides	04/03/2015	11/03/2015	height: 40 mm leaves: 1 leaf	1st 10/04/2015-12/05/2015	1st 10/04/2015-21/05/2015	low: 12/05/2015 high: 21/05/2015	low= 32 days high= 41 days
				2nd 18/05/2015-12/06/2015	2nd 27/05/2015-23/06/2015	low: 12/06/2015 high: 23/06/2015	low= 24 days high= 26 days
				3rd 18/06/2015-22/07/2015	3rd 29/06/2015-30/07/2015	low: 22/07/2015 high: 30/07/2015	low= 34 days high= 31 days

2.3. Results

2.3.1. Drought treatment

2.3.1. a. Effect of drought treatment on *A. myosuroides*

The drought treatments significantly affected plant height [Fig 3A, Table 3; $F_{(2, 50)} = 14.8$, $p = 8.97 \times 10^{-6}$], biomass [Fig 3B, Table 3; $F_{(2, 50)} = 42.1$, $p = 1.88 \times 10^{-11}$] and seed weight [Fig 3C, Table 3; $F_{(2, 49)} = 33.9$, $p < 1.00 \times 10^{-7}$] of *A. myosuroides*. This indicates that, with further increase in drought stress treatment from medium to high, both plant biomass and seed weight per plant were sharply reduced. The range of survival for plants in the medium drought treatment was >65% to 100% while the range in the high drought treatment was >75% to 95% (Fig. 3D), indicating marginal effects of the drought treatments on survival. Therefore, although large numbers of the plants died back completely during the drought treatments, almost all of them regrew upon re-watering. In terms of interaction between population and drought treatment no significant effect was observed on any of (Appendix 1; plant height: $F_{(4, 47)} = 0.40$, $p = 0.81$; biomass: $F_{(4, 47)} = 0.55$, $p = 0.70$ and seed weight: $F_{(4, 46)} = 1.65$, $p = 0.18$) therefore, the interaction terms for each growth parameters were not included in the main ANOVA table as the models with interactions were not altered the conclusions of the study's finding.

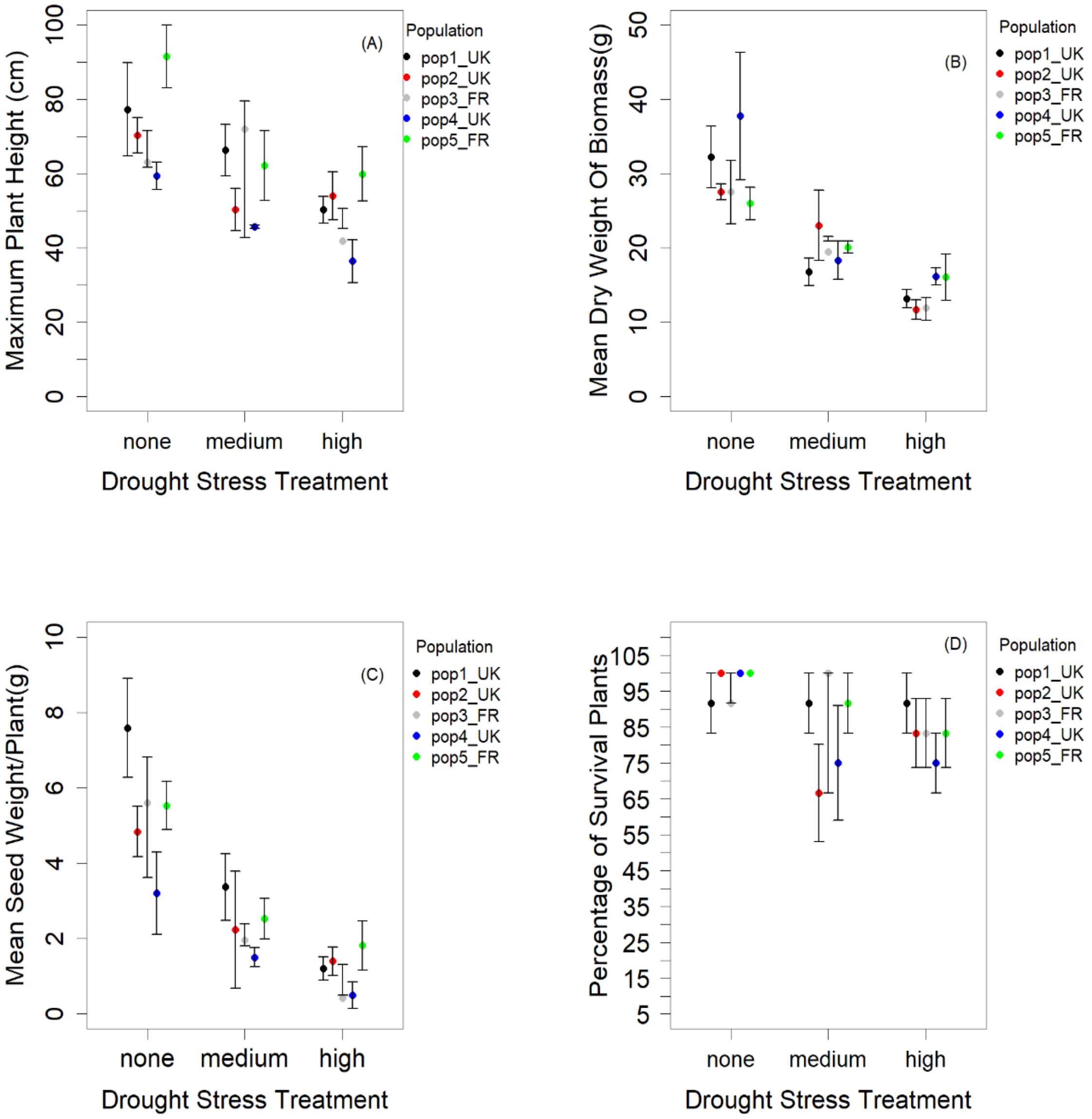

Figure 4. Effect of high (75%) and medium (25%) drought stress levels on *A. myosuroides* (A-D); average of maximum plant height (A) in centimetres, dryweight of aboveground biomass (B) in grams, seed production per plant (C) in grams and the percentage % of survival plants (D) across all the populations (P1-P5). Error bars represent standard errors.

2.3.1. b. Effect of drought treatment on *P. annua*

The drought treatments significantly affected plant height [Fig 3A, Table 4; $F_{(2, 27)} = 52.29$, $p = 5.18 \times 10^{-10}$], biomass [Fig 3B, Table 4; $F_{(2, 27)} = 12.71$, $p = 1.28 \times 10^{-4}$] and seed weight [Fig 3C, Table 4; $F_{(2, 27)} = 6.49$, $p = 0.005$] of *P. annua*. Despite the effects of the drought stress treatment on plant growth, no reduction in survival was observed (Fig 3D), and the species demonstrated very high tolerance to drought. Additionally, results show no interaction between drought stress and population for any of (Appendix 2; plant height: $F_{(4, 24)} = 0.48$, $p = 0.75$; biomass: $F_{(4, 24)} = 0.47$, $p = 0.76$, and seed weight: $F_{(4, 24)} = 0.25$, $p = 0.91$). Therefore, the interaction terms of drought and population omitted from the main ANOVA tables of the results as the models with interactions were not altered the conclusions of the study's finding. However, both main factors significantly affected the parameters in additive manner.

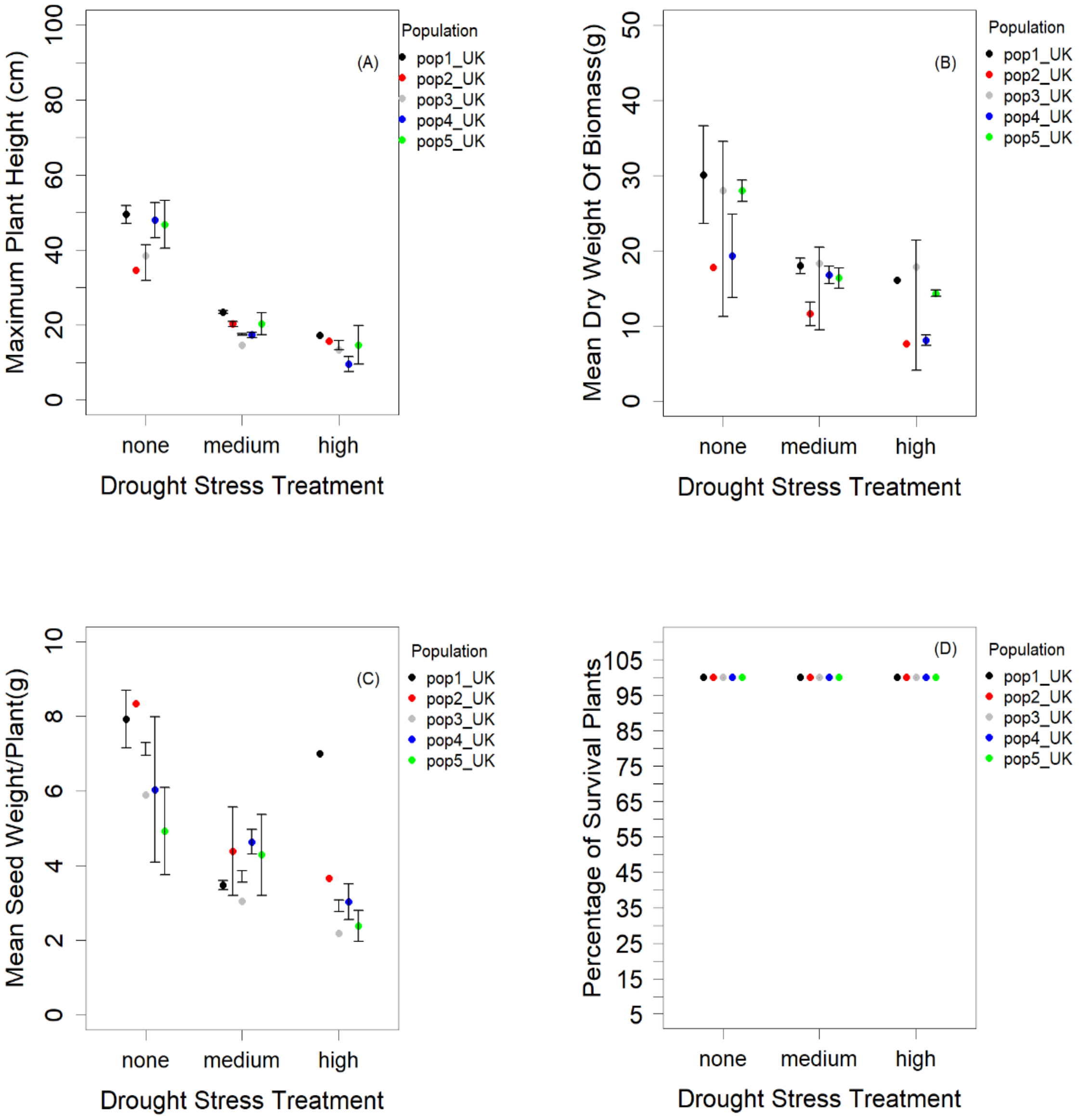

Figure 5. Effect of high (75%) and medium (25%) drought stress levels on P. annua (A-D); average of maximum plant height (A) in centimetre, dryweight of aboveground biomass (B) in grams, seed production per plant (C) in grams and percentage % of survival of plant (D). Error bars represent standard errors.

Table 3. Plant height, biomass and seed weight per plant of *A. myosuroides* under two different levels of drought stress medium and high (75% and 25% plant mortality). All the values are the logarithm of parameters value. Mean ± standard error are shown for *A. myosuroides* plants across all the populations, where the drought effect was significant. Asterisks indicate significant differences for both drought stress levels in comparison with controls. Significance levels from the ANOVAs are shown in the following way: $p < 0.05$, *, $p < 0.01$, **, $p < 0.001$, ***.

	Degrees of freedom	**Sum of Squares**	**Mean Square**	**F-value**	***p*-value**
Alopecurus myosuroides					
log (plant height)					
Replicate	3	0.297	0.099	1.797	0.160
Population	4	1.068	0.267	4.847	0.002**
Treatment	2	1.630	0.815	14.794	< 0.0001***
Residuals	50	2.755	0.055	-	-
log (biomass)					
Replicate	3	0.465	0.155	2.168	0.104
Population	4	0.206	0.052	0.723	0.581
Treatment	2	6.022	3.011	42.135	< 0.0001***
Residuals	50	3.573	0.072	-	-
log (seed weight)					
Replicate	3	0.446	0.149	0.286	0.835
Population	4	9.290	2.323	4.473	0.004**
Treatment	2	35.179	17.590	33.877	< 0.0001***
Residuals	49	25.441	0.519	-	-

Table 4. Results of ANOVA describing the effect of drought stress on the parental generation of *P. annua* including the treatment (medium and high drought) as well as controlling for origins (none drought), five populations and four replicates. Data on height, biomass and seed weight were log transformed. Significance of model terms indicated by asterisks: ‘***’ $p<$ 0.001, ‘**’ $p<$ 0.05, ‘•’ $p<$ 0.1.

	Degrees of freedom	**Sum of squares**	**Mean square**	**F-value**	***p*-value**
Poa annua					
Log (plant height)					
Replicate	3	1.203	0.401	5.078	0.006**
Population	4	1.390	0.348	4.401	0.007**
Treatment	2	8.259	4.130	52.288	<0.0001***
Residuals	27	2.132	0.079	-	-
log (biomass)					
Replicate	3	1.430	0.477	6.576	0.002**
Population	4	1.079	0.270	3.721	0.015*
Treatment	2	1.842	0.921	12.711	0.0001***
Residuals	27	1.957	0.072	-	-
log (seed weight)					
Replicate	3	1.047	0.349	1.770	0.177
Population	4	1.866	0.467	2.366	0.078 •
Treatment	2	2.558	1.279	6.487	0.005**
Residual	27	5.324	0.197	-	-

2.3.2. Herbicide resistance of F1 offspring

2.3.2.a. The response of *A. myosuroides* to herbicide application

There were significant effects of herbicide treatment ($x^2 = 31.30$, df = 1, $p< 1.00 \times 10^{-7}$) and the previous exposure of the P generation to drought treatments ($x^2= 7.86$, df = 2, $p = 0.02$) on the fraction of the F1 generation surviving following exposure to herbicides, measured as the number of plants surviving apparently intact (Fig 4a, Table 5a). When resistance was measured as plants that survived either damaged or intact versus those that were killed outright, (Fig 4b, Table 5a). However, this result was not significant when surviving plants measured as intact plants versus damaged and dead plants (Table 6a: $x^2 = 1.02$, df = 1, $p= 0.31$). On the other hand, the previous exposure of the P generation to drought treatment was significant ($x^2 = 13.09$, df = 2, p = 0.001).

There was significant interaction between previous drought exposure and herbicide treatment ($x^2= 28.36$, df = 2, $p < 1.00 \times 10^{-6}$). However, no significant interaction between herbicide and population was observed (Appendix 3: $x^2 = 3.42$, df = 4, $p =0.49$) thus, all the populations were combined together to analyse the effect of drought stress on the evolution of herbicide resistance in *A. myosuroides*. Therefore, the interaction term was omitted from the main ANOVA table as the models with interactions were not altered the conclusions of the study's finding. In terms of dry weight, the performance of surviving plants (dead and damaged) mirrored the outcome with respect to survival. There was a significant interaction between herbicide application and exposure of the parental generation to drought (Fig 4c, Table 7: $F_{(2, 67)} = 5.20$, $p = 0.008$) however, a significant interaction was observed between herbicide treatment and population (Appendix 5: $F_{(4, 63)} = 3.108$, $p = 0.021$).

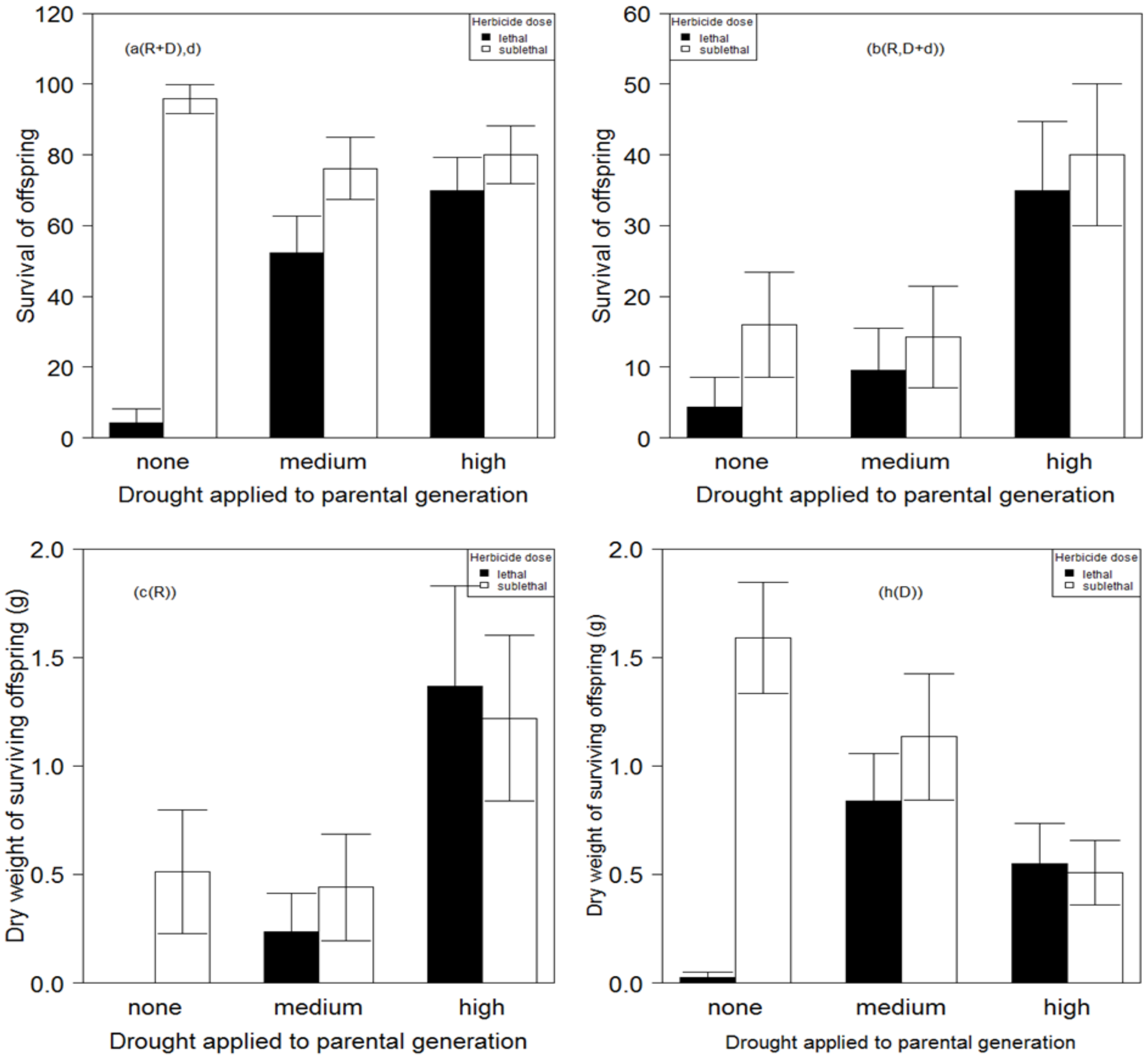

Figure 6. The effect of drought stress levels on the response of five *A. myosuroides* populations treated with lethal and sub-lethal doses of fenoxaprop-p-ethyl herbicide. (4a) percentage of survival of offspring when the resistant and damaged plants were combined. (4c and 4h) represent the effect of fenoxaprop-p-ethyl herbicide on the dry weight of intact and damaged plants, respectively. The error bars represent standard errors.

2.3.2.b. The response of *P. annua* to herbicide application

In contrast with *A. myosuroides*, there were no significant effects of herbicide treatment ($x^2 = 0.20$, df = 1, $p = 0.66$) and the previous exposure of the P generation to drought treatments ($x^2 = 1.57$, df = 2, $p = 0.46$) on the fraction of the F1 generation surviving following exposure to herbicides for *P. annua*; When resistance was measured as plants that survived either damaged or intact versus those that were killed outright (Fig 6A, Table 5b). In addition, there was also no significant effect when the resistance measured as plants survived (intact) versus those that were damaged and dead (Fig 6B, Table 5b). Furthermore, there was no significant interaction between previous drought exposure and herbicide treatment (Table 6b: $x^2 = 2.35$, df = 2, $p = 0.31$) as well as herbicide treatment and population (Appendix 4: $x^2 = 3.35$, df = 4, $p = 0.50$). In terms of dry weight, the performance of surviving plants showed significant effect of herbicide treatment (Fig 6C, 6D: $F_{(1,133)} = 7.50$, $p = 0.007$) on the *P. annua* plants. However, there was no significant effect of previous drought treatment in P generation on the evolution of herbicide resistance in F1 generation (Table 7: $F_{(2,133)} = 0.06$, $p = 0.94$). There was no interaction between herbicide application and exposure of the parental generation to drought (Table 7: $F_{(2, 133)} = 0.08$, $p = 0.92$). There was also no significant interaction between population and herbicide (Appendix 5: $F_{(4, 129)} = 1.06$, $p = 0.381$). Therefore, the interaction term between population and herbicide was omitted from the ANOVA of the main text and all the populations combined together to analyse the effect of drought stress on the evolution of herbicide resistance. As it would not alter the conclusion of the study's finding. In addition, these results confirmed that all the *P. annua* populations were highly resistant to the fenoxaprop-p-ethyl herbicide, irrespective of the drought treatment applied.

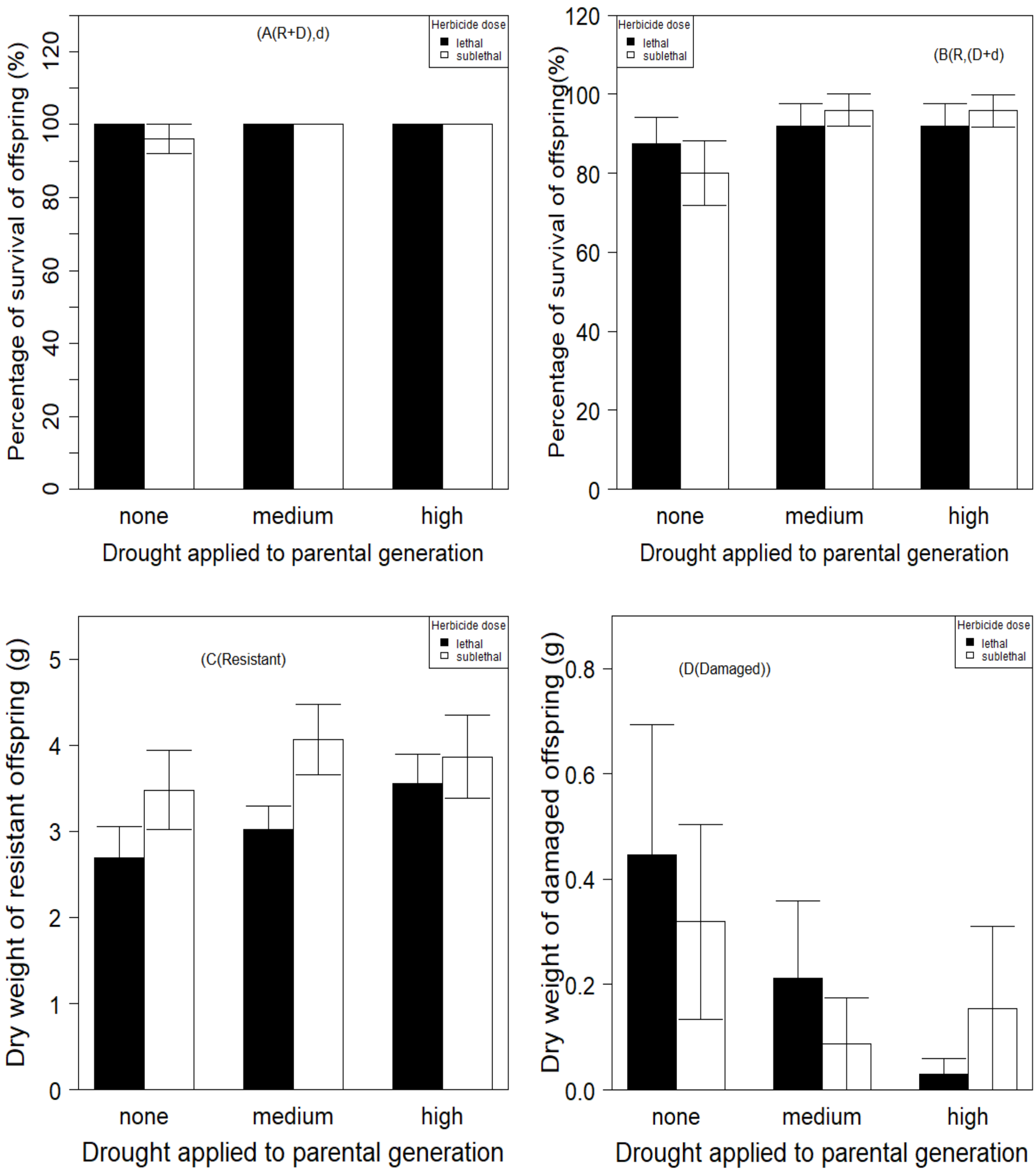

Figure 7. The effect of drought stress treatments on the response of five *P. annua* populations treated with lethal and sub-lethal doses of fenoxaprop-p-ethyl herbicide. (6A) percentage of survival of offspring when the resistant and damaged plants were combined. (6B) survival of offspring when the damaged and dead plants were combined. (6C and 6D) represent the effect of fenoxaprop-p-ethyl on dry weight of surviving and damaged plants. The error bars represent standard errors.

Table 5. Analysis of F1 generation plants that are resistant + damaged (i.e. survived) versus dead plants for (a) *A. myosuroides* and (b) *P. annua*. Results are from generalized linear models with binomial error and a log-link function. Significance of model terms indicated by asterisks: '*' *p*< 0.05 and '***' *p*< 0.001.

