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Understanding and combating the threat posed by

Lolium multiflorum as a weed of arable crops

Doctor of Philosophy

School of Plant Sciences

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Rothamsted Research

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Abstract

Lolium multiflorum (Italian rye-grass) is an increasing problem as a weed of arable crops in the UK and many other countries. However, there exists only limited knowledge on many basic aspects of this weed's agro-ecology and how it interacts with cultural and chemical control methods. Therefore, the aim of this project was to characterise better both the agro-ecology and the basis of resistance to ACCase inhibitors in this species.

Resistance in *L. multiflorum* to at least one ACCase-inhibiting herbicide was found to be widespread in England and was detected on 70% of the 50 farms surveyed. At least one ACCase mutation (Asp-2078-Gly (in 24.5% of 384 resistant plants assayed), Ile-1781-Leu (13.3%), Ile-2041-Asn (2.1%), Cys-2088-Arg (1.8%), Trp-2027-Cys (1.0%) and Trp-1999-Cys (0.3%)) was found in 40% of the resistant plants analysed. However, at present, the most common mechanism of resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations appears to be non-target site resistance, most likely enhanced metabolism.

Field studies showed that the majority (94%) of *L. multiflorum* plants in winter wheat fields emerged between October and December, with only 6% emerging in spring. Autumn emerging plants were much more competitive and produced on average 23 times as many seeds per plant as spring emerging cohorts. The success of *L. multiflorum* as a weed of winter cereals is due to its ability to produce high numbers of heads per plant (mean = 20) and seeds per head (mean = 295), even at low weed densities. *L. multiflorum* also had a highly detrimental effect on wheat yield with losses of up to 89%. *L. multiflorum* seed dormancy is short and determined by both genetics and weather conditions during seed maturation. By delaying the sowing date based on predictions of dormancy status and using resistance testing to help design an effective chemical control strategy in autumn, *L. multiflorum* infestations could be significantly reduced.

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signed:

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Chapter 1 General introduction

1.1 Weeds

There are many definitions of the term 'weed' but the most common is simply 'a plant growing where it is not desired'. In the context of agriculture, weeds are undesirable for several different reasons (Cobb, 1992, Zimdahl, 1993d). They compete with crop plants for resources such as nutrients, water and light, thereby causing significant yield losses. Weeds may act as reservoirs for pests and diseases, thus increasing the cost of crop protection in addition to the costs associated with weed control. Weeds also interfere with harvest operations and reduce the quality of the seed crop through contamination. Weeds can also interfere with water management in irrigated agriculture.

There are many characteristics that make a weed successful (Baker, 1974). Not all these characteristics are present in a given weed, but a combination of some of them in a given situation may provide the weed with a competitive advantage against the crop (Cobb, 1992). Successful weeds tend to be effective inter-specific competitors, and populations can often build up rapidly. Some weeds mature quickly and reproduce at a young age, thus completing their life-cycle before the crop is harvested. Many weeds reproduce by seeds but many are also capable of reproducing vegetatively. Often weeds have non-specific germination requirements, discontinuous germination, unspecialised cross-pollination mechanisms (e.g. wind) and great seed longevity. Weeds have environmental plasticity which allows them to adapt to adverse environmental conditions and they are often self-compatible.

Despite all their adverse effects, weeds may also have beneficial effects. Some weeds are valuable indicators of biodiversity as they provide food or shelter for animal

species. Weeds can protect the soil from erosion by wind and water and they may be more attractive to crop pests, thus reducing crop infestation levels.

However, the harmful aspects of weeds are much more important than their beneficial effects and for efficient crop production, appropriate weed management is essential.

1.2 Weed management strategies

Weed control includes many techniques with the aim of limiting weed infestations and minimizing competition with the crop. A balance between the cost of control and yield loss must be maintained (Zimdahl, 1993b). Weed management can essentially be divided into two strategies; chemical and non-chemical. Before the introduction of herbicides, weed management was largely dependent on a series of cultural and direct methods. Since the 1950's the greater reliance on modern selective herbicides has lead to a reduction in the use of 'traditional' weed control techniques and the consequent over-reliance on herbicides has resulted in the emergence of resistance in some weeds. To overcome problems with herbicide-resistant weeds, management through the rotation and integrated use of available weed control strategies has been shown to be effective. This approach, Integrated Weed Management (IWM), combines the use of a range of chemical and non-chemical control methods without excessive reliance on any single method (Naylor & Drummond, 2002). A rotational and integrated use of crop management, cultural techniques and chemical tools should help reduce the selection pressure imposed by herbicides and therefore the number of surviving weed plants. A number of studies in several different species have been carried out to prove this. The control of *A. myosuroides* by herbicides in France was found to be more efficient when combined with non-chemical practices (Chauvel *et al.*, 2001). Moss *et al.* (2007) recommend a series of strategies to prevent and manage herbicide resistant *A. myosuroides* populations, such as the adoption of more cultural control methods such as mouldboard ploughing, crop rotation, delayed drilling and non-

cropping and the use of herbicide mixtures and sequences to reduce the reliance on herbicides that belong to high resistance-risk mode of actions. To control resistant *L. rigidum* populations in Australia various strategies have been proposed such as delaying sowing to maximise pre-sowing weed kills, increasing crop density to suppress weed growth and the use of crop cultivars with superior weed suppression capacity (Gill & Holmes, 1997). However, farmers seem not to be motivated to use these approaches unless they have very high weed densities since they perceive it as a high cost investment (Chauvel *et al.*, 2001). In addition, many non-chemical methods are not as effective as herbicides or are more complex to manage, so these act as additional barriers to the adoption of IWM. However, the new EU Sustainable Use Directive, part of the EU Thematic Strategy for Pesticides, actively promotes Integrated Pest Management (IPM) with priority given wherever possible to non-chemical methods of plant protection (HSE, 2009).

1.3 Importance of arable crops in the UK and agrochemical inputs

The most recent available data showed that global agrochemical sales were $40.5 billion in 2008. The amount spent on herbicides accounted for 46.9% of this total, while the amount spent on insecticides and fungicides accounted for just 24.1% and 25.9% respectively of the global agrochemical market (AGROW, 2009).

In 2007 the European market for crop protection products increased by 5.2% to reach €7.08 billion ($9.51 billion). The amount spent on herbicides was €3.037 billion ($4.08 billion) which accounted for a 43% share of the total agrochemical spend, with fungicides making up 39% and insecticides and other pesticides the remainder (Figure 1.1) (ECPA, 2008). By crop, of the total amount spent on crop protection, 32.5% was used on cereals. The increase in Europe in herbicide sales was mainly driven by

France (6.5%), UK (19.3%), Poland (21.1%) and the Czech Republic (19.3%) (ECPA, 2007).

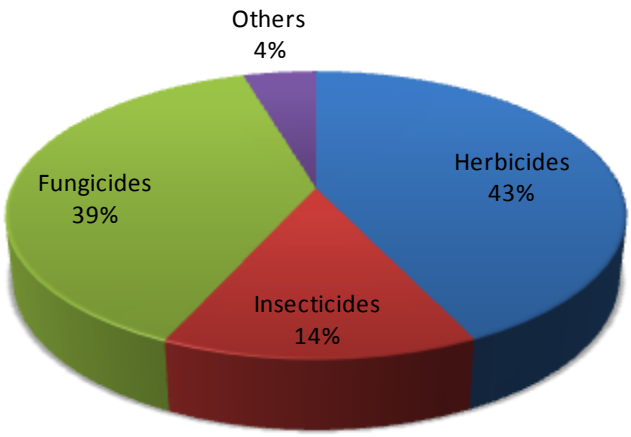

Figure 1.1. European agrochemical sales by category in 2007

In 2008, the UK produced 12% of the European Union's wheat, making it the third largest producer after France and Germany (DEFRA, 2008). Currently, cereals are the most widely grown arable crops in the UK.

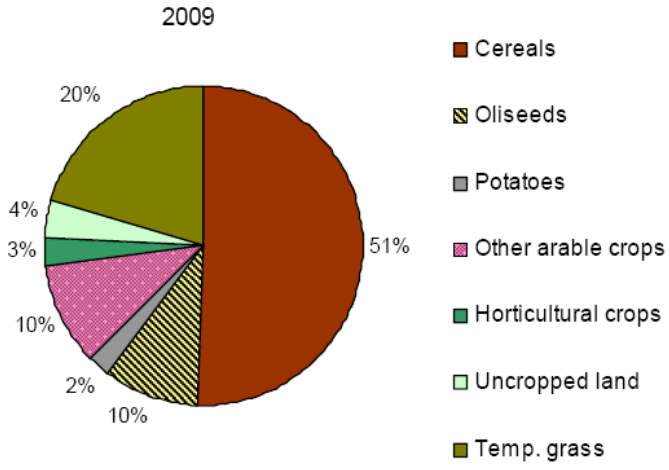

Figure 1.2. A breakdown of the total area cropped on agricultural and horticultural holdings in the UK on June 2009 (DEFRA, 2009b).

In June 2009 the total cropped area in the UK was estimated to be 6.2 million hectares, representing 36% of the total land on agricultural holdings (DEFRA, 2009b). 51% of the

croppable area, 3.1 million hectares, was occupied by cereal crops with wheat being the dominant cereal crop (Figure 1.2).

The area occupied by wheat in June 2009 was 1.8 million hectares. Barley, occupied just over 1 million hectares, which corresponds to 26% of the total cereal crop. The remaining area was occupied by a mixture of oats, rye, corn and triticale (Figure 1.3).

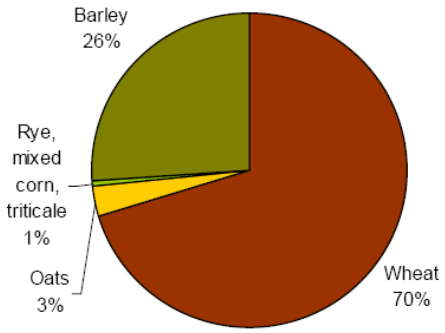

Figure 1.3. A breakdown of the total cereal area in the UK in June 2009 (DEFRA, 2009b).

Most cropping in the UK is in England, which in June 2009 had 4.9 million hectares classified as croppable land. Over half of that croppable area was taken by cereal crops with wheat being the main crop and occupying 1.7 million hectares (65% of the total cereal area) (DEFRA, 2009a). Most wheat in the UK is autumn-sown, planted in September and October and harvested the following August. Half of the barley is winter-sown, planted in September and harvested the following July and the other half is spring-sown, planted in March.

These figures highlight the importance of cereal cropping systems and in particular of wheat in the UK. However, despite growing concerns regarding the increase in the number of herbicide-resistant weeds and encouragement for farmers to practise integrated weed management strategies, high agrochemical inputs still exist in UK

cereal cropping systems. The continuation of these practices is, in part, responsible for the rise of herbicide-resistant weeds in UK agriculture.

1.4 Herbicides

Herbicides are chemicals that inhibit or interrupt normal plant growth and development. They can be classified according to their selectivity (non-selective, grass control, broadleaf control, etc.), time of application (pre-emergence or post-emergence), direct effects or translocation in the plant (contact or systemic), and mode of action.

Non-selective herbicides, such as glyphosate, are those that kill or suppress the growth of most plant species. In the past, such herbicides could not be used in conjunction with most crops unless used as a directed spray to avoid contact with foliage. Glyphosate resistant genetically modified (GM) crops have been developed to overcome this problem and are now widely grown in many countries, although not currently in Europe (James, 2007a, b).

However, the majority of herbicides in use today are selective, meaning that they control or kill targetted weed species while leaving the crop plants unharmed. Pre-emergence herbicides act by preventing weeds from emerging or establishing, while post-emergence herbicides control weeds that are already growing and easily visible. Herbicides can also act when they are applied to the soil, or when they are applied to a plant's foliage. Systemic herbicides are first taken in by a plant through its leaves, stems or roots, before translocating to a different part of the plant where they may exert their effect. In contrast, contact herbicides are not transported after they enter the plant and must be applied at the site of action to be effective.

Mode of action refers to the effect a herbicide has on a plant. They generally interfere with a process essential for normal plant growth and development. Herbicides can be

classified by mode of action based on how they work and the injury symptoms they cause (Reade & Cobb, 2002). The Herbicide Resistance Action Committee (HRAC) proposed the division of herbicides into 16 main modes of action which are in turn divided into further groups of chemical classes or families (HRAC, 2009). Some examples include:

- Inhibitors of acetyl CoA carboxylase (ACCase). This group of herbicides inhibit acetyl CoA carboxylase, the enzyme catalysing the first committed step in fatty acid biosynthesis. They belong to the group A/1. The chemical families included here are aryloxyphenoxypropionates ('fops'), cyclohexanediones ('dims') and phenylpyrazoline ('den'). Herbicides that belong to this group include clodinafop-propargyl, diclofop-methyl, fenoxaprop-P-ethyl, fluazifop-P-butyl, clethodim, cycloxydim, sethoxydim, tralkoxydim and pinoxaden.
- Inhibitors of acetolactate synthase ALS (acetohydroxyacid synthase AHAS). Acetolactate synthase inhibitors act by inhibiting the enzyme acetolactate synthase, a key enzyme in the biosynthesis of the branched-chain amino acids isoleucine, leucine and valine. They belong to the group B/2. Chemical classes here include sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinylthiobenzoates and sulfonylaminocarbonyl-triazolinone. Some of the herbicides in this group are chlorsulfuron, iodosulfuron, mesosulfuron, imazamethabenz-methyl, imazamox, imazapyr, cloransulam-methyl, diclosulam, florasulam, bispyribac-Na, pyribenzoxim and flucarbazone-Na.
- Ureas. This group of herbicides block electron transfer at photosystem II (PSII). These herbicides belong to the group C2/7, which also includes the amides. Some herbicides included here are chlorotoluron, diuron, isoproturon and linuron.
- Dinitroanilines. This group of herbicides inhibit microtubule assembly. These herbicides belong to the group K1/3, which also includes four other families.

Some herbicides included here are benfluralin, butralin, dinitramine, pendimethalin and trifluralin.

- EPSP synthase inhibitors. Only one chemical family, glycine, and one herbicide, glyphosate, are included in this group classified as G/9. Glyphosate is a non-selective post-emergence herbicide and its site of action is the enzyme 5-enoylpyruvyate shikimic acid 3-phosphate synthase (EPSP synthase) which is involved in the biosynthesis of the aromatic amino acids tryptophan, phenylalanine and tyrosine.

1.5 ACCase-inhibiting herbicides

ACCase-inhibiting herbicides are one of the most common groups of herbicides used to control grasses and in particular *L. multiflorum*. The first appearance of herbicide resistance in *L. multiflorum* populations was to this group of herbicides.

1.5.1 Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase is an enzyme that catalyzes the synthesis of malonyl-CoA, the first step in the synthesis of fatty acids. There are two forms of acetyl CoA carboxylase; a homomeric form consisting of three functional domains on a single polypeptide, biotin carboxylase (BC), carboxyltransferase (CT) and biotin carboxylase carrier protein (BCC), and a heteromeric form composed of four distinct subunits, the α- and β-CT subunits which constitute the CT catalytic domain and the BC and BCC subunits or domains (Nikolau *et al.*, 2003). In most plant species (but not grasses) the plastidic acetyl CoA carboxylase is the heteromeric isoform while the isoforms occurring in the cytosol in all plants and in the plastids and mitochondria in grasses are the homomeric enzymes (Konishi & Sasaki, 1994) (Figure 1.4).

The origin of plant ACCases was deduced from their subunit structure and by comparisons of amino acid sequence. During evolutionary time, fatty acid biosynthesis in most dicotyledons has been taken over by an endosymbiont of endobacterial origin (Huang *et al.*, 2002). Of the four genes encoding these subunits, three (*accA, B* and *C*) were transferred to the nucleus while the gene encoding the β-CT subunit (*accD*) remained in the chloroplast (or endosymbiont) genome (Figure 1.5).

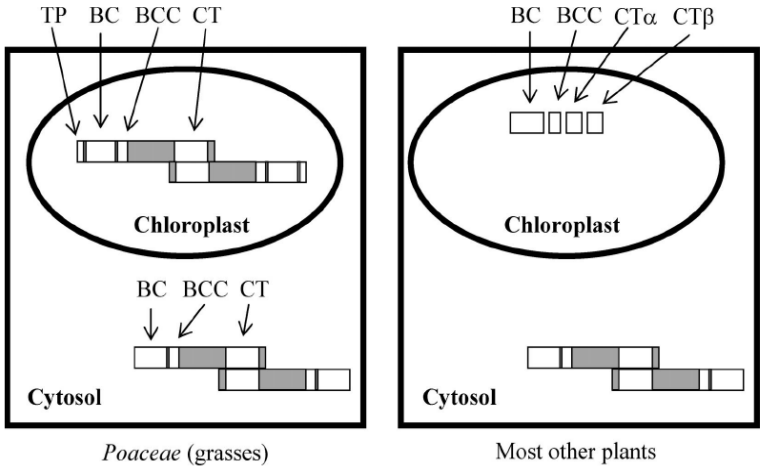

Figure 1.4. ACCase CoA carboxylase isoforms present in plants. TP, transit peptide; ACC functional domains: BCC, biotin carboxyl-carrier; BC, biotin carboxylase; CT, carboxyl transferase. Source: Délye (2005).

The nuclear encoded ACCase of the ancestral host eukaryote was retained in the cytosol. The duplication of the cytosolic ACCase gene (*Acc*) independently in grasses and in some dicotyledons led to the creation of a new eukaryotic-type gene encoding a multi-domain plastid ACCase (*Acc-1*). No bacterial-type multi-subunit has been found in grasses. Therefore, in grasses both plastid and cytosolic enzymes are homomeric or multi-domain and are encoded by the nuclear genes *Acc-1* and *Acc-2*, respectively. In the Triticea tribe more recent gene duplications created new copies of the *Acc-2* gene (Ψ-*Acc-2*).

The metabolic functions of these acetyl CoA carboxylases are different. The acetyl CoA carboxylase in plastids generate the malonyl-CoA that is used as the substrate for *de novo* fatty acid biosynthesis while the acetyl CoA carboxylase in the cytosol generates the malonyl-CoA that is used to elongate fatty acids and for the synthesis of a variety of secondary metabolites (Nikolau *et al.*, 2003). The plastidic acetyl CoA carboxylase (ACCase I) accounts for more than 80% of the total ACCase activity in green leaves in *L. multiflorum* and in other plants (Egli *et al.*, 1993, Evenson *et al.*, 1997, Menendez & De Prado, 1999, De Prado *et al.*, 2000).

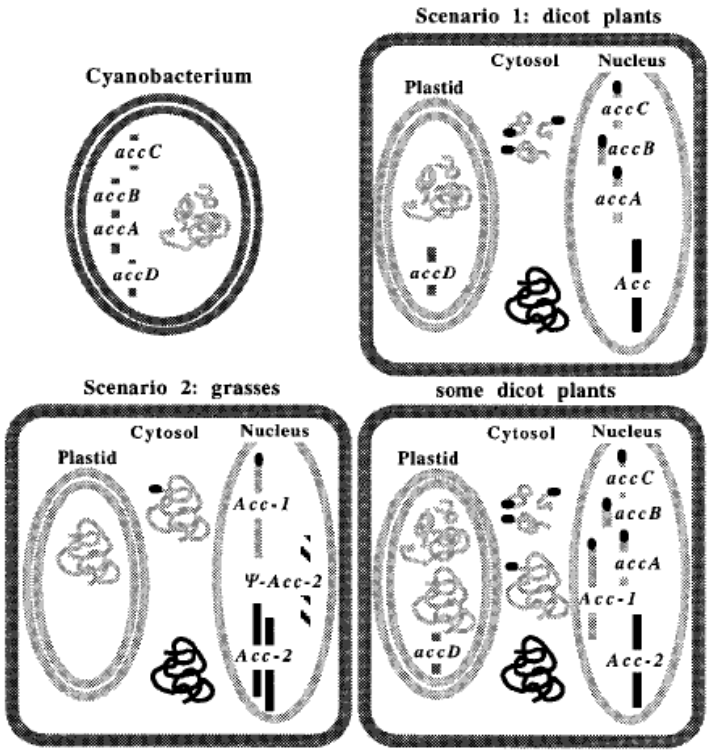

Figure 1.5. Origin of plant ACCases deduced from their subunit structure and amino acid sequence comparisons. Plastid ACCase in most dicot plants has a bacterial origin and is encoded by four different genes, three of them located in the nucleus and one in the chloroplast genome (*accA, B, C, D*). Plastid ACCase in grasses and some dicot plants was originated by a duplication of the host cytosolic ACCase (*Acc*) and is encoded by the nuclear gene *Acc-1*. Source: Huang *et al.* (2002).

1.5.2 **Chemistry and mode of action of ACCase-inhibiting herbicides**

There are three classes of ACCase-inhibiting herbicides which are the aryloxyphenoxypropionates (AOPP/APP/'fops'), cyclohexanediones (CHD/'dims') and phenylpyrazolines (ADs/'den'). As mentioned previously, these herbicides inhibit the eukaryotic acetyl CoA carboxylase found in the plastids of grasses, the enzyme that catalyses the first committed step in fatty acid biosynthesis. Structurally, the APP herbicides are derivatives of 2-phenoxypropionic acid, an aryl substituent in the *para* position of the phenoxy residue. APP herbicides are usually used as esters. Esters can be absorbed more effectively by the plant and are rapidly converted into the free acids which generally show low persistence in plants and soils. Further degradation appears to take place through hydroxylation. The CHD herbicides are bioactive compounds originally discovered in insects. They are weak acids whose solubility and partition properties are highly dependent on pH. They are unstable in aqueous solution and also photochemically (Roberts *et al.*, 1998). The phenylpyrazoline herbicide pinoxaden is different from any existing 'fop' or 'dim'. It is a derivative of hydroxyl-aryl-oxo-pyrazoline or, in a more general sense, the aryl-diones or ADs (Muehlebach *et al.*, 2007). Pinoxaden is hydrolized within a very short time to the parent acid, which is the metabolite with phytotoxic effect (Wenger & Niderman, 2007). It degrades quickly within one day and its degradation products are not considered to persist or accumulate in the soil (Hofer *et al.*, 2006).

The main advantage ACCase-inhibiting herbicides have is their specific activity against grass weeds. The plastidic homomeric acetyl CoA carboxylase (the relevant form since it accounts for most of the ACCase activity) present in grasses is sensitive to ACCase inhibiting herbicides while the heteromeric acetyl CoA carboxylase present in the plastid of most non-grass plant species is not sensitive (Konishi & Sasaki, 1994). Since plastidic ACCase accounts for 80% of the total ACCase activity, this activity is not

compromised by the sensitivity to herbicides of the homomeric form in the cytosol of most non-grass species. Acetyl CoA carboxylase inhibition by herbicides halts fatty acid synthesis and consequently membrane integrity is jeopardized causing metabolite leakage and rapid plant death (Incledon & Hall, 1997). ACCase inhibiting herbicides of the APP and CHD chemical classes exert their effect on the transcarboxylation reaction which suggests a binding site at the acetyl CoA carboxylase CT site (Burton *et al.*, 1991). This was confirmed by Zhang *et al* (2004) who determined the three-dimensional structure of yeast cytosolic acetyl CoA carboxylase CT domain dimers co-crystallized with bound APP molecules. When the APP was bound, conformational changes in the structure resulted, which were incompatible with the binding of acetyl CoA and malonyl CoA molecules for catalysis. Consequently, ACCase-inhibiting herbicides affect only the homomeric acetyl CoA carboxylase which explains the selectivity of these herbicides against grasses in broad-leaved crops. However, the selectivity against grass weeds in graminaceous crops such as wheat, rice and maize is due to the capacity of these cereal crops to metabolize some (but not all) ACCase-inhibiting herbicides. This capacity can be inherent to the cereals or enhanced by safeners that increase herbicide metabolism within the crop, and hence suppress the phytotoxic effects without altering their activity against weeds (Délye, 2005).

In summary, in most dicotyledonous plants tolerance is based on the inherited insensitivity to these herbicides of the plastid ACCase isoform present in these plants, while in monocotyledonous crops the selectivity is usually due to higher rates of herbicide detoxification.

1.6 Herbicide resistance

1.6.1 Definition of resistance

Herbicide resistance is an evolutionary process that can be defined as 'the inherited ability of a weed to survive a rate of herbicide which would normally result in effective control' (Moss, 2002b). It is an indication of an over-dependency on herbicides in a particular production system. The terms tolerance and resistance are often used interchangeably. However, resistance generally involves the concept of selection and evolution in response to a repeated exposure to herbicides, whereas tolerance would be due to naturally ocurring mechanisms (Holt & Lebaron, 1990). Tolerance is often used to describe crop responses to herbicides. There are two terms used to designate resistance depending on the number of resistance mechanisms involved. The term used to describe cases in which a weed population is resistant to two or more herbicides due to the presence of a single resistance mechanism is 'cross-resistance'. The term 'multiple resistance' is used to indicate resistance to a range of herbicides from different chemical classes, when resistant plants possess two or more resistance mechanisms (Moss, 2002b).

1.6.2 History of herbicide resistance

Prior to the widespread use of herbicides, cultural methods such as mechanical tillage, spring cropping, crop rotation and stubble burning were used to control weeds. The introduction of the first selective herbicides in the late 1940's allowed the reduction of manpower and more intensive production (Reade & Cobb, 2002). These first selective broad-leaved herbicides, 2,4-D and MCPA, belonged to a group of herbicides known as synthetic auxins that work by mimicking the action of this plant hormone. However, these auxin mimicry herbicides had some disadvantages including the need for high

application rates and their poor selectivity with the potential to damage non-target plants. Therefore, alternatives to these herbicides were soon developed and urea and carbamate herbicides, inhibitors of photosynthesis and cell division respectively, were marketed in the early 1950's. The triazines appeared in 1955 and the chloroacetamides in 1956. Most other herbicides with other modes of action in use today were released between 1955 and 1975 (Zimdahl, 1993a). Since 1980 only three new modes of herbicide action have been commercialised; inhibition of acetolactate synthase (ALS inhibitors), inhibition of glutamine synthase and inhibition of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD inhibitors) (Reade & Cobb, 2002).

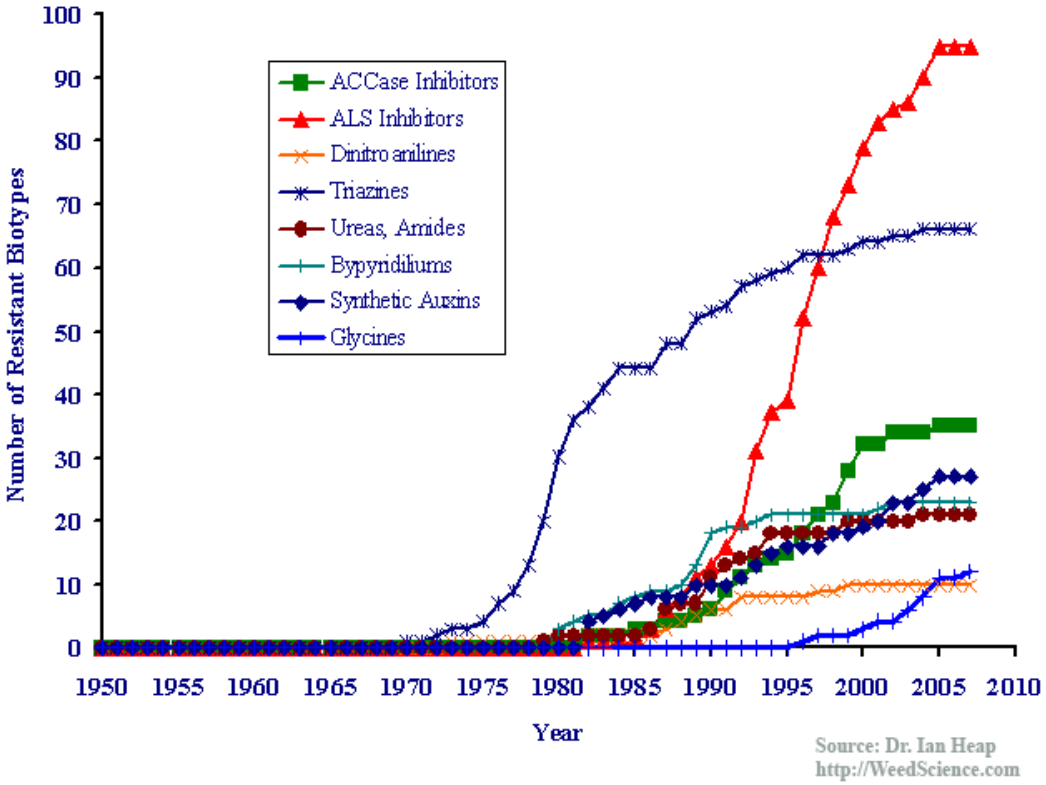

Figure 1.6. The chronological increase of herbicide resistant weeds worldwide since 1950, by mode of action.

The first case of high level herbicide resistance was recorded in simazine and atrazine resistant *Senecio vulgaris* in 1968 (Ryan, 1970), although a few cases of partial

resistance to synthetic auxins had already been reported, the first in *Commelina diffusa* in 1957 (Heap, 2009).

For several years resistance to triazines was the most prominent form of resistance but in the 1980's resistance to herbicides with other modes of action started to increase, especially the ALS inhibitors (Figure 1.6). Initially, problems with herbicide resistant weeds were limited mainly to the United States, Australia and Europe, but as herbicide inputs and intensive agricultural practices became more common in Asia, Africa and South America, resistance cases started to increase in those continents too.

Currently there are 337 confirmed resistant biotypes worldwide belonging to 191 species, of which 113 are dicotyledonous and 78 are monocotyledonous. Resistance to 19 different herbicide groups have been reported. Of these, the modes of action with the most cases of resistance are the ALS inhibitors (104 resistant species), the photosystem II inhibitors (68 resistant species) and the ACCase inhibitors (38 resistant species) (Heap, 2009).

1.6.3 **Evolution of resistance**

Resistance is usually associated with intensive agricultural or horticultural systems where there is a high dependency on herbicides to control weeds. In most cases the same herbicide or herbicides with the same mode of action have been used repeatedly for several years (Moss, 2002a). Weed populations therefore evolve resistance due to the selection pressure exerted by herbicides. Selection pressure is defined by Gressel (2002) as the relative ability exerted by different herbicides to decimate the wild type and leave the resistant individuals unaffected in a plant population. It is measured as the ratio of survival of resistant to susceptible propagules over a whole growing season or generation time and is the most important factor determining the rate of evolution of resistance in a population. If genetic variation for resistance is present in a population,

repeated herbicide application will result in a rapid increase in the frequency of resistant individuals. However, there are many other factors important in determining the evolution of resistance (Jasieniuk *et al.*, 1996):

- Genetic variation for resistance must be present in a susceptible population for herbicide resistance to evolve. The main source is likely to be gene mutation, which is more likely to occur spontaneously as opposed to being induced by herbicide application. Typical spontaneous mutation rates have been cited as $1.0x10^{-5}$ or $1.0x10^{-6}$ gametes per locus per generation.
- Initial frequency of resistant genes and individuals. The initial number of resistant individuals in a population has a direct influence on the rate at which resistance develops. Mathematical models for the evolution of resistance typically use the figure $1.0x10^{-6}$ to describe the initial frequency of resistant plants in a population. Considering this, a minimum of 3,000,000 seeds should be screened to be 95% confident of finding at least one resistant plant in a population where no selection pressure has been exerted.
- Inheritance of resistance. Depending on whether resistance is controlled by a single gene or by several (a polygenic trait), resistance will evolve in different ways. When resistance is controlled by a single gene, repeated application of highly efficient herbicides will impose a strong selection pressure and resistance will evolve rapidly. However, polygenic inheritance is more likely to occur under conditions of weak selection, as with sub-lethal herbicide applications. Therefore, the emergence of a highly resistant biotype would require recombination among individuals for many generations, meaning that the evolution of resistance will be slower.
- Herbicide rotations. If herbicide rotations are implemented, the evolution of resistance to a particular herbicide (or mode of action) will take place only during

the season when that herbicide is applied, meaning that resistance will evolve at a slower rate.

- Fitness. Fitness is the ability of a given genotype to compete with the wild type (Gressel, 2002). While herbicide-resistant individuals will possess a selective advantage when that herbicide is present, the mutation conferring resistance may carry a fitness penalty in its absence which may result in a decrease in resistance frequency.
- Weed mating systems. In general, natural selection strongly favours non-recessive favourable mutations in out-crossing species. In contrast, in autogamous plants the probability and rate of evolution of a favourable recessive mutation is increased.
- Interpopulation gene flow. Gene flow by pollen or seed from one field to another may provide an initial source of herbicide resistance. Therefore, as the rate of gene flow is believed to be higher than the rate of spontaneous mutation, the frequency of initial resistant genes would be higher and the evolution of resistance would be faster.

1.6.4 Mechanisms of resistance

Resistance to herbicides can broadly be divided into two classes; target site and non-target site based resistance.

1.6.4.1 Target site based resistance

A target site is a location within a weed where the active ingredients of herbicides bind to interfere with physiological processes. Often, a target site is an enzyme or protein that helps a weed to grow or reproduce. Once a herbicide binds to a target site, functionality is lost and the weed eventually dies.

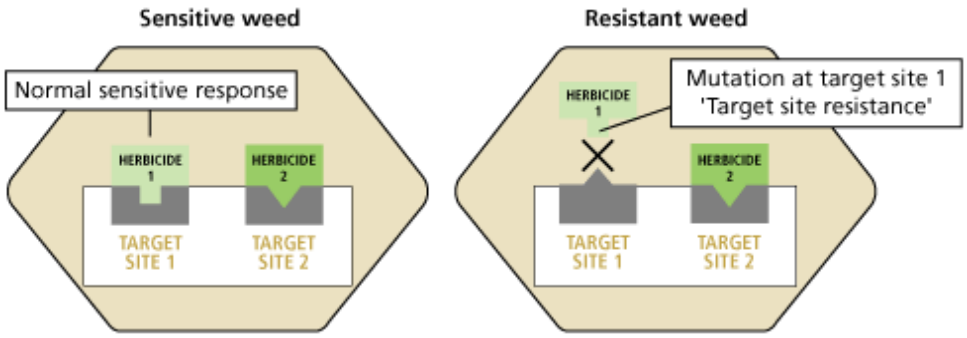

Figure 1.7. In susceptible plants, herbicides with different modes of action (1 and 2 in the scheme) can bind to their respective target sites causing plant death. In the resistant plant the target site for herbicide 1 is modified due to a mutation and the herbicide cannot bind. However, herbicide 2 can still bind as it has a different target site. Source: http://www.syngentacropprotection.com

Target site based resistance (TSR) to a herbicide usually occurs because one or more mutations in the gene encoding the herbicide target protein result in a change in the binding site of the protein, thus preventing the herbicide from binding or interacting (Figure 1.7). This mutation is a change in one nucleotide in the DNA sequence of the gene that results in a single amino acid change in the protein target site. Consequently, herbicides will have reduced effects on plants carrying this mutation.

This resistance mechanism can confer cross-resistance to herbicides targeting the same protein but not to herbicides with different modes of action (Figure 1.7).

It is relatively easy to study the molecular mechanisms of target site resistance, as this type of resistance is mostly monogenic and involves a single point mutated target enzyme. This form of resistance was the first to evolve due to the repeated usage of a herbicide (or group of herbicides with the same mode of action) and the exclusion of other weed control tactics (Yuan *et al.*, 2007, Gressel, 2009).

Target site mutations have been identified in weeds resistant to herbicides that inhibit Photosystem II (PSII), microtubule assembly, and the enzymes acetolactate synthase (ALS), acetyl-CoA carboxylase (ACCase) and 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Menne & Kocher, 2007, Gressel, 2009).

1.6.4.2 Non-target site based resistance

Plants have evolved sophisticated detoxification systems against toxic chemicals as a response to constantly changing environments. Herbicides are external factors that exert stresses on plants and as a consequence, trigger plant pathways in response to that stress (Délye, 2005). Non-target site resistance (NTSR) is caused by mechanisms that reduce the levels of a herbicide's active compound reaching the target site (Figure 1.8).

The most common mechanism is an enhanced metabolic degradation of herbicide molecules by the plant, preventing the action of the herbicide on the target protein (Eerd *et al.*, 2003, Yuan *et al.*, 2007). Furthermore, reduced uptake and translocation or sequestration of the herbicide may lead to insufficient herbicide transport to the target site. Enhanced metabolic resistance can occur across several herbicide modes of action (Cummins *et al.*, 1999) and can affect several herbicide groups such as ACCase inhibitors, PSII inhibitors, AHAS inhibitors, dinitroanilines, thiocarbamates and synthetic auxins (Powles & Yu, in press).

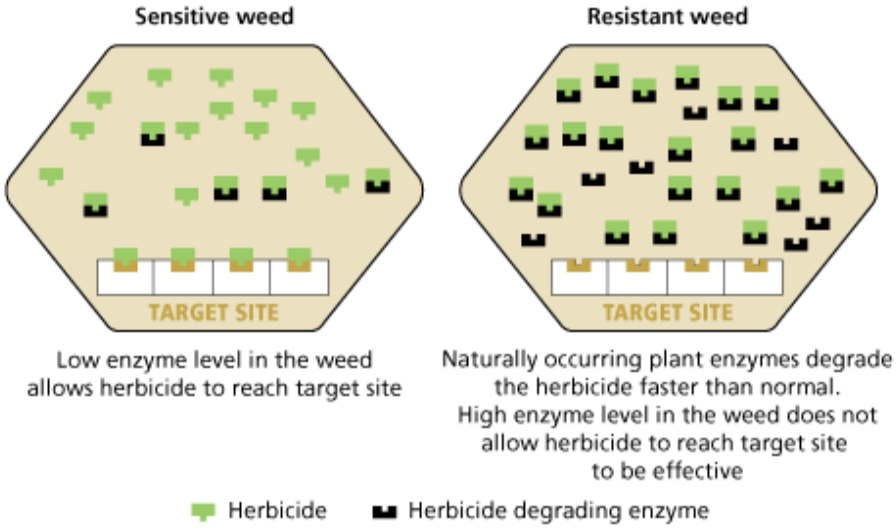

Figure 1.8. Example of a possible non-target site resistance mechanism. The herbicide is degraded by natural occurring plant enzymes. Source: http://www.syngentacropprotection.com

Non-target site resistance is thought to be multi-factorial, which means it would be controlled by several genes. This type of resistance could arise due to consecutive treatments with low herbicide doses. In this situation, plants only partially resistant to herbicides would survive and cross with other surviving plants. After many generations of exposure to low herbicide rates, surviving plants could accumulate a series of minor resistance genes that alone would confer only partial resistance to herbicides, but in combination would confer high levels of resistance. Therefore, high herbicide rates would initially control these minor genes and consequently prevent the appearance of non-target site resistance, whilst low rates of herbicide application would leave enough susceptible individuals to dilute major gene resistances (Gressel, 2009).

There may be many plant detoxifying proteins involved in non-target-site herbicide resistance but to date, participation in non-target herbicide resistance has been well established for only four gene families: P450s, GSTs, glycosyltransferases and ABC transporters (Yuan *et al.*, 2007).

P450 enzymes have been shown to be involved in metabolism-based resistance to ACCase-inhibiting herbicides in several weed species, including *Alopecurus myosuroides, L. rigidum, L. multiflorum, Avena sterilis, Echinochloa phyllopogon* and *Digitaria sanguinallis* (Christopher *et al.*, 1991, Maneechote *et al.*, 1997, Bravin *et al.*, 2001, Cocker *et al.*, 2001, Hidayat & Preston, 2001, Letouze & Gasquez, 2003, Letouzé & Gasquez, 2003, Yun *et al.*, 2005). ACCase-inhibiting herbicides that have been shown to be affected by this resistance mechanism include diclofop-methyl, clodinafop-propargyl, propaquizafop, haloxyfop, haloxyfop-R-methyl, fenoxaprop-P-ethyl, fluazifop-P-butyl and tralkoxydim (Powles & Yu, in press). The second group of enzymes often involved in metabolism-based resistance are the glucosyltransferases (GTs) and glutathione-S-transferases (GSTs). Cocker *et al* (2001) found greater GST activity in the resistant *L. multiflorum* populations Essex A1, Lincs A1 and Wilts B1 than

the standard susceptible populations, while the population Yorks A2, later reported to possess TSR, showed GST activity which was not significantly different from the susceptible standards. The herbicides affected in this case were diclofop-methyl, fluazifop-P-butyl and tralkoxydim. In studies on *A. myosuroides,* differences in GST activity were detected between resistant and susceptible plants in response to changes in temperature, which is thought to be linked to herbicide resistance in this species and would imply that resistant plants are more able to adapt to environmental stress (Milner *et al.*, 2007).

1.7 Biology of *Lolium* spp. and their relative importance in UK cereal cropping systems

The species classification within the genus *Lolium* has been a subject for continuous debate for many years as the variation within the species complicates their separation. The genus *Lolium* is classified within the family Poaceae, subfamily Pooideae and tribe Poeae. This genus is most closely related to *Festuca* with which it hybridises in nature forming x *Festulolium* (Chapman, 1996, Ryves *et al.*, 1996).

Based on morphological characters, the genus *Lolium* consists of eight recognised species native to Europe, North Africa and temperate regions of Asia, although they are widely distributed across temperate areas of the world (Loos, 1993, Chapman, 1996, Ryves *et al.*, 1996). There are seven species listed in 'GrassBase - The Online World Grass Flora': three autogamous species, including *L. persicum, L. remotum* and *L. temulentum*, three allogamous species, including *L. multiflorum, L. perenne*, and *L. rigidum*, and an intermediate species with respect to the mode of reproduction, *L. canariense* (Clayton *et al.*, 2006 onwards). Loos (1993) also includes within the genus *Lolium* the species *L. loliaceum*, which is an autogamous species in a somewhat isolated position, more closely related to *L. rigidum*.

Figure 1.9. *L. multiflorum* plants in a winter wheat field in Peldon, UK.

Nevertheless, the separation of *L. perenne, L. multiflorum* and *L. rigidum* into one, two or three species continues to cause disagreement. Morphological analyses have shown little differentiation between these three species although they were shown to be distinct (Loos, 1993, Bennett, 1997, Bennett *et al.*, 2000). However, more recent genetic studies showed that high molecular similarity strongly supported the classification of *L. multiflorum* and *L. perenne* as a single species and suggested that the differences between these two forms are related to the domestication process of *L. multiflorum* (Polok, 2007). It was then proposed that these two species be classified as subspecies. Regardless of their classification, in the UK the two most important species within the genus *Lolium* are *L. perenne* and *L. multiflorum,* as both have been grown for forage and for hay and silage throughout Europe. *L. rigidum* only occurs as an occasional introduced alien in the UK, and has never been reported as a serious weed of crops here as it is in other countries. In contrast, *Lolium multiflorum* Lam.

(Italian rye-grass) (Figure 1.9) is widespread in the UK and is a common weed affecting arable cropping systems on farms that have no grassland and have not sown *Lolium* spp. for many years (Figure 1.10).