	Degrees of freedom	**Deviance**	**Residual degrees of freedom**	**Residual Deviance**	***p*-value**
(a)					
Null			129	172.260	
Blocks	4	4.2517	125	168.008	
Population	4	9.6903	121	158.318	*
Herbicide	1	31.2973	120	127.020	***
Drought	2	7.8589	118	119.162	*
Herbicide*drought	2	28.3554	116	90.806	***
(b)					
Null			147	15.3737	
Blocks	4	4.8491	143	10.5446	
Population	4	3.0621	139	7.4625	
Herbicide	1	0.1968	138	7.2656	
Drought	2	1.5733	136	5.6924	
Herbicide*drought	2	2.3479	134	3.3445	

Table 6. Analysis of F1 generation plants that are resistant (i.e. survived intact) versus dead or damaged (a) *A. myosuroides* and (b) *P. annua*. Results are from generalized linear models with binomial error and a log-link function. Significance of model terms indicated by asterisks: *, $p<0.05$ and, **, $p<0.01$.

	Degrees of freedom	**Deviance**	**Residual degrees of freedom**	**Residual Deviance**	***p*-value**
(a)					
Null			129	127.283	
Blocks	4	6.0950	125	121.188	
Population	4	13.0558	121	108.133	*
Herbicide	1	1.0195	120	107.113	
Drought	2	13.0938	118	94.019	**
Herbicide*drought	2	1.2682	116	92.751	
(b)					
Null			147	92.660	
Blocks	4	4.5793	143	88.081	
Population	4	5.6043	139	82.477	
Herbicide	1	0.0000	138	82.476	
Drought	2	4.0262	136	78.450	
Herbicide*drought	2	1.3751	134	77.075	

Table 7. Results from analysis of variance from linear models describing the relationship between variance, drought, herbicide and drought*herbicide interaction for dry weight of surviving plants for both species *A. myosuroides* and *P. annua*. Significance of model terms indicated by asterisks: '*' $p < 0.05$ and, '**' $p < 0.01$.

	Degrees of freedom	Sum of Squares	Mean Square	F-value	*p*-value
Alopecurus myosuroides					
Blocks	4	9.0963	2.2741	8.2263	<0.001***
Population	4	1.1589	0.2897	1.0480	0.3892
Drought	2	0.5039	0.2519	0.9113	0.4069
Herbicide	1	0.0068	0.0068	0.0244	0.8762
Drought*herbicide	2	2.8734	1.4367	5.1972	0.0080**
Residuals	67	18.5216	0.2764	-	-
Poa annua					
Blocks	4	4.9695	1.2424	8.1087	<0.001***
Population	4	1.8382	0.4595	2.9993	0.0208
Drought	2	0.0195	0.0097	0.0636	0.9384
Herbicide	1	1.1488	1.1488	7.4978	0.007**
Drought*herbicide	2	0.0246	0.0123	0.0804	0.9228
Residuals	133	20.3776	0.1532	-	-

2.4. Discussion

2.4.1. The relationship between abiotic stress and herbicide resistance

This study has demonstrated that exposure to drought can increase the frequency of herbicide resistance across multiple grass weed populations with no previous herbicide exposure. The results suggest a close relationship between abiotic stress and the rapid acquisition of herbicide resistance in this grass weed, which appeared in the offspring of plants exposed to both drought treatments. Previous studies have suggested a relationship between the mechanism that endows resistance in weeds and resistance to abiotic stress, with the mechanism that typically governs NTSR in weeds being a subset of the mechanisms that govern responses to abiotic stresses (Cummins et al., 1997, Letouze and Gasquez, 2001, Rosenhauer et al., 2015). Interactions between stress resistances have been noted previously. For example, work with *Poa pratensis* established that, prior exposure to freezing significantly impacts survival and growth after subsequent exposure to drought (Kong and Henry, 2016). Furthermore, in a recent study it has been shown that under drought stress, repeated exposure to sublethal dose of herbicide decreases the *E. colona* (junglerice) sensitivity to herbicide, apparently due to “imprinted” upregulation of metabolic and protection genes in response to drought and herbicide stresses (Lariza et al., 2020). However, the current study is the first to identify the impact of different drought stress levels in conferring herbicide resistance in weeds. Results showed that drought stress resistance in the first generation of droughted parent plants can confer herbicide resistance in the subsequent generation. Further experiments would be required to establish whether the resistance is inherited across subsequent generations.

2.4.2. The role of oxidative-stress-related physiological pathways in response to biotic and abiotic stresses.

Plants have the ability to develop different types of antioxidants (Bakhsh and Hussain, 2015). Glutathione-s-transferases (GSTs) have the ability to coordinate tolerance to abiotic stress via their capability to control redox signalling pathways that transcriptionally stimulate resistance genes (Roxas et al., 1997). Reade et al., (2004) concluded that GSTs may defend against herbicides when their activity or abundance increases, even if they are not contributing directly to herbicide metabolism. Additionally, it has been confirmed that the contribution of GSTs in the evolution of multiple herbicide resistance (MHR) in *A. myosuroides* occurs through oxidative stress tolerance as well as detoxifying herbicides by stimulating their conjugation with glutathione (Preston et al., 1996, Cummins et al., 1999). Thus, this potential mechanism is presumed to be responsible for the evolution of herbicide resistance in grass weed populations that have been exposed to abiotic stress. Furthermore, (Morant et al., 2003) documented the responsibility of plant cytochrome P450s in mediating a wide variety of secondary metabolism in plants, participating in the majority of plant defences against biotic and abiotic stress.

2.4.3. Effect of drought stress on *A. myosuroides* and *P. annua*

Drought stress affected the growth, development and production of weed plants that led to significant reduction in the weight of seed produced. Abiotic stresses (e.g. drought, salinity, high temperature and frost) negatively affect the growth, development and output of plants and lead to significant reductions in plant productivity (Bakhsh and Hussain, 2015). An increasing level of drought stress resulted in greater reductions in plant height, biomass and seed weight of *A. myosuroides* and *P. annua*. However, survival of plants across all the populations was less affected by drought stress. All plants of *P. annua* demonstrated high resistance to the different drought stress levels, while to some extent there were differences between surviving plants of *A. myosuroides* in response to drought stress. Drought responses are mediated via continuous adjustment of morphological, physiology and biochemical characters during the life span within the framework of the genetic background leading to variation among genotypes and populations (Beck et al., 2007, Pastori and Foyer, 2002).

2.4.4. Herbicide dose response

The experimental herbicide treatment established that *A. myosuroides* can rapidly develop significant resistance to herbicide in the subsequent generation after pre-exposure to drought stress, irrespective of the herbicide dose. As predicted, exposure to drought stress in the parental generation increased both the survival and dry weight of the F1 generation of *A. myosuroides* after a lethal dose of herbicide. The approach of using different drought stress levels could also be of further use in assessing other arable weed species, for example other common grass weeds or broadleaved weeds, and using different herbicide modes of action to test for broader effects of drought stress on the evolution of herbicide resistance in weeds.

Furthermore, results for *P. annua* (self-pollinating grass species) demonstrated high frequencies of resistant plants pre-existing across all the populations irrespective of drought and no significant differences between herbicide doses (lethal and sub-lethal) in comparison with control plants. Consequently, data showed that high resistance to fenoxaprop-p-ethyl in the first generation of droughted stress treatment was already dominant in *P. annua* (self-compatible) populations with no previous history of herbicide treatment. The evolution of herbicide resistance in the self-pollinated species (*P. annua*) may have occurred at a high rate due to the high drought tolerance of this species, which leads to higher constitutive herbicide resistance, equivalent to that of the cross-pollinated species with exposure to drought stress. Additional reason for this resistance, is that may be the fenoxaprop-P-ethyl herbicide is not the correct herbicide to be used for controlling *P. annua* species.

Selection for herbicide resistance is typically thought to evolve through the action of natural selection on standing genetic variation for herbicide responses (survival and growth) that exists within plant populations (Neve and Powles, 2005). However, the extremely high survivorship of the parental generation after droughts in our experiment implies that natural selection was not responsible in this case, and instead indicates that herbicide resistance in the F1 generation may acquired via a non-genetic mechanism. Until now, the roles of very few non-genetic factors in the evolution of herbicide resistance have been investigated (Delye et al., 2013b). However, epigenetic mechanisms may mean that the environment can affect gene expression without influencing the DNA sequence (Germani Concenço, 2016). Environmental conditions undoubtedly impact plant physiology, and variation in the expression of resistance to herbicides with low temperature treatment has been recently confirmed (Vila-Aiub et al., 2013, Liu et al., 2015). Considering the interactions between genes and environment may consequently become important for forecasting and managing the acquisition of herbicide resistance. Non-genetic processes are widely involved in the regulation of stress responses (Boyko and Kovalchuk, 2008), and gene silencing is one of the epigenetic mechanisms of most concern for herbicide resistance in plants (Germani Concenço, 2016). The potential for epigenetic mechanisms will be the future focus of this project, further investigating its role in the evolution of herbicide resistance in grass weeds.

2.5. CONCLUSION

The result of the current study, suggests that environmental factors play a crucial role in the ability of grass weeds to resist herbicide. We especially underlined the importance of drought stress effects on improving the herbicide resistance in the F1 generation of *A. myosuroides* grass weed. Experimentally, we demonstrated that high drought stress can improve the mechanism of stress defence. In addition, exposure to drought stress has the potential to accelerate the frequency of herbicide resistance in different grass weed populations with no previous exposure to herbicide. This work has demonstrated a close relationship between environmental stress such as drought and the rapid evolution of herbicide resistance in grass weeds. Furthermore, the increased frequency of resistant plants confirmed that the potential for rapid selection pressure to fenoxaprop-p-ethyl in the first generation of droughted stress treatment was dominant in both species, with the consequence failed weed control. Further investigation in the role of epigenetic mechanisms in underpinning the evolution of herbicide resistance in grass weeds will help to understand the heritability of resistance to herbicide.

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CHAPTER THREE

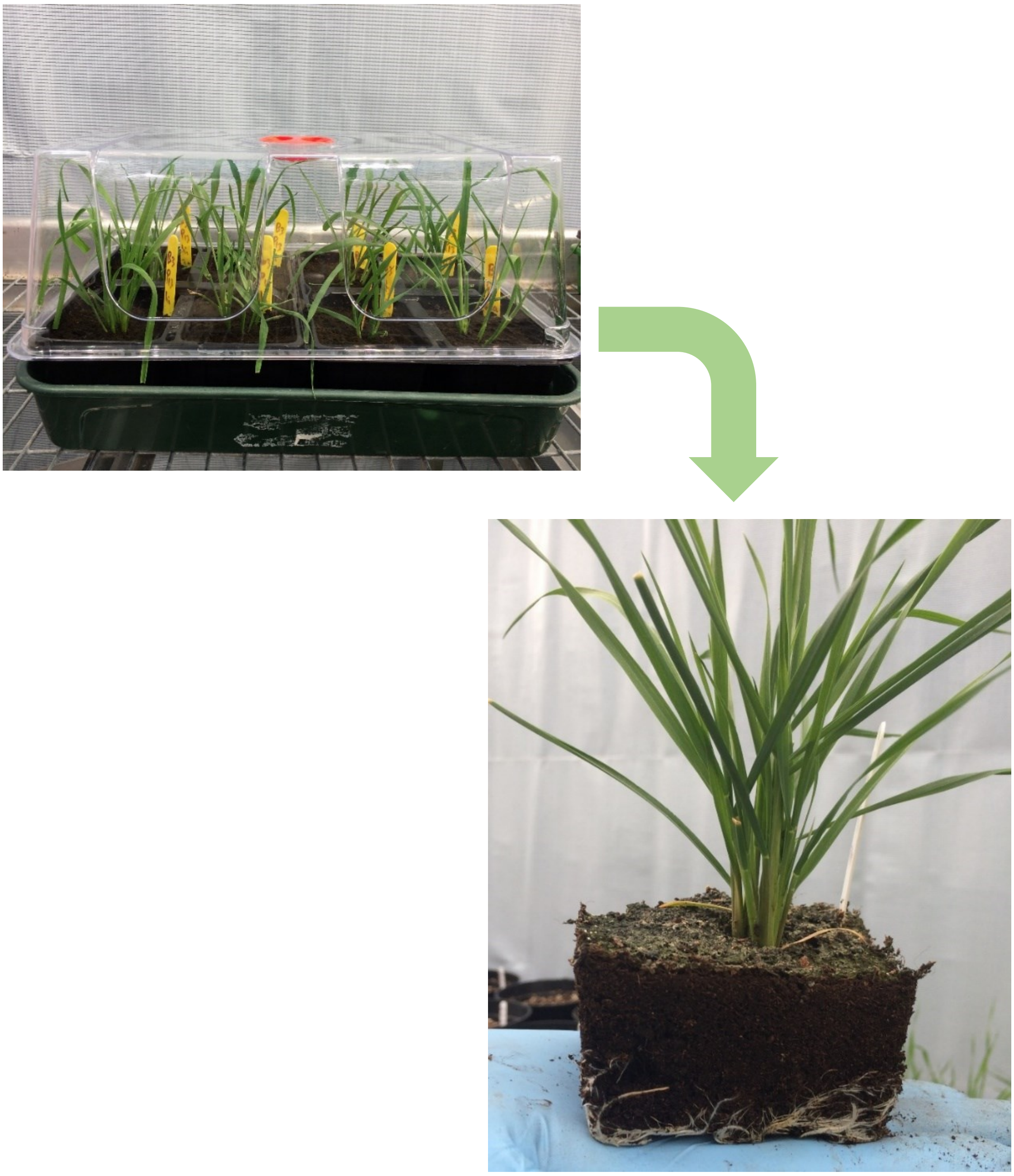

Figure 1. Pictures of cloned (epigenetically propagated) *A. myosuroides* for studding the role of epigenetic mechanism in the evolution of herbicide resistance in *A. myosuroides* population. The pictures are from the plants samples that used for this study.

STATEMENT OF CONTRIBUTIONS

The initial idea of this chapter were developed by Prof. Robert Freckleton, the candidate and Prof. Colin Osborne. The method which was used to conduct cloning process and the drought stress was constructed by the candidate. All the data collection and writing were carried out by the candidate. Data analysis was carried out by the candidate with editorial advice from Prof. Robert Freckleton. General editorial advice was provided by Prof. Robert Freckleton and Prof. Colin Osborne.

CHAPTER THREE

MECHANISMS THAT UNDERPIN THE EVOLUTION OF HERBICIDE RESISTANCE IN THE WEED *ALOPECURUS MYOSUROIDES*: EPIGENETICS AND THE ROLE OF ENVIRONMENTAL STRESS RESPONSES

Abstract

Long-term exposure of plants can confer evolved resistance to previous and present abiotic and biotic stresses. Plants have evolved physiological mechanisms in order to survive repeated stresses, and such responses may evolve quickly following exposure. Herbicide resistance is a trait that evolves quickly through the co-option of a suite of physiological mechanisms that originally arose to allow plants to survive environmental stress. Here two experiments were set up to identify whether (i) exposure to environmental stress (e.g. drought) in the parental generation promotes the evolution of resistance in the offspring; (ii) such evolution is mediated through inherited genetic change or via non-genetic (e.g. epigenetic) mechanisms. Individuals from different populations of black-grass (*Alopecurus myosuroides*) were experimentally cloned to produce pairs of genetically identical plants. One plant of the cloned pair was exposed to high drought stress, and the second was grown under well-watered condition (control group) as the first generation (F1) under a drought treatment. Then I tested whether there were consequent effects on herbicide resistance traits in the F1. In terms of survival, significant resistance was observed to lethal and sublethal herbicide doses in the F1 plants when the parental generation had been exposed to drought, in comparison with clones from the well-watered control treatment. Generally, the results suggest that exposure of the cloned grass weed populations to drought stress can confer herbicide resistance in subsequent generations and that the mechanism conferring heritability of herbicide resistance may be epigenetic. Knowledge that this herbicide resistance may be acquired epigenetically promotes better understanding of the evolution of the mechanism of herbicide resistance in grass weeds.

Keywords: fenoxaprop-p-ethyl herbicide, drought tolerance, epigenetic mechanism, drought-herbicide interaction, lethal dose, grass weed, physiological pathway

3.1. Introduction

Plants are unceasingly exposed to a large number of stresses, both biotic and abiotic, that adversely impact their growth, productivity and reproductive success. In order to survive stressful conditions, plants have evolved complex and sophisticated strategies (Goh et al., 2003,Anjum et al., 2011, Boyko and Kovalchuk, 2008, Wang et al., 2011, Golldack et al., 2011, Ding et al., 2012, Fu and Dong, 2013, Kinoshita and Seki, 2014).

Resistance to herbicide is an important example of rapid evolution to an environmental stress. When exposed to strong selection pressure, the selection of herbicide resistant biotypes occurs (Gressel, 2011). Although evolved resistance has resulted from selection for rare target site resistance alleles (Gressel, 2011), which is mainly linked with mutations in the gene that codes for the herbicide target enzyme, more commonly, selection for resistance results from sublethal doses, possibly via the repeated adaptation of number of secondary additive genes (Gressel, 2011). This latter form of resistance is termed metabolic resistance (Yu and Powles, 2014). Herbicide resistant biotypes can arise in only two or three generation, for example in experimental populations of *Lolium rigidum* (Neve and Powles, 2005b) and *Alopecurus myosuroides* (Chapter 2, this thesis).

Evolved herbicide resistance in weedy plant species due to increased metabolic ability to detoxify herbicides (metabolic resistance) is a growing problem (Yu and Powles, 2014). In weedy plant species, metabolic herbicide resistance was reported for the first time in ryegrass in Australia and in black-grass in the United Kingdom in the 1980s (Yu and Powles, 2014). It is currently progressively becoming a potential hazard in various crop-weed species and is an immediate danger to the sustainability of herbicides, and hence to world crop yield (Yu and Powles, 2014). Metabolic resistance is particularly problematic, because it endows herbicide resistance to groups of different chemicals and sites of action, and may spread to new herbicides. Cytochrome P450 monooxygenase, glycosyl transferase and S-transferase glutathione are frequently involved in this mode of herbicide resistance (Yu and Powles, 2014).

Throughout the world, studies have been carried out on both the molecular and physiological mechanisms mediating herbicide resistance in weedy plant species. this understanding aids the development of effective strategies to prevent resistance from evolving and furthermore to manage resistant weed populations (Powles and Yu, 2010). Herbicide resistance mechanisms are classified in nature as either 'target site (TS)' or 'non-target site (NTS)'. Non-target site mechanisms of herbicide resistance appear to be more complex than target site mechanisms and are frequently part of time-developing plant stress responses (Delye et al., 2013b). Non-target-site resistance includes mechanisms that decrease the amount of active herbicide reach the target site (e.g. decreased herbicide absorption or translocation, improved sequestration of herbicides or increased herbicide metabolism) (Powles and Yu, 2010, Han et al., 2014).

It is important to recognise that plants resist herbicides through a suite of mechanisms that originally evolved to deal with environmental stresses. Similar to several abiotic stresses, numerous herbicides cause oxidative stress in plants (Ian et al., 2013, Iwakami et al., 2014). Examples of such mechanisms include the detoxification of reactive oxygen species (ROS) caused by stress conditions (Ian et al., 2013, Iwakami et al., 2014). Previously, specific and other mechanisms of detoxification have been reported in major weed species (Kreuz et al., 1996, Delye et al., 2013b), where they can drive to herbicide resistance. These mechanisms underpin NTS resistance that is not linked with changes in the target site of the herbicides. NTS resistance has been documented repeatedly in grass weeds due to increased detoxification activity of glutathione S-transferases (GST) (Tal et al., 1995) or cytochrome P450 monooxygenases (CYT-P450) enzymes (Holtum et al., 1991, Han et al., 2013).

Plants have the ability to ‘remember’ previous environmental exposure and can benefit from this ability when exposed in the future (Kinoshita and Seki, 2014, Simpson et al., 2019). (Ding et al., 2012) in a study on *Arabidopsis* plants found that following exposure to drought stress conditions, plants respond to subsequent stress by increased rapid adaptive gene expression, compared with plants not previously exposed to a drought stress. Thus, previous experience of abiotic stresses can alter the response of individual plants to subsequent stresses (Tahkokorpi et al., 2007, Onate et al., 2011). This phenomenon can be described as the “priming effect” (Tanou et al., 2012) or “stress memory” (Ding et al., 2012, Walter et al., 2013). A series of mechanisms including epigenetic modifications assumed to be involved in such responses to previous exposure (Scholes and Paige, 2015), in addition to physiological, metabolic and morphological changes (Bruce et al., 2007, Walter et al., 2013).

Epigenetic mechanisms are thought to play an essential role in the regulation of the expression of stress response genes (Chinnusamy and Zhu, 2009), via small RNAs, histone modifications and DNA methylation. These can be passed on to the next generation, and hence this is a possible mechanism for stress ‘memory’ (Chinnusamy and Zhu, 2009, Kinoshita and Seki, 2014). Epigenetic mechanisms have been shown to regulate genetic functions such as replication, transcription, DNA repair, gene transposition and the cell differentiation. Both the generation of small RNAs and modifications in chromatin have been shown to contribute to transcriptional and post-transcriptional control of gene expression, which is crucial for environmental stress responses (Madlung and Comai, 2004, Angers et al., 2010).

Despite the evidence from stress responses, the possibility of whether plant responses to herbicides could be triggered by epigenetic changes is unknown (Markus et al., 2018). In an Investigation of atrazine in rice (*Oryza sativa*) showed that, in general, histone methyltransferases, DNA methyltransferases and DNA demethylases were differentially adjusted in response to the herbicide atrazine (Yi Chen et al., 2016). This indicates that epigenetic variations were involved in activation of particular genes responsible for the detoxification of atrazine. Consequently, epigenetic mechanisms could be involved in herbicide resistance in weeds, especially via NTS resistance (Markus et al., 2018).

Work in the previous chapter showed that herbicide resistance may be acquired in the F1 generation of *A. myosuroides* after exposure of the parental generation to drought. Here the possible role of epigenetic mechanisms was investigate in this acquired herbicide resistance. Plants of different populations of *A. myosuroides* were cloned to control for genetic background, then exposed the cloned plants to different levels of drought stress (i.e. none and high). Offspring of the survivors were exposed to two levels of fenoxaprop-p-ethyl herbicide, testing whether an epigenetic mechanism could successfully explain the evolution of herbicide resistance in the first generation of cloned-droughted parental generation.

A. myosuroides is an annual grass weed of arable habitats and within cereal crop fields, and is the most important herbicide resistant weed occurring throughout European countries (Moss et al., 2007). In the United Kingdom, it is the major annual grass weed through the main arable area of England (Preston et al., 2002).

3.2. Materials and methods

3.2.1. Plant material

In July 2016, seeds of *A. myosuroides* (black-grass) populations from a set of 15 arable winter cereal fields across England were collected (Figure 3). Following collection, seeds were threshed and cleaned to eliminate unfilled seeds and debris, then stored in a paper bag in dark/cool conditions (i.e. a fridge at 5 °C) after collection until conducting the current experiment. A population was defined as *A. myosuroides* plants growing in a single field and seeds were collected randomly.

3.2.2. Germination test

In September, 2016, germination tests of all 15 populations of *A. myosuroides* conducted were in growth cabinets. Ten seeds of each population were placed in 90 mm-diameter petri dish containing two layers of Whatman Grade 1 filter paper (Whatman International Ltd., Maidstone, U.K.). 5 ml of distilled water were delivered to each petri dish. Each petri dish was sealed with a piece of Parafilm to reduce evaporation of water, and the petri dishes were placed in the growth incubator for three weeks. Temperatures were set to 21/15 °C day/night fluctuations with 8 hour daylight and 16 hour dark periods regime. The photoperiod was set at 8 h to coincide with the high temperature photoperiod. As the germination rate was very low, consequently in October 2016, the seeds of all populations were located in an incubator at 30 °C for six weeks to break primary seed dormancy. After exposing the seeds to heat shock, in December, 2016, we repeated the germination test as described previously, and the germination rate was 100 percent.

3.2.3. Plant cloning and drought stress treatment

The response of cloned plants of *A. myosuroides* to drought stress was investigated in a greenhouse environment (Arthur Willis Environment Centre) at The University of Sheffield. The objective was to propagate first generation plants (F1) from parents (P) that had been cloned and exposed to different drought stress levels.

In March 2017, nine seeds of each population were sown at a planting depth of 50 mm below the soil surface in 100 mm in diameter, 215 mm depth and 4 L volume circular plastic pots. Pots contained a 1:1 mixture of standard potting of compost and vermiculite. Saucers were used for each pot to avoid leaching soil nutrients. The pots were maintained in a greenhouse with a 14 h day length and supplementary lighting. Temperature was set to 23 °C during daylight hours, and 15 °C during night-time. After seed planting, pots were well-watered thoroughly from above to ensure seed germination. Following emergence, seedlings similar in height (40 mm) and number of leaves (1 leaf) were thinned to three plants per pot to ensure sufficient plant material.

In April 2017, five weeks after sowing, at the 3-4 tiller stage each plant was divided into two clones (Figure 1). The root of cloned plants was cut to approximately one centimetre and the plant shoots were trimmed into 4-5 cm. The cloned plants were replanted in a clone-propagator tray, and maintained in the green house for two weeks. On April, 24th 2017 after two weeks of replanting, all the cloned plants were re-potted in 100 mm in diameter, 215 mm depth and 4 L volume circular plastic pots. Pots contained a 1:1 mixture of standard potting compost and vermiculite, and allowed to establish for one week before initiating the drought stress treatment.

The experiment was conducted as randomized complete-block design with six replicates (blocks). A high drought treatment was applied to half of the pots by withholding water until the shoots of approximately 75% of plants had died back. The first period of high drought treatment was started on 1st May, 2017 until 23rd May, 2017 (approximately 3 weeks); the second period was started on 27th May, 2017 and continued until 22nd June, 2017 (roughly 4 weeks) and the last period of drought treatment was applied from 26th June, 2017 until 28th July 2017 (more than 4 weeks).

After each period of drought treatment, the plants were re-watered as normal (twice per week) until the appearance of shoots. Visual assessment by the same observer was made using consistent criteria to monitor the growth and mortality rate for the drought group.

Plant height, above ground biomass and seed weight were measured, in addition to the number of surviving and dead plants. Plant height was recorded before the harvest of plants in August, 2017. The shoots of all plants were measured from the soil level to the end of the longest flowering shoot. During the anthesis stage and before pollen emission, the *A. myosuroides* plants were covered (3 pots together) by a pollen-proof bag to ensure that cross-pollination only occurred among members of the same population (Neve and Powles, 2005).

At harvest time, after 6 weeks of withholding irrigation the aboveground biomass of a single mature plant per pot was harvested on 15th September, 2017. This allowed the impact of water stress on aboveground biomass production to be evaluated. Aboveground biomass was hand-harvested and directly weighed with a scale (EP 6102C, max 100 g, decimal (d) =0.01 g, Ohaus Corporation, Parsippany, NJ, USA). After harvesting, seeds of each plant were separated and weighed using a high precision scale (GH-252-EC, max = 250 g, min= 1 mg, d=0.01/0.1 mg, A&D Instruments, Abingdon, UK). Seeds were stored in dark and cool conditions (fridge 4 °C) until further use. The percentage reductions in plant height, biomass and seed production were calculated relative to the unstressed control plants for drought treatment level.

During July, 2017, some plants were infested with aphids. Aphiline biological control agent containing the Braconid parasitoide wasp *Aphidius colemani* was used to control the aphids (Aphiline Mix, Bioline, AgroScience). This wasp stings and parasitizes smaller aphid species. Additionally aphids from infested leaves were removed by hand.

3.2.4. Response to herbicide treatment

Germination tests were carried out in March 2018 for all the F1 offspring of the parental clone x drought experiment. These were undertaken in growth cabinets by placing 10 seeds of each population in 90 mm-diameter petri dish containing two layers of Whatman Grade 1 filter paper (Whatman International Ltd., Maidstone, U.K.), and 5 ml of water. Each Petri dish with its cover was sealed with a piece of Parafilm to reduce evaporation of distilled water and the petri dishes were placed in growth incubator for five weeks. Temperatures were set to 21/15 °C day/night fluctuations with 8 hour daylight and 16 hour dark periods. The photoperiod was set at 8 h to coincide with the high temperature photoperiod.

As previously, the germination rate was very low, and consequently the seeds of all populations were located in an incubator at 30 °C for six weeks to break primary seed dormancy. After exposing the seeds to heat shock, in June, 2018 the germination test was repeated as described previously, in addition to viability test to ensure that the seed dormancy was broken and seeds were shown to be viable. Following the incubation for three weeks all the seeds were removed from the incubator to record the germinated seeds and to conduct seed viability tests. Each non-germinated seeds was subjected to the simple pressure test, seed was put between two fingers to evaluate viability. White, firm embryos were considered viable, while brown, soft embryos were considered non-viable or dead (Baskin and Baskin, 2014). Out of 15 populations of the F1 generation (high drought treatment) seeds of five populations were viable and germinated. Therefore, the herbicide experiment was carried out using the five populations that possessed high viability and germination percentages in both treatments (“none” and “high” drought).

Herbicide dose-response trials were conducted to investigate the effect of exposure to drought in the P generation on herbicide resistance in the F1 generation. At initiation of the dose–response experiments in August 2018, nine seeds from the F1 generation of the five populations of *A. myosuroides* were planted in circular plastic pots (100 mm in diameter, 215 mm depth and 4 L volume). Pots contained a 1:1 mixture of standard potting of compost and vermiculite. Saucers were used for each pot to avoid leaching soil nutrients. The pots were maintained in a greenhouse environment (Arthur Willis Environment Centre, University of Sheffield). Temperature was set to 23 °C during daylight hours, and 15 °C during night-time with a 14 h day length and supplementary lighting. Following seed sowing, pots were thoroughly watered from above to ensure seed germination and through the course of the experiment, plants were watered as required. After seedling emergence, seedlings similar in height and number of leaves (height: 40 mm, and 1 leaf) were thinned to three plants per pot.

Fenoxaprop-p-ethyl was used because resistance to this herbicide is linked with selection for NTSR (Letouze and Gasquez, 2001; Delye et al., 2007; Delye et al., 2015) or enhanced metabolism, this is where the target plants are able to detoxify the herbicide before it reaches the target site. Additionally, the occurrence of NTSR is predicted to be derived by exposure to abiotic stress. Also the herbicide is used very widely to control *A. myosuroides*. For example, in Germany, fenoxaprop-p-ethyl is one of the most common herbicides used in cereal crop fields for selective control of *A. myosuroides* over long periods (Rosenhauer et al., 2015). Furthermore, the herbicide was used in the previous study (chapter 2) to investigate how rapidly the evolution of herbicide occurs, thus all the factors mostly kept the same in this study (epigenetic role in the evolution of herbicide resistance).

At the 3-4 leaves stage in September 2018, the seedlings were sprayed with fenoxaprop-p-ethyl herbicide (“Puma Super” – 69 g a.i. L^{-1}, Bayer Crop Science) using two different doses: a lethal dose of 40 g a.i. h^{-1}, and a sub-lethal dose 20 g a.i. h^{-1}. There were four replicate pots per population for both drought treatment by dose combination, and there were two pots per dose per drought treatment with the five *A. myosuroides* populations. There were 20 pots per block (10 blocks in total, giving 200 pots) and the pots were completely randomised within blocks. One set of F1 plants for each clone x drought treatment (cloned then exposed to drought) from each population was sprayed with fenoxaprop-p-ethyl herbicide at 40 g a.i. h^{-1} (lethal dose) and the other set of F1 plants from clone x drought treatment was sprayed with fenoxaprop-p-ethyl herbicide at 20 g a.i. h^{-1} (sublethal dose), using a spray nozzle sprayer 3L of capacity delivering herbicide in Max 45 PSI, 3BAR.

Four weeks after herbicide application (October 2018) dead and damaged plants were assessed. This was carried out by the same observer for all plants. Plants were scored as dead if they had yellow/burned leaves following herbicide treatment. Surviving plants were categorised in two ways to account for the differential outcomes of exposure to herbicide: plants were categorised as surviving if they showed no visible effects of herbicide exposure, or damaged if they survived but with obvious effects on above ground tissues.

To evaluate the impacts of herbicide exposure, we calculated survival and damage in two ways. First, surviving and damaged individuals were combined, and we expressed these numbers as a proportion of the plants treated. Second, the number of plants that survived was expressed relative to those that died or were damaged. These two approaches measure resistance in slightly different ways. The first measures the plants that survive application, whether they are damaged or not; the second measures those plants that are unaffected by the herbicide application. One plant per pot was harvested at the soil surface and dried at 50 °C for three days. The dry weight of each surviving and damaged plant was measured.

After harvest the surviving and damaged plants (1 plant pot^{-1}) were re-arranged in the greenhouse. Plants were grown to maturity in the greenhouse condition, to allow production of seed for each surviving plant within each population after herbicide treatment. To determine the effect of fenoxaprop-p-ethyl herbicide on growth parameters then plant height, biomass and seed weight were recorded. Plant height was recorded before harvesting of plants in January 2019. The shoots of all plants were measured from the soil level to the end of the longest flowered shoot. During the anthesis stage and before pollen emission plants were covered (same treatment within same population pots together) by a pollen-proof bag to ensure that cross-pollination only occurred among members of the same population (Neve and Powles, 2005a). During harvest time and after 4 weeks of withholding irrigation the aboveground biomass of a single mature plant per pot was harvested 28th February, 2019. Because some tillers regrew during withholding irrigation period, the harvested plants were dried in a drying cabinet at 50 °C for 72 hours. The dry weight of each harvested plant was measured using high precision scales (GH-252-EC, A&D Instruments). Following drying, separating and cleaning seeds were weighed using precision scale (GH-252-EC, max = 250 g, min= 1 mg, decimal=0.01/0.1 mg, A&D Instruments, Abingdon, UK). Seeds were stored in paper bags in dark and cool conditions (fridge 4 °C) until further use. The protocol used in this study are shown in a diagram (Figure 2) for further clarification.

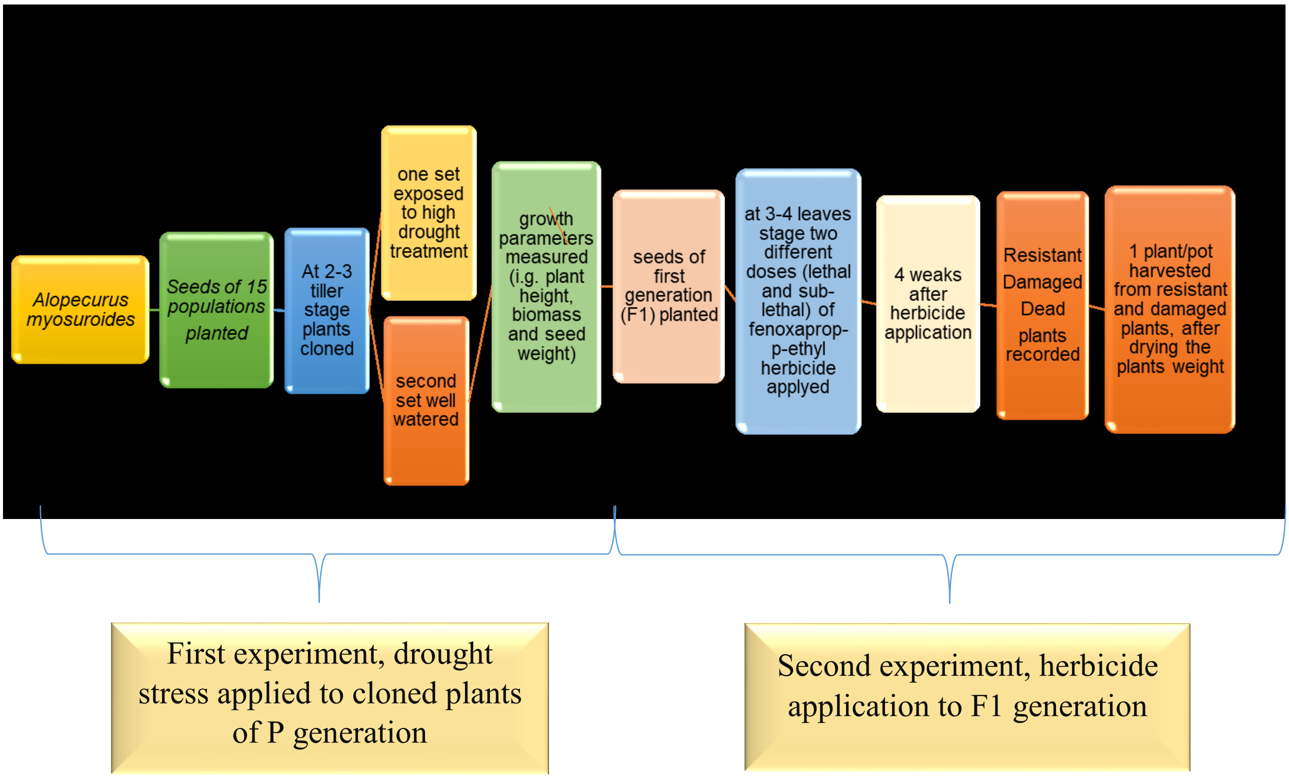

Figure 2. Shows the summary of the methods of both experiments for this study. First part of the experiment: seeds of 15 different populations of *A. myosuroides* were planted. At the 2-3 tillers stage, plants were cloned into two identical plants. After repotting the cloned plants, one set of the cloned plants were exposed to high drought treatment and the second set were grown under well-watered condition. Growth parameters and survival were recorded for both sets. Second part of the experiment: seeds of first generation of cloned and droughted parental plants were planted and then at the 3-4 leaves stage plants were exposed to two different doses of fenoxaprop-p-ethyl herbicide. Four weeks after herbicide application resistant, damaged and dead plants were recorded. In addition, one plant per pot was harvested and dried to record the plant biomass.

3.2.5. Statistical analysis

R (R Core Team, 2019) and *lme4* (Douglas et al., 2015) were used to perform a linear mixed effects analysis of the response of cloned P generation to drought treatment (first experiment). Restricted maximum likelihood (REML) was used to estimates the parameters. To test the effects on plant height, biomass and seed production we assumed a Gaussian error distribution. Drought was entered into the model as a fixed effect. Clone ID was included as random effects. *P*-values were obtained by running the function of lmertest of the full model. Data on the effect of drought in cloned plants on plant height, biomass, seed mass and the survivorship were analysed.

For the herbicide spray experiment (second experiment) Generalized Linear Mixed-Effect Models fit by restricted? maximum likelihood (Laplace Approximation) and *lme*4 (Douglas et al., 2015) were used to explore the effect of drought stress in parental cloned plants upon the evolution of herbicide resistance in the F1 generation for all survived populations. A binomial error structure was assumed in this experiment as the dependent variable was a binary outcome (e.g. a yes/no response). Herbicide and drought (with an interaction term) were entered as fixed effects in the model. Blocks and clone ID were entered as random effects.

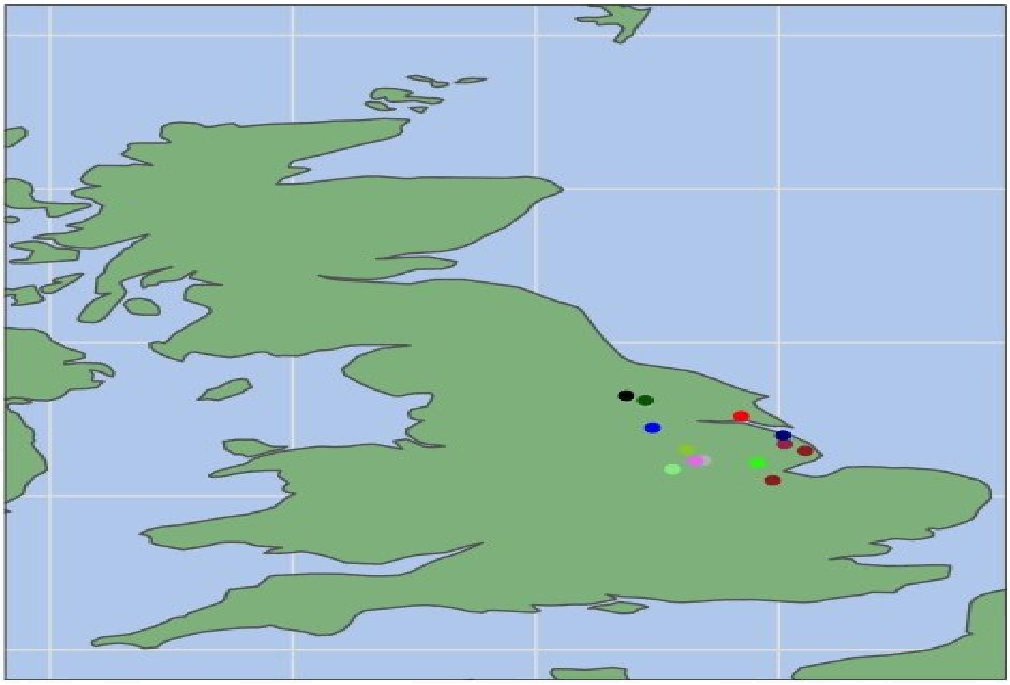

Figure 3. The dots on the map represent the locations of sampling the *A. myosuroides* weed. Each dot represent a population "Pop= Population" and each population is indicated by a colour, Pop1= Black; Pop2= Red; Pop3= Grey; Pop4= Blue; Pop5= Green; Pop6= Turquoise; Pop7= Violet; Pop8= Maroon; Pop9= Dark-orange; Pop10= Dark-red; Pop11= Yellow-green; Pop12= Orange; Pop13= Brown; Pop14= Dark-green and Pop15= Dark-blue.

3.3. Results

3.3.1. Drought stress treatments

Effect of drought stress on survivorship and growth parameters in cloned plants.

Here we confirm that the drought stress treatment significantly impacted plant performance. Drought stress significantly affected plant height, biomass and seed weight of *A. myosuroides*. Plant height is lower in high drought treatment, by about -0.47 mm ± 0.06, $p = 6.08 \times 10^{-12}$ (Fig 4A, Table 1). For plants harvested at the end of the experiment as plant biomass -0.70 g ± 0.1, $p = 4.16 \times 10^{-11}$ (Fig 4B, Table1). In addition significant reduction were observed in seed weight -1.55 g ± 0.21, $p = 7.22 \times 10^{-11}$ (Fig 4C, Table 1).

Exposure to the drought treatment resulted in increased mortality in each population of cloned plants -2.27 ± 0.28, $p = 1.04 \times 10^{-15}$ (Fig 4D, Table 2).

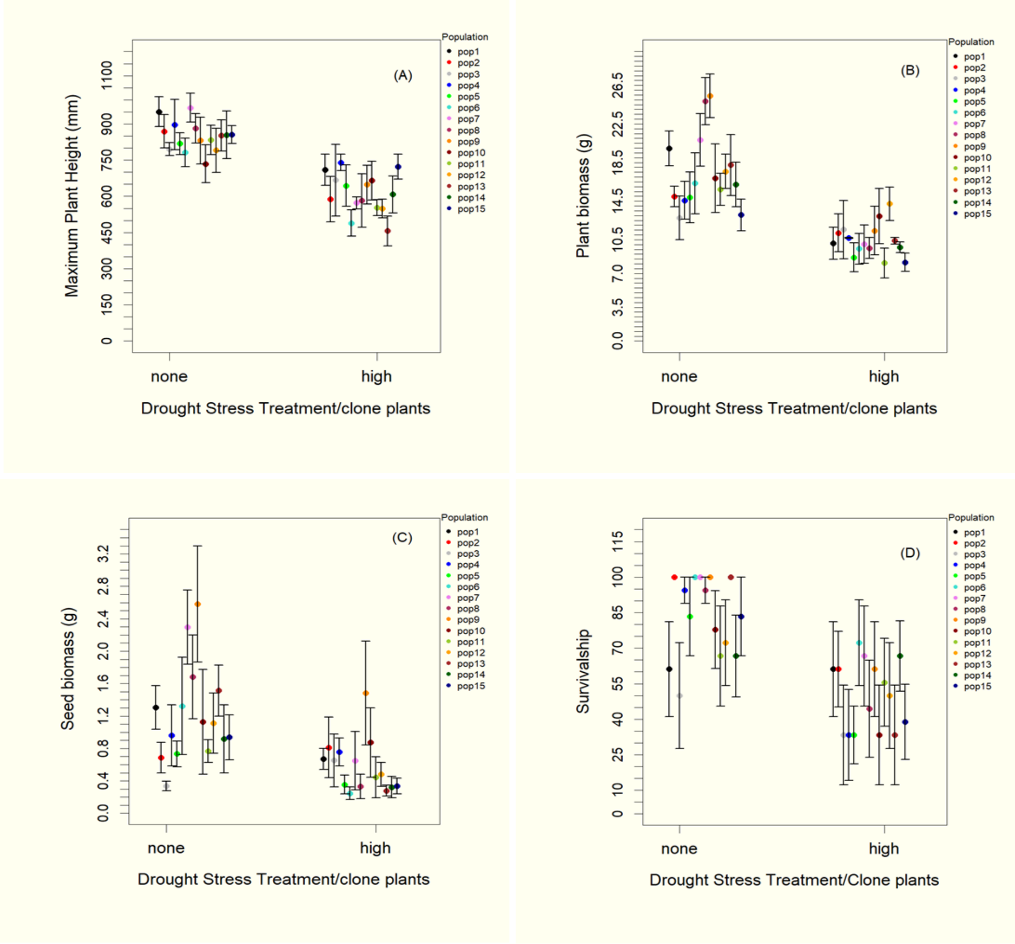

Figure 4. Effect of drought stress on cloned parent populations (Pop1 - Pop15) of *A. myosuroides*: maximum plant height (A) in percentage, Dryweight of aboveground biomass (B), seed production per plant (C) and percentage of survival of plants that exposed to drought. Error bars represent standard errors.

Table 1. Results of Linear Mixed Models fit by REML; t-test use Satterthwaite's method describe the effect of high drought stress treatment on growth of cloned parental generation of 15 populations of *A. myosuroides*. The model included drought treatment as a fixed effects and clone.ID as a random effects. Data on (A) plant height, (B) biomass and (C) seed weight were log transformed. Significance of model terms indicated by asterisks: *** $p < 0.001$.

Fixed effects	**Estimate**	**Std. Error**	**t value**	**Satterthwaite *p***
A) Plant height				
drought	-0.47	0.06	-7.67	6.08×10^{-12} ***
B) Plant biomass				
drought	-0.70	0.10	-7.29	4.16×10^{-11} ***
C) Seed weight				
drought	-1.55	0.21	-7.48	7.22×10^{-11} ***

Table 2. Results of generalized linear mixed model fit by maximum likelihood (Laplace approximation) with binomial error (logit), for the effect of drought stress treatment on survivorship of cloned parental generation of 15 populations of *A. myosuroides*. The first column: Estimate, refers to the estimated value of the coefficient. The second column, Std. Error, is the standard error of the estimate betas. The last two columns characterise z-values of the estimated beta coefficients and the p-value. Significance of the model terms indicated by asterisks: ***$p < 0.001$.

Fixed effects	**Estimate**	**Std. Error**	**z value**	***p* (>|z|)**
intercept	1.70	0.22	7.61	2.74×10^{-14} ***
drought	-2.27	0.28	-8.02	1.04×10^{-15} ***

3.3.2. Herbicide treatment study

The response of generation F1 to herbicide

Drought treatment applied to the parental generations generated herbicide resistance in the F1. Herbicide treatment significantly affected the evidence of herbicide resistance in F1 plants from parents that experienced high drought (3.67 ± 0.45, $p = 2.67 \times 10^{-16}$) in comparison to well-watered plants. This was also true for the number of plants surviving apparently intact versus those that were combined together as damaged and dead (Fig 5A, Table 3). Additionally, when resistance was measured as plants that survived either damaged or intact together (combining survived and damaged plants and count as surviving plants) versus those that were dead (Fig 5B, Table 3) in offspring of highly droughted plants following the herbicide treatment there was also a strong effect (2.12 ± 0.48, $p = 1.04 \times 10^{-5}$). Furthermore, there was a significant interaction between drought applied to the parental generation and herbicide application in the F1 (-1.86 ± 0.58, $p = 0.0013$) in both combinations (resistant vs damaged+dead) and (resistant+damaged vs dead). This is an indication of a significant impact of drought stress exposure upon the evolution of herbicide resistance in the F1 generation (Table 3).

The dry weight of resistant plants was analysed, i.e. the dry weight of F1 plants that survived the herbicide application. A linear model of resistant dry weight was constructed as a function of clone.ID, herbicide and drought. There was no significant effect of herbicide application on dry weight of resistant F1 plants (Fig 6C, Table 4: $F_{(1,103)} = 0.30$, $p = 0.58$), while drought treatment as a factor had a significant effect on the dry weight of resistant plants ($F_{(1,103)} = 81.27$, $p = 1.14 \times 10^{-14}$). There was no significant interaction between herbicide application and exposure of parental generation to drought for *A. myosuroides* (stats). To analyse the dry weight of damaged plants a linear mixed effects analysis was performed and there was no significant effect of herbicide application on the dry weight of damaged plants in F1 generation, except for drought treatment which had a significant impact as one of the fixed effect 1.36 ± 0.39, $p = 0.001$ (Fig 6D, Table 5). Additionally, there was no significant interaction between drought applied to parental generation and herbicide application in F1 generation.

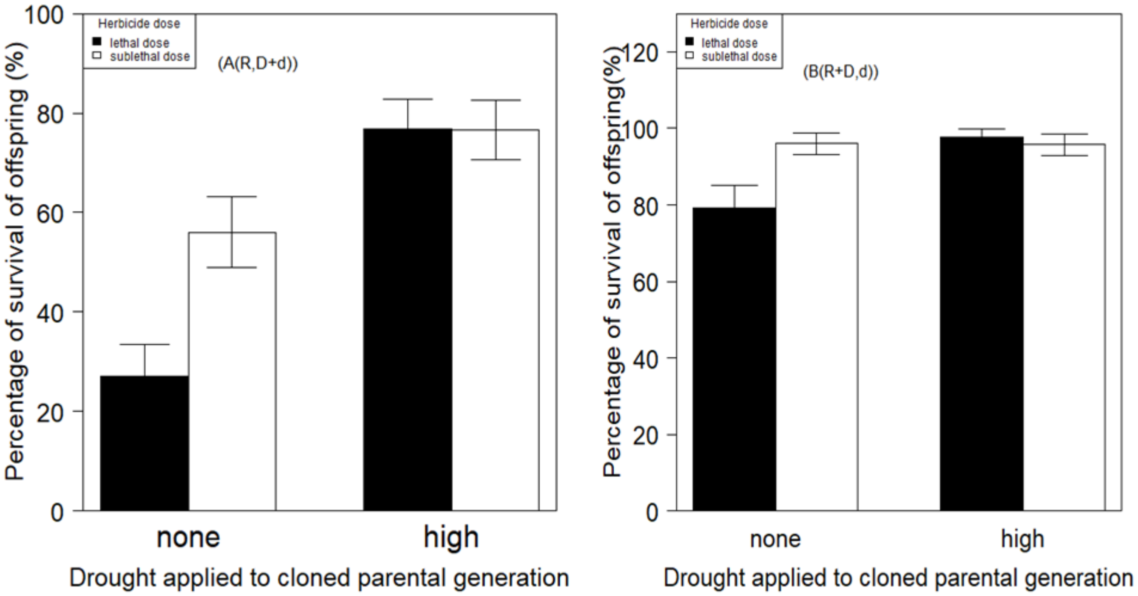

Figure 5. The effect of high drought stress treatment on the survival cloned parental populations of *A. myosuroides* (A, B); populations treated with lethal and sub-lethal doses of fenoxaprop-P-ethyl herbicide. (A) Percentage of survival (R) of offspring when the damaged (D) and dead (d) plants were combined (R, D+d). (B) Percentage of survival of offspring when the resistant and damaged plants were combined (R+D, d). Error bars are ± one standard error of the mean.