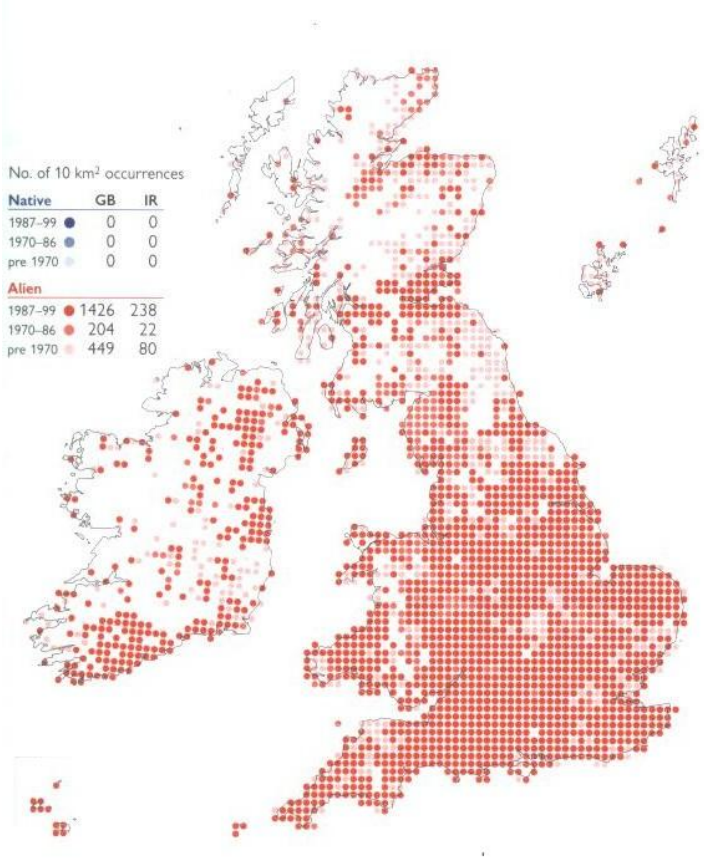

Figure 1.10. The distribution of *Lolium multiflorum* in the British Isles. Each dot represents at least one record in a 10 km square of the National Grid. Taken from "New Atlas of British and Irish Flora" (Preston *et al.*, 2002).

Lolium multiflorum is a caespitose, fibrous-rooted glabrous plant with annual, winter-annual, and biennial forms. It began to be used in significant quantities in agriculture in Britain after about 1830, and until recently it was often grown in temporary leys for hay or silage (Preston *et al.*, 2002). It is an outcrossing species and is generally observed as a diploid taxon with the chromosome constitution 2n=2x=14. However, some commercial cultivars can be tetraploid as the artificial doubling of diploids is a common

practice among grass breeders. Flowering occurs from June to August and reproduction occurs only by seed. It is a copious seed producer but the caryopses are fairly heavy and, therefore, unlikely to be dispersed far from the site of the parent (Beddows, 1973, Hubbard, 1984). Seeds, however, may persist for up to seven years in the soil (Rampton & Ching, 1970). *L. multiflorum* tends to dominate any companion species due to its vigorous seedling and plant growth (Beddows, 1973).

Lolium perenne L. (Perennial rye-grass) is a caespitose, fibrous-rooted glabrous perennial plant. Its longevity is variable within and between populations and ranges from the annual/biennial life span to extremely persistent. Field populations are diploid (2n=2x=14) but tetraploid plants can be obtained. Flowering occurs from May to August and reproduction depends almost exclusively on seed although vegetative self-propagation is possible (Beddows, 1967, Hubbard, 1984). *L. perenne* seeds can survive for up to four years in the soil (Rampton & Ching, 1970). It has been cultivated since at least the 17th century and cultivars developed by plant breeders are among the most commonly sown agricultural grasses (Preston *et al.*, 2002). The most reliable character for distinguishing these two species is the rolling of the young leaves in the sheath in *L. multiflorum*, while in *L. perenne* they are folded (Figure 1.11). The base of the stem is green or purplish in *L. multiflorum* and green or pinkish in *L. perenne*. *L. multiflorum* has ligules 1-2 mm long with narrow spreading auricles while *L. perenne* ligules are up to 2 mm long and the auricles are small and narrow. In both species, spikelets are arranged alternately along the length of the head and are awned in *L. multiflorum* while they are not in *L. perenne* (Figure 1.11) (Hubbard, 1984).

L. multiflorum readily crosses with *L. perenne* to give fertile progeny and hybrids have a combination of characteristics from both parents. Hybrids are annual to perennial, and have rolled young leaves and intermediate spikelet structure (with awned or awnless lemmas) (Beddows, 1973, Hubbard, 1984, Stace, 2001). Fertile hybrids have

been also recorded from hybridization with *L. temulentum* and *L. rigidum* (Ryves *et al.*, 1996).

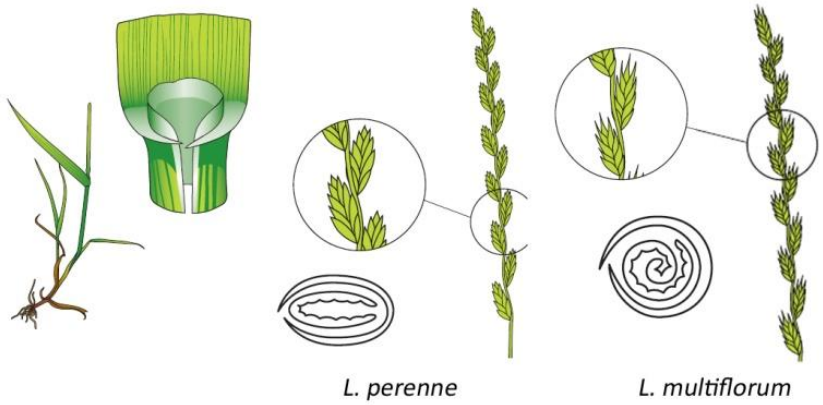

Figure 1.11. Some of the traits used to differentiate between *L. multiflorum*, *L. perenne* and other *Lolium* spp.: Base of the stem, ligules, auricles, position of the spikelets on the head, young leaves rolled or folded in the sheath and presence or absence of awn in the seeds. (Source: http://www.weedfocus.com/pdfs/Ryegrass_Expert_Guide_2006.pdf)

Both *L. multiflorum* and *L. perenne* are distributed thoroughout the UK. However, it is *L. multiflorum* that probably represents the greater weed threat to arable crops since, although it is a common weed, *L. perenne* is not a problem in arable crops in the UK. In a survey conducted in cereal crops in Southern England after the completion of herbicide programmes in 1981, approximately 8% of fields were infested with *L. multiflorum* while less than 1% were infested with *L. perenne* (Froud-Williams & Chancellor, 1982). By 2004, a total of 324 farms in England had been shown to possess herbicide resistant *L. multiflorum* populations while no reports of resistance in *L. perenne* were received (Moss *et al.*, 2005). *L. perenne* does not persist as a weed of arable crops which may be connected to the fact that, as a weed of cereals, it has to act as an annual species and be propagated mainly by seed, rather than act as a perennial species (Moss, 2005). In this respect, *L. multiflorum* is better adapted since it can be biennial and annual, so can produce larger quantities of viable seeds.

L. rigidum is a glabrous, annual grass generally observed as a diploid (2n=2x=14) (Monaghan, 1980). It is also allogamous and can cross with other *Lolium* spp. Seeds generally germinate with the first autumn rains although a proportion of seeds can remain ungerminated. Some cold is necessary for flowering initiation which occurs in spring. Development between flower initiation and heading is hastened by warmth and long days. As in *L. multiflorum*, young leaves are rolled along the long axis, but spikelets have no awns as in *L. perenne*. As is the case with *L. multiflorum*, *L. rigidum* is very competitive and a prolific seed producer. *L rigidum* tends to be more common in warmer climates, such as Southern Europe and Australia, but is a rather frequent alien from grain, wool and other sources in waste ground in the UK (Stace, 2001).

Other *Lolium* spp. that can be found in the British Isles are *L. temulentum, L. persicum* and *L. remotum* although all are uncommon (Stace, 2001).

1.8 Herbicide-resistant *Lolium multiflorum*

In a survey carried out throughout Europe in 2003, *Lolium* spp. were rated as the second most important herbicide-resistant weed after *A. myosuroides,* with *Papaver rhoeas* and *Avena* spp. in third and fourth place (Moss, 2004). In 2005, a compilation exercise for herbicide-resistant weeds in the UK was published which showed that the three major resistant grass weeds in the UK are *A. myosuroides*, *L. multiflorum* and *Avena* spp (Moss *et al.*, 2005). Worldwide, resistance has been found in other *Lolium* spp. too, including *L. rigidum*, *L. persicum* and *L. perenne* (Heap, 2009). *L. rigidum* was the first *Lolium* sp. with confirmed resistance to herbicides in 1979, in Israel, and subsequently in Australia, in 1982. It presents cross-resistance to ten chemical groups and is present in eleven countries. According to the international survey of herbicide resistant weeds *L. rigidum* is the worst resistant grass-weed worldwide (Heap, 2009).

The first case of herbicide resistant *L. multiflorum* populations appeared in Oregon (USA) in 1987 (Stanger & Appleby, 1989) where two populations were found to be resistant to the herbicide diclofop-methyl, an ACCase inhibitor. In 1991, just four years later, the first resistant populations appeared in the UK (Moss *et al.*, 1993). Resistance to diclofop-methyl was found in five farms in England and cross-resistance to other herbicides from the same chemical class was also demonstrated, including fenoxaprop-ethyl and fluazifop-P-butyl. There were indications of resistance to tralkoxydim and partial resistance to isoproturon, an inhibitor of photosynthesis from the group of ureas and amides, was also detected. Since then, the number of cases of resistant *L. multiflorum* populations have increased and are currently present in eight countries in the world; Argentina, Brazil, Chile, France, Italy, Spain, UK and USA (Heap, 2009). The modes of action affected by resistance in *L. multiflorum* are ACCase inhibitors (30 cases reported worldwide) ALS inhibitors (16 cases), glycines (13 cases), chloroacetamide (1 case) and ureas and amides (1 case).

The threat that *L. multiflorum* poses to UK arable cropping systems is increasing. This is probably due to increased autumn cropping, especially of wheat and oilseed rape, greater use of minimum tillage, a lack of good rotations and early drilling as well as the rise of herbicide resistant varieties (Moss, 2005). Herbicides from ten different groups can be used to control *L. multiflorum* (HSE, 2008), but those most commonly used in cereal crops in the UK have traditionally been the ACCase inhibitors. Options for the control of *L. multiflorum* in winter wheat are: flufenacet + pendimethalin applied pre-emergence of the crop and weed, and chlorotoluron, diflufenican + flurtamone + isoproturon or iodosulfuron +/- mesosulfuron applied post-emergence of the crop and weed (Orson, 2007b). Since *L. multiflorum* is a highly competitive and prolific weed, high levels of control are needed to maintain crop yields and to avoid a high seed return. The appearance of resistant populations makes it necessary to use non-chemical control methods in an integrated system. There is a lack of knowledge of *L.*

multiflorum biology and how it responds to cultural control in UK conditions. In addition, the extent of this resistance and the mechanisms responsible for it need to be known in order to develop strategies to counter this problem. Although much research has been carried out on *Lolium* spp. in other countries, these data cannot be extrapolated to UK *L. multiflorum* populations, since cropping systems and growing conditions in those countries are different to those in the UK. Therefore, there is a need for more research on the chemical and cultural control of *L. multiflorum* that is relevant to the agronomic systems in the UK.

1.9 Project aims and objectives

This project aimed to use a combination of field, glasshouse and molecular studies to characterise both the agroecology and the basis of resistance to acetyl CoA inhibiting herbicides in *L. multiflorum* populations from the UK. Discovering the mechanisms of resistance in field populations and studying the agro-ecology of *L. multiflorum* should allow the development of better weed management strategies. The project focused on the following topics:

1. The seed production potential of different *L. multiflorum* populations from the UK were determined to discover how this is affected by weed density and the implications it has for population dynamics.
2. The agro-ecology of *L. multiflorum* and *A. myosuroides* plants in competition with wheat was evaluated. The effects of both crop and weed densities on weed seed production and on crop yield were examined.
3. Seedling emergence patterns. The proportion of *L. multiflorum* plants emerging in autumn and in winter/spring was investigated. The aim was to determine the point when the majority of plants have emerged and thus, the optimum time for herbicide applications.

4. *L. multiflorum* dormancy and its implications for weed control. The relative influence of weather conditions, in particular temperature and soil moisture, during seed maturation on the initial dormancy of UK *L. multiflorum* populations was investigated, with the aim of predicting the emergence of *L. multiflorum* seedlings based on weather conditions.
5. Whole plant resistance assays were carried out to provide a better understanding of the extent of resistance to ACCase-inhibiting herbicides and the cross-resistance pattern within UK *L. multiflorum* populations.
6. Studies of the molecular basis of resistance in UK *L. multiflorum* populations to determine the most common mechanisms of resistance to ACCase-inhibiting herbicides and to identify the most frequent mutations responsible for target site resistance.

Chapter 2 Materials and methods

2.1 *Lolium multiflorum* and *Alopecurus myosuroides* biotypes

Table 2.1. List of *L. multiflorum* populations collected to be tested for dormancy in 2006, 2007 and 2008. Populations from different fields of the same farm have the same number in brackets. Populations in italic and italic + bold were used in the glasshouse assay described in section 6.2.1.2 (except Bald, Barnfield and Lovatt). Populations in italic+bold were used in molecular assays. (*)The population from Peldon 'Geedon' was called 'Peldon' in the seed production potential studies from Chapter 4.

County	Populations
Buckinghamshire	***High Wycombe***
Cambridgeshire	***Chat1, Chat2, Galons, Pidley*** (1), Roadside (1)
Durham	***Adams, Hay Shed***
East Riding of Yorkshire	***Low, Smiths***
Essex	***B.LandsL2*** (2), B.LandsQ2 (2), ***G.Rising*** (3), ***Geedon**** (3), ***Wallraven*** (3), *Bald* (3), *Barnfield* (3), *Lovatt* (3), ***North Benfleet, Wickford***
Gloucestershire	*Cinder*
Herefordshire	*Lewis* (4), Redman (4)
Hertfordshire	***20AC*** (5), ***Hitchin*** (5), ***Berkhamsted***, ***Dairy, Pub, Roedowns***, *Rothamsted*
Kent	***Court, Oaktree, Paddock, Ripple, Walette***
Lincolnshire	***Cott*** (6), ***Long*** (6), ***Fish***, ***Grum, Louth***
Norfolk	***Belaugh, Chapel, Heacham, Thornham***
North Yorkshire	***Hill, Ken***
Northamptonshire	***Maid***
Nottinghamshire	***Clements***
Oxfordshire	***Corner, Green Ball, Parson***
Shropshire	***3in1, Bridgnorth***
Stockton-on-Tees	***Port***
Suffolk	***School*** (7), ***Wood*** (7)
Tyne and Wear	***Eppleton***
Warwickshire	***Aviary***
West Yorkshire	***Charleston, Raven***
Wiltshire	***Manor***
Unknown	08054, 08056, 080118, 080140, 080119

Most of the UK *L. multiflorum* seed samples used in the experiments described in this project were collected in 2006, 2007 and 2008 to be tested initially for dormancy (Table 2.1). Subsequently, most of these samples were used for resistance testing on both a whole plant and molecular basis. Consequently, the samples can be considered to have been collected semi-randomly, as they were not from fields targeted specifically because there was a problem with control by herbicides. A good geographical distribution was obtained (see Figure 2.1), with samples collected in winter cereal fields from 55 farms in 22 counties in England.

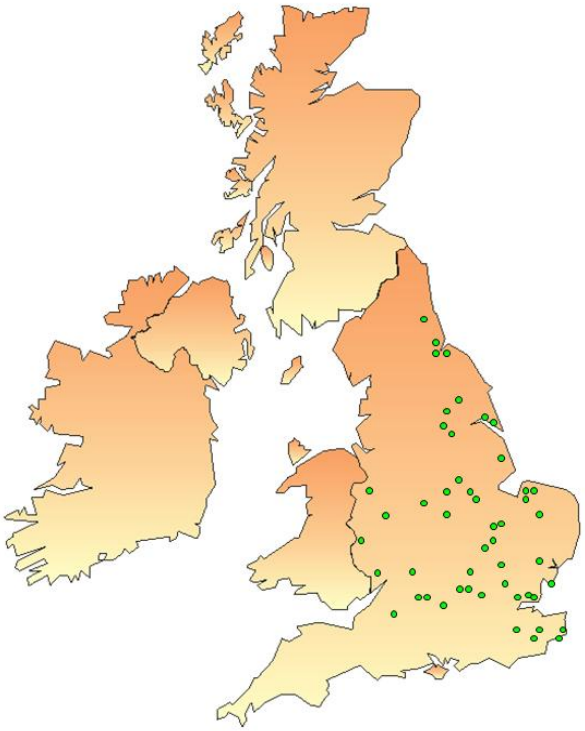

Figure 2.1. Map showing the distribution of farms where the *L. multiflorum* seed samples used for dormancy tests, herbicide resistance assays and/or molecular studies were collected.

The standard susceptible population used, Trajan, was a commercial *L. multiflorum* cultivar that had been used in previous studies (Cocker et al., 2001, White et al., 2005).

The *L. multiflorum* and *A. myosuroides* populations used for comparative reasons are listed in Table 2.2.

Table 2.2. List of *L. multiflorum* and *A. myosuroides* seed samples used as standard populations in different experiments ((1) Cocker *et al.* (2001); (2) White *et al.* (2005); (3) Cocker *et al.* (1999)).

Population	Collection year	County	Details
Wilts B1	1997	Wiltshire	Resistant *L. multiflorum* biotype that shows enhanced metabolic resistance to a wide range of herbicides [(1)].
Yorks A2	1998	Yorkshire	Resistant *L. multiflorum* biotype known to possess the Ile-1781-Leu mutation that confers resistance to ACCase-inhibiting herbicides [(1) (2)].
Pyl	1999	Nottinghamshire	Resistant *L. multiflorum* biotype known to possess resistance to ACCase-inhibiting herbicides [(2)].
G. Rising01	2001	Essex	Resistant *L. multiflorum* biotype from Peldon Hall farm known to possess resistance to ACCase-inhibiting herbicides.
Roth04	2004	Hertfordshire	Susceptible *A. myosuroides* biotype. Collected from no herbicides section of the Broadbalk long term experiment on Rothamsted Estate [(3)].
Roth07	2007	Hertfordshire	Susceptible *A. myosuroides* biotype. Collected from no herbicides section of the Broadbalk long term experiment on Rothamsted Estate [(3)].
Peldon03	2003	Essex	Resistant *A. myosuroides* biotype that shows enhanced metabolic resistance to a wide range of herbicides. No ACCase target site resistance present. Samples collected from Patch C of Hams field on Peldon Hall farm [(3)].
Peldon07	2007	Essex	Resistant *A. myosuroides* biotype that shows enhanced metabolic resistance to a wide range of herbicides. No ACCase target site resistance present. Samples collected from Patch C of Hams field on Peldon Hall farm [(3)].

2.2 Collection and storage of seed material

Ripe seeds were collected from winter wheat fields in July or from the glasshouse by gently rubbing heads of individual plants over a tray or envelope so that only mature seeds were collected. After collection, samples were labelled with details of their

source and date of collection. Seeds were air-dried in plastic trays for 2 or 3 days and then cleaned using an air column seed cleaner (Figure 2.2). The seed cleaner ensured removal of chaff and debris from samples, as well as most non-viable seeds. After this, seeds were stored in labelled envelopes at 18°C and 50% relative humidity. The cleaner was cleaned thoroughly between each sample to prevent cross-contamination.

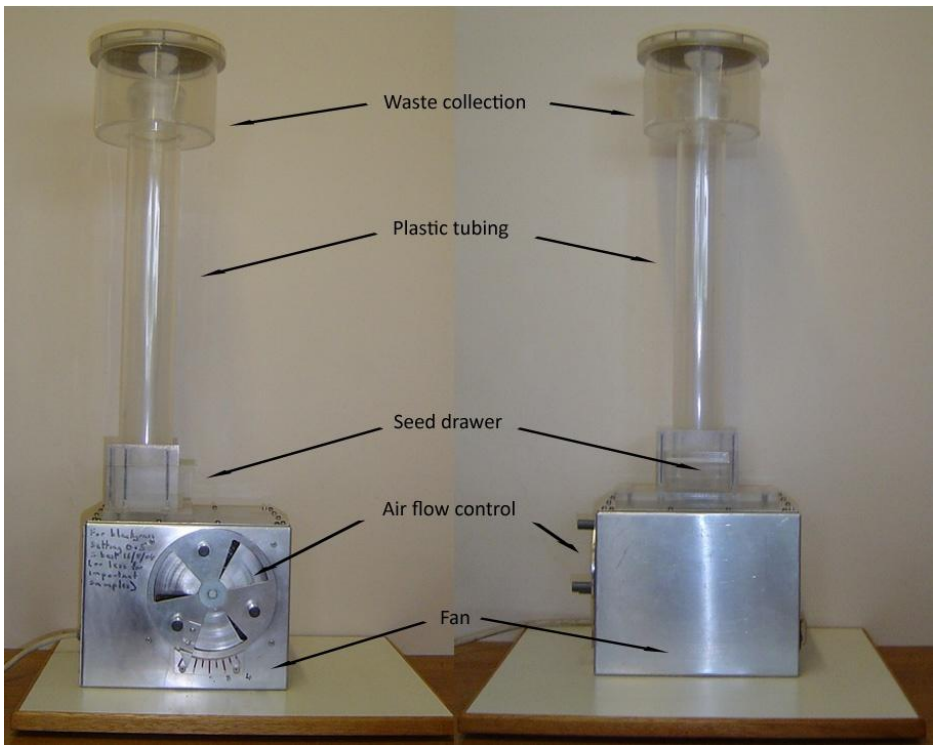

Figure 2.2. Views of the seed cleaner.

2.3 Pre-germination and sowing seeds

For establishing plants for evaluation in glasshouse experiments, seeds (approximately 80 per dish) were placed in 9cm diameter Petri dishes containing three Whatman cellulose filter papers covered with a single Whatman glass fibre paper to reduce growth of fungal infections and to minimise damage to roots on removal of germinated seeds. Dishes were labelled with a population code which was also used on pot labels. Normally 2-4 times as many seeds were placed in dishes than the number of plants required, depending on the germination rate of the population to be tested.