Table 3. Reports the results of generalized linear mixed model fit by maximum likelihood (Laplace approximation) with binomial error (logit), for F1 generation plants that are (A) resistant vs damaged + dead plants, and (B) resistant + damaged vs dead plants of *A. myosuroides*. The first column: Estimate, refers to the estimated value of the coefficient. The second column, Std. Error, is the standard error of the estimate betas. The last two columns characterise z-values of the estimated beta coefficients and the p-value. Significance of model terms indicated by asterisks: **, $p< 0.01$ and ***, $p< 0.001$.

Fixed effects	**Estimate**	**Std. Error**	**z-value**	***p* (>|z|)**
A) resistant vs (damaged + dead) plants				
Intercept	-1.376	0.528	-2.607	0.009 **
herbicide-sublethal dose	1.592	0.291	5.476	4.36 x 10^{-8} ***
drought	3.669	0.448	8.188	2.67 x 10^{16} ***
herbicide-sublethal dose: drought	-1.863	0.580	-3.211	0.0013 ***
B) (resistant + damaged) vs dead plants				
Intercept	2.10	0.76	2.72	0.007 **
herbicide-sublethal dose	2.12	0.48	4.41	1.04 x 10^{-5} ***
drought	3.47	0.84	4.16	3.21 x 10^{-5} ***
herbicide-sublethal dose: drought	-3.67	1.15	-3.20	0.001 **

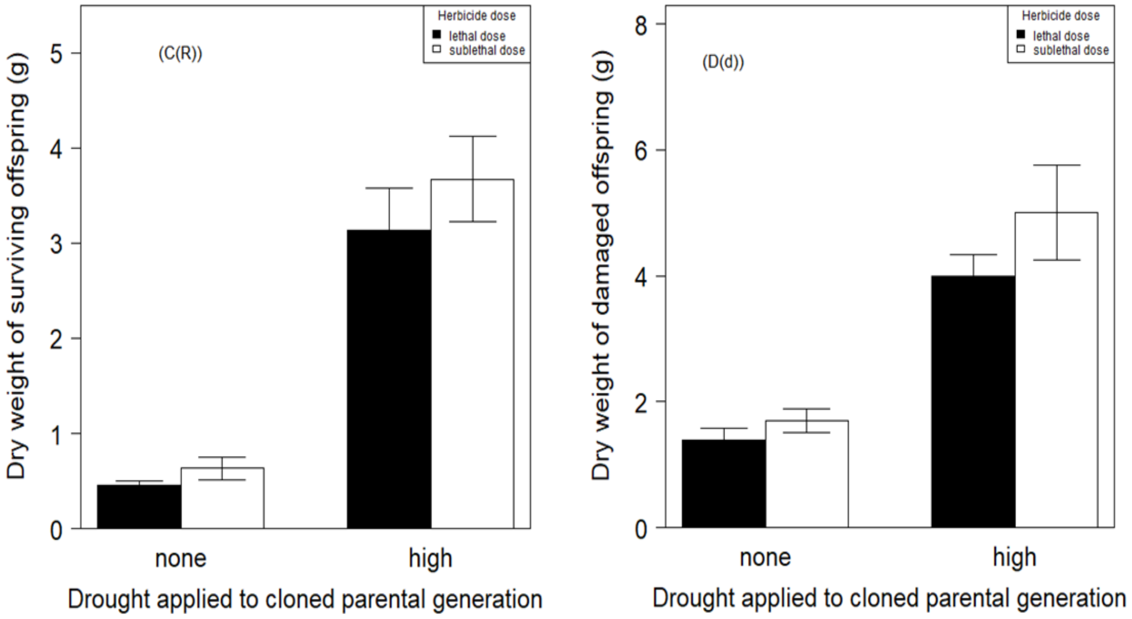

Figure 6. Effect of high drought stress in cloned parental generation and herbicide treatment lethal and sublethal doses of fenoxaprop-p-ethyl herbicide on the dry weight of resistant (C (R)) and damaged plants (D (d)) in first generation of *A. myosuroides*. Error bars represent standard errors.

Table 4. Results of ANOVA from Linear Model describing the effect of drought stress in cloned parental generation on the response of F1 resistant dry weight after fenoxaprop-p-ethyl herbicide application (lethal and sublethal doses). Data on dryweight of resistant plants were log transformed. Significance of model terms indicated by asterisks: '***' , $p< 0.001$.

	Degrees of freedom	Sum of squares	Mean of squares	F-values	*p*-value
log (resistant. Dryweight)					
Clone.ID	4	5.03	1.26	1.39	0.24
Drought	1	73.57	73.57	81.27	1.14×10^{-14} ***
Herbicide	1	0.28	0.28	0.30	0.58
Residuals	103	93.24	0.91		

Table 5. Results of Linear Mixed Models fit by REML; t-tests use Satterthwaite's method describe the effect of High drought stress treatment in cloned parental generation on the response of F1 damaged dry weight after fenoxaprop-p-ethyl herbicide application (lethal and sublethal doses). Data on dryweight of damaged plants were log transformed. The model included drought and herbicide treatment as a fixed effects and clone ID as a random effects. Significance of model terms indicated by asterisks: **, *p*< 0.01.

	Estimate	**Std. Error**	**t value**	***p*(>|t|)**
log (dryweight of damaged plants)				
Herbicide lethal dose	-0.19	0.26	-0.74	0.489
Herbicide sublethal dose	-0.05	0.28	-0.19	0.852
drought	1.36	0.39	3.46	0.001 **

3.5. Discussion

Herbicide resistance in weeds is growing rapidly throughout the world (Heap, 2014). It is important to understand the genetic bases and evolutionary mechanisms underpinning the emergence of herbicide resistance in order to generate solutions, as well as to prevent future problems. Despite the fact that target site resistance can endow high levels of herbicide resistance (Preston et al., 2009), the current rapid increase in the abundance of herbicide resistance is thought to be mainly due to non-target site resistance (NTSR) (Ge et al., 2010, Delye et al., 2013b, Shaner et al., 2012). The results from our study using cloned plants indicate that epigenetic mechanisms may be involved in herbicide resistance, especially via NTSR. Epigenetic mechanisms have been highlighted an important mediators of interactions between plants and their response to the environment, largely linked with stress adaptation (Markus et al., 2018). Environmental conditions impact on plant physiology, which can lead to difference in herbicide resistance expression (Markus et al., 2018).

Epigenetic mechanisms also currently recognised to have a fundamental role in the control of gene expression via small RNAs, histone modifications and DNA methylation. These are inherited via mitotic cell divisions and, in a number of situations, can be transferred to the next generation. Hence they provide a possible mechanism for stress memories in plants (Kinoshita and Seki, 2014). The primary objective of this study was to determine whether the evolution of herbicide resistance will occur through an epigenetic mechanism, and further to determine the role of environmental stresses (e.g. drought stress) in the evolution of herbicide resistance through this mechanism.

3.5.1.1. Effect of drought stress on cloned *A. myosuroides* growth parameters

Water availability is essential to life and is a major molecule in the majority of cellular processes; thus, particularly in plants, its availability has a direct influence on growth and survival. Here, we wished to establish how different cloned populations of *A. myosuroides* will response to high drought stress in terms of survivorship, the drought stress effects upon growth parameters and further to produce a generation following drought stress exposure. Our results demonstrated that a high drought treatment in the cloned parental generation differently caused a significant reduction in plant height, above ground biomass and reduction in seed weight across all the populations. Previously the reduction of growth and reproduction parameters in response to drought stress have been evaluated in an *E. colona* (Junglerice) weed in a rice field (Chauhan and Johnson, 2010). In addition, Brown, (2009) indicated that exposing to drought after weed emergence may strengthen plants which leads to increasing their survivorship. The ability of *A. myosuroides* to regrow and produce seeds at a high level of drought treatment would aid ensure survival of the population in a changeable environment. Furthermore, the significance of water availability has been established in *Arabidopsis thaliana*, in which signals of drought are transduced into impacts on gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). As variations in gene expression patterns are largely accompanied by alterations in the chromatin circumstances (Campos and Reinberg, 2009), in response to desiccation, the modification of histone tails, has been analysed in the chromatin surrounding drought stress-responsive genes (Kim et al., 2008, Kim et al., 2012).

Out of 15 populations that we used in this study, we could use just five populations for the herbicide spray study because the germination rate was high (100%) in those 5 populations, while the other 10 populations were unviable and the germination rate was (0%). This observation then added another pathway to our interest for more investigations about the effect of previous management on seed germination, viability and other seed characteristics of *A. myosuroides* seeds (chapter 4). (Cordeau et al., 2018) previously showed that drought stress would reduce weed emergence and the early growth of weed seedlings. This could explain how the viability of *A. myosuroides* seeds was lost during the drought treatment.

3.5.1.2. Effect of drought stress on survivorship

The response of *A. myosuroides* populations to drought stress was highly significant. This indicates that the *A. myosuroides* plants were able to tolerate drought stress, because the mortality rate in the drought treatment was low. The differential responses of the 15 populations may partially account for their success or failure in their ability to tolerate high drought stress. Applying high drought stress at early stage of the plants life allowed us to observe the strong ability of *A. myosuroides* plant to withstand the drought treatment. All the plants in our study were showing a significant tolerance to the drought treatment after re-watering them in compare to their previous period of drought. This was more likely related to remembering the stress, as a previous study has indicated that recovery rate depend on experienced stress level (Resco et al., 2009).

3.5.1.3. The evolution of herbicide resistant in weeds through physiological pathways.

In the current study, we wished to experimentally investigate the possible role of physiological pathways in the evolution of herbicide resistance in *A. myosuroides*. The dire danger of resistance to metabolic herbicides is that they can confer resistance across herbicides from various groups of chemicals (Yu and Powles, 2014). High level of survivorship in highly droughted individuals following the application of different doses of herbicide in *A. myosuroides* populations in our study showed "cross-resistance" to fenoxaprop-p-ethyl herbicide and previous exposure to drought stress. Previously it has been reported that occurrence of metabolic "cross-resistance" in *Lolium rigidum* to different herbicides may be either via the P450 or other metabolism genes (e.g. glutathione transferases (GT) and glutathione S-transferases (GST)). This can unexpectedly metabolise a number of herbicide chemical structures responsible for the resistance (Busi and Powles, 2013, Yu and Powles, 2014). Furthermore, environmental conditions also play a role in metabolic resistance evolution, as the enzymes involved (e.g. P450s and GSTs) can respond to biotic or abiotic stresses (Marrs, 1996, Schuler and Werck-Reichhart, 2003). Plant GSTs bind glutathione to electrophilic xenobiotics, which marks them for sequestration with vacuole impact. GSTs ' role in metabolism is uncertain, nonetheless their complicated regulation by environmental stimuli suggests that they have vital defensive functions (Edwards et al., 2000). As the GSTs are cytosolic proteins which have many physiological functions. In normal plant growth and plant stress responses the plant GSTs perform a number of key catalytic and non-enzymatic functions (Dixon et al., 2002). Thus, it can be presumed that NTSR is part of the response to abiotic stress. This is supported by research on the impact on plant transcriptome or proteome of herbicide applications, which indicate that response to herbicide stress can be correlated with response to other stresses (Das et al., 2010, Unver et al., 2010, Vivancos et al., 2011). Previous studies have shown that environmental conditions (e.g. high temperature) have a major effect on the evolution of resistance to different herbicides through metabolic detoxification mechanisms (Yu et al., 2009, Ge et al., 2011, Matzrafi et al., 2016). The results of the current study indicate that drought stress can affect the efficiency of herbicide in controlling weed species. For example, the results of survival and dry weight of offspring both in resistant and damaged plants presented here suggest that exposure to high drought stress can result in failed weed treatment. These results underline the importance of environmental conditions after application of herbicide (Matzrafi et al., 2016).

3.5.3. The response of F1 of droughted-cloned parental generation to herbicide treatment

3.5.3.1. Survivorship of the drought-herbicide treatment

We wished to establish whether evolution of herbicide resistance might be underpinned via an epigenetic mechanism and how the first generation of cloned droughted plants will then respond to a herbicide treatment as the next stress exposure. Our study provides clear evidence that drought stress experienced by cloned parental generation of *A. myosuroides* can elicit herbicide resistance in the F1.

It has been reported that plants; previous experience of stress may change subsequent responses towards the next stress by producing more rapid and/or stronger responses, which means plants practice a form of 'stress memory' (Ding et al., 2012, Walter et al., 2013). In addition, evolved tolerance for abiotic stress after previous exposure to stress has been called the 'priming effect' (Tanou et al., 2012), and has been reported for drought and inundation in previous studies (Li et al., 2011, Onate et al., 2011). This effect has also been described by (Boyko and Kovalchuk, 2011) as a phenomenon known as plants hardening, in which low or medium stress can act as a signal for more severe stress in future, stimulating mechanisms to produce superior stress tolerance (Beck et al., 2004).

Our results for the first time clearly provide new evidence that exposing cloned *A. myosuroides* plants to high drought stress would evolve stronger defence mechanism to resist lethal and sublethal doses of fenoxaprop-p-ethyl herbicide as a subsequent stress in the first generation plants than in well watered plants. This explains the potential ability of grass weed *A. myosuroides* to inhibit or minimize damage or mortality through a resistance mechanism and/or increase the ability to recover from damage through a tolerance mechanism. Furthermore, the resistance trait can be transmitted to the next generation. Consequently, there is a great possibility of involving physiological pathways in *A. myosuroides* plants response to stresses.

3.5.3.2. Effect of herbicide treatment on dryweight

We investigated whether dry weight of resistant plants (intact or damaged) would be affected by herbicide treatment in the F1 generation of *A. myosuroides*. As well as surviving, we also showed that resistant plants can grow normally after herbicide application, especially in plants previously exposed to drought stress, as the herbicide treatment did not cause any reduction in dry weight of resistant and/or damaged plants. Thus, there was no significant effect of herbicide treatment on dry weight of *A. myosuroides* plants. Furthermore, we could not confirm significant interactions between the drought applied to parental generation and herbicide treatment in F1 generation. (Walter et al., 2011) in research on *Arrhenatherum elatius* showed that above ground biomass significantly increases in the plants that previously exposed to drought stress. Interestingly, we observed variation in the response of the plants to both herbicide doses, as the dry weight of resistant and damaged plants were higher in sublethal dose both in none and high drought treatments. These results provide evidence that the grass weed *A. myosuroides* grow healthier than their growth under well-watered conditions, when they evolve resistance under stress conditions.

3.6. Conclusion

Plants are persistently exposed to different stresses, and their responses are extremely dynamic include crosstalk. Our results of the drought treatment in the cloned parental generation (first part of the experiment) demonstrate that drought stress significantly achieved a reduction in growth of cloned *A. myosuroides* plants. Significant differences observed for survivorship in the high drought treatment suggest the potential ability of rapid evolution of defence mechanism (resistance) in *A. myosuroides* grass weed populations. Furthermore, our results after herbicide application to the F1 generation (the second part of the experiment) demonstrate a strong relationship between exposure to drought stress in the cloned parental generation and the ability of *A. myosuroides* F1 to withstand herbicide application. These findings support the vital role of an epigenetic mechanism that may induced by drought stress in the evolution of herbicide resistance in *A. myosuroides*. Knowledge that this herbicide resistance may arises epigenetically could promotes better understanding of the evolution of the mechanism of herbicide resistance in grass weeds. Further investigations across different generations for example, F2 will provide more evidence for this response.

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CHAPTER FOUR

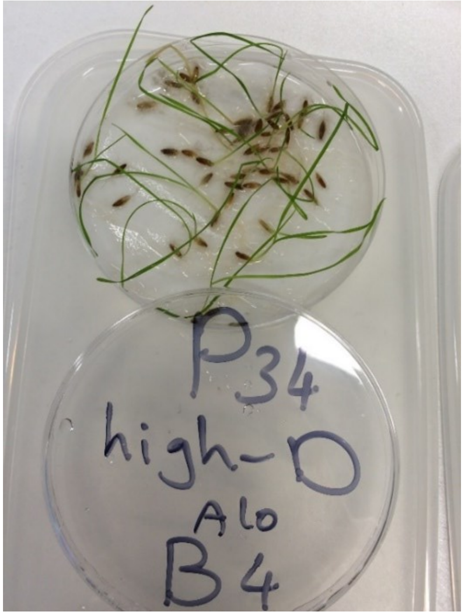

Figure 1. Samples of seeds from different densities of *A. myosuroides* that used in this study "the effect of different environmental conditions in which the mother plant grow on germination behaviour": top-left seeds from parent that grew under high density and bottom-left seeds from par-

STATEMENT OF CONTRIBUTIONS

The initial idea of this chapter were developed by Prof. Robert Freckleton. The method which was used to conduct germination test and emergence characteristics test was constructed by the candidate. All the data collection and writing were carried out by the candidate. Data analysis was carried out by the candidate with editorial advice from Prof. Robert Freckleton and Prof. Colin Osborne. General editorial advice was provided by Prof. Robert Freckleton and Prof. Colin Osborne.

CHAPTER FOUR

THE IMPACT OF DENSITY, SOIL TYPE AND EXPOSURE TO HERBICIDE ON GERMINATION AND EMERGENCE CHARACTERISTICS OF POPULATIONS OF *ALOPECURUS MYOSUROIDES*

Abstract

Seed germination is a key process in weed population dynamics, and is predicted to be under strong selection pressure to ensure germination under circumstances that increase plant survival. Furthermore, the selective pressure applied by environmental conditions and/or agriculture practices on arable weeds populations stimulate the evolution of adaptive traits. The focus of this chapter is to determine whether there are correlations between environmental factors such as herbicide management, soil type, density and other aspects of biology, specifically germination behaviour. I analyse the effects of the environmental conditions in which mother plants grow on seeds and germination behaviour. Specifically, I analyse previous management, soil types and density in which parent plants had grown on size, germination and emergence characteristics of seeds of 43 populations of the weed black-grass (*Alopecurus myosuroides*). I show that, seeds produced in high density parent populations are significantly larger in weight and size, with higher viability and germination, compared with seeds produced at low parent population density. Furthermore, I show that there is a significant relationship between density and previous herbicide applications experienced by parent plants. Herbicides in interaction with high density caused an increase in seed germination, viability, seed weight and seed size across populations. The results also suggest an increase in seed germination, viability, seed weight and seed size in response to density in interaction with the soil type in which the parent plants grew. Overall these results suggest that the environmental conditions in which the parental plants develop can affect seed germination and seed characteristics of weed seeds, and is influenced by the history of herbicide application.

Keywords: Seed characteristics, Previous management, Soil type, Black-grass, Seed germination, Density state.

4.1. Introduction

The parental environment (abiotic and biotic) in which plants grow can have a considerable impact on the phenotypes of offspring, in terms of seed morphology (Alexander and Wulff, 1985, Keigley and Mullen, 1986, Violle et al., 2009) and germination (Sawhney and Naylor, 1982, Lacey, 1984, Alexander and Wulff, 1985, Colbach et al., 2005, Orrock and Christopher, 2010). Variation in these characteristics can have a subsequent impact on the growth of seedlings (Weis, 1982). Weed populations frequently show heritable differences in life history traits that may possibly reflect phenological adaptations to environmental effects (Mortimer, 1997). One of the strongest environmental impacts experienced by weed species populations is that of management practices that result in increased weed mortality, and the decreased fertility of survivors. Consequently, weed management practices may form agents of natural selection given heritable genetic variation for life history traits. As a result of past selection, individuals within populations may become adapted to the prevailing management (Mortimer, 1997). Moreover, it has been recognized that genes endowing adaptation to novel environments can also affect phenotypic characteristics through pleiotropic effects (Sarah, 2004, Pavlicev and Wagner, 2012).

There is considerable variation in the density of weeds both within and between fields (Gabriel et al., 2005). This variation can also be observed at the national scale, as some areas contain more weeds compared with others (Marshall, 2009). Such differences mirror the combined imprint of environmental conditions and the history of management (Fried et al., 2008). Therefore, agricultural management and environmental conditions are both considered to be important factors that affect density and distribution of weeds. For example, cropping management such as tillage and crop rotation have been shown to affect the variations of weed density within fields (Santín-Montanyá et al., 2013, Freckleton et al., 2017).

Soil conditions, another environmental condition, also have a particular effect on weed density across agricultural fields (Radosevich et al., 2007). For example, clay soil has been shown to result in a high density of some weeds (Lousada et al., 2013, Metcalfe et al., 2016), and *A. myosuroides* is conventionally thought to be associated with heavy soil. However changing cropping management may have permitted it to enlarge its range into lighter soil (Holm et al., 1997). In addition to these relationships with specific soil types, there is evidence that the occurrence of *A. myosuroides* populations within individual fields also correlated with variation in soil properties (Metcalfe et al., 2016, Metcalfe et al., 2019).

Given these impacts of soil properties on the density of weeds (Metcalfe et al., 2016, Metcalfe et al., 2019), there should be a possibility that they will also impact on the efficacy of herbicides. For example, a high content of clay and organic carbon in soil can drive the sorption of most herbicides (Gaston et al., 2001) and consequently the level of control that is attained can be affected by soil type (Metcalfe et al., 2016, Metcalfe et al., 2019).

A wide range of studies have been conducted on weed populations. Of those: the impact of weed density on crop productivity (Firbank and Watkinson, 1986), the impact of controlling weeds on the type and number of weed (McCloskey et al., 1998, Colbach et al., 2005). Characterising the life history features (like seed output) of single weed species (Freckleton and Watkinson, 1998), and those of arable weeds mainly and the cost of weed species management (Doyle et al., 1986, Maxwell and Luschei, 2005) studied. However, less is known about how weed density affects responses to herbicides, specifically germination behaviour.

Weed control often becomes unsuccessful due to the evolution of resistance in populations owing to the historical use of herbicides (Ghersa and Martinez-Ghersa, 2000, Powles and Yu, 2010). Moreover, weeds adapt to herbicides via phenological changes (Mortimer, 1997, Martinez-Ghersa et al., 2000). For example, in a grass weed species, increased seed dormancy could be an adaptive response to application of herbicide, with plants showing periodic germination from a short-lived seed bank (Batlla et al., 2020). In addition, Batlla et al., (2020) showed that seed dormancy is one of the seed characteristics that has been recognised as having great adaptive value to modify weed biology in relation to agriculture practices. In an annual grass weed such as *A. myosuroides*, generally associated with autumn-sown cereal crops in the UK, seedling groups which is related with autumn cultivation crop may be conscripted for several months into spring. Therefore, during post emergence herbicide application, different cohorts of seedling populations may extend the time over which applications are required (Mortimer, 1997, Martinez-Ghersa et al., 2000). In addition, comparisons between resistant and susceptible biotypes have shown that weed populations can be different in physiological characteristics as well, such as the rate of relative growth, photosynthetic rate or germination rate (Vila-Aiub et al., 2005).

It has been suggested that genes conferring herbicide-resistance can have various effects on weed life histories (Vila-Aiub et al., 2009b, Vila-Aiub et al., 2011). A number of previous studies have proposed possible links between variation in seed germination, emergence or survival in the soil and resistance to the main class of grass specific selective herbicides inhibiting acetyl-coenzyme A carboxylase (ACCase) (Ghersa et al., 1994, Gill et al., 1996, Vila-Aiub et al., 2005, Gundel et al., 2008, Wang et al., 2010). A direct relationship between herbicide resistance and seed germination or survival in the soil would means that herbicide applications could promote the simultaneous evolution of herbicide resistance and other traits that drive the persistence of weeds in agricultural ecosystems (Vila-Aiub et al., 2005). There is evidence that certain herbicide resistance alleles are linked with pleiotropic effects contributing to loss of plant fitness costs (Vila-Aiub et al., 2009a). Therefore, to predict the evolutionary dynamics of herbicide resistance, an understanding of the fitness consequences of herbicide resistance alleles in the presence and absence of a herbicide is important (Neve et al., 2003).

Although it is frequently presumed to be relatively constant (Harper et al., 1970) seed size is a characteristic that can be affected by environmental conditions. Variation in seed size has been reported together within (Wulff, 1973, Waller, 1982) and among plant populations (Baker, 1972, Schimpf, 1977), and even at the level of individual plants (Janzen, 1977). Both seed size and seed germination characteristics may differ with the environmental conditions experienced by the parent plant (Fenner, 1991, Munir et al., 2001, Luzuriaga et al., 2006).

Seed size and germination are important factors in determining plant establishment (Winn, 1988, Alcocerruthling et al., 1992, Dyer et al., 1993, Thompson et al., 1994). Optimal timing of germination timing permits seedlings to escape pre-planting weed control and to develop under the best possible conditions for growth (Mortimer, 1997, Andersson and Espeby, 2009). The ability to resist the effects of herbicide is an additional major adaptive trait (Powles and Yu, 2010). Therefore, the success of a weed species will depend on its germination strategy, herbicide resistance or both, under agricultural conditions (Delye et al., 2013c).

A. myosuroides is one of the major grass weeds of winter cereal crops in the UK and other North Western European countries since 1960 (Moss, 1983, Marechal et al., 2012). The spread of this species is facilitated by intensive managements, as well a large seed output (Chauvel et al., 2002). For farmers, controlling this weed has been a major priority, especially because of its potential impact on crop yield. Herbicides were historically the most effective weed control tool for farmers. However, herbicide resistance evolved in the 1980s, and since then it has been a major concern (Moss, 1983, Marechal et al., 2012). Understanding the effect of different environmental conditions in which the weed species develop such as weed density, soil type and weed management history on variations of *A. myosuroides* population's offspring characteristics in agricultural fields is important for the development of effective management (Lousada et al., 2013).

The overall objective of this study was to analyse the effect of soil type, exposure to herbicides and variations in weed density in which the mother plants grow on the germination and seed characteristics of the arable weed *A. myosuroides*. We analysed variations in seed characteristics across different populations of *A. myosuroides* in relation to plant density, previous herbicide history management as well as variation in soil types. The questions posed in this chapter are (i) Is there an interaction between weed densities, soil type and previous herbicide exposure history affect germination and emergence characteristics? (ii) Do germination and emergence characteristics vary across populations relative to their previous herbicide exposure history and soil type? I answered these questions by experimentally determining whether these factors impact upon the seed size, seed weight, germination and emergence characteristics across populations that differed in their previous management history.

4. 2. Materials and methods

4.2.1 Study system

A. myosuroides Huds. (black-grass) in winter cereal crops across the lowland arable region of the UK was chosen as a study system, because of its agronomic importance. Seeds of *A. myosuroides* were collected in July 2015. The study sites were selected to represent the range of farming management typical of the area. 24 farms were selected and, at each farm, two fields of winter wheat were selected: one field was assessed to contain high density of *A. myosuroides* population on the field, the other containing a low density of the species.

For each field we recorded the previous history of herbicide treatment, as well as soil type. Furthermore, seeds were characterized by production conditions (i.e. *A. myosuroides* density) in the field where the seeds were collected (48 fields x density). Figure 2, shows the sampling sites of all the populations' seeds were used in the study. In addition, details about previous management (i.e. herbicide treatment), soil type, and density condition/field number of the seed conditions for each population are provided in (Appendix 6).

4.2.2. Previous herbicide management history and soil type

Historical management data was requested for each field. Complete data was not available for all the fields and consequently I was able to analyse management data only where available. Herbicide application data was derived (based on the modes of action for each herbicide (MOAs)) for the year of seed sampling. These data was used to investigate how different herbicides used by crop growers for controlling the parental plants affected the seed characteristics of offspring. The herbicides used by farmers were classified according to their MOAs as follow:

- MAI: Microtubule assembly inhibition.
- CBI: Carotenoid biosynthesis inhibition, bleaching: inhibition of carotenoid biosynthesis at the phytoene desaturate step.
- EPSP: synthase inhibition, this mode of action is specific to glyphosate (glycines), which are nonspecific herbicides that act by inhibiting the amino acid synthesis (EPSP I).
- AHAS: inhibits plant amino acid synthesis-acetohydroxyacid synthase (A.A.S.I).
- SA: Synthetic auxin, foliar uptake causing auxin-type response.
- GSI: Glutamine synthetase inhibitor, accumulates ammonium ions, inhibits photosynthesis.

In addition to the history of management, we analysed the effect of the soil type in which the parent plants grew on seed germination and emergence characteristics of offspring. For each field, soil type was extracted from the National Soil Resources Institution NATMAP1000 database, (using, the Soilscapes Viewer dataset www.landis.org.uk). We extracted the type of soil within each field (e.g. medium-heavy, clay-loam, silty-loam, heavy-clay, peaty-loam, medium, deep-clay, clay-gravel and heavy-fen).

4.2.3. Weed density

A density-structured approach was used to determine the density state of the weed population within each fields (Freckleton et al., 2011, Queenborough et al., 2011). We analysed the impact of different parent plant densities on seed germination and emergence characteristics of offspring. Density censuses were undertaken by a team of three trained observers. Each field was divided into quadrats (20 × 20 m) in which *A. myosuroides* density assigned to a density state (absent, low, medium, high, very high). The density corresponded to the estimated number of the plants per 20 m^2 in groups of 0, 1-160, 160-450, 450-1,450 and >1,450 for the absent, low, medium, high and very high density state observations respectively (Hicks et al., 2018). For this experiment, at each site, two fields of winter wheat were selected, one field estimated to contain the highest densities of weeds on the field, the other selected to contain the lowest densities.

4.2.4. Seed sampling

Seed samples were collected within each field by dividing the fields into ten linear sections. An individual location was selected in each section, and the seeds were sampled from *A. myosuroides* plants at this point. Heads of *A. myosuroides* were selected at random at each location. The selected heads with mature seeds were collected by carefully shaking into a bag. All the heads were collected around the collection point not more than five metres away from the collection point (Hicks et al., 2018). Following threshing and cleaning, the seeds of all populations of *A. myosuroides* that used in this study, were located in an incubator at 30 °C for 6 weeks to break primary seed dormancy. Until needed, the seeds were stored in dark/cool conditions in a fridge at 5 °C.

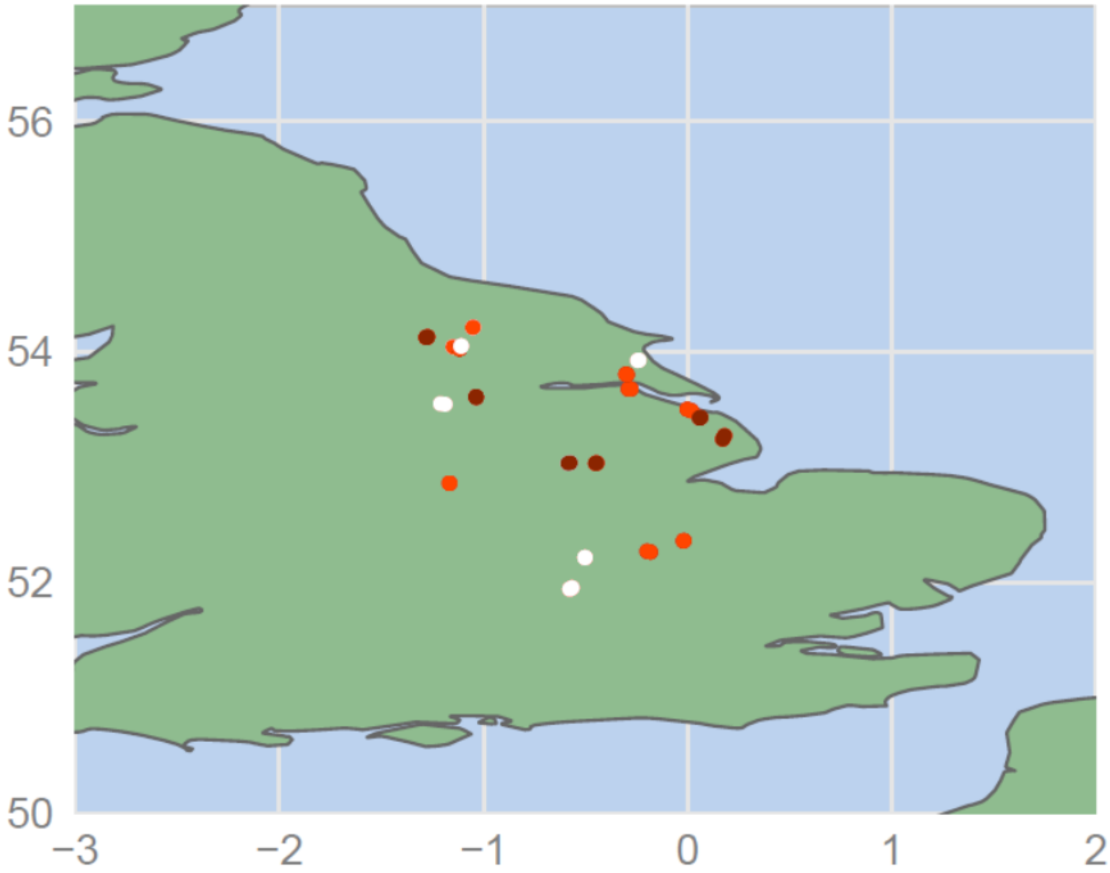

Figure 2. The location of sampling sites for the seeds of *A. myosuroides* populations. The dark orange circles ● on the map represent the sampling sites for the populations at high density of *A. myosuroides* in each farm. The light orange circles ● represent the sampling sites for the populations at low density of *A. myosuroides* in each farm. The white circles ○ on the map represent unknown sampling sites in which no seeds for those sits were collected, either for high or low density.

4.2.5. Seed size

All seeds were measured individually by placing the seed on a ruler under a microscope (MICROTEC, WF10X/20) (Figure 3). The average of the 50 seeds per population was calculated. All the 50 seeds per population were selected randomly.

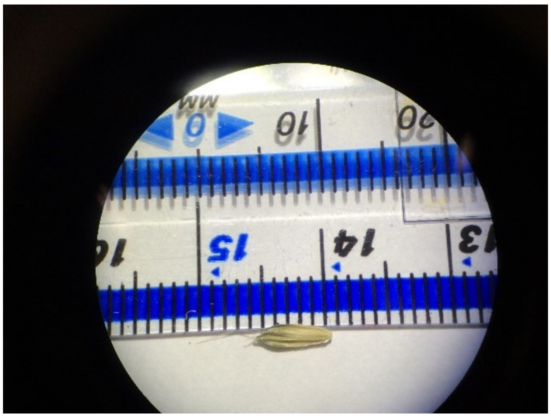

Figure 3. Shows an example of the measurement taken for the size of each seed of the 50 seeds per population of *A. myosuroides* under microscope (MICROTEC, WFX/20).

4.2.6. Seed weight

Ten seeds of the 50 seeds per population were selected randomly and weighed. Seeds were weighed using a high precision scale (GH-252-EC, max = 250 g, min= 1 mg, d=0.01/0.1 mg, A&D Instruments, Abingdon, UK).

4. 2.7. Seed germination

In January 2016, the germination experiment was carried out at the University of Sheffield in laboratory growth cabinets. The germination and viability of seeds were determined by placing 50 seeds of each population in 90 mm-diameter petri dish containing two layers of Whatman Grade 1 filter paper (Whatman International Ltd., Maidstone, U.K.) (Figure 1). 5 mL of distilled water was delivered to each petri dish. Each petri dish with its cover was sealed with a piece of parafilm to reduce evaporation of distilled water. The petri dishes were arranged on the shelf of incubator in a randomised block design (4 blocks). Temperatures were set to 21/15 °C day/night with 8 hour daylight and 16 hour dark period.

The germination tests were initiated within six months of seed collection. All seeds were incubated for four weeks. Germination of seed was determined based on the emergence of the radicle and the appearance of the first leaf. The number of germinated seeds was monitored daily after first record of germinated seeds (four days), and up to four weeks from being placed in the incubator. Germinated seeds were removed from the petri dishes. Seeds that did not germinate were further assessed for viability. Germination tests were terminated when no further germination took place (after four weeks), at which time the total number of germinated seeds was determined. The number of seeds which had germinated after four weeks was recorded and used as a measure of the proportion of viable seeds in the samples.

4. 2.8. Mortality and viability

After the termination of the standard germination test, ungerminated seeds were checked for viability. A simple pressure test was used to determine whether the differences in germination in *A. myosuroides* were because of varying degrees of dead or differences in viability of seeds that had not germinated. Each non-germinated seed was subjected to the simple pressure test, a seed was put between two fingers to evaluate viability/dead. Seeds that were not damaging when pinched and had white firm embryos were considered viable seed, while seeds that were damaging easily when pinched and had brown soft embryos were considered nonviable or dead seeds (Baskin and Baskin, 2014). In addition, seeds that were covered with fungi and damaged when pinched softly between fingers counted as dead seed.

4.2.9. Statistical analysis

The proportion of germinated, viable, dead seeds were analysed using generalized linear models (GLM), assuming a binomial response variable (viable or dead) and logit link function. The effect of different herbicide and soil type upon the proportion of seeds that germinated, dead or viable seeds were also analysed using Generalized Linear Models (GLM), assuming a binomial response variable (viable or dead) and logit link function. An ANOVA with likelihood-ratio chi-square tests were used to test the significance of effects of density, population, soil type and herbicide. We used Linear Models (LM) to analyse the effect of density, herbicide and soil type on the seed weight and seed size, followed by an ANOVA. Note that in most cases there were significant interaction terms, therefore to preserve statistical marginality, tests on main effects are not reported. All the statistical analyses were performed using R 3.6.0 (R Development Core Team, 2019). Log-likelihood ratio (LR) tests were used to test the significance of individual terms and marginality dealt with using Anova in package (car).

4.3. Results

4.3.1. Effect of density

4.3.1.1. Seed fate

There was a significant increase in germination of seeds from the parent populations that developed at high density, as the percentage of germination suggests that germinability increased by 10% for the seeds from the parent populations that developed at high density in compared to the parent populations from low density (Figure 4A) with a significant interaction between density and population (Table 1: $\chi^2 = 277.14$, df = 29, $p = 2.2 \times 10^{-16}$). Among the seeds that did not germinate, the proportion of viable seeds showed a strong dependence on weed density, with a significant increase in viability at high density compared to low density (Figure 4B). There was a statistically significant interaction between the effect of density and populations in which the parent plants grow on viable seeds (Table1: $\chi^2 = 89.83$, df = 29, $p = 3.7 \times 10^{-8}$). Finally, in contrast to germination and viability of seeds, which increased at high density, the proportion of dead seeds decreased at high density. Dead seeds at low density showed a significant increase (Figure 4C) and there was similarly significant interaction between the effect of density and populations on the proportion of dead seeds (Table1: $\chi^2 = 225.15$, df = 29, $p = 2.2 \times 10^{-16}$).

4.3.1.2. Effect of weed density on seed weight and seed size

A positive effect of weed density was found on seed weight and seed size. The weight of seeds showed a significant increase at high density (Figure 5D), as did seed length (Figure 5E), compared with seeds of low density plants: the interaction between density and population was significant in both cases ($F_{(30, 216)} = 16.16$, $p = 2.2 \times 10^{-16}$; $F_{(30, 216)} = 19.83$, $p = 2.2 \times 10^{-16}$).

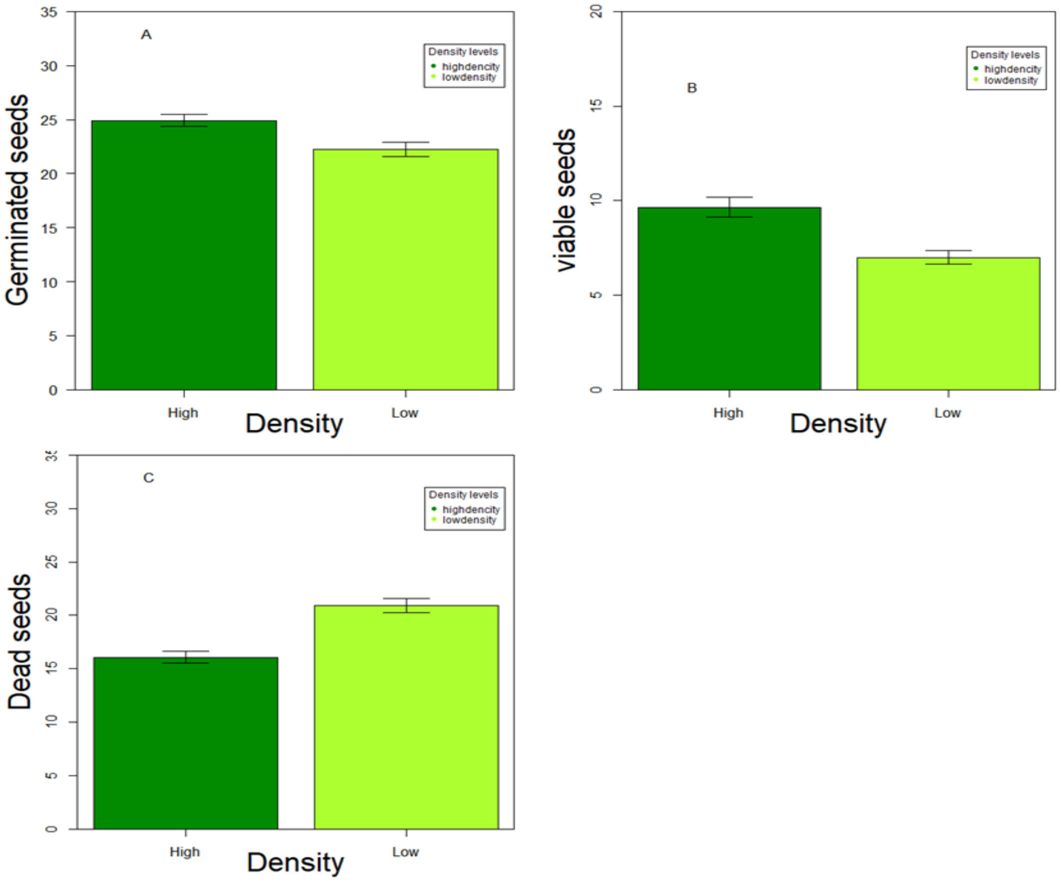

Figure 4. The effect of high and low densities of different populations of *A. myosuroides* plants on (A) percentage of seed germination, (B) viable seed (seeds that not germinated) and (C) dead seeds. Dark green represents seeds of all populations produced at high density, while light green represents seeds of all populations produced at low density. Error bars represent standard errors.

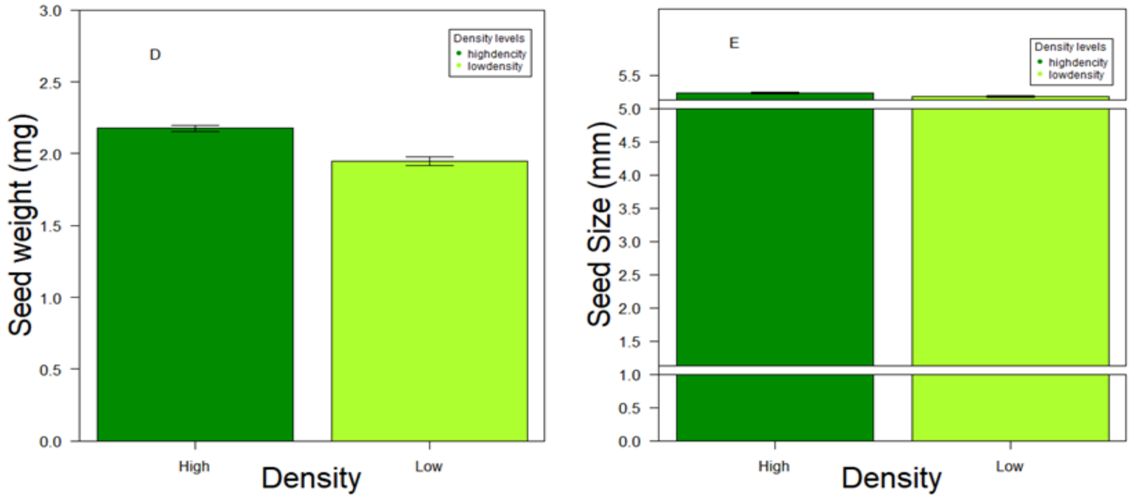

Figure 5. The effect of density on seed weight (D) and seed size (E) of different populations of *A. myosuroides* plants. Dark green represent seeds of all populations produced at high density, while light green represent seeds of all populations together that produced at high density, while light green represent seeds of all populations together that produced at low density. Error bars represent standard errors.

Table 1. Analysis of response of different populations of *A. myosuroides* seeds to levels of weed density (high and low). Results are from generalised linear models with binomial error and a log-link function of analysis of deviance table. Significance of model terms indicated by asterisks: $p < 0.001$, ***.

Alopecurus myosuroides	**LR Chisq**	**Df**	***p*(>Chisq)**
Germinated seeds			
blocks	20.00	3	0.0002 ***
population	874.51	42	$< 2.2 \times 10^{-16}$ ***
density	31.38	1	2.1×10^{-8} ***
population*density	277.14	29	$< 2.2 \times 10^{-16}$***
Viable seeds			
blocks	22.85	3	4.3×10^{-5}***
population	327.51	42	$<2.2 \times 10^{-16}$***
density	27.66	1	1.7×10^{-7}***
population*density	89.83	29	3.9×10^{-8}***
Dead seeds			
blocks	60.70	3	4.2×10^{-13}***
population	725.93	42	$<2.2 \times 10^{-16}$***
density	98.28	1	$<2.2 \times 10^{-16}$***
population*density	225.15	29	$<2.2 \times 10^{-16}$***

Table 2. Results from linear models of analysis of deviance table. Describing the effect of density (high and low) on seed characteristics (size and weight) of *A. myosuroides* for all the populations. Data on seed weight and size were log transformed. Significance of model terms indicated by asterisks: NS, $p<0.001$, ***.

Alopecurus myosuroides	**Sum of Squares**	**Df**	**F-value**	***p*(>F)**
log (seed weight)				
blocks	0.14	3	11.51	4.5×10^{-7}***
population	5.36	42	30.97	$<2.2 \times 10^{-16}$***
density	1.10	1	266.35	$<2.2 \times 10^{-16}$***
population*density	1.91	30	16.16	$<2.2 \times 10^{-16}$***
Residuals	0.89	216	-	-
log (seedsize)				
blocks	0.001	3	1.66	0.178^{NS}
population	0.21	42	31.34	$<2.2 \times 10^{-16}$***
density	0.01	1	44.81	1.8×10^{-10}***
Population*density	0.09	30	19.83	$<2.2 \times 10^{-16}$***
Residuals	0.03	216	-	-

4.3.2. Effect of historical herbicide treatments

4.3.2.1. Seed fate

The germination of seeds was significantly affected by historical herbicide treatments. However, this effect depended on density, population and the MOA of herbicide (Figure 6A). The interaction between density and herbicide treatment on seed germination showed a significant effect (Table 3: $\chi^2 = 92.25$, df = 7, $p = 2.2 \times 10^{-16}$) in addition to the significant effect from interaction between herbicide and population on germinated seeds (Table 3: $\chi^2 = 32.46$, df = 5, $p = 1.88 \times 10^{-10}$).

The effect of herbicide type on the viability of seeds was also significant (Figure 6B). However, this effect also depended on density and the MOAs. Furthermore, there was a significant interaction between density and herbicide treatment for viable seeds (Table 3: $\chi^2 = 15.66$, df = 6, $p = 0.016$) however, there was a marginal significant effect from the interaction between population and herbicide on seed viability (Table 3: $\chi^2 = 11.04$, df = 5, $p = 0.05$). Finally, the proportion of dead seed seeds were significantly affected by density and herbicide treatment (Figure 6C). However, this effect also depended on density and the type of herbicide. The interaction between the effect of herbicide and density on the proportion of dead seeds was highly significant (Table 3: $\chi^2 = 106.97$, df = 7, $p = 2.2 \times 10^{-16}$) this is in addition to the significant interaction effect between population and historical herbicide treatment on the proportion of dead seed seeds (Table 3: $\chi^2 = 35.10$, df = 5, $p = 1.4 \times 10^{-6}$).

4.3.2.2. Seed weight and seed size

Seed weight was significantly affected by density and historical herbicide treatments, which led to a significant increase in seed weight especially at high density (Figure 7A, table 4: $F_{(6, 236)} = 4.26$, $p = 0.0004$), compared with low density. The interaction between previous herbicide management and density showed no-significant effect (Table 4: $F_{(6, 236)} = 1.02$, $p = 0.414$) furthermore, the interaction between population and previous herbicide treatment showed significant effect on seed weight (Table 4: $\chi^2 = 4.31$, df = 5, $p = 0.0009$). In terms of seed size, there was a significant effect of different types of MOAs and density (Figure 7B, Table 4: $\chi^2 = 5.9$, df = 6, $p = 2.2 \times 10^{-6}$), the impact of different types of MOAs and population showed a marginal significant effect (Table 4: $\chi^2 = 2.77$, df = 1, $p = 0.1$) and the interaction between herbicide type and density showed a significant effect on this character (Table 4: $F_{(6, 236)} = 4.21$, $p = 0.0005$) further to the significant interaction effect between previous herbicide treatment and population (Table 4: $\chi^2 = 4.68$, df = 5, $p = 0.0004$) on seed length.

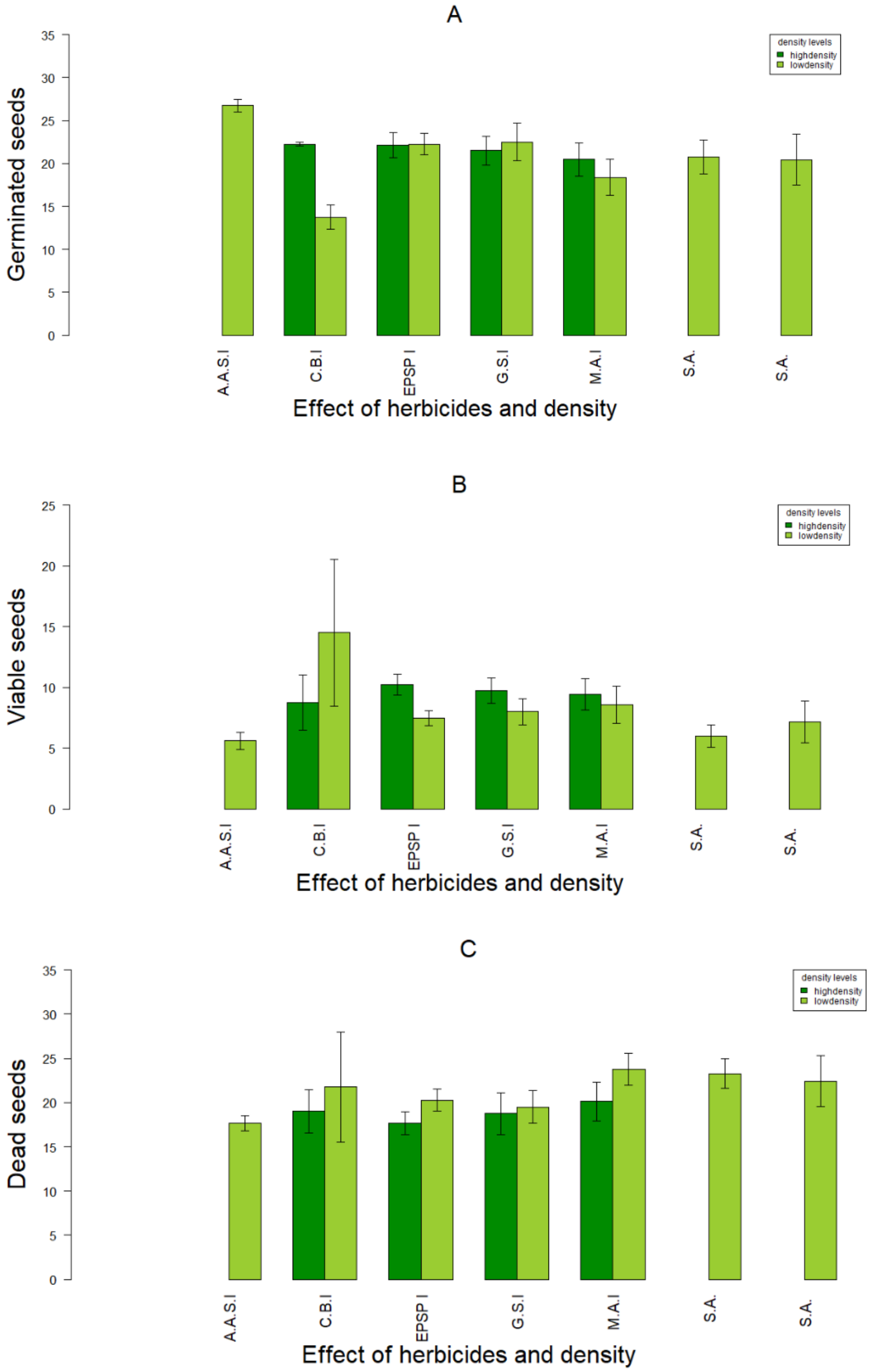

Figure 6. Effect of different MOAs of each herbicide that used (prior the seed collection) to control *A. myosuroides* populations within each field, in interaction with density (high and low) on seed germination (A), seed viability (B) and dead seeds (C). The MOAs are MAI, CBI, EPSP I, AASI, SA and GSI. Error bars represent standard errors.