Immediately after placing the seeds, 7ml of KNO_3 (2g L^{-1}) was added to each dish in order to help overcome dormancy (Hilton, 1984, 1985, Beckie *et al.*, 2000, Alboresi *et al.*, 2005, ISTA, 2010). Dishes were placed in stacks of up to 20 dishes in polythene bags which were sealed to minimize moisture loss. Bags were then placed in an incubator running at settings of 17°C 14 hour day; 11°C 10 hour night. After 5-7 days seeds had just germinated and were in a stage suitable for sowing. Two to ten germinated seeds (depending on the experiment) were then placed in pots of compost, covered with a further 1cm of compost and pots placed in the glasshouse. The compost used was prepared by Petersfield Products for Rothamsted Research and consisted of the following components: 75% medium grade (L&P) peat, 12% screened sterilised loam, 3% medium grade vermiculite and 10% grit (5mm screened, lime free).

2.4 Germination test in Petri dishes

For each population to be assessed precise number of seeds were counted out (50 per dish) and placed in Petri dishes following the procedure explained above. For dormancy tests two different procedures were carried out; half of the samples were tested as described above by adding 7ml of potassium nitrate solution to each dish while in the other half 7ml of deionised water was added to each dish. Stacks of Petri dishes with a blank dish on top were placed in an incubator running at settings of 17°C 14 hour day; 11°C 10 hour night. Assessments of number of germinated seeds per Petri dish were made after two weeks.

2.5 Agroecology studies

2.5.1 Collection of plant material

Twelve to twenty-four three-sided quadrats were placed randomly in winter wheat fields to ensure that *L. multiflorum* plants collected would be representative of the whole field. Quadrat size varied from 0.05mx0.05m to 0.5mx0.5m and 0.1mx0.1m depending on the weed density. Plants from the same quadrat were carefully removed from the soil with a trowel, labelled when necessary and placed in a polythene bag labelled with the quadrat number. Bags from every quadrat were then placed in a cold-storage room at 4°C until assessment was carried out.

2.5.2 Assessment of plant material

The number of *L. multiflorum* plants per bag/quadrat was counted to estimate the number of plants per unit area. The number of tillers and heads per plant in every quadrat was also counted. Two plants from each quadrat were randomly selected and head length and number of spikelets per head was measured on five random heads on each plant. If the number of heads on those plants was lower than five, then more plants would be randomly selected. The number of seeds per spikelet was also measured on those heads by taking the average of the number of seeds on two spikelets in the lower third, two in the middle and two in the upper third of the head. To assess the validity of this method, seeds were also counted in every spikelet per head assessed in one quadrat in each of the 2005/06 fields. Mean number of seeds was determined and then compared with the mean number derived from the previous method. As results did not differ by more than 3.5%, the first method was used in the following two years.

Plants that were labelled were placed overnight in an oven at 80°C and dry weight determined the next day.

2.6 Herbicide resistance studies

2.6.1 Track sprayer calibration and spraying conditions

Pots were sprayed in an enclosure with extractor fans housing a laboratory track sprayer (see Figure 2.3). The sprayer was calibrated before every herbicide application. The calibration was done by weighing five Petri dishes before and after spraying water and then converting weight of water intercepted into litres ha^{-1}.

Figure 2.3. The Rothamsted track sprayer.

The Petri dishes were placed so that there were three along the central line of the room and two more one off to each side to check for lateral evenness of spraying. The mean output from at least two calibrations was used for herbicide dose calculations.

Commercial formulations of herbicides treatments were applied at the two- to four-leaf stage (BBCH growth stage 12-14) using the laboratory sprayer delivering between 225 and 250 L spray solution ha^{-1} at a pressure of 234kPa through a flat fan ceramic nozzle 'Teejet' TP110015VK. The speed of the sprayer was 0.33m s^{-1}. All pots were placed along the central line of the room under the path of the spray nozzle which was at a height of 0.5m above the pots.

2.6.2 Commercial formulations

Commercial herbicide formulations and adjuvants were used according to manufacturers' recommendations.

2.6.2.1 Diclofop-methyl ('Hoegrass')

Diclofop-methyl (2-(4-(2, 4-Dichlorophenoxy) phenoxy) propanoic acid), CAS number (*) 51338-27-3, is a selective post-emergence herbicide used for the control of annual grasses in grain and vegetable crops. It belongs to the chemical group of aryloxyphenoxypropionates or 'fops' within the ACCase inhibitors. It was applied as the 'Hoegrass' formulation (Bayer) an emulsifiable concentrate (EC) containing 378g a. i. L^{-1} (37.8% w/w) diclofop-methyl.

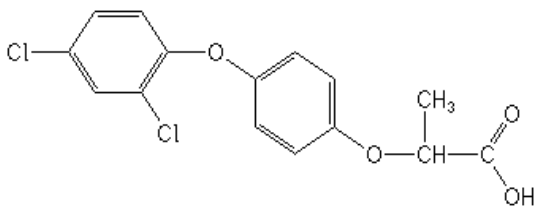

Figure 2.4. Chemical structure of diclofop-methyl (From http://www.alanwood.net/pesticides/).

(*)CAS numbers are unique numerical identifiers for chemical elements, compounds, polymers, biological sequences, mixtures and alloys that are assigned to every chemical described in the literature by *Chemical Abstracts Service* (CAS), a division of the *American Chemical Society*.

2.6.2.2 Fluazifop-P-butyl ('Fusilade 250 EW')

Fluazifop-P-butyl ((R)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionate), CAS number 69806-50-4, is a translocated grass herbicide used for post-emergence control of annual and perennial grass weeds in broadleaved crops. It is an aryloxyphenoxypropionate or 'fop' herbicide within the ACCase inhibitors. It was applied at the 'Fusilade 250 EW' formulation (Syngenta) containing 250g a. i. L^{-1} (25% w/v) fluazifop-P-butyl in mixture with the adjuvant 'Partner' at 0.5% spray volume.

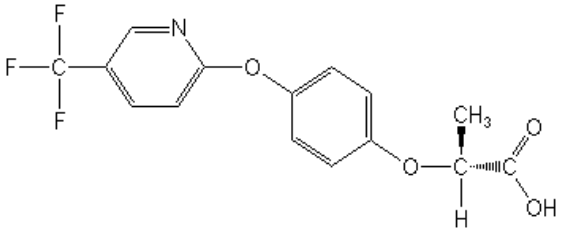

Figure 2.5. Chemical structure of fluazifop-P-butyl (From http://www.alanwood.net/pesticides/).

2.6.2.3 Tralkoxydim ('Grasp')

Tralkoxydim (2-[1-(ethoxyimino) propyl]-3-hydroxy-5-(2, 4, 6-trimethylphenyl)-2-cyclohexen-1-one), CAS number 87820-88-0, is a foliar applied herbicide for grass weed control in cereals. It is an ACCase inhibitor that belongs to the chemical group of cyclohexanediones or 'dims'. It was applied at the 'Grasp' formulation (Syngenta) containing 250g a. i. L^{-1} (25% w/v) tralkoxydim in mixture with the adjuvant 'Output' used at 0.375% spray volume.

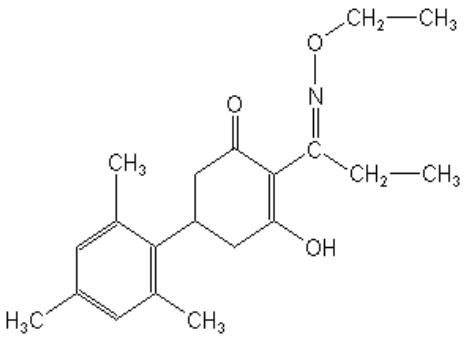

Figure 2.6. Chemical structure of tralkoxydim (From http://www.alanwood.net/pesticides/).

2.6.2.4 Cycloxydim ('Laser')

Cycloxydim (2-[1-(ethoxyimino) butyl]-3-hydroxy-5-(tetrahydro-2*H*-thiopyran-3-yl)-2-cyclohexen-1-one), CAS number 101205-02-1, is a systemic post-emergence cyclohexanedione herbicide for grass weed control. It was applied as the 'Laser' formulation (BASF), an emulsifiable concentrate (EC) containing 200g a. i. L^{-1} (21% w/w) cycloxydim, in mixture with the mineral oil adjuvant 'Actipron' used at 0.8% spray volume.

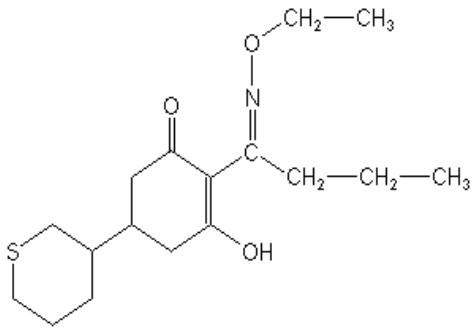

Figure 2.7. Chemical structure of cycloxydim (From http://www.alanwood.net/pesticides/).

2.6.2.5 Pinoxaden ('Axial')

Pinoxaden (8-(2,6-diethyl-4-methylphenyl)-1,2,4,5-tetrahydro-7-oxo-7*H*-pyrazolo [1,2-d] [1,4,5] oxadiazepin-9-yl 2,2-dimethylpropanoate, CAS number 243973-20-8, is a herbicide for post-emergence control of annual grass weeds in some cereal crops. It belongs to the chemical class phenylpyrazoline or 'den' within the ACCase inhibitors. It

was applied as the 'Axial' formulation (Syngenta) which is an emulsifiable concentrate containing 100g a. i. L^{-1} (10% w/v) pinoxaden and 25g L^{-1} (2.45 % w/w) safener cloquintocet-mexyl (1-methylhexyl 5-chloro-8-quinolinoxyacetate), CAS number 99607-70-2. Applications were in mixture with the adjuvant 'Adigor' used at 0.5% spray volume.

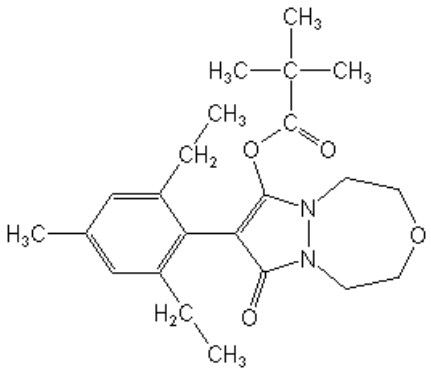

Figure 2.8. Chemical structure of pinoxaden (From http://www.alanwood.net/pesticides/).

2.6.3 Conditions after spraying

After spraying, plants were left to dry and then moved to a glasshouse that provided 14h, 16°C day with supplementary lighting providing 200-250µmol sec^{-1} m^{-2}, and a 10h, minimum 8°C night phase. All plants were not watered for at least 12 hours post spraying, but then watered from above daily. Two days after spraying, pots were randomised within replicates.

2.6.4 Injury scoring

About 21 days after spraying, and just before harvesting, a visual score was carried out for phenotypic response to herbicide treatment for all the plants, using untreated plants as controls. Four categories were used (see Figure 2.9):

1) Plants basically unaffected showing minimal or no characteristic symptoms of herbicide damage compared to untreated plants.

2) Plants showing visible symptoms of herbicide damage, including different levels of discolouration, leaf curling and stunting, but still alive, recovering and exhibiting new growth.
3) Plants with some green material present but with serious symptoms including discolouration, leaf curl, severe stunting and lack of new growth.
4) Complete plant death with no green leaf material present.

Figure 2.9. Examples of *L. multiflorum* plants falling into each category of the injury scoring scale.

2.6.5 Plant storage for molecular studies

One leaf from each surviving plant was cut, placed in individual paper envelopes and left to dry for subsequent DNA extraction and molecular studies. Leaves from plants belonging to the same population and sprayed with the same herbicide were stored together in two envelopes; one for plants classified in category number 1 and another one for those classified as category 2 (see Injury scoring).

2.6.6 Plant harvest and fresh weight determination

Plants grown in the glasshouse were harvested and assessed about 21 days after herbicide treatment. This corresponded with the date when plants from the susceptible

standard were completely controlled. After determining the injury score and taking leaf samples from each plant, the fresh foliage weight per pot was measured. This assessment was carried out cutting plants at soil level and weighing them to three decimal places in a metal tray. All pots were well watered the previous evening but not on the same day of assessment to ensure that no plants were wilting and also that there was no surface water present.

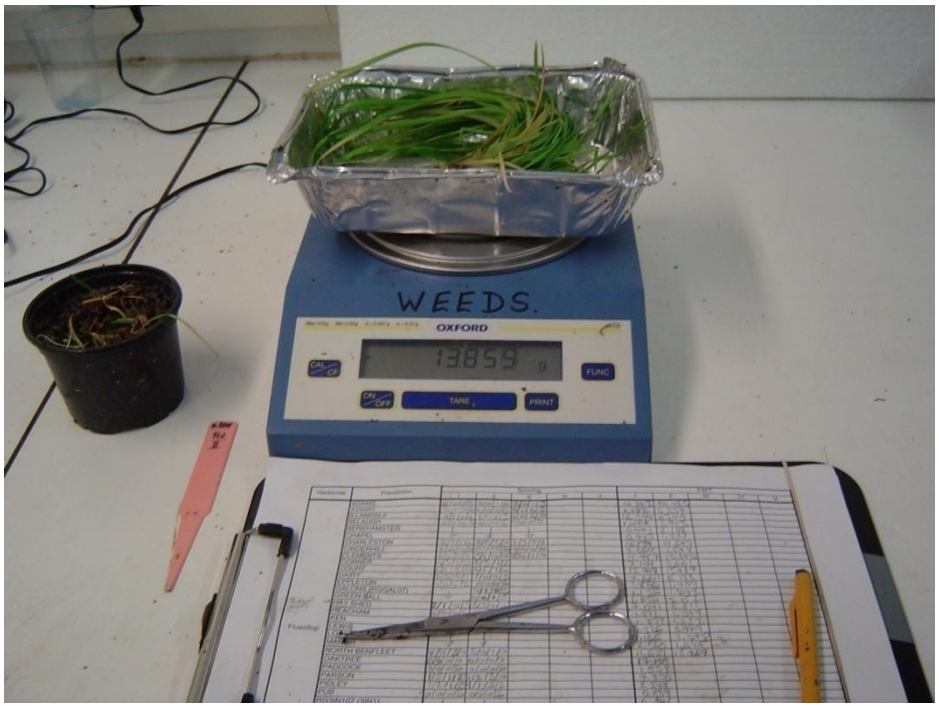

Figure 2.10. Equipment used for fresh weight determination.

2.6.7 System for designating resistance in screening assays

The system used in the experiments in this project was described by Moss *et al*, (2007) and called the “R” resistance rating system. It is appropriate for other grasses as well as *Lolium multiflorum*. This system requires the inclusion of a susceptible standard population in every test. The percent reduction values are divided into five equal categories, between zero and the value for the susceptible standard. One of these

categories is later subdivided into two smaller categories 1* and S. The values for each category will differ between tests as each one is one fifth of the value of the susceptible standard. Therefore, if the percent reduction for the susceptible standard is 95%, each category will be 19% of the total (95% divided by 5). Plants with up to 19% reduction in fresh weight will be classified as 5*, plants with a percent reduction between 19 and 38% will be classified as 4*, and so on (Figure 2.11). For practical reasons, these six different categories are grouped in four new categories, RRR (5*/4*), RR (3*/2*), R? (1*) and S. For these categories there is a colour code assigned. The description of each of these categories is given in Figure 2.11.

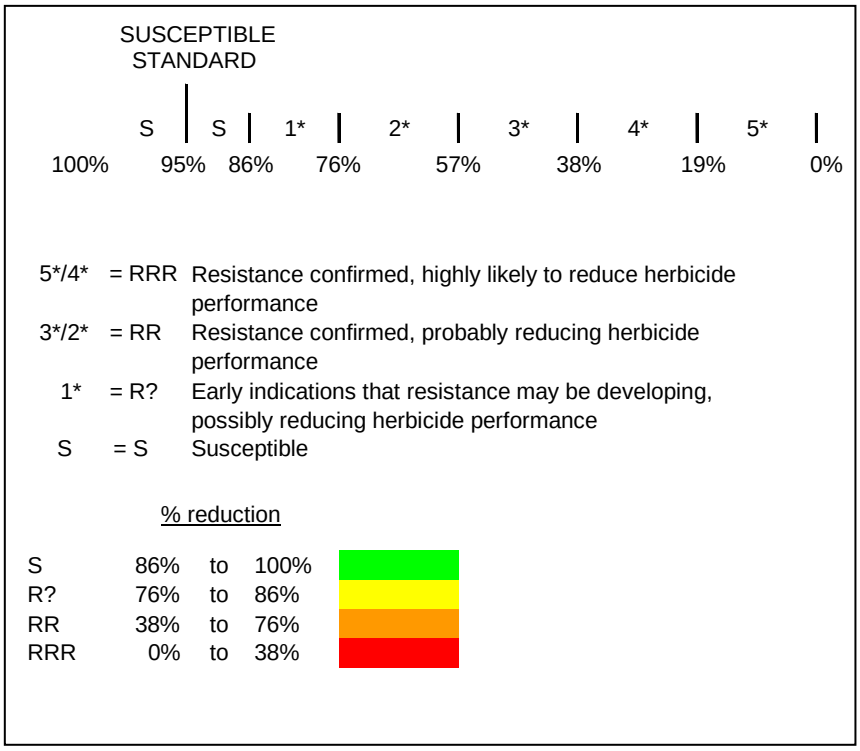

Figure 2.11. Description of each category in the "R" system for designating resistance in screening assays.

2.7 Molecular biology studies

2.7.1 Molecular biology standard solutions

10x TBE buffer	0.89M Tris-borate, pH 8.3 0.025M EDTA
10x loading dye	0.25% (w/v) bromophenol blue 40% (v/v) glycerol
6x loading dye	10mM Tris-HCl (pH 7.6) 0.03% (w/v) bromophenol blue 0.03% (w/v) xylene cyanol FF 60% (v/v) glycerol 60mM EDTA
1x TE buffer	10mM Tris-HCl, pH 8.0 1mM EDTA
LB Medium	2.5% (w/v) LB Broth
LB Agar	LB medium 2% (w/v) Bacto Agar
2% X-Gal (per 10ml)	Dimethylformamide (DMF) 2% (w/v) 5-bromo-4-chloro-3-inodlyl-β-Dgalactopyranoside (X-Gal)
LB–Ampicillin Agar	LB agar

0.1% (v/v) 100mg ml^{-1} filter-sterilized ampicillin solution
0.14% (v/v) 2% X-Gal

2.7.2 **DNA and RNA extraction**

DNA extractions were carried out using the “DNeasy 96 Plant Kit” (Qiagen) when work was carried out at Rothamsted Research and the “Promega Plant Magnesil Kit” at Jealott’s Hill, Syngenta. For the DNA extractions at Rothamsted, approximately 10mg of dry leaf was used per sample. For the work carried out at Jealott’s Hill, seeds were pre-germinated in Petri dishes and 2cm of shoot tissue was taken after 10 days to extract the DNA. Extractions were performed according to the manufacturers’ instructions. DNA was eluted in 100µl buffer AE (Qiagen).

RNA extractions were carried out using the “RNeasy Plant Mini Kit” (Qiagen) at Rothamsted and “TRIzol Kit” (Invitrogen) at Jealott’s Hill. In both cases a maximum 100mg of fresh leaf tissue was used. RNA was extracted according to the manufacturers’ instructions. RNA was eluted in 30µl RNase-free water.
Ribonuclease (RNase) is an enzyme that catalyzes the degradation of RNA into smaller components. These enzymes are present everywhere. They are highly stable and can survive prolonged boiling or autoclaving. That makes RNA more susceptible to degradation than DNA. To prevent the RNA from RNase contamination RNase-free solutions were used and the initial material and the bench were cleaned with the RNase decontamination solutions RNase Zap (Applied Biosystems) or RNase AWAY (Molecular Bio Products).

2.7.3 **DNA and RNA quantification**

All DNA and RNA samples were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). 2µl of each sample was loaded to measure concentration and quality against a blank with dilution buffer or water.

2.7.4 **Derived cleaved amplified polymorphic sequence (dCAPS) markers and cleaved amplified polymorphic sequence (CAPS)**

To detect three of the seven Single Nucleotide Polymorphisms (SNPs) known to confer resistance to ACCase-inhibiting herbicides (Zagnitko *et al.*, 2001, Brown *et al.*, 2002, Christoffers *et al.*, 2002, Délye *et al.*, 2002a, Délye *et al.*, 2002b, Délye *et al.*, 2002c, Délye *et al.*, 2003, Tal & Rubin, 2004, Délye & Michel, 2005, White *et al.*, 2005, Zhang & Powles, 2006b, a, Hochberg *et al.*, 2007, Liu *et al.*, 2007, Yu *et al.*, 2007, Hochberg *et al.*, 2009), dCAPS markers were used (Neff *et al.*, 1998). This method allows discrimination between homozygous and heterozygous individuals and involves three steps: PCR, selective restriction digestion and electrophoresis. For PCR, two primers are used and one of them is modified to create a diagnostic restriction site for either wild-type or mutant DNA sequences.

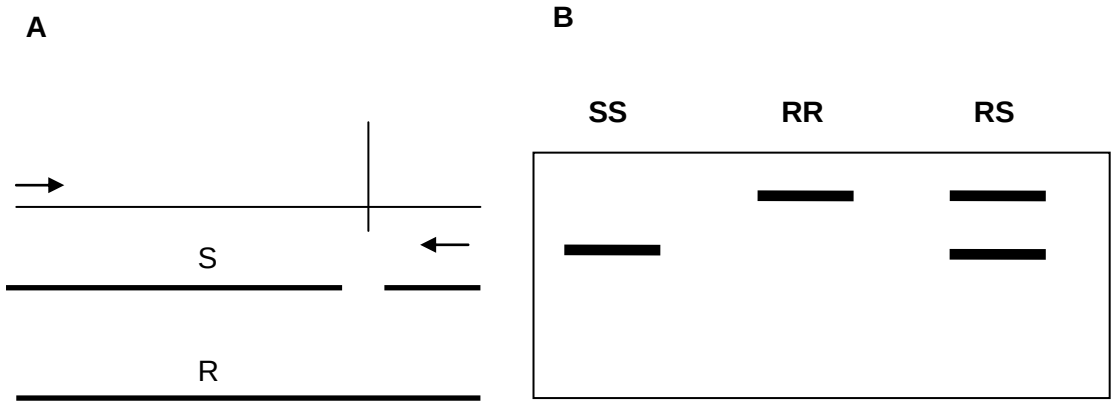

Figure 2.12. Possible results after PCR and digestion with the restriction enzyme for a dCAPs marker. Example of the restriction digest pattern for a heterozygous sample (A) and example of the different possible results in a gel electrophoresis (B).

Thus, if the diagnostic restriction site is created in the wild DNA sequence (susceptible), after the digestion with the restriction enzyme there will be (Figure 2.12):

- One digested band (smaller size) if the sample is SS.
- One undigested band (larger size) if the sample is RR.
- One digested band and one undigested if the sample is RS.

The three SNPs of particular interest were Ile-1781-Leu, Asp-2078-Gly and Cys-2088-Arg as they are the most common SNPs conferring resistance to ACCase inhibitors in the UK. Primers were provided by Syngenta and the procedure carried out as described by Kaundun & Windass (2006).

In the case of CAPS markers, there is no need to introduce a mismatch in one of the primers as the nucleotide change creates a restriction site for the enzyme in either wild or mutant DNA sequences.

2.7.4.1 Polymerase Chain Reaction (PCR)

PCR amplifications were carried out using "2x PCR Master Mix" (Promega) which is a pre-mixed ready-to-use solution containing 50units ml^{-1} of *Taq* DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dNTPs and 3mM $MgCl_2$, optimal concentrations for an efficient amplification of DNA templates. Reactions were carried out in Thermo-fast 96 PCR plates (Thermo Fisher Scientific).

25µl reactions consisted of the following components:

PCR Master Mix	12.5µl
Sterile distilled water (SDW)	7.1µl
Forward primer (100µM)	0.2µl
Reverse primer (100µM)	0.2µl
DNA (10-50ng)	5µl

PCR reactions were prepared on ice and cycled in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the following standard cycling conditions:

- 94°C 2 minutes
- 40 cycles of:
    - 94°C 30 seconds
    - 60°C 30 seconds
    - 72°C 1 minute
- 72°C 10 minutes
- Hold at 4°C

2.7.4.2 Restriction digestion

PCR reactions were digested using the appropriate enzyme and buffer from New England Biolabs:

*Nsi*I for Ile-1781-Leu

*Rsa*I for Asp-2078-Gly

*Hha*I for Cys-2088-Arg

Reaction mix:

1x NEBuffer	4µl
Enzyme (10units μl^{-1})	0.5µl
SDW	31.5µl
PCR product	4µl

96 wells plates were then placed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) at 37°C for 2 hours.

2.7.4.3 Gel electrophoresis

After the restriction digestion PCR products were run on a 2-3% (w/v) agarose gel with thin-combed wells to facilitate sufficient resolution of DNA bands.

2.7.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse Transcriptase Polymerase Chain Reaction, abbreviated as RT-PCR, is a two-step process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) followed by a standard PCR amplification of this cDNA.

2.7.5.1 First-Strand reaction: cDNA synthesis

The cDNA synthesis was carried out using the "SuperScript III First-Strand Synthesis System for RT-PCR" (Invitrogen) according to the manufacturer's instructions.

2.7.5.2 Second-Strand reaction: PCR

The second-strand reaction is a standard PCR amplification. It was carried out using the "Go*Taq* Flexi DNA Polymerase" and the "PCR Nucleotide Mix" (Promega). 25mM $MgCl_2$ was supplied with the "Go*Taq* Flexi DNA Polymerase".

50µl reactions consisted of the following components:

SDW	33.75µl
Buffer (5x)	10µl
$MgCl_2$ (25mM)	2µl
dNTPs (10mM)	1µl
Forward primer (10µM)	1µl
Reverse primer (10µM)	1µl
Go*Taq* Flexi DNA Polymerase	0.25µl

cDNA (100ng) 1µl

PCR reactions were prepared on ice and cycled in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the following standard cycling conditions:

- 95°C 2 minutes
- 35 cycles of:
    - 95°C 30 seconds
    - 57°C 30 seconds
    - 72°C 2 minutes
- 72°C 10 minutes
- Hold at 4°C

2.7.5.3 Gel electrophoresis

10µl of each PCR reaction was run on a 1% (w/v) agarose gel.

2.7.6 Gel extraction of DNA fragments and purification of PCR reactions

PCR products of interest were excised from the agarose gel using a scalpel and the DNA band purified using the "QIAquick gel extraction" kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 30µl buffer EB (Qiagen).

PCR reactions were purified using the "PCR purification kit" (Qiagen) according to the manufacturer's instructions. PCR products were eluted in 30µl buffer EB (Qiagen).

2.7.7 Cloning and sequencing

2.7.7.1 Ligating the insert and transforming the competent cells

Insert ligation and transformation into competent cells were carried out using "Strataclone PCR Cloning Kit" (Stratagene) according to the manufacturer's instructions. The insert was obtained by PCR using *Taq* DNA polymerase and then extracted and purified from a 1% (w/v) agarose gel.

50µl and 200µl samples of transformed cells were pipetted onto pre-warmed agar plates containing 0.028mg ml^{-1} X-GAL and 0.1mg ml^{-1} ampicillin and incubated overnight at 37°C.

2.7.7.2 Collecting colonies / colony PCR

Positive colonies were selected using blue-white screening and colony PCR was carried out to confirm that they contained the correct insert.

Colonies were picked up with a tip and pipetted directly into 15µl reactions consisting of the following components:

PCR Master Mix	7.5µl
SDW	6.5µl
Forward primer (10µM)	0.5µl
Reverse primer (10µM)	0.5µl

PCR reactions were then cycled in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the following standard cycling conditions:

- 95°C 2 minutes
- 35 cycles of:
    - 95°C 30 seconds
    - 57°C 30 seconds

    - 72°C 2 minutes
- 72°C 10 minutes
- Hold at 4°C

Each PCR product was run on a 1% (w/v) agarose gel to confirm the presence of the insert.

2.7.7.3 Culturing bacterial colonies

Eight positive colonies per sample were then picked from each plate and incubated overnight at 37°C with agitation in 5ml LB medium containing 0.1mg ml^{-1} ampicillin.

2.7.7.4 Plasmid DNA purification

Plasmid DNA was purified using the "QIAprep plasmid mini kit" (Qiagen) according to the manufacturer's instructions. DNA was eluted in 50µl buffer EB (Qiagen). Positive cloned inserts were again confirmed by *Eco*RI digestion followed by agarose gel electrophoresis.

2.7.7.5 Sequencing

Purified DNA samples were sent to be sequenced with M13 and custom-designed sequencing primers using the commercial service provided by Eurofins MWG Operon (London).

2.7.8 SNaPshot Multiplex Method for SNP genotyping

Seven SNP polymorphisms within the carboxyl transferase (CT) domain of the chloroplastic ACCase gene that are known to confer resistance to ACCase-inhibiting herbicides were genotyped using the SNaPshot® Multiplex System (Applied

Biosystems). This is a primer extension-based method that enables multiplexing of up to ten SNPs at known locations on multiple DNA templates in a single tube.

2.7.8.1 Amplification of DNA samples

A DNA template of 1976 bp length was amplified using the "Go*Taq* Flexi DNA Polymerase" and the "PCR Nucleotide Mix" (Promega). 25mM $MgCl_2$ was supplied with the "Go*Taq* Flexi DNA Polymerase".

25µl reactions consisted of the following components:

SDW	14.25µl
Buffer (5x)	5µl
$MgCl_2$ (25mM)	1µl
dNTPs (10mM)	0.5µl
Forward primer (10µM)	0.5µl
Reverse primer (10µM)	0.5µl
Go*Taq* Flexi DNA Polymerase	0.25µl
DNA	3µl

PCR reactions were then cycled in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the following standard cycling conditions:

- 95°C 2 minutes
- 35 cycles of:
    - 95°C 30 seconds
    - 57°C 30 seconds
    - 72°C 2 minutes
- 72°C 10 minutes
- Hold at 4°C

2.7.8.2 EXOSAP treatment

7.5µl of each PCR product was incubated with 0.014µl *Exo*I (20unit μl^{-1}) from New England Biolabs and 0.71µl shrimp alkaline phosphatase (1unit μl^{-1}) from GE Healthcare at 37°C for 1h and then inactivated by incubating at 80°C for 15 minutes.

2.7.8.3 SNaPshot Multiplex reaction

The SNaPshot single base extension assay was performed in a 5µl reaction containing:

SDW	2.25µl
SNaPshot Multiplex Master Mix	0.25µl
Pooled primers (2.5µM each)	0.5µl
Exosap-treated PCR product	2µl

Reactions were cycled in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the following cycling conditions:

- 30 cycles of:
    - 96°C 10 seconds
    - 50°C 5 seconds
    - 60°C 30 seconds
- Hold at 4°C

2.7.8.4 Phosphatase treatment

5µl SNaPshot multiplex reactions were incubated with 0.5µl calf intestinal phosphatase (1unit μl^{-1}) from Promega for 1h at 37°C.

2.7.8.5 Sequencing

One microliter of product was mixed with 8.8µl HiDi formamide and 0.2µl Genescan™ LIZ-120 size standard (Applied Biosystems), denatured at 95^{o}C for two minutes and electrophoresed on an ABI PRISM® 3100 Genetic Analyser according to the manufacturer's instructions.

2.7.9 **DNA sequencing and purification**

Sequencing reactions were performed in 10µl total volumes comprising:

BigDye® v1.1 Terminator Reaction Mix (Applied Biosystems)	1µl
5x BigDye dilution buffer (Applied Biosystems)	2µl
Primer (100ng µl^{-1})	1µl
DNA template from gel purification step (see 2.7.6)	2µl
SDW	to 10µl volume

Thermocycling conditions were as follows:

- 30 cycles of:
    - 95°C 30 seconds
    - 50°C 15 seconds
    - 60°C 3 minutes
- Hold at 4°C

Products were precipitated using the ethanol/sodium acetate protocol supplied by the manufacturer (Applied Biosystems) and resuspended in 10µl of HiDi formamide (Applied Biosystems)

2.7.10 **Preparation of samples to be sent for sequencing**

DNA samples and sequencing primers were sent to Eurofins MWG Operon (London) to be sequenced.

Purified plasmid DNA was diluted to 50-100ng μl^{-1} in a minimum volume of 15µl for each reaction. Purified PCR products (>1000bp) were diluted to 10ng μl^{-1} in a minimum volume of 15µl for each reaction. Primer concentrations were 2pmol μl^{-1} in a minimum volume of 15µl.

2.7.11 **Data analysis**

Sequencing data was analysed using the ContigExpress feature of the Vector NTI software package (Invitrogen).

Genotypes from the SNaPshot Multiplex assay were determined using GeneMapper® 4.0 software (Applied Biosystems).

2.7.12 **Primer design**

Primers for sequencing were designed using a coding sequence from *L. multiflorum* (EMBL accession number AF310684) and the Vector NTI software package (Invitrogen).

SNaPshot primers were examined using a web-based oligonucleotide calculator tool (www.sigma-genosys.com/calc/DNACalc.asp) to ensure that they had melting temperatures greater than 60°C. Primer sequences were also analysed for secondary structure.

2.7.13 **Measurement of ploidy levels**

Three leaf samples of three different plants from each *L. multiflorum* population used in the molecular studies were sent to the company “Plant Cytometry Services” (Postbus 299, 5480 AG Schijndel, The Netherlands) for determination of ploidy level.

Samples were analysed using “Flow Cytometry”, by which the quantity of chemical and physical characteristics of small particles can be measured in a small liquid stream. The technique is based on the measurement of the intensity of the fluorescence of nuclei, stained with a DNA specific dye. The quantity of the fluorescence is representative of the amount of DNA in a cell nucleus. The intensity of the fluorescence of the cell nuclei of an unknown plant is compared with that of a control plant with a known chromosome number, thus providing an accurate indication of the number of chromosomes of the plant.

Chapter 3 Dormancy studies

3.1 Introduction

Seed dormancy is an important element in the survival of a species (Peters, 1982). Seed dormancy in grasses is a condition referring to the temporary suspension of germination. It is an adaptive trait that optimizes the distribution of germination over time within a population of seeds and is part of a strategy that ensures survival in a changing environment (Simpson, 1990). It is determined partially by genetics but the seed's environment also has a major influence (Foley, 2001). Two different types of dormancy mechanisms are known; coat imposed dormancy and embryo dormancy (Hilhorst, 1995). In the first, dormancy is imposed by the structures surrounding the embryo which prevent embryo germination by interference with water uptake, interference with gaseous exchange, the presence of chemical inhibitors, modification of light reaching the embryo, mechanical restrain and by exerting a barrier for the release of inhibitors from the embryo (Bewley & Black, 1994). The control of embryo dormancy involves cotyledons and germination inhibitors (Desai *et al.*, 1997). The growth regulators abscisic acid and gibberellin are known to be involved in dormancy regulation, with gibberellins promoting and abscisic acid inhibiting seed germination respectively.

Dormancy can be considered an evolved mechanism in the plant to avoid germination when environmental conditions are unfavourable for plant establishment and reproductive growth. Thus, there are often differences in dormancy characteristics between species. However, environmental factors that are also important include the seed maturation environment and changes after shedding determined by the ambient environment (Finch-Savage & Leubner-Metzger, 2006). Dormancy may be classified by the timing of occurrence of two types of dormancy, primary and secondary. Primary

dormancy is possessed by freshly shed seed and can be divided into innate and enforced dormancy, and secondary dormancy is induced in non-dormant seeds by unfavourable conditions for germination (Benech-Arnold *et al.*, 2000). Primary dormancy in *Lolium multiflorum* is low although a prolonged germination in the field has been noted in some cases (Orson, 2007b). Under favourable weather conditions the period of germination will coincide with sowing of winter wheat. The pattern of seedling emergence is important as before drilling they can be easily destroyed by cultivations or non-selective herbicides, while if they emerge after drilling an application of selective herbicides will be required. For this reason a better knowledge of seed dormancy in *L. multiflorum* populations would be helpful when planning control strategies, particularly in relation to cultivation timing and crop drilling date.

The difference between 'dormant' and 'non-dormant' seeds is the degree of specificity of conditions necessary for germination. The environmental conditions during seed development and maturation both play an important role in meeting these requirements (Stearns, 1960). It has been shown that seed dormancy in *Avena fatua* is influenced by temperature during seed development, with high temperature and low levels of soil moisture during this period reducing the duration of primary dormancy (Sexsmith, 1969, Sawhney & Naylor, 1979, 1982). Swain *et al.* (2006) observed similar effects for *Alopecurus myosuroides* seeds with temperature having a greater effect than soil moisture. In contrast, in dormancy studies carried out on *Cenchrus ciliaris*, high temperatures during the seed maturation period had the same effect as mentioned above, decreasing dormancy, while soil moisture had the opposite effect and seeds from water stressed plants during seed maturation periods exhibited greater dormancy (Sharif-Zadeh & Murdoch, 2000). In studies carried out with *L. multiflorum* and *Lolium perenne* high temperatures during seed development and maturation decreased seed dormancy (Wiesner & Grabe, 1972) but no effect was found from soil moisture condition in *Lolium rigidum* seeds (Steadman *et al.*, 2004). These studies were carried

out in North America and Australia respectively, but there is still a lack of information concerning seed dormancy in UK populations of *L. multiflorum*.

Thus, the aim of the studies described in this chapter was to investigate the relative influence of weather conditions, in particular temperature and soil moisture, during seed maturation on the initial dormancy of UK *L. multiflorum* populations. Particular emphasis was placed on conditions likely to be experienced in the UK and other countries with similar climatic conditions. Hence, depending on the weather conditions during this period, time of emergence of *L. multiflorum* seedlings could be approximately predicted allowing appropriate control strategies to be carried out according to this prediction.

3.2 Materials and methods

3.2.1 Dormancy studies of *Lolium multiflorum* seeds from field populations

Experiments were carried out in three successive years (2006-2008) to study the initial proportion of dormant seeds in a range of freshly collected *Lolium multiflorum* field populations. Mature seeds were collected from 20, 38 and 5 winter wheat fields in 22 counties in England in 2006, 2007 and 2008, respectively. Seeds were mainly collected in July during the time of peak seed shedding. Seed samples were air-dried, cleaned and tested for dormancy in Petri dishes within seven days of the collection date as described in Chapter 2. Four replicates for each population and for each type of Petri dish test (in a KNO_3 solution (2 g L^{-1}) and in deionised water only) were used. Seeds lying on the soil surface are likely to be imbibed with water containing inorganic ions, thus, seeds in a KNO_3 solution would be likely to reflect field conditions. Roberts and Benjamin (1979) conclude that the choice of anion has little significance in terms of its effects on seed germination and that differences in stimulation were inconsistent

over the range 0.001-0.1M, even though 0.1M nitrate could be supra-optimal. The choice of nitrate concentration was based on soil nitrate analysis at Sonning Farm, University of Reading, over two consecutive years which ranged from 0.001-0.01M. However, the use of KNO_3 could have an effect on breaking dormancy. Therefore, tests with deionised water were also carried out for comparative purposes. The populations used are those listed in Table 2.1 in Chapter 2. In 2006 and 2008 all the populations were also tested for germination two months after the collection date using the same methodology. In 2007, due to the high number of populations, a representative number only were tested for germination two months later. During the two months between the initial and subsequent tests seeds were stored in labelled envelopes at 18°C and 50% relative humidity. Seeds were expected to lose dormancy after two months but the possibility of an increase in the proportion of dormant seeds was also contemplated, as it has been shown in other species. In studies on *Setaria chevalieri*, seeds were more dormant after ten weeks of dry and dark storage, probably due to the induction of secondary dormancy (Erasmus & Vanstaden, 1983). For comparative reasons, in 2007 two *A. myosuroides* populations were also tested for dormancy. These populations were Peldon07, from Patch C of Hams field on Peldon Hall farm, Essex and Roth07, an *A. myosuroides* sample collected from the Broadbalk experiment at Rothamsted, Hertfordshire.

3.2.2 Pot experiments on the effects of temperature and soil moisture on seed dormancy

Two experiments were conducted in two successive years (2007 and 2008) to investigate the effects of temperature and soil moisture conditions during spike emergence and seed maturation phase on the dormancy of *L. multiflorum* seeds produced by plants growing in pots. In the experiment carried out in 2007, plants were grown from seeds from two populations of *L. multiflorum*, Wickford and Fish (the most

and least dormant populations respectively in the study carried out in 2006) and two populations of *A. myosuroides* for comparison, Peldon03 and Roth04, from Patch C of Hams field on Peldon Hall farm, Essex and the Broadbalk experiment at Rothamsted, Hertfordshire; collected in 2003 and 2004 respectively. In the experiment carried out in 2008, plants were grown only from *L. multiflorum* seeds collected in July 2007. The populations used were the two most dormant in the studies carried out in 2007, Belaugh and Dairy, collected at Litcham (Norfolk) and Bishops Stortford (Hertfordshire), respectively; and the two least dormant, Rothamsted and Cinder, collected at Geescroft field at Rothamsted (Hertfordshire) and Pauntley (Gloucestershire), respectively. In both experiments, seeds were pre-geminated in Petri dishes with a potassium nitrate solution, sown in individual 5 cm pots with compost and then transplanted at the two- to three-tiller stage into 16 x 25 cm diameter pots (four plants per pot) filled with a Kettering loam: grit (4:1) mix containing a slow release fertiliser (Osmocote mini, 5-6 months, 2 kg m^{-3}; Petersfield Products, Leicestershire, UK). Pots were kept in an open-sided polytunnel over winter. In June and July, when heads started to emerge, sets of 16 plants, in four pots were subjected to four different combinations of air temperature and soil moisture: hot/wet (HW), hot/dry (HD), cool/wet (CW) and cool/dry (CD). The HW and HD treated pots were kept in a heated glasshouse with an average temperature of approximately 30°C during the day. The CW and CD treatments were kept in the open-sided polytunnel to be maintained close to ambient conditions. The 'wet' treated pots were stood inside large containers of water in order to maintain the soil close to field capacity. The 'dry' treated pots were kept drought stressed supplying only enough water to keep plants alive. Soil samples were collected at regular intervals, weighed, dried at 110°C overnight and weighed again to determine soil moisture content. The mean % soil moisture contents for the two years were 36.2% and 41.2% in the 'wet' (W) treatments and 11.5% and 13.3% in the 'dry' (D) treatments respectively. The minimum and maximum temperatures each day during the seed maturation period were recorded in both the

heated glasshouse and polytunnel. The mean daily temperatures (mean of maximum and minimum) recorded in 2007 and 2008 were 27 and 28°C in the 'hot' (H) treatments and 19 and 21°C in the 'cool' (C) treatments respectively. Seed samples were collected when an estimated 10%, 50% and 90% had shed and were air dried, cleaned and tested for viability and dormancy within seven days of the collection date. Viability was estimated by dissecting 50 random seeds per sample and recording the total number of caryopses present. On average 98% of cleaned seeds contained a caryopsis. Germination tests were carried out in Petri dishes with and without KNO_3, as described in Chapter 2 and in trays with compost kept in the open-sided polytunnel to mimic field conditions. Four replicates were used for each sample tested and for each type of test in Petri dishes. Two replicates were used for each sample tested in trays using 50 seeds per tray. Seeds from every collection were tested in Petri dishes but only seeds of *L. multiflorum* from the second collection were tested in trays. The test in trays was not carried out with the *A. myosuroides* seeds and with seeds from the HW treatment from Fish in the 2007 experiment, due to a lack of seeds. In the tests in trays seedlings were counted and removed every two weeks in 2007 and every week in the experiment carried out in 2008. These tests in trays were continued for 12 weeks in 2007 and for 35 weeks in the experiment started in 2008. Tests were started in July-August and pots kept in the glasshouse/polytunnel until three collections were made for each population and treatment.

3.2.3 **Data analysis**

In the dormancy tests in Petri dishes, the number of seeds germinated after two weeks in each dish was recorded and expressed as % non-dormant seeds relative to the total number of seeds assessed (50 seeds per dish). An average for each sample was taken from the four dishes set up. In the experiments carried out in pots, results for all

the collection dates were pooled for each treatment. In the tests carried out in trays, an average for each sample was taken from the two trays.