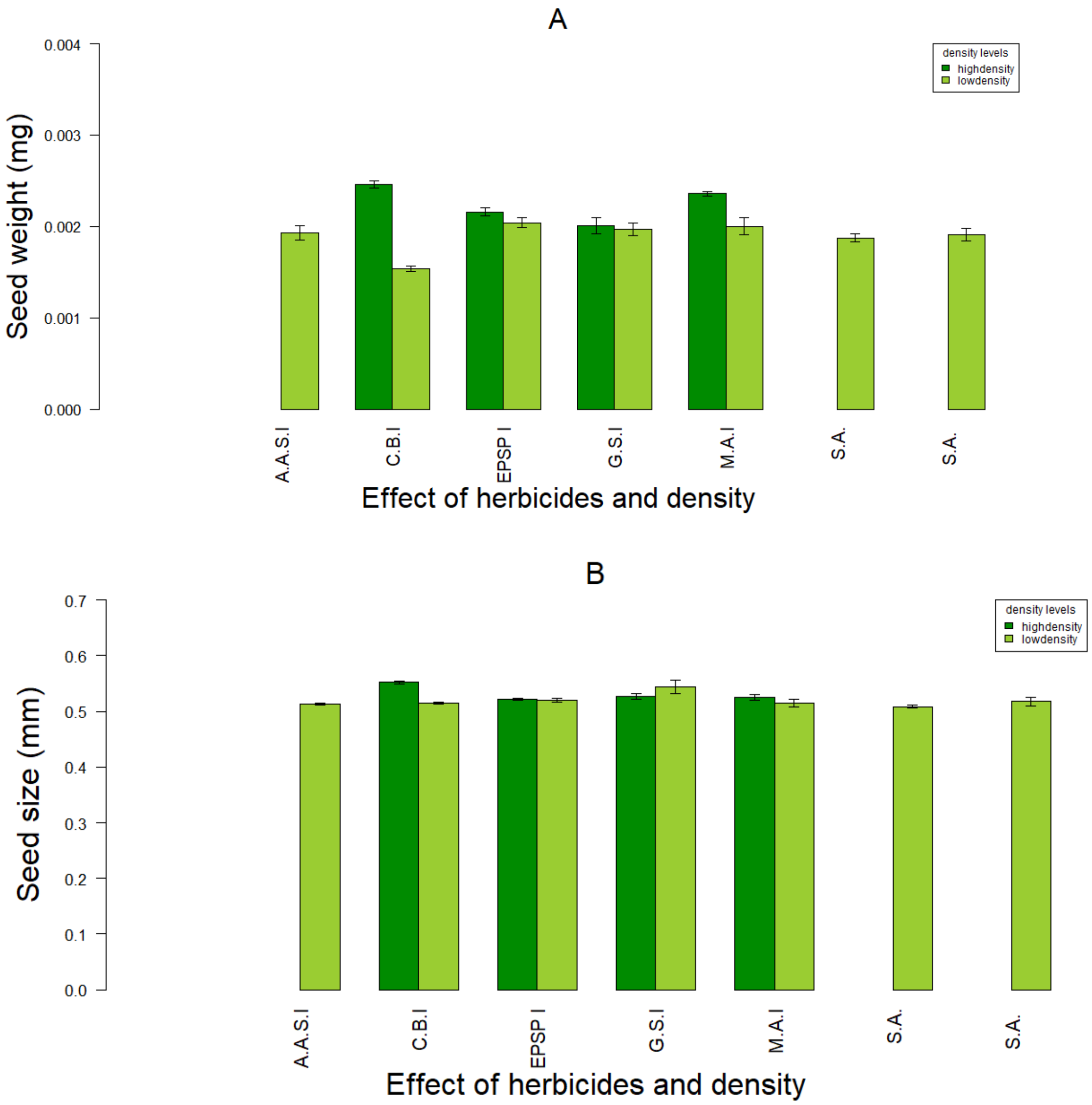

Figure 7. Effect of different MOAs of each herbicide that applied (prior collection of the seeds) to control *A. myosuroides* populations within each field, in interaction with density on seed weight (A) and seed size (B). The MOAs are: MAI. = Microtubule assembly inhibition, CBI = Carotenoid biosynthesis inhibition (Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturate step (PDS)), EPSP I = EPSP synthase inhibition (this mode of action is specific to glyphosate (glycines), which are nonspecific herbicides that act by inhibiting the amino acid synthesis), AASI = Inhibits plant amino acid synthesis- acetohydroxyacid synthase AHAS, SA = Synthetic auxin (Foliar uptake causing auxin-type response) and GSI = Glutamine synthetase inhibitor (accumulates ammonium ions, inhibits photosynthesis). Error bars represent standard errors.

4.3.3. Effect of different soil types

4.3.3.1. Seed fate

Germination of seeds was significantly affected by soil type (Figure 8A). However, this relationship was affected by an interaction with population and density that was highly significant (Table 3: χ^2= 97.97, df = 7, p = 2.2 x 10^{-16}) (Table3: χ^2= 10.63, df = 2, p = 0.004). The highest germination was recorded in the seeds of populations that grew in silty-loam soil, whilst the lowest germination was observed in those of populations that grew at low density and in heavy-clay soil type.

Seed viability was also significantly affected by soil type (Figure 8B, Table 3: χ^2 = 211.95, df = 11, p = 2.2 x 10^{-16}). The highest seed viability was observed within the populations that grew in clay-gravel soil followed by heavy-clay and heavy-fen soil regardless of density. The interaction between soil type and density was not significant (Table 3: χ^2 = 9.17, df = 6, p = 0.165) as well as no significant interaction was observed between soil type and population (Table3: χ^2 = 0.03, df = 2, p = 0.99). Finally, the proportion of dead seeds was affected significantly by soil types (Figure 8C). There was a significant interaction between the effect of soil type and density on dead seeds (Table 3: χ^2 = 104.47, df = 7, p = 2.2 x 10^{-16}) in addition to a significant interaction between soil type and population (Table 3: χ^2 = 11.95, df = 2, p = 0.002). In general, this effect tended to increase at low density in compared to high density.

4.3.3.2. Seed weight and seed size

Seed weight was significantly affected by soil type, which led to a significant increase in seed weight especially for the populations at high density (Figure 9A), such that the weight of *A. myosuroides* seeds was affected by the soil type (Table 4: $F_{(12, 236)}$ = 2.39, p = 0.006) however, the effect of population showed no significant effect on seed weight (Table 4: $F_{(1, 236)}$ = 0.034, p = 0.854) in which the mother plant grew. For example, seed weight was greatest for the populations that grew in heavy soils such as clay. The interaction between soil type and density also showed a significant impact on seed weight of seeds (Table 4: $F_{(6, 236)}$ = 6.54, p = 2.11 x 10^{-6}) while the interaction between soil type and population also showed no significant effect on seed weight (Table 4: $F_{(2, 236)}$ = 0.429, p = 0.652). Furthermore, seed size showed a significant response to the variation in soil type (Figure 9B, Table 4: $F_{(12, 236)}$ = 16.222, p = 2.2 x 10^{-6}) nonetheless, the effect of population showed a marginal significant effect on seed size (Table 4: $F_{(1, 236)}$ = 2.77, p = 0.01). The maximum length of seed was observed in the populations that grew in soil with a high clay content. The interaction between soil type and density showed a significant impact on the length of seeds (Table 4: $F_{(6, 236)}$ = 6.40, p = 2.92 x 10^{-6}) in addition, the interaction between soil type and population showed a significant effect on this character (Table 4: $F_{(2, 236)}$ = 10.03, p = 6.6 x 10^{-5}).

The results confirm the important role of environmental conditions, specifically density, herbicide management and soil type, in which the mother plants grow in determining the seed characteristics. All these factors separately and their interaction (e.g. density x herbicide, density x soil type) showed a significant impact upon variations in seed germination and seed characteristics of offspring, causing adaptive phenological changes, which enabling its successfulness survival and evolutionary response in grass weed *A. myosuroides*.

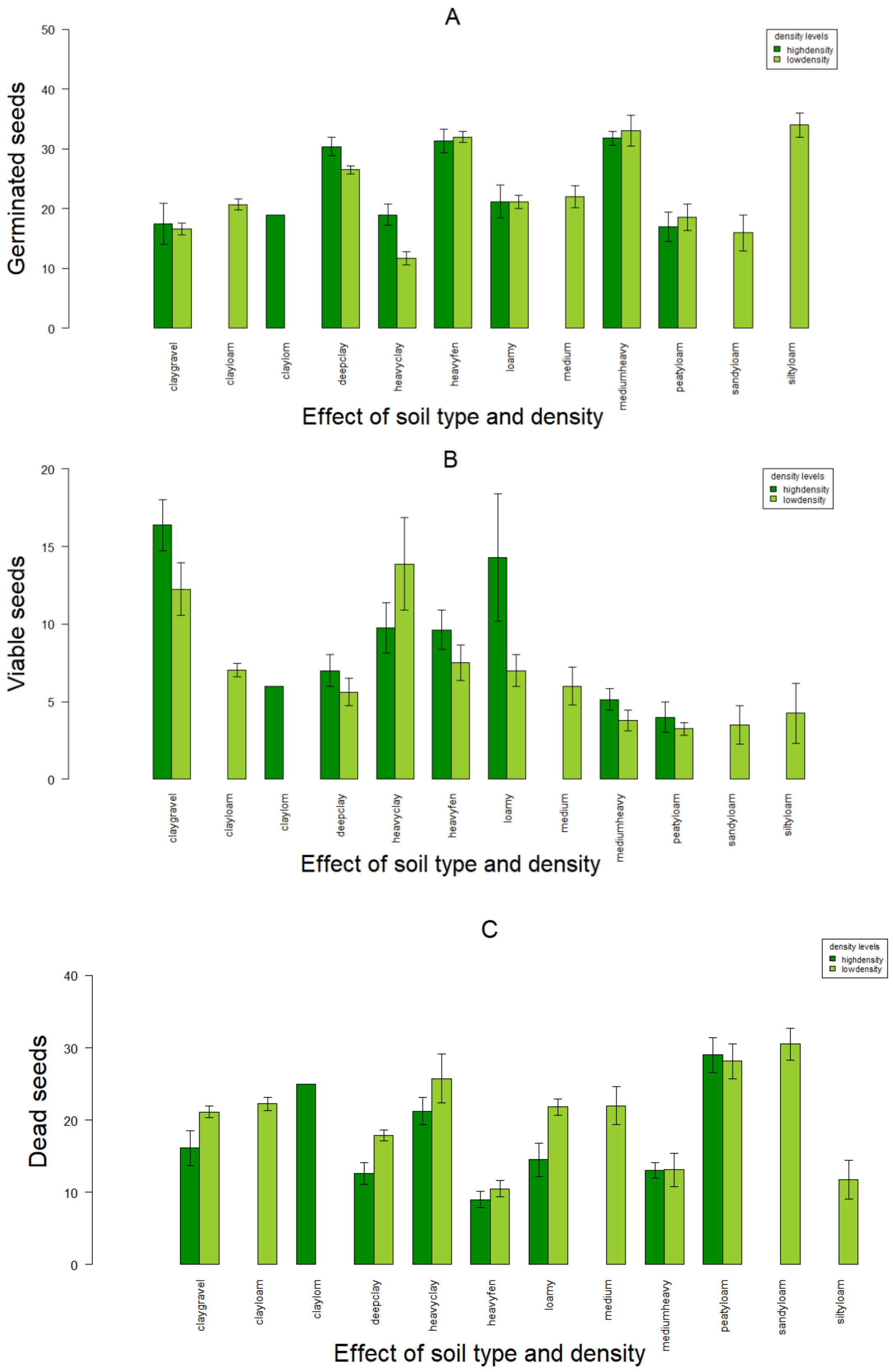

Figure 8. Shows the effect of different soil types in interaction with density levels on different populations of *A. myosuroides*, A) seed germination, B) viable seeds and C) dead seeds. Error bars represent standard errors.

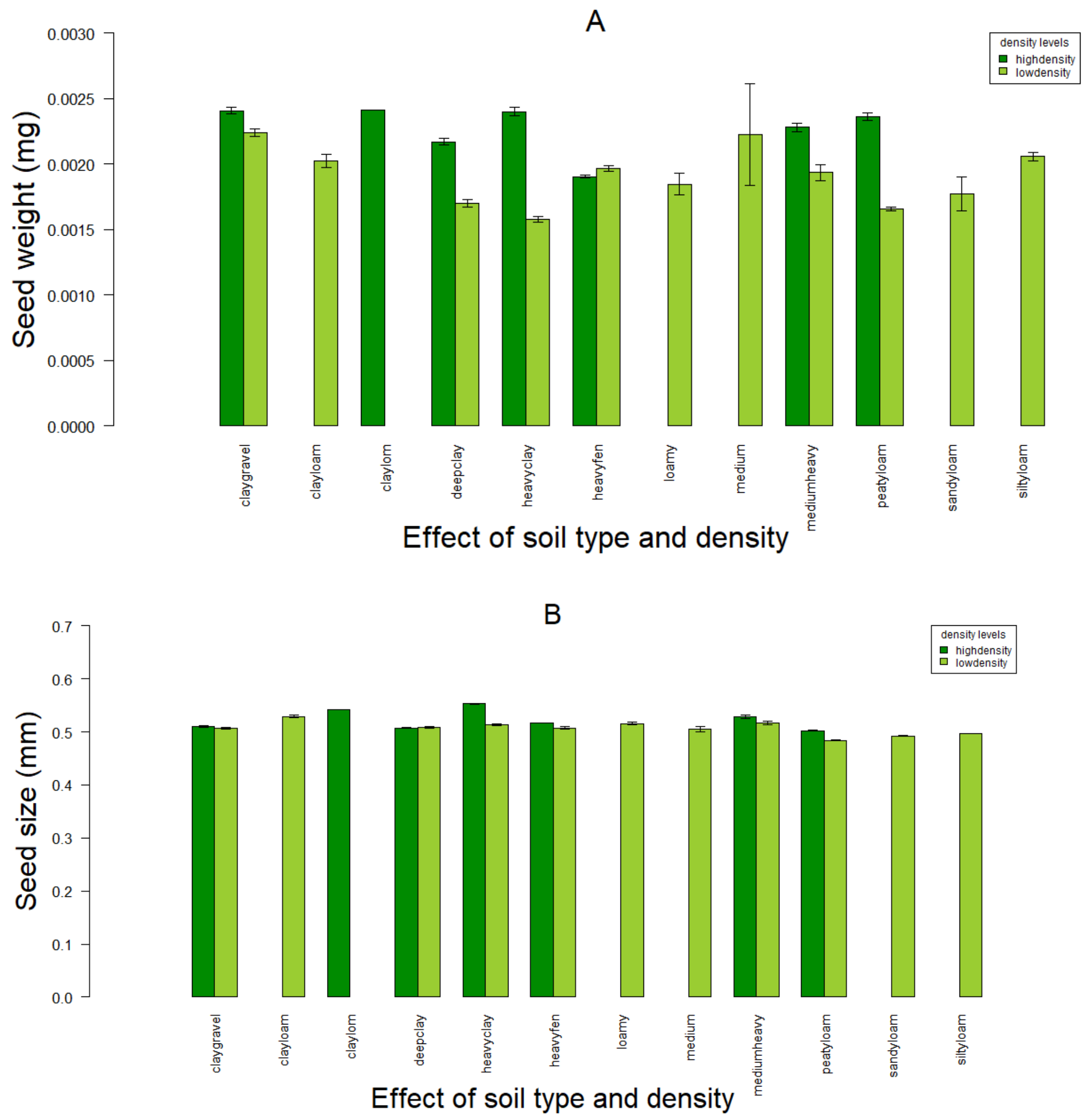

Figure 9. Shows the effect of different soil types in interaction with density on *A. myosuroides* populations seed weight (A) and seed size (B). Error bars represent standard errors.

Table 3. Analysis of characterising different populations of *A. myosuroides* seeds with historical herbicide managements and soil type in interaction with density. Results are from generalized linear models with binomial error and log-link function. Significance of model terms indicated by asterisks: ‘•’ $p < 0.1$, ‘**’ $p < 0.01$ and ‘***’ $p < 0.001$.

Alopecurus myosuroides	**LR Chisq**	**Df**	***P-value***
Germinated seeds			
Blocks	17.46	3	0.0006 ***
population	11.59	1	0.0007 ***
Soil.type	432.47	11	<2.2 x10^{-16} ***
Herbicide	56.94	6	1.8 x10^{-10} ***
Density	23.26	1	1.4 x10^{-6} ***
Soil.type:herbicide	23.88	7	0.0012 **
Herbicide:density	92.25	7	<2.2 x10^{-16} ***
Herbicide:population	32.46	5	<4.8 x10^{-6} ***
Soil.type:density	97.97	7	<2.2 x10^{-16} ***
Soil.type:population	10.63	2	0.005 **
Viable seeds			
Blocks	22.28	3	5.7 x10^{-5} ***
Population	2.05	1	0.152
Soil.type	211.95	11	<2.2 x10^{-16} ***
Herbicide	44.84	6	5.1 x10^{-8} ***
Density	44.725	1	<2.2 x10^{-11} ***
Soil.type:herbicide	30.42	7	7.9 x10^{-5} ***
Herbicide:density	15.66	6	0.016 *
Herbicide:population	11.04	5	0.051 •
Soil.type:density	9.17	6	0.99
Soil.type:population	0.03	2	0.985
Dead seeds			
Blocks	47.57	3	<2.6 x10^{-10} ***
Population	19.84	1	<8.4 x10^{-6} ***
Soil.type	349.56	11	<2.2 x10^{-16} ***
Herbicide	47.08	6	1.8 x10^{-8} ***
Density	127.67	1	<2.2 x10^{-16} ***
Soil.type:herbicide	15.26	7	0.033 *
Herbicide:density	106.97	7	<2.2 x10^{-16} ***
Herbicide:population	35.10	5	1.4 x10^{-6} ***
Soil.type:density	104.47	7	<2.2 x10^{-16} ***
Soil.type:population			

Table 4. Results from analysis of variance of linear models describing the effect of historical herbicide treatments and soil type on seed characteristics (size and weight) of *A. myosuroides* for all the populations. Data on seed weight and seed size were log transformed. Significance of model terms indicated by asterisks: 'NS' *p*< not significant, '•' *p*< 0.1, '*' *p* < 0.05, '**' *p*< 0.01 and '***' *p*< 0.001.

Alopecurus myosuroides	**Sum of Squares**	**Df**	**F-value**	***p-value***
log (seed weight)				
blocks	0.15	3	2.333	0.075 •
population	0.001	1	0.034	0.854 $^{\text{NS}}$
herbicide	0.55	6	4.261	0.0004 ***
soil.type	0.62	12	2.391	0.0062 **
density	1.07	1	49.753	$1.9\text{x}10^{-11}$ ***
herbicide:soil.type	0.09	7	0.628	0.732 $^{\text{NS}}$
herbicide:density	0.13	6	1.018	0.414 $^{\text{NS}}$
herbicide:population	0.463	5	4.32	0.0008 ***
soil.type:density	0.84	6	6.54	$2.1\text{x}10^{-6}$ ***
soil.type:population	0.02	2	10.03	0.652
Residuals	5.1	236	-	-
log (seed size)				
blocks	0.001	3	0.437	0.726 $^{\text{NS}}$
population	0.002	1	2.772	0.097 •
herbicide	0.02	6	5.895	$9.4\text{x}10^{-6}$ ***
soil.type	0.12	12	16.222	$<2.2\text{x}10^{-16}$ ***
density	0.004	1	7.195	0.008 **
herbicide:soil.type	0.01	7	1.229	0.287 $^{\text{NS}}$
herbicide:density	0.01	6	4.213	0.0004 ***
herbicide:population	0.013	5	4.681	0.0004 ***
soil.type:density	0.02	6	6.399	$2.9\text{x}10^{-6}$ ***
soil.type:population	0.01	2	10.03	$6.6\text{x}10^{-5}$ ***
Residuals	0.13	236	-	

4. 4. Discussion

Since the beginning of conversion of land for cultivation, agricultural management practices such as herbicide application, plant density, crop rotation and tillage system have applied selection pressures on weed populations (Delye et al., 2013c, Owen et al., 2015, Batlla et al., 2020). Consequently, changes in management over time have led weeds to evolve adaptive traits that allow them to survive and persist in novel environments (Delye et al., 2013c, Owen et al., 2015, Batlla et al., 2020). The experiments described in this chapter have highlighted variation in traits among seeds from populations of *A. myosuroides* with variation in density, historical herbicide management and soil types. Our results provide evidence that selection in response to environmental conditions may cause adaptive phenological change in offspring.

4.4.1 Effect of weed density on germination and emergence characteristic

This study has demonstrated a significant effect of maternal density on the germination and viability in seeds of different populations of *A. myosuroides*. This change in seed characteristics, particularly the seed viability response to high density, was observed previously in *A. myosuroides* populations (Chauvel et al., 2005). The novelty of this study was to show the positive relationships between density states and germination behaviour and emergence characteristics of *Alopecurus* progeny. Therefore, this study confirms the potential importance of maternal environmental conditions for the change of germination and viability of *Alopecurus* offspring. A significant response of germination and viability to maternal density states were found to be positive across all the populations, as both germination and viability were found to be higher with increasing density. However, increasing density did not show any significant effect on seed mortality. When the density is of high intensity (e.g. in presence of high number of *A. myosuroides* plants), seed provisioning is important to maximize germination and viability in response to current and future density environments. Thus, these observed positive responses across all the populations to the maternal density support the phenological adaptive response hypothesis under high density. Density effects on germination and other seed traits occur in several arable species and could potentially have a strong influence on overall population dynamics (Saayman-duToit, 2000, Violle et al., 2009, Orrock and Christopher, 2010). As an out-crossing grass species, the fertilisation of *A. myosuroides* might be expected to be sensitive to weed density, as typically seed fertilisation shows an increase at greater weed densities. For this reason, the negative response of dead seeds also expected to increased density, because as the number of *Alopecurus* plants reduces, the chances of fertilization may also decline.

Even though seed weight is a comparatively conservative trait within many species (Harper et al., 1970), it can vary depending on a range of factors, including the conditions to which the mother plant is exposed. Previously, Waller, (1982) found variation in seed weight within populations as well as in individual plants. However, the direct effects of competition on the seed characters of parent plants are poorly reported in spite of their possible effect on plant fitness (Violle et al., 2009). Alteration in seed size, another seed trait, has also been shown not essentially have to be adaptive responses to environmental conditions, since they might also mirror impacts of resource variation during fruit development, as was established for *Convallaria majalis* L

(Eriksson, 1999). However, the results show that maternal environmental conditions such as density had a significant effect on seed weight as well as seed size. As with increasing density states, we observed an increase in seed weight and seed size. This change in seed weight and size in response to maternal density, across all the populations of *Alopecurus* offspring, provides evidence for the importance of maternal environment for the phenological adaptive response of seeds to a novel environment. Therefore, the directional selection for other characteristics apart from resistance, may result from herbicide control causing adaptive phenological change.

4.4.2. Effect of different historical herbicide application in an interaction with density on seed germination and the characteristics of seed.

We found a significant relationship between both historical herbicide management and the germination behaviour and emergence characteristics of *A. myosuroides* seeds. The relationship between herbicide management and germination behaviour was also previously investigated by Gundel et al. (2008) in *Lolium multiflorum* seeds, who showed that herbicide management causing phenological alteration may lead to create directional selection for other traits apart from resistance. Our results showed that seed germination and viability varied with the different herbicides previously applied to parental plants. The highest response of germination was observed in the populations that treated with AASI MOAs, but the lowest response of germination was in the populations treated with CBI. Conversely, the highest response of seed viability was observed in the populations that previously treated with CBI in compare to the lowest response, which was in the populations treated with AASI. Thus, our results support the hypothesis that historical herbicide application in *A. myosuroides* may select for changes in adaptive phenological traits (Mortimer, 1997).