Analysis of variance using Genstat 11.1 (Payne, 2004) was carried out on the results to determine differences between years and between treatments, and ultimately, which environmental factors were significant in affecting seed dormancy.

3.2.4 **Meteorological data**

Under UK conditions *L. multiflorum* heads emerge mainly in June and seeds are shed in July. As the environmental conditions (temperature and soil moisture) during both seed development and seed maturation periods play an important role in seed dormancy (Stearns, 1960), meteorological data for June and July were recorded each year. Meteorological data were recorded from the meteorological stations at Rothamsted Research, Harpenden (Hertfordshire) and ADAS Boxworth (Cambridgeshire). Data from ten additional stations was also obtained from the UK's national weather service website (http://www.metoffice.gov.uk/climate/uk/stationdata/). These stations were located in Oxford (Oxfordshire), Cambridge NIAB (Cambridgeshire), Hurn (Dorset), Sutton Bonington (Nottinghamshire), Sheffield (South Yorkshire), Ross On Wye (Herefordshire), Shawbury (Shropshire), Durham (Durham), Newton Rigg (Cumbria) and Eastbourne (East Sussex). The meteorological parameters used were maximum and minimum daily temperatures (°C), daily rainfall (mm) and daily sunshine (h). Daily sunshine was available from only eight stations. Number of days with rain (>0.2 mm) were also used, but only data from Rothamsted and Boxworth were available. For comparative purposes the departure from the thirty year means for the months of June and July in 2006, 2007 and 2008 was used for each parameter. The thirty year means used for Rothamsted Research and for the ten stations from the UK's national weather service were those calculated for the years

1971-2000 (http://www.metoffice.gov.uk/climate/uk/averages/19712000/). For Boxworth the thirty year means used were those for 1974-2004.

3.3 Results

3.3.1 Dormancy studies of *Lolium multiflorum* seeds from field populations

The mean proportion of non-dormant seeds was highest in 2006 and lowest in 2007 with 2008 intermediate (Table 3.1). The mean proportion of non-dormant seeds in 2006 was 72% and 39% in the Petri dish tests carried out with and without KNO_3 solution, respectively. In 2008 mean values were lower with 68% and 35% of non-dormant seeds in tests with and without KNO_3 solution, respectively. The year 2007 had the lowest values with 53% and 26% of non-dormant seeds in tests with and without KNO_3 solution, respectively.

Although the mean % values varied considerably (26-97%), this trend (2006>2008>2007) was consistent for seeds tested both shortly after collection and two months later, both with and without KNO_3. The range of values between these three years overlapped (Figure 3.2), however, the overall difference between years was highly significant ($F_{2, 246}$=34.63, p<0.001 and $F_{2, 246}$=12.88, p<0.001 for the tests with and without KNO_3 solution, respectively).

Table 3.1. Percentage of germinated *L. multiflorum* seeds (non-dormant seeds) in 2006, 2007 and 2008 obtained from the dormancy and germination tests in Petri dishes with and without KNO_3 solution. Values in brackets are the standard errors of the means.

Collection year	Dormancy test (within 7 days of collection)		Germination test (after 2 months of collection)	
	(+)KNO_3	(-)KNO_3	(+)KNO_3	(-)KNO_3
2006	72 (±3.4)	39 (±5.0)	97 (±1.0)	88 (±1.9)
2007	53 (±2.7)	26 (±2.6)	90 (±1.9)	70 (±4.1)
2008	68 (±6.6)	35 (±7.1)	92 (±2.8)	80 (±5.4)

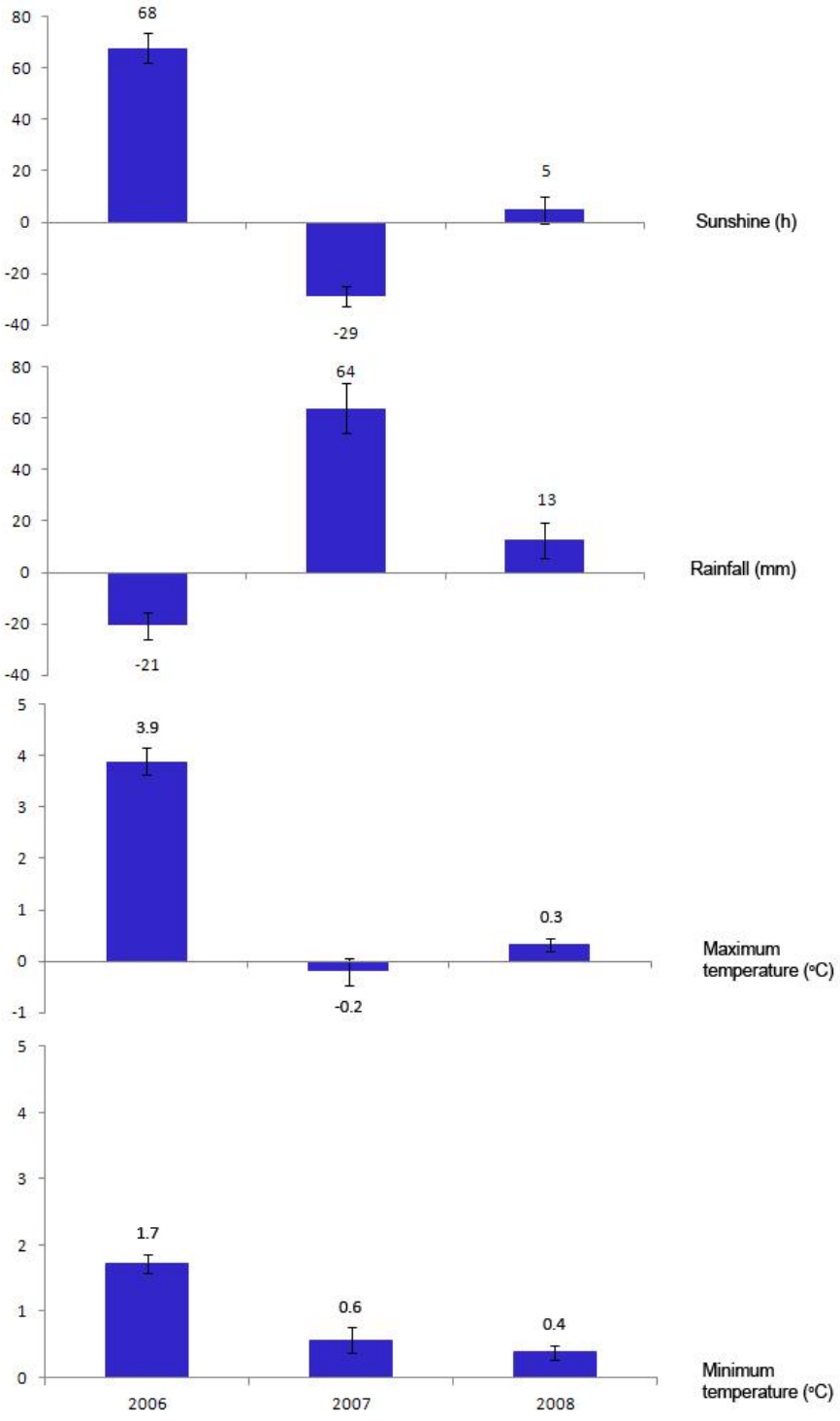

Figure 3.1. Meteorological data for June and July for 2006, 2007 and 2008 from 12 meteorological stations. Values shown are the average of the differences for the parameters relative to the 30 year mean values (1971-2000).

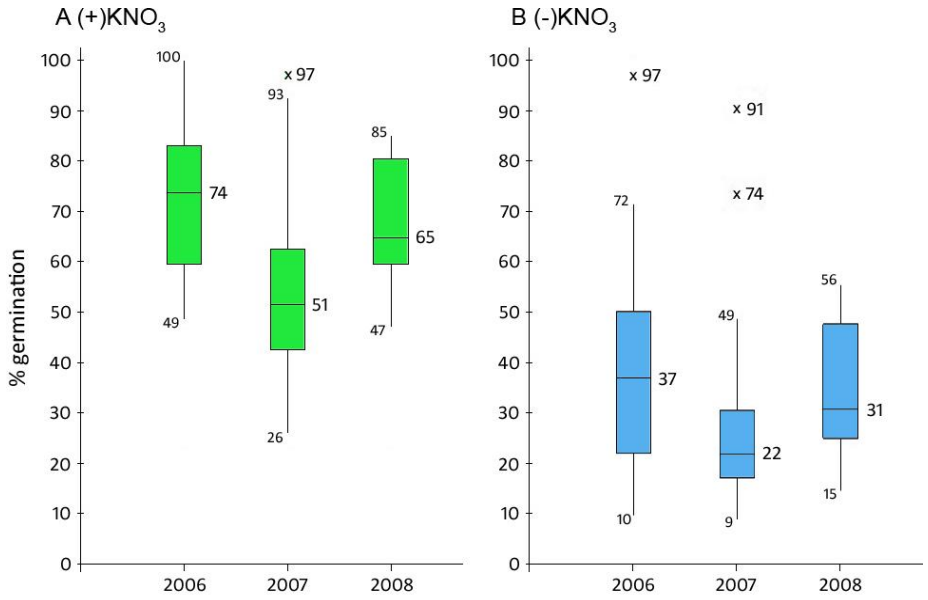

Figure 3.2. Box plot with the data obtained from the dormancy tests in Petri dishes with (A) and without (B) KNO_3 solution carried out within 7 days of collection of *L. multiflorum* seeds collected from winter wheat fields in England in 2006, 2007 and 2008. The central horizontal line represents the median, boxes encompass 50% of values and whiskers delineate the range. The symbol 'x' indicates extreme outliers.

The year 2006, in which a higher proportion of seeds were non-dormant, was hotter than average with mean maximum and minimum temperatures in June and July well above the long-term mean (Figure 3.1). Hours of sunshine were also above average and rainfall was below average. The year 2007 in which there was a lower proportion of non-dormant seeds was the coolest of the three years with rainfall above average and hours of sunshine below average. Mean minimum temperatures were also above the 30 year mean but closer to the average than in 2006. Mean maximum temperatures in June and July 2007 were near or below average.

The year 2008 had intermediate weather conditions and was cooler than 2006 but hotter than 2007. Total rainfall in June and July was lower than in 2007 and the total number of hours of sunshine was higher than 2007 but lower than 2006. Mean minimum and maximum temperatures for June and July 2008 were near or above average.

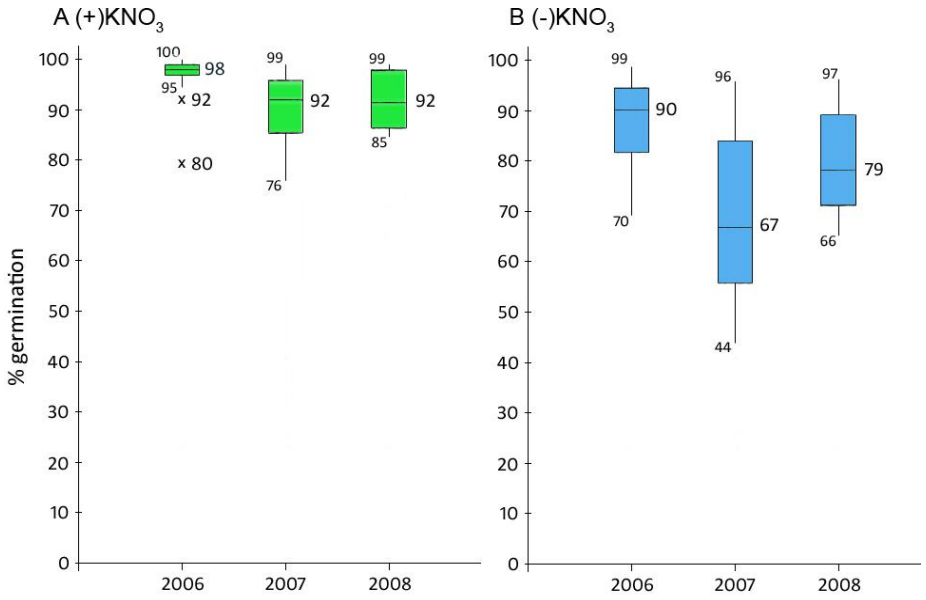

Figure 3.3. Box plot with the data obtained from the germination tests in Petri dishes with (A) and without (B) KNO_3 solution carried out 2 months after collection with *L. multiflorum* seeds collected from winter wheat fields in England in 2006, 2007 and 2008. The central horizontal line represents the median, boxes encompass 50% of values and whiskers delineate the range. The symbol 'x' indicates extreme outliers.

As expected, after these intermediate weather conditions, the proportion of non-dormant seeds in 2008 had intermediate values between those obtained in 2006 and 2007, for both type of tests in Petri dishes (Table 3.1, Figure 3.2). If we also consider the number of days in June and July on which rain fell (>0.2 mm) there appears to be a relationship between the number of days with rain and the proportion of non-dormant seeds. In June and July there were 21, 40 and 30 days with rain for the years 2006, 2007 and 2008, respectively at Rothamsted and 17, 34 and 24, respectively at Boxworth. The proportion of non-dormant seeds were 72%, 53% and 68% in the tests carried out with KNO_3 and 39%, 26% and 35%, in the tests carried out without KNO_3 for 2006, 2007 and 2008, respectively (Table 3.1). It appears that the greater the number of days with rainfall, the lower the proportion of non-dormant seeds and thus the greater the dormancy.

Weather conditions during the seed's maturation period seem not only to affect the proportion of non-dormant seeds but also the duration of dormancy. The mean proportion of germinated (non-dormant) seeds in 2006 after two months of collection were the highest of all three years with values of 97% and 88%, for the test carried out with and without a KNO_3 solution respectively (Table 3.1). The year 2008 with intermediate weather conditions had mean germination values of 92% and 80% for both tests respectively (Table 3.1). And the coolest year, 2007, had the lowest germination values; 90 and 70% of seeds germinated in the tests carried out (with and without a KNO_3 solution respectively) two months after the collection date (Table 3.1). Every year, most dormancy was lost within two months of shedding, but trends remained the same (Figure 3.3).

The tests carried out with *A. myosuroides* seeds indicate that this species is more dormant that *L. multiflorum* and that its dormancy lasts longer than for *L. multiflorum* seeds (Figure 3.4).

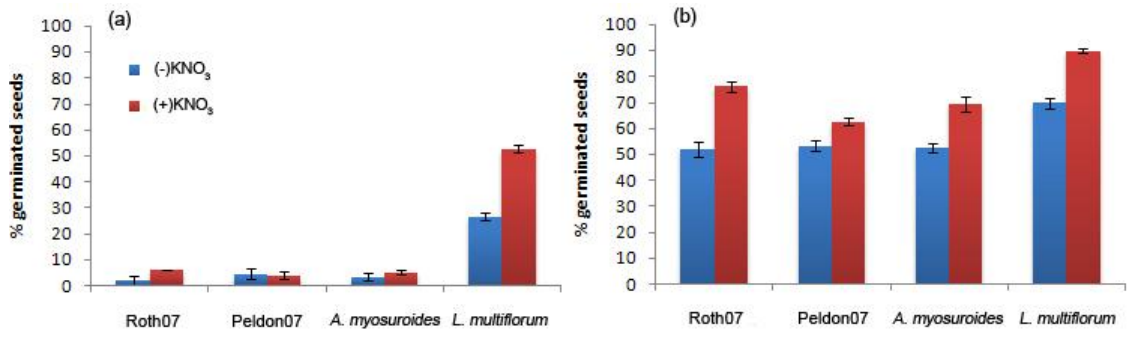

Figure 3.4. Proportion of non-dormant seeds obtained from dormancy tests within seven days of collection (a), and germination tests two months after collection (b), in Petri dishes with and without KNO_3 solution. Data showed are the mean values of the number of germinated seeds of two *A. myosuroides* populations, Broadbalk and Peldon, the mean values for these two populations combined and the mean values of all the *L. multiflorum* populations collected in 2007.

To provide more robust results these tests should be carried out with more populations as here only two were tested. In any case, none of the *L. multiflorum* populations within the wide range studied in 2007 (38) gave such low germination values. These tests

also confirm that the use of KNO_3 has little or no effect on breaking the dormancy of freshly shed *A. myosuroides* seeds as indicated by Swain *et al.* (2006), while it does have an effect on *L. multiflorum* seeds. However, trends using both types of test were similar and, as the aim of the experiment was to make comparisons between years, this can be achieved with both tests.

3.3.2 Pot experiments on the effects of temperature and soil moisture on seed dormancy

In both experiments, germination tended to be uniformly high, regardless of temperature and soil moisture treatment for the three populations selected for their non-dormancy based on previous studies (Fish, Rothamsted and Cinder). For these populations all treatments gave similar results with a high percentage of germinated seeds and values between 75 and 99% germination (Figure 3.5, Figure 3.6) for all treatments and both tests in Petri dishes, with the single exception of Cinder. Seeds produced under HD conditions from this population had less than 50% of germinated seeds for both tests in Petri dishes (Figure 3.6). The high temperature and water stress to which these plants were exposed could have made these seeds less viable. This high germination in non-dormant population despite the weather conditions implies a strong genetic basis for their non-dormancy.

Of the previously dormant *L. multiflorum* populations (Wickford, Belaugh and Dairy) and the *A. myosuroides* populations (Roth04 and Peldon03) the different environmental treatments clearly had a larger effect on dormancy (Figure 3.5, Figure 3.6).

Plants grown under hot conditions produced a significantly higher proportion of non-dormant seeds than those grown under cooler conditions. Soil moisture did not seem to have any appreciable effect on dormancy as results did not follow any particular

trend and differences between wet and dry treatments within the hot and cool treatments, were not significant, except for Cinder.

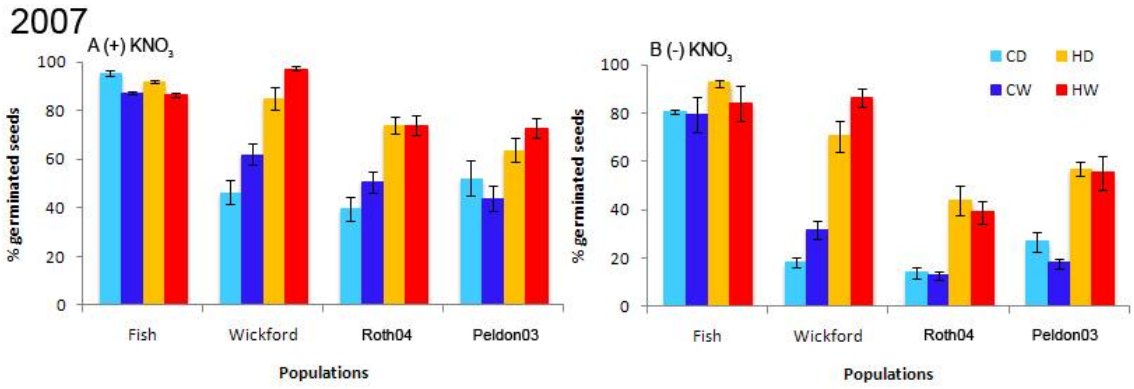

Figure 3.5. Effect of four temperature and soil moisture regimes applied during the period of spike emergence and seed maturation on the proportion of non-dormant seeds obtained on tests carried out on Petri dishes with (A) and without (B) a KNO_3 solution of two *L. multiflorum* populations (Fish and Wickford) and two *A. myosuroides* populations (Roth04 and Peldon03) in 2007.

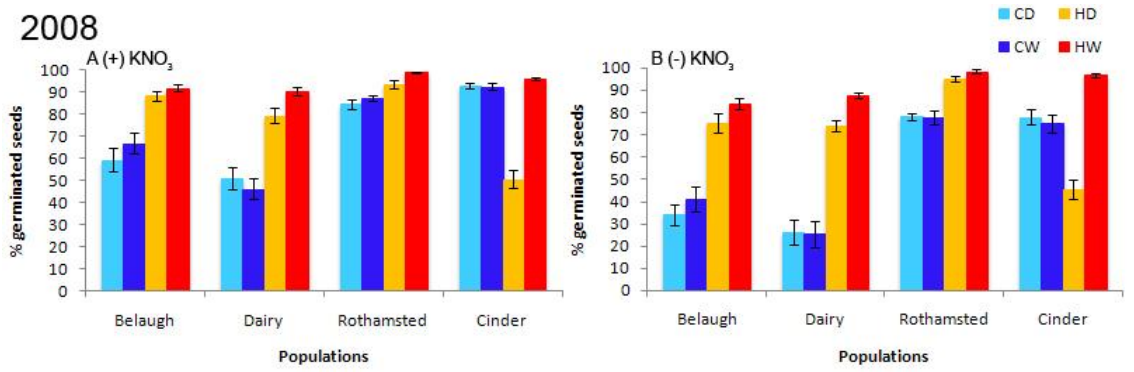

Figure 3.6. Effect of four temperature and soil moisture regimes applied during the period of spike emergence and seed maturation on the proportion of non-dormant seeds obtained on tests carried out on Petri dishes with (A) and without (B) a KNO_3 solution of four *L. multiflorum* populations, Belaugh, Dairy, Rothamsted and Cinder in 2008.

A visible effect of the hot treatments in all the populations was a reduction in seed production. Inclusion of KNO_3 had the effect of breaking dormancy especially on 'cool' sets that seemed to be more stimulated by KNO_3 than 'hot' sets. In populations like Belaugh, Dairy or Wickford germination in 'cool' treatments increased from values between 18 and 40% without KNO_3 solution to 46-62% with a KNO_3 solution, while in 'hot' treatments the range of values increased from 71-88% to 80-97%. As trends

remained the same for both types of test and the aim of these experiments was to compare trends between treatments, both tests appear to be valid.

Tests carried out in trays gave similar trends to those carried out in Petri dishes but with more protracted germination. Twelve weeks after sowing seeds the proportion of non-dormant seeds from every treatment for Fish was similar and relatively high. By then 85-90% of the sown seeds had germinated (Figure 3.7).