In evolutionary terms, increased resistance to herbicides may be linked with a fitness cost (Vila-Aiub et al., 2009b). It is a fundamental principle of evolutionary genetics that adaptation to a new environment is likely to have adverse effects on fitness in the original environment, known as the “cost of adaptation” (Strauss et al., 2002, Vila-Aiub et al., 2011). In agricultural ecosystems, the success of a weed species depends on its capability to successfully resist or escape pre-planting weed management and post-planting herbicide applications (Mortimer, 1997, Froud-Williams et al., 1984, Delye et al., 2013c, Batlla et al., 2020). In several cropping-systems, weed control by herbicide application has caused the selection of weed populations with altered seed germination characteristics, combined with herbicide resistance traits (Batlla et al., 2020). The first possibility for this alteration in seed germination characteristics of the populations’ resistance to herbicides either results from the selection of herbicide resistance alleles with direct pleiotropic impacts, associated to germination-affecting alleles, or solely co-selection. A second possibility is that there is a ‘founder’ effect, whereby small populations that survive herbicide applications are subject to subsequent genetic change (Batlla et al., 2020). This was demonstrated for *A. myosuroides* plants with target-site resistance by (Delye et al., 2013c), who showed that mutant acetyl-CoA carboxylase (ACCase) alleles that endow the ability to resist the application of extensively used herbicides based on the ACCase inhibitor mechanism have a pleiotropic impact, and directly result in several phenotypic and phenological changes in weeds, specifically in the dynamics of seed germination. Furthermore, Vila-Aiub et al., (2009b) showed that several herbicide resistance alleles are linked with pleiotropic impacts on plant fitness. There is substantial interest in fitness costs for resistance to herbicide because these may reduce the incidence of resistant genotypes in weed populations when the herbicide selection is stopped, and may underpin strategies for managing resistant weeds (Vila-Aiub et al., 2009). The results of this study suggest that the changes in germination and emergence characteristics of *A. myosuroides* seeds due to maternal environmental conditions such as herbicide application may endow adaptive changes to weed populations, allowing them to survive and persist in a novel cropping management.

4.4.3. Effect of soil type in interaction with density

Our results support previous results showing that soil type is an important determinant of the within-field distribution of *A. myosuroides* (Metcalfe et al., 2019). Historically, it has been found in water-retentive, poorly-drained, heavy-textured soil (clay or silt) (Lutman et al., 2013). Altering cropping management, as well as evolved herbicide resistance, however, may have allowed it to broaden its range into lighter soil (sandy soil). However, overall it is more likely to be a problem on heaver soil such as clay or silt, rather than on lighter soil (Lutman et al., 2013).

Weed distributions have been shown to be associated to other soil variables such as carbon, water, and macronutrients (Lutman et al., 2002). The impact of different soil factors on weed proliferation can frequently be linked back to their impact on soil moisture and water retention capability (Dieleman et al., 2000). Soil water availability is important in numerous aspects of the weeds' life-cycle, especially in the primary growing season (Dieleman et al., 2000). Furthermore, soil conditions may have an important influence in improving the efficacy of certain herbicides (Lutman et al., 2002). Large quantities of clay and organic carbon in soil may cause the absorption of most herbicides (Gaston et al., 2001). Thus herbicide absorption in the soil will decrease the amount of herbicide that the plant takes up and, by this process, various types of soil can influence the degree of management obtained by herbicide application. The results showed the important role of different soil types in interaction with density in which the parental plants grew, in variation in seed germination and emergence characteristics of *Alopecurus* offspring. Consequently, we showed significant impacts upon variations in seed germination and seed characteristics of the offspring, causing adaptive phenological changes in different soils, which may enable successful survival and evolutionary responses in the grass weed *A. myosuroides*

The mean seed weight of *A. myosuroides* was significantly affected by soil type and density level across all the populations. The heaviest seed weight was recorded for populations produced in heavy-clay, clay-gravel, medium-heavy, deep-clay and peaty-loam soil from high density level. Thus our results suggest that heavier seeds are produced under high population densities, in interaction with heavy and well-drained soil types (Lutman et al., 2013). The largest seed size was observed in populations from high density and heavy-clay, medium-heavy, heavy-fen followed by clay-gravel and deep-clay respectively. Nevertheless, populations from both density levels (high and low) showed the same response of seed size to clay-gravel and deep-clay.

4.5. Conclusion

The results of the current study suggest important impacts on germination and seed characteristics of the growing environment of plants of *A. myosuroides*, specifically density, crop management (e.g. different herbicide application) and soil type. Based on the results, this study suggest that variations in seed germination and seed characteristics in response to altered environmental conditions may help *A. myosuroides* adapt to a different environments. Further investigations into the effect of density and other factors that affect seed characteristics in field conditions will help develop improved management practices. The study shows that environmental conditions, specifically weed density, herbicide treatment and soil type, in which the mother plant grew, significantly affect the germination and emergence characteristic of the offspring.

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CHAPTER FIVE

General discussion

STATEMENT OF CONTTRIBUTIONS

The general discussion was written by the candidate with editorial advice from Prof. Colin Osborne and Prof. Robert Freckleton.

CHAPTER FIVE

GENERAL DISCUSSION

Discussion

Plants are subjected to a range of abiotic and biotic stresses. Adaptive local plant responses to these stresses may arise via the evolution of defensive strategies, which confer resistance to previous and current stresses. The mechanisms of plant defence generate constitutive and inducible characters that inhibit or limit damage or mortality, which can be defined as resistance, while those improving the ability to recover from damage are known as tolerance (Núñez-Farfán et al., 2007, Vila-Aiub et al., 2004, 2011). In addition, plants' vigour and responsiveness to environmental stress are the products of the re-modification of physiology and metabolism throughout genetic history and over the life span of the individual. Plants have evolved unique strategies for adapting to changing environmental conditions, monitoring their environments exhaustively and changing their metabolism to sustain balances (Pastori and Foyer, 2002). Plant’s everyday survival or death can be determined by the stress severity, plant genetic background and its individual history. The destiny of any individual is dictated by these factors. Thus, the interaction of genome and environment is an important subject for elucidating the nature of the phenotypic variability contributing to the effective response of plants to environmental changes (Pastori and Foyer, 2002). Through the stress response relationship, plants make use of common mechanisms and components. The understanding of how plants identify environmental stimuli has improved dramatically over the past few decades, for example to survive these perturbations, plants have evolved elaborate mechanisms of environmental detection, which are mediated by cellular signalling cascades and gene transcription links (Kinoshita and Seki, 2014). The ultimate evidence that weeds have an exceptional potential to evolve under stress conditions is herbicide resistance. Although herbicide resistance mutations in agroecosystems under herbicide selection have conferred an exceptional advantage to plant fitness, resistance mutations are expected to exhibit an adaptation cost in untreated herbicide conditions compared to the susceptible wild-type (Vila-Aiub, 2019). Resistance mechanisms in plants therefor provide an excellent model for addressing basic concerns about the cost of stress adaptation (Vila-Aiub et al., 2011). On a global scale, the widespread and repeated use of herbicides has imposed selection for the evolution of weeds resistant to these chemicals, since triazine-resistant weeds were first reported in the mid-1960's (Busi et al., 2013). Herbicide resistance in weeds is most frequently caused by a cellular level alteration of the herbicide's site of action. In comparison, herbicide tolerance, i.e. low doses of herbicides, classically results either from variations in herbicide absorption and translocation at plant level or from variations in plant metabolism and detoxification of herbicides (Warwick, 1991).

The aims of this thesis were to identify the effect of abiotic stress particularly drought stress on the evolution of herbicide resistance in grass weeds and to identify how rapid selection for herbicide resistance occurs in grass weed species. The thesis further investigated the effect of environmental factors upon aspects of grass weed biology, particularly germination behaviour. This understanding would add a new perception to our knowledge of environmental stresses and their effects on the evolution of herbicide resistance in weeds. The following questions were asked. How does rapid selection for herbicide resistance occur in grass weed species through the interaction of abiotic stress, such as drought and a xenobiotic such as herbicide exposure (Chapter 2)? Is the mechanism of inheritance for herbicide resistance acquired in this way genetic or epigenetic (Chapter 3). How does drought stress impact on the growth and morphological traits of the grass weed species (Chapters 2 and 3)? And how do environmental conditions in which mother plants develop (especially weed density, soil type and herbicide applications) affect the biology of offspring (e.g. seed weight, seed size and germination behaviour)? The main findings of this thesis are summarised in figure 1, which shows the effect of environmental conditions on the evolution of herbicides resistance as well as seed characteristics.

The results of this study reveal that exposure to abiotic stress, particularly drought stress, underpins the rapid evolution of herbicide resistance in subsequent generations. The work also shown that exposure to drought stress highly impacts the growth of grass weeds, which leads to a substantial decrease in growth parameters. The absence of high rates of variations in resistance in F1 plants of *Alopecurus myosuroides* confirms the rapid evolution of resistance to selective herbicide doses, implying a non-genetic mechanism rather than natural selection on standing genetic variation. Crucially, the exposure to drought stress increased the level of resistance in several previously untreated populations to different doses of herbicide, revealing the potential for rapid selection of cross-resistance (Chapters 2). Through the work presented in chapter 3, I show new evidence that epigenetic mechanisms are involved in the stress response modification. The transmission of pre-modified expression patterns to the offspring of plants that have exposed to drought stress, and subsequently survived herbicide application without the requirement for mutations, therefore accelerates the evolution of resistance in the F1 generation (Chapter 3). This thesis also reveals that germination behaviour and other biological aspects (i.e. seed size and seed weight) are correlated with variation in weed density levels, soil type and herbicide applications in outcrossing grass populations (Chapter 4). Vitally these results indicate that understanding physiological pathways that are potentially involved in stress-responses and detoxification of herbicides require a deeper consideration of the evolution of herbicide resistance under global climate change.

Plants have to combine a variety of environmental and metabolic signals; they do this by linking interacting signal transduction mechanisms that, for the duration of stress, modify gene expression together (Pastori and Foyer, 2002). As a consequence, genes coding for other functions in stress responses are selected on in order to achieve more rapid detoxification of the herbicides. There are lots of physiological pathways that are potentially involved in such responses, including those that are concerned with detoxification and stress responses. For example, the glutathione S-transferase (GST) pathway in plants is a physiological pathway that has both been implicated in the response to drought as well as in herbicide and other stress tolerance. Thus, it was expected that exposing several previously untreated populations of grass weeds (*A. myosuroides* Huds and *Poa annua*) to an abiotic stress such as drought would increase resistance levels to herbicide as a subsequent stress exposure. High frequency of survival in the F1 generation of droughted parental plants reveal that enhanced herbicide detoxification mechanism is probably responsible for resistance across all the populations. This response was depending on the dose of fenoxaprop-p-ethyl herbicide. In general exposure to a high dose of fenoxaprop-p-ethyl herbicide showed increased rate of survivorship in *A. myosuroides* compared to low-dose of fenoxaprop-p-ethyl herbicide, irrespective to the level of drought stress (high or low) (chapter 2). However, there was no difference between *Poa annua* populations relative to their response to any of the herbicide dose that applied to F1 generation (chapter 2) as such, thus this species was omitted from further investigations and replaced with larger numbers of *A. myosuroides* populations (Chapter 3). Additional reason for omitting the *P. annua* from further investigation is that may be the fenoxaprop-P-ethyl herbicide is not the right herbicide to be used for controlling *P. annua* species. Since there was no effect of low drought treatment on herbicide resistance in chapter 2, the work in chapter 3 exposed weed populations to a single level of drought stress (high drought treatment). It has been reported that environmental conditions play an important role in the evolution of metabolic herbicide resistance, especially in *A. myosuroides*, as the two major enzymes glutathione S-transferase (GST) and cytochrome P450 monooxygenases (P450) that have been implicated in herbicide resistance due to increased detoxification, can respond to both abiotic and biotic stresses (Marrs, 1996, Schuler and Werck-Reichhart, 2003). Furthermore, it has been established that GST enzymes can contribute to multiple herbicide resistance by playing a role in oxidative stress response and/or herbicide detoxification by catalysing herbicide conjugation with glutathione (Cummins et al., 1999, Powles and Yu, 2010).

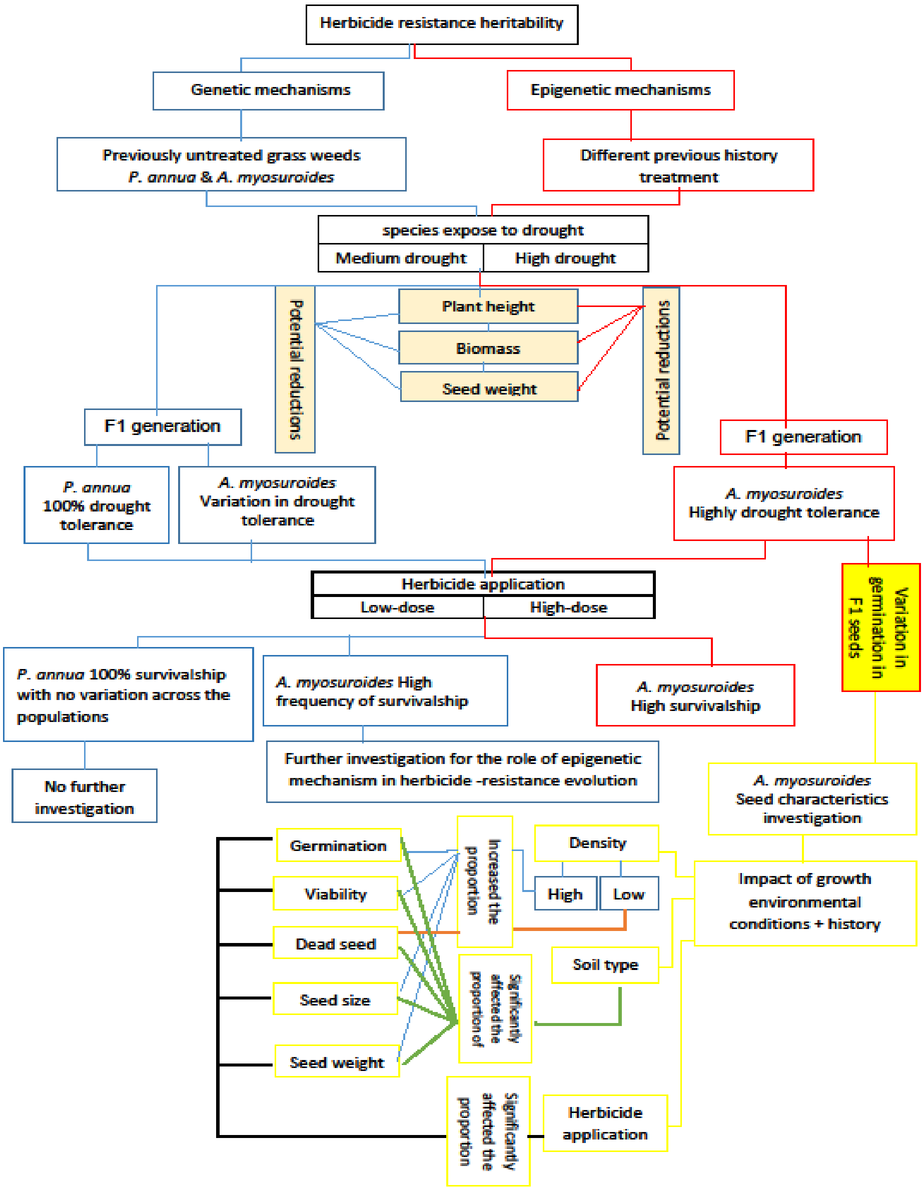

Figure 1. Shows the main findings of the thesis, the evolution of herbicide resistance in the grass weed *A. myosuroides* and *P. annua*, with the genetic mechanisms shown in blue, epigenetic mechanisms in red, and the effect of environmental conditions (i.e. density, soil type and herbicide application) on seed germination and emergence characteristics in yellow. The effect of population density (high and low) on germination and seed characteristics shown in blue, soil type in green and herbicide in black. The effect of low density shown in brown, only significantly affected seed mortality. Boxes that are shared between genetic and epigenetic mechanisms indicated that the same procedure was implemented for each mechanism.

Water is an important factor that affects the growth and reproduction of plants. Nevertheless, the weed species *A. myosuroides* and *P. annua* showed a great ability to withstand the drought treatments in which they could grow and produce seeds. However, the drought treatment caused a great reduction in plant height, biomass and seed weight across all the populations. Based on these observations, drought stress can count as a limiting factor that affects weed growth, therefore, understanding the impact of drought stress upon weed growth is important for better understanding of evolutionary mechanisms and then the deleterious impacts of weeds (Chapters 2 and 3). This effect of drought stress upon growth of other species was also previously observed (Saayman-duToit, 2000, De Abelleyra et al., 2008, Chauhan and Johnson, 2010, Li et al., 2011).

As mentioned in previous sections, plants have to adapt to both biotic and abiotic stressors via evolving complex adaptation and defence mechanisms. Chromatin modifications, nucleosome positioning, and DNA methylation have been known as vital components in these adaptations. Assumed its possible epigenetic nature, these modifications may provide the basis for the mechanism of stress memory, making plants to respond more efficiently to repeated stress or even to prepare their progeny for potential upcoming stresses (Lämke and Bäurle, 2017, Markus et al., 2018). These mechanisms are inherited via mitotic cell divisions and, in some circumstances, can be passed to the subsequent generation. They consequently confer a possible mechanism for stress memories in plants (Kinoshita and Seki, 2014). Until now, the role of epigenetic mechanisms in the evolution of herbicide resistance have not been widely investigated (Delye et al., 2013b). However, previous studies have indicated that epigenetic mechanisms are involved in stress response regulation (Chinnusamy and Zhu, 2009, Kinoshita and Seki, 2014, Markus et al., 2018). The evolution of herbicide resistance could be accelerated through transmission of pre-modified expression patterns to the offspring of plants having survived herbicide stress without the requirement for mutation (Delye et al., 2013b). Thus, the role of epigenetic mechanisms in underpinning the evolution of herbicide resistance in a broader number of *A. myosuroides* populations was investigated (Chapter 3). I was able to identify that exposure to high drought stress in clonal parental plants could evolve a rapid or more potent response to fenoxaprop-p-ethyl herbicide application as a subsequent stress (Chapter 3), because exposing the parent plant to stressors can impact the genetic formation of the progeny. A response to environmental conditions such as drought stress in one generation can therefore cause epigenetic variations with the potential to be inherited for multiple generations. This transmission of pre-adjusted expression patterns to the offspring of plants having survived drought stress without the requirement for mutations accelerated the evolution of herbicide resistance in the F1. Therefore, the transgenerational priming “transgenerational stress memory” (Lämke and Bäurle, 2017, Markus et al., 2018) is the most possible responsible mechanism for the evolution of herbicide resistance in *A. myosuroides* via epigenetic mechanisms as a conduit for environmental cues that initiate any changes in gene expression in response to stress. In each generation, epigenetic variations are developmentally controlled, whereas stress-induced alterations are likely more random and normally revert rapidly after their incidence (Pecinka and Mittelsten Scheid, 2012). Nevertheless, under certain conditions, it is expected that, chromatin adjustments (such as: DNA methylation, histone modifications and/or exchange of histone variants) may continue for an extended time after stress exposure, providing a 'stress memory', and may be inherited via mitotic or even meiotic cell divisions (Chinnusamy and Zhu, 2009). Usually, global eradication of epigenetic alterations occurs in germline cells, denoted to as epigenetic reprogramming. Nonetheless, specific genomic regions can escape eradication, permitting the existence of epigenetic stats to be carried to offspring, resulting in transgenerational epigenetic inheritance (Heard and Martienssen, 2014).

Cloned *A. myosuroides* plants were able to tolerate drought stress. Although, I observed some mortality in the droughted plants, but it was not high. The differential responses of the 15 populations may partially account for their success or failure in their ability to tolerate high drought stress. The method for applying high drought stress at an early stage of the plant's life (after seedling establishment from the cloning process) followed by a recovery treatment (re-irrigation) until the drought treatment was stopped before harvesting the plants, allowed me to observe the stronger ability of *A. myosuroides* plants to withstand the drought stress after each time of re-watering the plants. An altered ability of *A. myosuroides* F1 generation's seeds to germinate following exposure to drought stress illustrates the concept of primary dormancy or an inhibition of full seed development that may have been caused by the drought treatment. These results show that environmental stress (i.e. drought stress) readily may triggers the mechanisms of stress response in a grass weed at an epigenetic scale. Hence, the observed herbicide resistance in the offspring of stressed plants may correlated with the parental environment. Current study in the field of epigenetics is adding to an increasing understanding of how epigenetic mechanisms may have an important role in underpinning the evolution of herbicide resistance in *A. myosuroides* populations and the potential role it may has to play in adaptation to environmental conditions. Both heritable and non-heritable epigenetic variability are major causes of heterogeneity in ecologically relevant traits such as reproduction and resistance to stress. The epigenetic variations between different ecosystems are further evidence of the role of epigenetic processes in natural populations in plant response to the environment (Robertson and Wolf, 2012).

Considerable variation was observed in seed germination across the populations of *A. myosuroides* that were collected from different farms with different previous histories of herbicide applications (Chapter 3) leading to interesting questions regarding the environmental factors that may affect the progenies behaviour. Detailed study was conducted in Chapter 4 to determine the importance of density, historical herbicide management, and also the effect of different soil types, in which the mother plants are developed upon the progenies germination behaviour and other biological aspects (e.g. seed size and seed weight). This can then be used as the basis for better understanding the consequences of density, herbicide application and soil type on germination behaviour and emergence characteristics. Like other annual weeds, the only way for *A. myosuroides* populations to survive and proliferate is via seed production (Moss, 2017). Thus understanding the seed biology and evolution of this weed is important for predicting its responses to environmental conditions and cropping systems. Weed populations frequently show heritable difference in life history traits that may possibly reflect phenological adaptations (Mortimer, 1997). One of the strongest environmental fluctuations that is experienced by weed species populations is the net impact of weed management practices that result in individual's mortality and the decreased fertility of survivors. Consequently, in weed species, weed management practices will form agents of natural selection given variation of heritable genetic for traits of life history. As a result of past selection, populations possibly become adapted in that individuals possess adaptive features (Mortimer, 1997). Density effects on germination and other seed traits were found to occur in several arable species and could potentially have a strong influence on overall population dynamics (Saayman-duToit, 2000, Violle et al., 2009, Orrock and Christopher, 2010). As an outcrossing grass species the fertilisation of *A. myosuroides* might be expected to be sensitive to weed density, as typically seed fertilisation shows an increase with increases in weed density. We also expected the negative response of dead seeds to increased density, because as the number of *Alopecurus* plants reduce the chance of fertilization of the plant may also reduce. This change in seed characteristics, especially the seed viability response to density, was observed previously in *A. myosuroides* populations (Chauvel et al., 2005).

Remarkable differences were also observed in the interaction between different herbicide treatments previously applied to the mother plants and the density in which the seed developed. An increase in germination, viability, seed weight and seed size were observed relative to the type of herbicide that applied to the mother plants in interaction with density. Thus the efficacy of the herbicide type in interaction with density is expected to impact upon the *A. myosuroides* progeny's germination, and seed characteristics, which could lead to further adaptation to environmental conditions and agriculture practices during subsequent seasons. As a result, the herbicide may therefore causes high resistance to evolve in the population and thus these plants developed normally depending on the herbicide type (Chapter 4). The relationship between herbicide management and germination behaviour was also previously investigated by (Gundel et al., 2008). Young, (1984) also showed that different herbicides will differently reduce seed germination and growth of grass weed seedlings. Resistance to herbicides is an evolutionary process that may include variations in physiological and ecological characters associated to plant fitness (Torres-Garcia et al., 2015). It is a fundamental principle of evolutionary genetics that adaptation to a new environment can always have adverse effects on fitness in the original environment, known as the “cost of adaptation” (Strauss et al., 2002).

Additionally, the germination and seed characteristics of *A. myosuroides* were affected by both soil type and density and their interaction. Increase in germination, viability, seed weight and seed size mostly occurred in heavy to clay soil types in interaction with high density of *A. myosuroides* populations. These results were potentially expected, as seeds of *A. myosuroides* require a low amount of oxygen in the soil to germinate and also germination and growth of *A. myosuroides* were previously found to be successful in such soils. Nevertheless, altering cropping managements and reduced management due to evolved herbicide resistance may have allowed it to broaden its range into lighter soil (sandy soil). However, the weed is more likely to be a problem on heaver soil such as clay or silt rather than on lighter soil (Lutman et al., 2013).

The only seed character that showed a potential response to low density populations of *A. myosuroides* was the seed mortality (dead seeds). However, at low density, resources are less likely to be limiting. As *A. myosuroides* behaves as an allogamous species (Chauvel et al., 2005) and it is mainly a wind pollinated species, it is expected that an increased number of dead seeds will occur in low density populations due to incomplete pollination. Consequently, germination is less likely to be accelerated in low population densities in the *A. myosuroides* species. This notable observation is more likely to be associated with the ability of fertilization and also the pollen dispersal. It has been documented that the mean dispersal distance of *Alopecurus* seed is 51cm (Colbach and Sache, 2001), and this is why it was expected that density could be one of the most important factors that affecting seed mortality. As it was found, the ability of *A. myosuroides* to be limited in terms of self-dispersal and pollen grain (Marechal et al., 2012), therefore, in the absence of the appropriate number of pollen donors there will be an increased number of unfertilized plants, which is more likely to occurs at low population densities. These results are in contrast with the data of (Saayman-duToit, 2000) with other weed species such as common thorn-apple (*Datura stramonium* L.), who showed that dead seeds increase at greater weed densities.

For more than 400 million years, plants have been subjected to both biotic and abiotic stresses arising from environmental conditions. Through evolving complex networks of stress detection, signalling and response mechanisms that trigger both general and specific responses, they have survived these stresses, with a modification of the response over time (Vaahtera and Brosche, 2011). Herbicides are a strong xenobiotic stress and are expected as such to trigger some of these pathways. Thus, non-target site resistance (NTSR) can be assumed to be associated with a general plant stress response in weed species. This is supported by studies on the effects on plant transcriptome or proteome of herbicide applications, which demonstrate that the response to herbicide stress can be compared with responses to other stresses (Das et al., 2010). The NTSR mechanism in weeds is complex and its incidence is increasing, which means that weed management may become even more problematic and challenging in times of global warming, by accelerating the evolution of herbicide resistance.