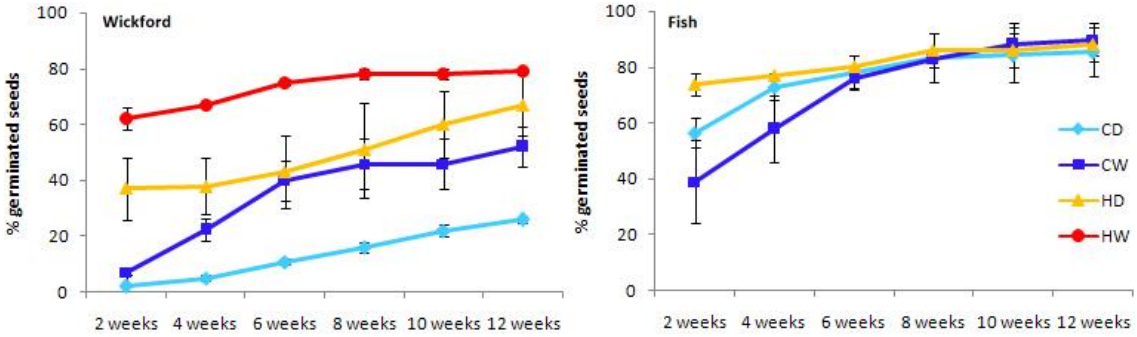

Figure 3.7. Acumulated percentage of germinated seeds on trays with compost of two *L. multiflorum* populations subjected to four temperature and soil moisture regimes during the period of spike emergence and seed maturation. Test in trays started in July and August 2007 and finished in November 2007.

After the same interval, seeds from Wickford had lower and different percentage of emergence for each treatment, 26, 52, 67 and 79% for CD, CW, HD and HW treatments respectively (Figure 3.7). Tests carried out in Petri dishes had similar trends with the percentage of germination ordered in the same way: CD<CW<HD<HW.

In the 2008 experiment, twelve weeks after sowing, the populations Belaugh, Dairy, Cinder and Rothamsted showed similar trends to those tests carried out in Petri dishes. In all cases germination stopped during winter months and started again in spring reaching values between 60% and 98% of germination; with the exception of seeds from Cinder from HD treatments that achieved only 38% germination. In the case of the non-dormant populations, Cinder and Rothamsted, and with the exception

of seeds from the HD treatment of Cinder, most of the sown seeds had germinated by autumn (76-94%).

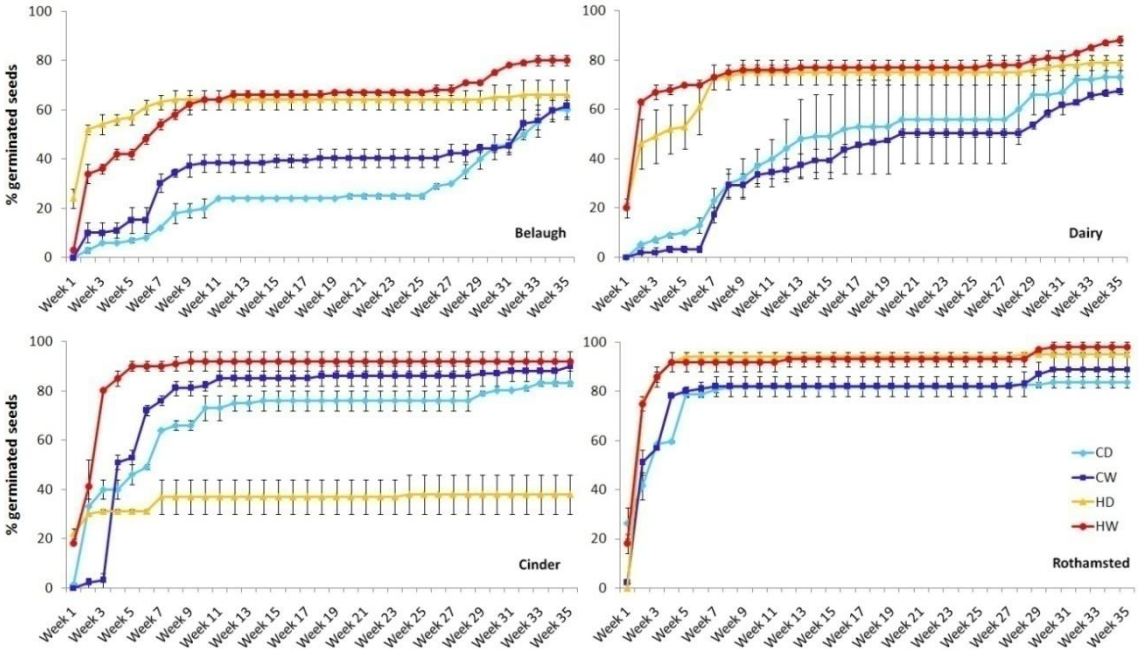

Figure 3.8. Acumulated percentage of germinated seeds in trays of four *L. multiflorum* populations subjected to four temperature and soil moisture regimes during the period of spike emergence and seed maturation. Tests in trays started in July and August 2008 and finished in June 2009.

For the populations Belaugh and Dairy, the germination values in autumn were lower with 25% and 40% of seeds germinating for CD and CW treatments respectively for Belaugh and 56% and 50% for CD and CW treatments respectively for Dairy. For these populations, a high proportion of the total number of seeds that were sown (17%-35%) emerged in spring. These values are much higher than those obtained in the emergence pattern studies under field conditions (Chapter 4). In greenhouse conditions, probably due to the lack of seed predation and other factors leading to seed loss, the proportion of seeds that survive overwinter and germinate in spring would be expected to be higher than under field conditions.

In summary, results in this experiment indicate that in non-dormant populations, emergence is rapid and independent of environmental conditions. In dormant populations, environmental conditions are critical and there appears to be a strong genetic basis. Hot weather conditions during seed maturation result in a fairly rapid emergence whereas cool weather conditions lead to a much more protracted emergence pattern. With all non-dormant populations and seeds from dormant populations which have undergone a 'hot' treatment, 50% emergence was achieved in 2-6 weeks, while it took 12-32 weeks to achieve this stage with seeds from dormant populations which had undergone a 'cool' treatment.

3.4 Discussion

The aim of these studies was to investigate the effect of different weather conditions, in particular temperature and soil moisture, during seed maturation on the initial dormancy of *L. multiflorum*. The dormancy studies of different field populations of *L. multiflorum* during three sequential years indicated that the most important parameter affecting the initial dormancy was temperature during spike emergence and the seed maturation period. Years that were warmer than average had a higher proportion of non-dormant seeds (72 and 39% for the tests with and without KNO_3, respectively) while cooler than average years had the lowest proportion of non-dormant seeds (53 and 26% with and without KNO_3, respectively). Years that were wetter than average and with a higher number of days of rain appear to be associated, to some extent, with the production of a greater proportion of dormant seeds. These results agree with those published by Swain *et al*. (2006) where temperature had a greater effect on seed dormancy in *A. myosuroides* seeds than soil moisture. In these studies the number of days of rain during the seed maturation period appeared to be more influential on seed dormancy than the amount of precipitation. Years with a higher proportion of non-

dormant seeds had fewer days with rain than years with a low proportion of non-dormant seeds.

The germination values from each year were the average values of the percentage of germination of a wide number of *L. multiflorum* populations. All these field populations were grown under similar weather conditions during the same growing season but differed in their degree of dormancy. They could be divided into two groups as mentioned by Wiesner & Grabe (1972); populations that were nearly non-dormant and which were unaffected by weather conditions and populations considered dormant in which weather conditions during seed development and maturation had an important effect. This was demonstrated in the pot experiments, where populations such as Fish or Rothamsted that were found to be less dormant in previous studies, with 90%-100% non-dormant seeds, were consistently non-dormant in subsequent studies in pots, regardless of the temperature and moisture treatment they received, with 78%-99% non-dormant seeds. However, populations such as Wickford, Dairy or Belaugh that were found to have some degree of dormancy in previous studies, with 9%-49% non-dormant seeds, were found in subsequent studies in pots to be greatly affected by temperature during seed development and maturation.

Although the average proportion of non-dormant seeds varied between years, there was great within year variation between field samples and values from the different populations between years also overlapped. This variability might be explained by an intrinsic genetic dormancy in some populations. Thus, although in a hot year low dormancy levels would be expected and this would probably be the general trend, populations should also be considered individually as some of them could be genetically non-dormant and acting independently of weather conditions. Conversely, others could present less dormancy in a cooler year but still have some intrinsic genetic dormancy. This could be the case with populations like Wickford which, in a hot

year such as 2006, when low dormancy would be expected, had only 49% and 10% of non-dormant seeds in tests with and without KNO_3, respectively. This population has been shown in pot experiments to be affected by temperature during spike emergence and the seed maturation period. The low dormancy levels found during 2006 may indicate that this population has high intrinsic genetic dormancy despite being affected by weather conditions.

Results indicated that high temperatures during seed development and maturation in the dormant populations of *L. multiflorum* decreased initial seed dormancy. There was no clear correlation between soil moisture and dormancy. In the study published on dormancy in *Cenchrus ciliaris* by Sharif-Zadeh & Murdoch (2000) dormancy increased if water stress was imposed during maturation, but in contrast, other studies on *Avena fatua* (Sexsmith, 1969, Peters, 1982, Sawhney & Naylor, 1982) and *Sorghum halepense* (Benech-Arnold *et al.*, 1992) found that a lower level of soil moisture during seed maturation decreased dormancy. The reason for the higher dormancy of *C. ciliaris* seeds from water stressed plants could be due to its glumes which are less permeable to water and oxygen. These glumes may thus protect seeds in times of drought and impose dormancy for survival – potentially an adaptive characteristic for arid areas (Sharif-Zadeh & Murdoch, 2000). Benech-Arnold *et al.* (1992) suggested that dormancy of *S. halepense* seeds from water stressed plants was reduced due to modifications in the properties of the glumes that result in an enhancement of their permeability to oxygen diffusion. Sawhney & Naylor (1982) found that when both conditions, drought and high temperature, were imposed during seed development the water content of mature seeds was reduced, and this could be correlated with reduced dormancy through a reduction in abscisic acid levels, a plant hormone which inhibits germination. In *Fraxinus excelsior* seeds, a decline in abscisic acid concentration is also associated with warm temperatures and with the release of dormancy (Finch-Savage & Leubner-Metzger, 2006). Germination was induced in *Hordeum vulgare*

seeds that had suffered heat stress in mature stages of seed development. The increased germination levels were related to a thinner seed coat, increased permeability and decreased water soluble inhibitor content of the seed (Khan & Laude, 1969). Higher temperatures and a water deficit during seed development and maturation would likely cause a reduction in abscisic acid levels, increase permeability of the glumes to oxygen diffusion and the seed coat to water soluble inhibitors of *L. multiflorum* seeds, and consequently, increase germination and therefore decrease dormancy. This could explain the results in the studies carried out on field samples during three years. As results indicate, the effect of temperature on seed dormancy is much greater than the effect of soil moisture, so temperature appears to be the parameter most responsible for changes in dormancy in *L. multiflorum* seeds. The role of temperature as the main factor in the regulation of dormancy in temperate species has been reviewed by Vleeshouwers *et al.,* (1995).

The *A. myosuroides* populations Roth04 and Peldon03 studied in the pot experiment appeared to be affected by temperature during seed development and had significant differences in % non-dormant seeds between 'hot' and 'cool' treatments as was also recorded for the dormant *L. multiflorum* populations. However, germination values for *A. myosuroides* seeds from plants which had undergone 'hot' treatments were still lower than those values obtained for *L. multiflorum*, with less than 80% non-dormant seeds. In addition, the two field *A. myosuroides* populations showed much higher levels of dormancy than any of the *L. multiflorum* populations studied. This indicates that *A. myosuroides* despite being affected by weather conditions during seed development, retains a higher degree of dormancy than *L. multiflorum*. Dormancy also lasted longer in *A. myosuroides* seeds and two months after the collection date the proportion of non-dormant seeds was lower than for *L. multiflorum*. A decrease in the proportion of dormant seeds was not necessarily expected, as it has been shown that

A. myosuroides seeds were more dormant from later collections and after dry-storage (Froud-Williams, 1985).

In the tests carried out in Petri dishes with *A. myosuroides* seeds, it appeared that KNO_3 had a lesser effect than with *L. multiflorum* on releasing dormancy of freshly shed seeds. Two different Petri dish tests were carried out alongside a test in trays in order to determine which one would most closely represent field conditions, thus providing more accurate dormancy values. Tests in Petri dishes are faster and more practical than tests in trays as they are carried out in two weeks while tests in trays take much longer. Although no conclusions could be drawn regarding which one would be more accurate, they all gave similar trends and the comparison of trends between years or treatments could be achieved using all approaches.

In conclusion, it seems that, as has been shown in *A. myosuroides* (Swain *et al.*, 2006), the critical period when environmental factors affect dormancy is during seed maturation. Warmer temperatures during this period, which in the UK can occur in June and July, could result in a lower dormancy. These non-dormant seeds are likely to germinate as soon as the weather conditions are favourable. In contrast, cooler temperatures during this period will result in a higher proportion of non-dormant seeds with a delayed and prolonged germination time. A prediction of the dormancy status of *L. multiflorum* seeds according to the weather conditions during this period would help in the development of control strategies. In a hot year when weather conditions will be suitable for germination, it is expected that most seeds would be non-dormant, meaning that most seeds would germinate before autumn drilling, provided there was sufficient soil moisture, resulting in a lower crop infestation. Conversely, in cooler years dormancy would be higher and drilling should be delayed to a later time when most weeds have emerged or a more robust herbicide programme should be used to reduce the potentially greater numbers of emerging plants. During the three year studies, the

proportion of germinated or non-dormant seeds varied within a wide range indicating genetic differences between populations. Commercial cultivars of *L. multiflorum* would be expected to have a low dormancy as a uniform germination would be required. It would be interesting to know the field history in each case and, if the *L. multiflorum* population present in a field is expected to be a commercial cultivar sown in previous seasons, it is likely to be a non-dormant population with most of the seeds germinating early regardless of the weather conditions during previous months. The herbicide history of each field would also be helpful as selection by herbicides might have an effect in other traits such as dormancy (Gundel *et al.*, 2008).

3.5 Chapter summary

- Years when the months of June and July were warmer and drier than average and with fewer days of rain were associated with the production of a higher proportion of non-dormant seeds. Thus, in these years a high proportion of *Lolium multiflorum* seeds would be expected to germinate in early autumn with favourable weather conditions.
- In years when there are cooler and wetter than average conditions in June and July and with more days with rain than average, a higher proportion of non-dormant seeds and a longer dormancy will be expected. Thus, germination of *L. multiflorum* seeds will be predictably delayed.
- Two different kinds of *L. multiflorum* populations were found. Those which are non-dormant and not affected by weather conditions during seed development and maturation and those which are dormant and affected by weather conditions during that period.
- The great variability in % non-dormant seeds found between field samples grown under similar weather conditions might be explained by an intrinsic genetic dormancy.

- High temperature during seed development and maturation in dormant cultivars of *L. multiflorum* decreased initial seed dormancy and seemed to be the most important parameter responsible for changes in dormancy.
- Soil moisture was not clearly correlated with dormancy.
- With similar weather conditions during the seed maturation period *A. myosuroides* seeds were more dormant than those of *L. multiflorum*.

Chapter 4 Emergence pattern and seed production potential studies

4.1 Introduction

Knowledge of the population dynamics of the weed species affecting a crop is essential to predict the long-term consequences of different levels of weed control. To make weed management programmes as effective as possible we need to understand all the factors involved in the population dynamics of weeds. While the occurrence of a few weed plants may not result in significant crop yield loss, they can lead to an increase in subsequent weed population which can reduce future yields (Naylor, 2002). To understand the population dynamics of a weed it is important to consider factors such as flowering and seed rain, pre- and post-dispersal losses, seed production per plant, periodicity of germination, longevity and the effects of agricultural practice (Froud-Williams, 1999).

However, this knowledge must be linked to practical management problems. One good example of linking these studies with weed management was published by Van Acker (2009) who created a table of common weeds where the potential problem derived from a weed escape is rated from 1 (low) to 7 (high) based on classifications of ranges for seed longevity and seed production. The objective was to help farmers decide which control strategies should be carried out.

For this same reason, mathematical models to study the long-term dynamics of weed populations in agriculture have been developed. A recent review cited 134 publications on weed dynamic models, highlighting their importance in weed ecology (Holst et al., 2007). These models can be relatively simple and estimate just one of the factors involved in population dynamics, such as seed production or the weed emergence

pattern of one or several weed species (Moss, 1990b, Lutman, 2002, Figueroa *et al.*, 2007, Martinson *et al.*, 2007, Lutman *et al.*, 2008, Leguizamon *et al.*, 2009). Others are more complicated and can simulate weed population dynamics, describe the behaviour of a weed and predict the effect of various control strategies and cultivation systems in the long-term (Gonzalez-Andujar & Fernandez-Quintanilla, 2004) or predict the evolution of resistance depending on the management strategies carried out (Cavan *et al.*, 1999).

In this chapter results are shown from studies of two key population dynamic parameters, emergence pattern and seed production potential for a range of *Lolium multiflorum* populations on unsprayed areas of winter wheat fields. A simple mathematical model to estimate the average seed production potential of a single *L. multiflorum* plant depending on the weed density was developed from these data by using and modifying an existing model relating yield loss to weed density (Cousens, 1985). The model presented here does not consider factors such as emergence time or the spatial distribution of plants. Plants emerging later or too close to other plants will produce less seeds as there will be greater competition for nutrients and light. This model gives an average value of the number of heads per plant of a population of plants which are heterogeneously distributed and have different emergence times.

Studies carried out in other weeds like *Chenopodium album* showed that, even in competition with crops, there was a significant increase in seed number per plant when weed plant density was lower (Grundy *et al.*, 2004). The number of seeds produced per plant for several common weeds can vary from 50 to 223,200 (Zimdahl, 1993c). This means in some cases that just a few survivors can cause a rapid increase in the number of seeds in the soil bank and, consequently, the number of weed plants in the next season if no adequate control measure is taken. *Lolium multiflorum* behaves like an annual weed in cereal crops and can produce large quantities of viable seeds, while

Lolium perenne is not a major problem in arable crops and this is probably related to the fact that it is not adapted to act like an annual weed (Moss, 2005).

Knowing the percentage of plants emerged in autumn and in spring can help in the decision of when to apply herbicides. However, even when spraying in autumn, the small number of plants that emerge in spring might be important as they could still contribute to seed return and cause significant yield losses. The emergence patterns of *Galium aparine* and the biology of individuals emerging on different dates have been studied (Cussans & Ingle, 1999). The results showed that the small percentage of plants emerging in spring (7%) had significantly reduced vigour compared to the plants emerging in autumn. These plants did not have a significant effect on wheat yield and the number of seeds per plant produced was significantly reduced. Studies conducted with *Lolium rigidum* in Spain showed that 90% of seedlings emerged after a very short period (Fernandez-Quintanilla *et al.*, 2000).

In the work described in this chapter the proportion of *Lolium multiflorum* plants in UK winter wheat fields that emerged in autumn and in winter/spring was determined. The seed production potential of different UK *Lolium multiflorum* populations was also determined. From this data we wanted to know how rapidly a population of *L. multiflorum* plants can increase and to determine the optimum time for herbicide application.

4.2 Materials and methods

Experiments were carried out in the 2005/06, 2006/07 and 2007/08 cropping years to study the emergence pattern and seed production potential of a range of *Lolium multiflorum* (Italian rye-grass) populations in winter wheat fields in England. All the fields were selected to have similar and typical wheat densities (210-283 plants m^{-2}) for

UK conditions, but widely different weed densities in order to provide more information about this weed's population dynamics. Fields were drilled from 21st September to 5th October.

4.2.1 Emergence pattern studies of five populations of *Lolium multiflorum*

To measure the proportion of plants emerging in autumn and spring, quadrats were placed randomly in untreated areas in four winter wheat fields in October 2006, in the counties of Buckinghamshire (High Wycombe), Essex (North Benfleet) and Hertfordshire (Hitchin and Berkhamsted07), and one field in October 2007 in Hertfordshire (Berkhamsted08).

Figure 4.1. Labelled *L. multiflorum* plants in one of the quadrats placed in an untreated plot of a winter wheat field.

The assessment was carried out between October and April in 24 x 0.1m^2 quadrats per site. Plants were counted every four weeks in each quadrat. For a more accurate assessment of emergence, newly emerging plants were labelled every month in four quadrats per site in 2006/07 (Figure 4.1) and in every quadrat in 2007/08, using plastic

coffee stirrers numbered from one to seven depending on the month the plant emerged. The growth stage of all plants in the 2007/08 field and a few plants per site in the 2006/07 fields (two plants per quadrat labelled in autumn 2006 and all new emergences labelled in spring 2007) were monitored. These plants were also assessed in June to study the seed production potential (described below) and also dried and weighed, with the aim of comparing the effect of time of emergence on plant vigour and seed production potential.

4.2.2 Seed production potential studies of ten populations of *Lolium multiflorum*

In June 2006, 2007 and 2008 plants of *Lolium multiflorum* with intact inflorescences ('heads') were collected from untreated areas of winter wheat crops, in Lincolnshire (Louth), Essex (Peldon, Wickford and North Benfleet), Cambridgeshire (Chatteris 1 & 2), Buckinghamshire (High Wycombe) and Hertfordshire (Hitchin, Berkhamsted07 and Berkhamsted08). Sampling was carried out using 12 to 24 random quadrats per site (size varied from 0.05mx0.05m to 0.5mx0.5m and 0.1mx0.1m depending on the weed density) and all plants within each quadrat were collected. This system was used to ensure that a truly representative sample of plants was collected. The numbers of plants m^{-2}, tillers and heads $plant^{-1}$, head length, spikelets $head^{-1}$ and seeds $spikelet^{-1}$ were measured as described in Chapter 2. A total of 1985 plants were assessed in these studies. Assessments are explained in more detail in Chapter 2.