Herbicide resistant weeds are an increasing problem worldwide with a number of species displaying these traits, such as *A. myosuroides* and *P. annua*. The evolution of herbicide resistance is a classic example of accelerated contemporary adaptation when confronted with new environmental stresses (David et al., 2019). Resistance to herbicide in *A. myosuroides* populations can be due to a mutations at the herbicide target site and/or from changes in the pathways of weed metabolism such as enhanced herbicide detoxification. Metabolic resistance frequently endows resistance to herbicides of various chemical groups and sites of action and can prolong to new chemicals (Yu and Powles, 2014). Therefore, evolved herbicide resistance due to enhanced metabolic capability of herbicide detoxification in weedy plant species are the main problem. Glutathione S-transferase, glycosyl transferase and cytochrome P450 monooxygenase are the enzyme families that are implicated in herbicide metabolism resistance (Cummins et al., 1997, Reade et al., 2004, Restif and Koella, 2004, Delye, 2005, Powles and Yu, 2010, Cummins et al., 2011, Yu and Powles, 2014). These enzyme families also found to be involved in biotic and abiotic stress response (Marrs, 1996, Schuler and Werck-Reichhart, 2003). Especially, in plant development, it has been shown that GSTs have different roles such as endogenous metabolism, stress tolerance, and xenobiotic detoxification (Nianiou-Obeidat et al., 2017). These enzyme families, as detoxification systems, are expressed both constitutively and stimulated (upregulated) in response to herbicide safeners. Plant GSTs are families of multifunctional enzymes that catalyze the conjugation of glutathione to a range of electrophilic and hydrophobic substrate. Previously, studies with *A. myosuroides* as a multiple resistant biotypes have shown that they have higher GST activity with enhanced P450-catalyzed herbicide metabolism, while for GST-catalyzed herbicide conjugation there is finite evidence of higher capacity (Cummins et al., 1999, Reade et al., 2004, Restif and Koella, 2004). Increased GST activity possibly has a secondary role in moderating against oxidative stress in these biotypes. Therefore, GSTs may have a direct role in herbicide conjugation or indirect role in stress response in evolved resistance to herbicide (Powles and Yu, 2010). This shows the potential role of detoxification systems in the evolution of herbicide resistance in weeds, in the process of adapting to repeated abiotic stresses.

As sessile organisms, plants are required to constantly regulate their responses to exterior environmental changes to survive the ever-altering growth conditions (Boyko and Kovalchuk, 2011). In addition to herbicides, abiotic stresses (e.g. drought, heat and limitation in CO_2) similarly impart selection pressure on plants and, therefore, can accelerate the evolution of herbicide-resistant plants. Recent related research on different weed species *E. colona* (junglerice) (Lariza et al., 2020), and the results of this thesis support this hypothesis. The results of this thesis provide clear evidence that pre-exposure to abiotic stress particularly drought stress can change the responses of plants to forthcoming stress from herbicide application by producing a rapid and/or powerful heritable response in grass weed populations such as *A. myosuroides*. This mechanism of stress response was previously described as a form of "stress memory" by (Ding et al., 2012). Pastori and Foyer, (2002) have additionally suggested that in the stress response relationship, plants make use of common mechanisms and components. They further identified this phenomenon as "cross-tolerance", which allows plants to adapt to a number of different stresses following exposure to a particular stress. Therefore, exposure to drought stress may act as a signal for forthcoming stress from other xenobiotic agents, including herbicides, which can stimulate mechanisms resulting in greater stress resistance (Beck et al., 2004). Consequently, the results of this thesis suggest that environmental conditions play a crucial role in the herbicide detoxification capacities of weeds. Particularly, the thesis underline the significance of drought stress, and demonstrate that drought stress can enrich herbicide degradation pathways.

By addressing these hypotheses I have met my main objective, which was to identify the effect of environmental stresses (i.e. drought stress) on the evolution of herbicide resistance especially in grass weed *A. myosuroides* and use this to identify the possible role of epigenetic mechanism in this process. I have developed our understanding of the role played by abiotic factors in determining the acceleration of the evolution of herbicide resistance in *A. myosuroides*, both through observed rapid acquisition of herbicide resistance genetically and via possible role of epigenetic mechanism. In order to investigate these effects further it would be possible to conduct further research such as molecular biology to test the hypothesis of an epigenetic mechanism's role in the evolution of herbicide resistance and how this mechanism will evolve without the need for mutation. Does this defence mechanism exist in the plant prior to the stress exposure? And does this mechanism persist in the subsequent generations? These hypotheses can be tested through exposing different generations to drought stresses and screening the progeny of each generation under herbicide treatment along with plants that grow under normal conditions (well-watered); to find whether the resistant trait will occur continuously in the subsequent generations or the resistant trait will revert and the plant become a non-resistant plant. Overall, further works needed in order to investigate the fate of this resistant trait in different generation and under different environmental conditions and herbicide treatments. It would also be interesting to run selection experiments on different herbicides where plants are exposed to arrange of herbicides after drought exposure to see if the evolution of resistance is accelerated under any particular herbicide treatment.

My work has gone a long way towards identifying other environmental conditions such as: density, soil type and historical herbicide treatment responsible for determining the germination behaviour and emergence characteristics of *A. myosuroides* seeds. However, there is still more work to do on determining the mechanistic response of the plant adaptation to those environmental conditions, and identifying how knowledge of density condition, soil type and herbicide treatment can be used to locate weed adaptation and evolution of resistance within fields.

The results from my experiments could potentially be translated into the development of herbicide compounds by the agrochemical industries. Most herbicide screening in the agrochemical industry should not just characterize by a protocol where solutions of test chemicals spray over sets of plants in pots or over seeded bare soil in the case of pre-emergent screens. In addition to these tests, the agrochemical industry might need to screen chemicals in different environmental conditions for example, stressed and unstressed environmental conditions. In the light of the results of this study it might be essential for screening the chemical compounds under drought and none droughted conditions. However, if the herbicide efficacy can be affected by environmental conditions then perhaps this needs to be taken into consideration when developing such chemical compounds.

Under field condition *A. myosuroides* encounters different environmental conditions throughout their life cycle, especially, in summer season during the anthesis stage and seed development black-grass plants exposes to natural drought stress within the field. Therefore, farmers should consider the evolution of resistance after seasons. Consequently, the study also reveals that farmers might need consider trans-generational phenotypic adaptation to drought stress and its association with herbicide tolerant/resistant phenotypes.

Conclusion

In order to improve our understanding of how environmental stresses, particularly drought, underpin the evolution of herbicide resistance in grass weeds, it is essential to identify the mechanisms of stress response that involve in the evolution of herbicide resistance as well as in drought stress. Plants utilize and can evolve powerful biological strategies for adjusting their metabolic systems and defence mechanisms. Unravelling the involvement of physiological pathways and their complexities in metabolic-based resistance mechanisms, is a challenge that has the potential to cause a fundamental change in our understanding and approach to weed resistance management. I provide evidence that, in grass weeds with no previous history of herbicide application, drought stress can act as a signal for forthcoming severe stress such as herbicide application, which can underpin mechanisms produce rapid and/or strong stress resistance (i.e. herbicide resistance) in the F1 generation. I also show that an epigenetic mechanism may plays a vital role in response to drought stress in the evolution of herbicide resistance in the F1 generation of *A. myosuroides*. An epigenetic mechanism may also involve in the phenotypic response and offspring traits, specifically germination behaviour of parental plants to drought. These results suggest that drought stress can accelerate the evolution of herbicide resistance in grass weeds as quickly as in the first generation.

Seed germination is predicted to be under strong selection pressure to occur under circumstances that increase subsequent plant survival. Furthermore, variation in seed characteristics and germination behaviour between weed populations enables the weed species to continue through changing environmental conditions and is therefore an important constituent of weediness. Therefore, the identification of maternal impacts in *A. myosuroides* as an out-crossing species arising from their growth environment such as density condition, cropping management practices (e.g. different herbicide application) and soil types on germination and seed characteristics can be of importance for thinking about management strategies as well as future research. Through this study, I identify that variation in the response of *A. myosuroides* seeds to their growth environment will increase the population's ability to effectively resist applications of herbicide. These results contribute to the identification of the important role of drought stress in the evolution of herbicide resistance in weeds that can help in better predict and manage the spread of herbicide resistance. Furthermore, they can improve our understanding of how cropping managements in interaction with environmental conditions where the populations develop will affect the offspring characteristics. In turn, this knowledge can contribute to better understand the consequences of weed management practices and environmental stresses and improve expectation of how grass weeds and their offspring may respond to changes in their environment.

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List of APPENDICES:

Chapter two: Drought exposure leads to rapid acquisition and inheritance of herbicide resistance in the weed *Alopecurus myosuroides*

- **Appendix 1.** Plant height, biomass and seed weight per plant of A. myosuroides under two different levels of drought stress medium and high (25% and 75% plant mortality). All the values are logarithm of parameter's value. Mean ± standard error are shown for A. myosu-roides plants across all the populations, where the drought effect was significant. Asterisks indicate significant differences for both drought stress levels in comparison with controls. Significance levels from the Anova are shown in the following way: '**' $p< 0.01$ and '***' $p< 0.001$.
- **Appendix 2.** Results of ANOVA describing the effect of drought stress on the parental generation of P. annua including the treatment (medium and high drought) as well as con-trolling for origins (none drought), five populations and four replicates. Data on height, biomass and seed weight were log transformed. Significance of model terms indicated by asterisks: '***' $p<0.001$, '*' $p<0.05$, '**' $p<0.01$ and '•' $p<0.1$
- **Appendix 3.** Analysis of F1 generation plants that are resistant + damaged (count as survived) versus dead plants for (a) A. myosuroides and (b) P. annua. Results are from gener-alized linear models with binomial error and a log-link function. Significance of model terms indicated by asterisks: '*' $p<0.05$, and '***' $p<0.001$.
- **Appendix 4.** Analysis of F1 generation plants that are resistant (i.e. survived intact) versus dead or damaged (a) A. myosuroides and (b) P. annua. Results are from generalized linear models with binomial error and a log-link function. Significance of model terms indicated by asterisks: '*' $p< 0.05$ and '**' $p< 0.01$.
- **Appendix 5.** Results from analysis of variance from linear models describing the relation-ship between variance, drought, herbicide, drought*herbicide and herbicide*population interaction for dry weight of surviving plants for both A. myosuroides and P. annua. Sig-nificance of model terms indicated by asterisks: '*', $p< 0.05$, '**' $p< 0.001$, and '***' $p<0.001$,

Chapter four: The impact of density, soil type and exposure to herbicide on germination and emergence characteristics of populations of *Alopecurus myosuroides*

- **Appendix 6.** Previous history of cropping management practices (i.e. herbicide application), soil types in which parental plant populations developed and density conditions (high and low) for the fields where seeds of *Alopecurus myosuroides* populations (P) were collected in 2015, for conducting this study.

Appendix 1. Plant height, biomass and seed weight per plant of *A. myosuroides* under two different levels of drought stress medium and high (25% and 75% plant mortality). All the values are logarithm of parameter's value. Mean ± standard error are shown for *A. myosuroides* plants across all the populations, where the drought effect was significant. Asterisks indicate significant differences for both drought stress levels in comparison with controls. Significance levels from the Anova are shown in the following way: '**' $p< 0.01$ and '***' $p< 0.001$.

	Degrees of freedom	**Sum of Squares**	**Mean Square**	**F-value**	***p*-value**
Alopecurus myosuroides					
log (plant height)					
Replicate	3	0.297	0.099	1.712	0.178
Population	4	1.068	0.267	4.616	0.003**
Drought	1	1.574	1.574	27.209	< 0.0001***
Population*Drought	4	0.092	0.023	0.400	0.810
Residuals	47	2.720	0.058	-	-
log (biomass)					
Replicate	3	0.465	0.155	1.941	0.136
Population	4	0.206	0.052	0.650	0.632
Drought	1	5.668	5.668	71.039	< 0.0001***
Population*Drought	4	0.176	0.044	0.552	0.699
Residuals	47	3.750	0.080	-	-
log (seed weight)					
Replicate	3	0.446	0.149	0.291	0.832
Population	4	9.290	2.323	4.543	0.004**
Drought	1	33.731	33.731	65.970	< 0.0001***
Population*Drought	4	3.370	0.842	1.648	0.179
Residuals	46	23.520	0.511	-	-

Appendix 2. Results of ANOVA describing the effect of drought stress on the parental generation of *P. annua* including the treatment (medium and high drought) as well as controlling for origins (none drought), five populations and four replicates. Data on height, biomass and seed weight were log transformed. Significance of model terms indicated by asterisks: '***' p<0.001, '*' p<0.05, '**' p<0.01 and '•' p<0.1

	Degrees of free-dom	**Sum of Squares**	**Mean Square**	**F-value**	***p*-value**
Poa annua					
log (plant height)					
Replicate	3	1.203	0.401	2.892	0.056 •
Population	4	1.390	0.348	2.506	0.069 •
Drought	1	6.799	6.799	49.012	< 0.0001***
Population*Drought	4	0.264	0.066	0.476	0.753
Residuals	24	3.330	0.139	-	-
log (biomass)					
Replicate	3	1.430	0.477	5.755	0.004**
Population	4	1.079	0.270	3.256	0.029*
Drought	1	1.660	1.660	20.012	0.0001***
Population*Drought	4	0.154	0.039	0.465	0.761
Residuals	24	1.990	0.083	-	-
log (seed weight)					
Replicate	3	1.047	0.349	1.528	0.233
Population	4	1.866	0.467	2.043	0.120 •
Drought	1	2.179	2.179	9.43	0.005**
Population*Drought	4	0.225	0.056	0.246	0.909
Residuals	24	5.479	0.228	-	-

Appendix 3. Analysis of F1 generation plants that are resistant + damaged (count as survived) versus dead plants for (a) *A. myosuroides* and (b) *P. annua*. Results are from generalized linear models with binomial error and a log-link function. Significance of model terms indicated by asterisks: '*' $p<0.05$, and '***' $p<0.001$.

	Degrees of freedom	Deviance	Residual degrees of freedom	Residual Deviance	*p*-value
(a)					
Null			129	127.260	
Blocks	4	4.252	125	168.008	0.373
Population	4	9.690	121	158.318	0.046 *
Herbicide	1	31.297	120	127.020	2.21×10^{-8}***
Drought	2	7.859	118	119.162	0.0197 *
Herbicide*drought	2	28.355	116	90.806	6.9×10^{-7} ***
Herbicide*population	4	3.420	112	87.386	0.490
(b)					
Null			147	15.374	
Blocks	4	4.8491	143	10.545	0.303
Population	4	3.0621	139	7.463	0.548
Herbicide	1	0.1968	138	7.266	0.657
Drought	2	1.5733	136	5.692	0.455
Herbicide*drought	2	2.3479	134	3.345	0.309
Herbicide*population	4	3.3445	130	0.000	0.502

Appendix 4. Analysis of F1 generation plants that are resistant (i.e. survived intact) versus dead or damaged (a) *A. myosuroides* and (b) *P. annua*. Results are from generalized linear models with binomial error and a log-link function. Significance of model terms indicated by asterisks: '*' $p < 0.05$ and '**' $p < 0.01$.

	Degrees of freedom	**Deviance**	**Residual degrees of freedom**	**Residual Deviance**	***p*-value**
(a)					
Null			129	127.283	
Blocks	4	6.0950	125	121.188	0.1923
Population	4	13.0558	121	108.133	0.0110 *
Herbicide	1	1.0195	120	107.113	0.3127
Drought	2	13.0938	118	94.019	0.0024 **
Herbicide*drought	2	1.2682	116	92.751	0.5304
Herbicide*population	4	4.1586	112	88.593	0.3850
(b)					
Null			147	92.660	
Blocks	4	4.5793	143	88.081	0.333
Population	4	5.6043	139	82.477	0.231
Herbicide	1	0.0000	138	82.476	0.997
Drought	2	4.0262	136	78.450	0.134
Herbicide*drought	2	1.3751	134	77.075	0.503
Herbicide*population	4	5.3615	130	71.714	0.252

Appendix 5. Results from analysis of variance from linear models describing the relationship between variance, drought, herbicide, drought*herbicide and herbicide*population interaction for dry weight of surviving plants for both *A. myosuroides* and *P. annua*. Significance of model terms indicated by asterisks: '*', p< 0.05, '**' p< 0.001, and '***' p<0.001,

	Degrees of freedom	**Sum of Squares**	**Mean Square**	**F-value**	***p*-value**
Alopecurus myosuroides					
Blocks	4	9.0963	2.2741	8.2263	5.96x10^{-6}***
Population	4	1.1589	0.2897	1.0480	0.3283
Drought	2	0.5039	0.2519	0.9113	0.3643
Herbicide	1	0.0068	0.0068	0.0244	0.8688
Drought*herbicide	2	2.8734	1.4367	5.1972	0.0047 **
Population*herbicide	4	3.0529	0.7632	3.1084	0.02127 *
Residuals	63	15.4687	0.2455	-	-
Poa annua					
Blocks	4	4.9695	1.2424	8.1087	7.12x10^{-6}***
Population	4	1.8382	0.4595	2.9993	0.0208
Drought	2	0.0195	0.0097	0.0636	0.9382
Herbicide	1	1.1488	1.1488	7.4978	0.007 **
Drought*herbicide	2	0.0246	0.0123	0.0804	0.9227
Population*herbicide	4	0.6466	0.1617	1.0569	0.3807
Residuals	129	19.7309	0.1530	-	-

Appendix 6. Previous history of cropping management practices (i.e. herbicide application), soil types in which parental plant populations developed and density conditions (high and low) for the fields where seeds of *Alopecurus myosuroides* populations (P) were collected in 2015, for conducting chapter four's study.

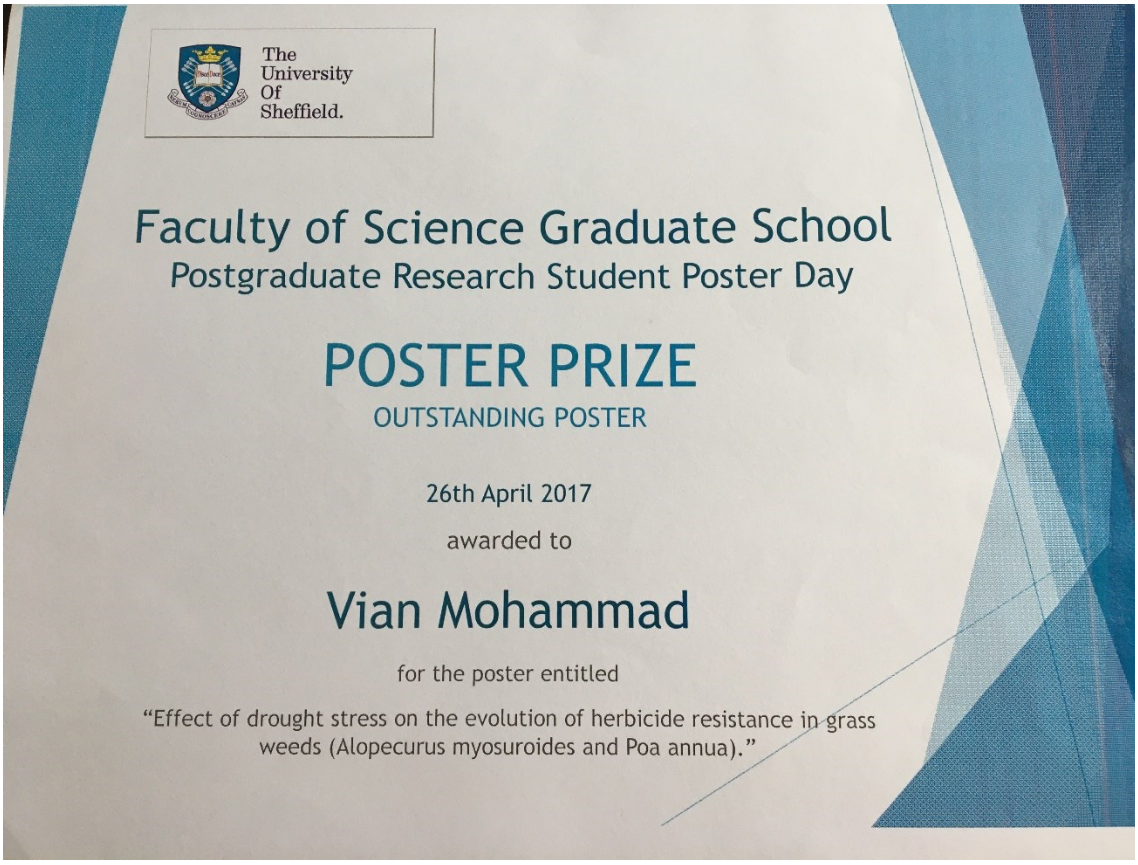
Species	**populations (P)**	**herbicide (MOAs)**	**soil types**	**density state/field number(F)**
Alopecurus myosuroides	P1	EPSP inhibition	clay-loam	highdensity/F1
		EPSP inhibition	clay-loam	lowdensity/F1
	P2	-	medium-heavy	highdensity/F1
		-	medium-heavy	lowdensity/F1
	P3	-	clay-loam	highdensity/F1
		-	clay-loam	lowdensity/F1
	P4	EPSP inhibition	clay-loam	highdensity/F1
		EPSP inhibition	clay-loam	lowdensity/F1
	P5	Synthetic auxin	sandy-loam	highdensity/F1
		Synthetic auxin	sandy-loam	lowdensity/F1
	P6	EPSP inhibition	silty-loam	highdensity/F1
		EPSP inhibition	silty-loam	lowdensity/F1
	P7	A.A.S. inhibition	loamy	highdensity/F1
		A.A.S. inhibition	loamy	lowdensity/F1
	P8	M.A. inhibition	heavy-clay	highdensity/F1
		M.A. inhibition	heavy-clay	lowdensity/F1
	P9	M.A. inhibition	peaty-loam	highdensity/F1
		M.A. inhibition	peaty-loam	lowdensity/F1
	P10	EPSP inhibition	clay-loam	highdensity/F2
		EPSP inhibition	clay-loam	lowdensity/F2
	P11	-	medium-heavy	highdensity/F2
		-	medium-heavy	lowdensity/F2
	P12	Synthetic auxin	loamy	highdensity/F2
		Synthetic auxin	Loamy	lowdensity/F2
	P13	M.A. inhibition	clay-loam	highdensity/F2
		M.A. inhibition	clay-loam	lowdensity/F2
	P14	Synthetic auxin	clay-loam	highdensity/F2
		Synthetic auxin	clay-loam	lowdensity/F2
	P15	EPSP inhibition	clay-loam	highdensity/F2
		EPSP inhibition	clay-loam	lowdensity/F2
	P16	G.S. inhibition	clay-loam	highdensity/F2
		G.S. inhibition	clay-loam	lowdensity/F2

	P17	-	loamy	highdensity/F2
		-	loamy	lowdensity/F2
	P18	EPSP inhibition	medium	highdensity/F2
		EPSP inhibition	medium	lowdensity/F2
	P19	A.A.S. inhibition	deep-clay	highdensity/F3
		A.A.S. inhibition	deep-clay	lowdensity/F3
	P20	EPSP inhibition	clay-gravel	highdensity/F3
		EPSP inhibition	clay-gravel	lowdensity/F3
	P21	-	heavy-fen	highdensity/F3
		-	heavy-fen	lowdensity/F3
	P22	G.S. inhibition	clay-loam	highdensity/F3
		G.S. inhibition	clay-loam	lowdensity/F3
	P23	A.A.S.inhibition	clay-loam	highdensity/F3
		A.A.S.inhibition	clay-loam	lowdensity/F3
	P24	-	clay-loam	highdensity/F3
		-	clay-loam	lowdensity/F3
	P25	-	clay-loam	lowdensity/F3
		-	clay-loam	highdensity/F3
	P26	EPSP inhibition	loamy	highdensity/F3
		EPSP inhibition	loamy	lowdensity/F3
	P27	Synthetic auxin	medium-heavy	highdensity/F1
		Synthetic auxin	medium-heavy	lowdensity/F1
	P28	EPSP inhibition	clay-loam	highdensity/F1
		EPSP inhibition	clay-loam	lowdensity/F1
	P29	C.B. inhibition	heavy-clay	highdensity/F1
		C.B. inhibition	heavy-clay	lowdensity/F1
	P30	Synthetic auxin	peaty-loam	highdensity/F1
		Synthetic auxin	peaty-loam	lowdensity/F1
	P31	EPSP inhibition	clay-loam	highdensity/F1
		EPSP inhibition	clay-loam	lowdensity/F1
	P32	Synthetic auxin	medium-heavy	highdensity/F2
		Synthetic auxin	medium-heavy	lowdensity/F2
	P33	EPSP inhibition	clay-loam	highdensity/F2
		EPSP inhibition	clay-loam	lowdensity/F2
	P34	Synthetic auxin	clay-loam	highdensity/F2
		Synthetic auxin	clay-loam	lowdensity/F2
	P35	Synthetic auxin	clay-loam	highdensity/F2
		Synthetic auxin	clay-loam	lowdensity/F2

	P36	-	loamy	highdensity/F2
		-	loamy	lowdensity/F2
	P37	A.A.S. inhibition	deep-clay	highdensity/F3
		A.A.S. inhibition	deep-clay	lowdensity/F3
	P38	M.A. inhibition	clay-gravel	highdensity/F3
		M.A. inhibition	clay-gravel	lowdensity/F3
	P39	EPSP inhibition	heavy-fen	highdensity/F3
		EPSP inhibition	Heavy-fen	lowdensity/F3
	P40	-	clay-loam	highdensity/F3
		-	clay-loam	lowdensity/F3
	P41	-	clay-loam	highdensity/F3
		-	clay-loam	lowdensity/F3
	P42	-	clay-loam	highdensity/F3
		-	clay-loam	lowdensity/F3
	P43	EPSP inhibition	loamy	highdensity/F3
		EPSP inhibition	loamy	lowdensity/F3

Prizes

I participated with the chapter 2 in postgraduate research student poster day, and I won the poster prize for outstanding poster, April 2017.



The University Of Sheffield.

Faculty of Science Graduate School

Postgraduate Research Student Poster Day

POSTER PRIZE

OUTSTANDING POSTER

26th April 2017

awarded to

Vian Mohammad

for the poster entitled

“Effect of drought stress on the evolution of herbicide resistance in grass weeds (Alopecurus myosuroides and Poa annua).”