4.2.3 Statistical analysis

Dry weight and seed production data from the emergence pattern studies were logarithmically transformed and analysed by a general ANOVA, having

year/field/quadrat/plant as block structure and season as treatment. The variates were the log_{10} transformations of seeds plant^{-1} and dry weight plant^{-1}.

To give an insight into how the plant populations were grouped a canonical variate analysis (CVA) was performed with the previously transformed data from the seed production potential. This allows for comparisons between populations based on multiple variables. Correlation coefficients between variables were calculated to determine relations between different traits. A regression analysis was also performed with some of these data. All analyses were carried out using Genstat 11.1 (Payne, 2004).

4.3 Results

4.3.1 Emergence pattern studies of five populations of *Lolium multiflorum*

The results of the emergence pattern studies of five *L. multiflorum* populations showed that the vast majority of plants emerged in the autumn (Figure 4.2B).

On average, by November 88.1% of plants had emerged. 6.2% emerged in December and the remaining 5.8% in January, February, March and April.

Fields were selected to have a wide range of *L. multiflorum* plant densities, from Berkhamsted08 (3.0 plants m^{-2}) to High Wycombe (195.4 plants m^{-2}). The other fields North Benfleet, Berkhamsted07 and Hitchin had 45.4, 86.5 and 103.3 plants m^{-2}, respectively. Despite these big differences in plant density all the fields exhibited similarly low spring emergence of *L. multiflorum* (Figure 4.2A).

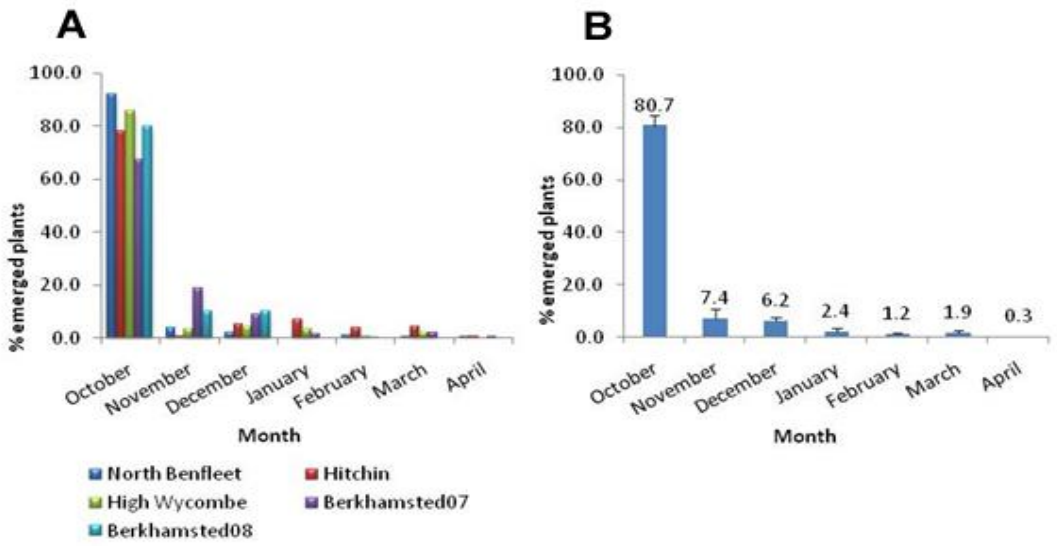

Figure 4.2. Percentage of emergence of *L. multiflorum* plants per month, in five winter wheat fields (A) and average values of emerged plants per month (B).

L. multiflorum plants can potentially produce a large number of seeds, so even low rates of spring emergence may contribute significantly to the weed population. To estimate the potential threat from plants emerged in spring, the seed production potential and dry weight of plants emerged in winter/spring was measured and compared to those of plants emerged in autumn.

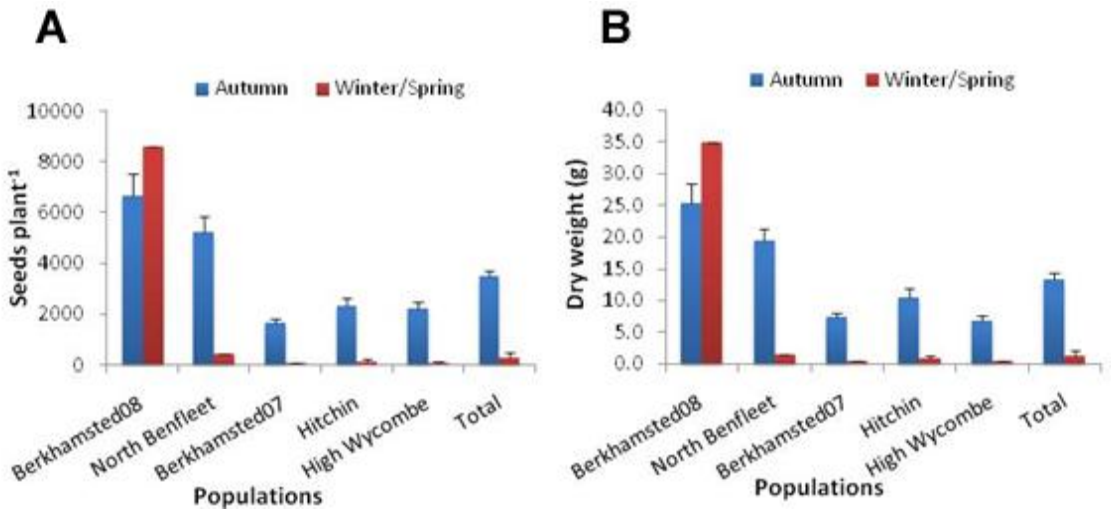

Figure 4.3. Mean seed production potential (A) and mean dry weight (g) (B) per plant for plants emerged in autumn and in winter/spring from the five *L. multiflorum* populations studied.

Plants were classified as either autumn or winter/spring emergers. Those plants which emerged in October or November were counted as autumn emergers and those which emerged between December and April were included as winter/spring emergers.

Plants were assessed in June. In North Benfleet and Berkhamsted08 only one winter/spring emerger survived until June. There was an average seedling mortality of 12%.

Table 4.1. Mean, maximum and minimum values of seeds plant^{-1} for autumn and winter/spring emerging *L. multiflorum* plants.

	Autumn emergence (Oct/Nov)					Winter/Spring emergence (Dec-April)				
Field	No plants	Mean seeds plant^{-1}	Min seeds plant^{-1}	Max seeds plant^{-1}	SEM	No plants	Mean seeds plant^{-1}	Min seeds plant^{-1}	Max seeds plant^{-1}	SEM
Berkhamsted08	38	6,674	312	17,846	857.3	1	8,607	8,607	8,607	0.00
North Benfleet	45	5,249	0	19,832	596.1	1	426	426	426	0.00
Berkhamsted07	48	1,658	147	5,449	170.0	15	93	0	285	23.78
Hitchin	46	2,325	308	10,061	313.3	9	159	0	736	93.45
High Wycombe	46	2,213	0	7,862	254.4	17	114	0	650	40.76

Table 4.2. Mean, maximum and minimum values of dry weight for autumn and winter/spring emerging *L. multiflorum* plants.

	Autumn emergence (Oct/Nov)					Winter/Spring emergence (Dec-April)				
Field	No plants	Mean weight plant^{-1}	Min weight plant^{-1}	Max weight plant^{-1}	SEM	No plants	Mean weight plant^{-1}	Min weight plant^{-1}	Max weight plant^{-1}	SEM
Berkhamsted08	38	25.3	0.9	85.1	3.2	1	35.0	35.0	35.0	0.00
North Benfleet	45	19.4	0.9	53.1	2.0	1	1.6	1.6	1.6	0.00
Berkhamsted07	48	7.4	0.4	23.4	0.7	15	0.5	0.0	2.0	0.12
Hitchin	46	10.5	1.3	46.9	1.4	9	0.9	0.1	3.5	0.47
High Wycombe	46	6.9	0.2	22.7	0.7	17	0.4	0.1	1.7	0.10

The plants which emerged in autumn had a much lower mortality than those plants which emerged in spring. In Berkhamsted08 this single winter/spring emerger was first detected in December and had a growth stage of 2 leaves, GS1.2 in the Zadoks scale

(Zadoks et al., 1974). This and the lack of competition because of the very low weed density in this field (3.0 plants m^{-2}) could explain why the seed production potential and dry weight for this plant is higher than the mean values for these two variables in autumn (Figure 4.3).

However, the maximum values in autumn for this population were much higher than the values for this single plant. In the rest of the fields, mean values of dry weight and seeds per plant were much higher in autumn than in winter/spring (Table 4.1 and Table 4.2). As the growth stage of some of the plants was monitored and recorded every month from November, comparisons of the seed production potential and the dry weight of plants with different growth stages in November were carried out.

There was information about the growth stage in November of a total of 171 plants. Plants were grouped in growth stages, plants with one shoot to two leaves (GS1.0, GS1.1, GS1.2 or GS1.3), one tiller (GS2.1), two tillers (GS2.2) and three tillers (GS2.3). Only one plant had a growth stage of three tillers, while 14, 54 and 102 plants had two tillers, one tiller and from one shoot to two leaves, respectively (Table 4.3).

Table 4.3. Mean, maximum and minimum values of dry weight (g) and seeds per plant in June for groups of plants with different growth stages in November. GS1.X represents growth stages between first shoot and three leaves, GS2.1 one tiller, GS2.2 two tillers and GS2.3 three tillers.

Variable	Growth stage in November	Minimum	Maximum	Mean	No of plants	SEM
Dry weight (g)	GS1.X	0.18	45.8	8.2	102	0.78
in June	GS2.1	0.94	52.82	13.9	54	1.65
	GS2.2	2.15	53.14	17.1	14	3.41
	GS2.3	40.39	40.39	40.4	1	0.00
Seed production	GS1.X	0	14,893	2,161	102	238.43
(seeds $plant^{-1}$)	GS2.1	0	19,832	3,779	54	477.90
	GS2.2	599	13,305	3,865	14	879.94
	GS2.3	8,620	8,620	8,620	1	0.00

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As can be seen in Table 4.3 there were evident differences between these groups of plants for the mean values of seed production potential and dry weight, although the range of values was very wide for every group. The only plant with a growth stage GS2.3 had a dry weight of 40.39g and a seed production potential of 8,620 seeds plant^{-1}, whilst, even if the mean values were lower, there were plants with much higher values than that within the other groups of plants. Since the data was skewed a $\log_{10}$ transformation was needed to carry out the statistical analysis.

The conclusions were that the differences in growth stage in November had a highly significant effect on plant dry weight and seed production potential in June ($F_{3, 167}$=7.36, p<0.001 for dry weight and $F_{3,167}$=5.26, p=0.002 for seed production potential).

A general ANOVA was also carried out to compare dry weight and seed production potential of plants which emerged in different seasons. It indicated that the seed production and dry weight of plants emerged in autumn compared with winter/spring were statistically different. As values were skewed, a $\log_{10}$ transformation was needed ($z=log_{10}(y)$). Within the seed production data there were some plants with zero heads and consequently zero seeds. Because zero values are problematic in a $\log_{10}$ transformation we had to add an offset *c* onto every data value. In this case *c* was calculated as half of the minimum value measured. The minimum value measured was 45 seeds plant^{-1}, therefore *c*=22.5. To back transform the data we used the formula $y=10^z$ for dry weight data and $y=10^z - c$ for the seed production potential data. Each stratum used to obtain the information about differences between seasons gave a different percentage of information. The bottom stratum represented the effects between plants from the same quadrat and accounted for 65% of the information. The effects between quadrats from the same field accounted for 27% of the information and the effects between fields accounted for just 5.2% of the information.

The differences between seed production potential mean values were significant in all strata ($F_{1,145}$=209, p<0.001 and $F_{1,114}$=55.96, p<0.001 in the plant and quadrat strata,

respectively, and $F_{1,2}$=29.42, p=0.032 in the field stratum). A similar result was found for the dry weight ($F_{1,145}$=272.31, p<0.001 and $F_{1,114}$=58.83, p<0.001 in the plant and quadrat strata, respectively, and $F_{1,2}$=115.44, p=0.009 in the field stratum). This means that the season when the plant emerges has a significant effect on seed production and plant dry weight. The table of means would give us only 65% of the information about variability. But with the table of combined means (Table 4.4) we use the information about variability in every stratum as it brings this 65% of information together with the information of the other strata. In other words, the effects of every stratum were combined.

Table 4.4. Tables of combined means for $\log_{10}$(Seeds+45/2) and $\log_{10}$(Dry weight). Values in brackets are the back transformed values for this variables, mean dry weight and mean number of seeds per plant.

Table of combined means for $\log_{10}$(Seeds+45/2)		
Season	Winter/Spring	Autumn
Combined means	2.032 (85.1)	3.300 (1,972.8)
rep.	43	223
Standard errors of differences of combined means		
s.e.d.	0.0768	
effective d.f.	222	

Table of combined means for $\log_{10}$(Dry weight)		
Season	Winter/Spring	Autumn
Combined means	-0.354 (0.44)	0.906 (8.05)
rep.	43	223
Standard errors of differences of combined means		
s.e.d.	0.0685	
effective d.f.	214	

The back transformed values for the mean seed production potential and mean dry weight per plant were approximately 23 and 18 times greater, respectively for autumn emergence than for winter/spring emergence. This indicates that even at low plant densities where there is less competition for nutrients, only plants emerging in October

and November will be able to produce a large amount of seeds. Plants emerging in spring are unlikely to undergo full development and produce a high number of seeds. The correlation coefficient between dry weight plant^{-1} and seeds plant^{-1} was 0.95. This indicates that there is a strong positive correlation between the dry weight of the plant and the seed production and can help when estimating the differences in number of seeds plant^{-1} depending on plant vigour.

4.3.2 Seed production potential studies of ten populations of *Lolium multiflorum*

The number of plants m^{-2}, tillers and heads plant^{-1}, head length, spikelets head^{-1} and seeds spikelet^{-1} were determined in quadrats placed in untreated areas of ten winter wheat fields. Mean values of these variables per quadrat were calculated and from them an overall mean of the number of heads m^{-2}, seeds m^{-2} and seeds plant^{-1} was estimated.

Table 4.5. Mean number of plants m^{-2}, heads m^{-2}, heads plant^{-1}, tillers plant^{-1} and seeds plant^{-1} for the ten *L. multiflorum* populations studied.

Population	Plants m^{-2}	Heads m^{-2}	Heads plant^{-1}	Tillers plant^{-1}	Seeds plant^{-1}
Berkhamsted08	3.0	54.8	20.5	25.4	6,816
Chat2	12.8	205.7	16.7	12.8	5,620
Peldon	25.0	410.0	21.5	26.5	7,220
Louth	27.3	347.7	16.4	21.8	4,467
North Benfleet	45.4	482.9	11.1	15.5	4,209
Berkhamsted07	86.5	571.7	7.3	16.2	2,050
Chat1	94.2	356.7	3.9	6.4	909
Hitchin	103.3	524.6	5.7	10.7	1,961
High Wycombe	195.4	1,099.2	6.5	10.0	1,568
Wickford	488.3	1,333.9	2.9	3.9	546

The mean values for all these variables for each site were also calculated. *L. multiflorum* plant density had a range of values from 3 to 488 plants m^{-2} between the

ten sites and so represented a good range in densities, from very low to very high (Table 4.5, Figure 4.4). The maximum and minimum mean values of seeds spikelet^{-1} for all the populations were 12.47 (Chat2) and 8.53 (Wickford), respectively.

The maximum and minimum mean values of spikelets head^{-1} were 32.19 (North Benfleet) and 21.93 (Wickford), respectively. The maximum and minimum average values of head length were 32.19 (Peldon) and 22.14 (Wickford), respectively (Table 4.6).

Table 4.6. Mean number of head length (cm), spikelets head^{-1} and seeds spikelet^{-1} for the ten *L. multiflorum* populations studied.

Population	Head length (cm)	Spikelets head^{-1}	Seeds spikelet^{-1}
Berkhamsted08	27.3	26.7	11.7
Chat2	29.8	27.2	12.5
Peldon	32.2	26.3	12.4
Louth	26.7	23.0	11.6
North Benfleet	29.5	32.2	11.6
Berkhamsted07	24.2	27.2	10.1
Chat1	25.2	25.2	9.3
Hitchin	28.8	30.6	10.8
High Wycombe	27.1	28.2	8.5
Wickford	22.1	21.9	8.5

However, the differences between populations for these variables were relatively small considering the large differences in population density. In contrast, the number of tillers and heads per plant differed greatly depending on weed density. Those populations with a high density of plants had fewer heads per plant than those with a lower plant density (Table 4.5).

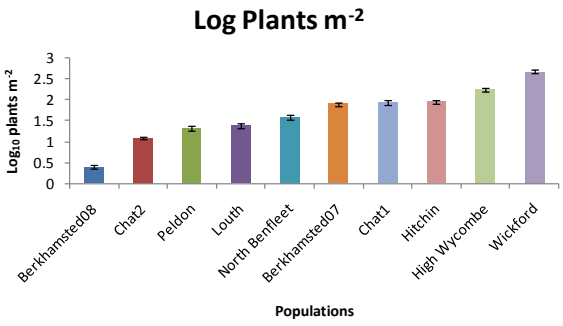


Figure 4.4. Log_{10} transformed mean values of the number of plants m^{-2} for the ten *L. multiflorum* populations studied.

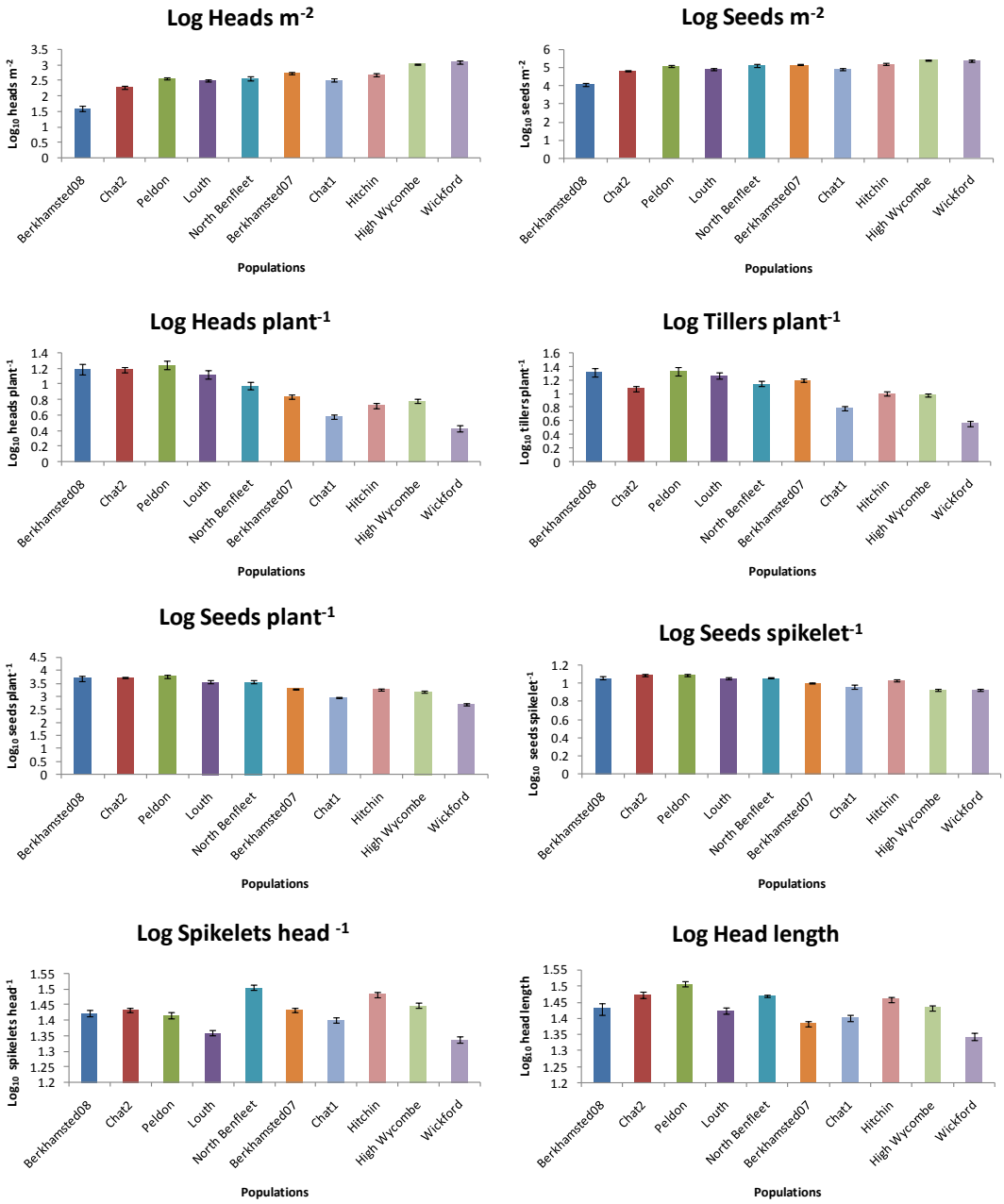


Figure 4.5. Mean values for the log_{10} transformed values of the number of heads m^{-2}, seeds m^{-2}, heads $plant^{-1}$, tillers $plant^{-1}$ and seeds $plant^{-1}$, seeds $spikelet^{-1}$, spikelets $head^{-1}$ and head length (g) for the ten *L. multiflorum* populations studied. Populations are arranged from low to high plant density.

Histograms and graphs from raw mean data from all the quadrats showed that most variables were quite skewed and that the variance increased along with the mean, so would benefit from a log_{10} transformation. After the log_{10} transformation the correlation

coefficients for all these variables were calculated (Table 4.7) to see which variables were most affected by the plant density.

Table 4.7. Correlation coefficients for plants m^{-2}, heads m^{-2}, seeds m^{-2}, heads $plant^{-1}$, tillers $plant^{-1}$ and seeds $plant^{-1}$, seeds $spikelet^{-1}$, spikelets $head^{-1}$ and head length calculated from log_{10} transformed mean values from every quadrat for the ten *L. multiflorum* populations studied.

	Plants m^{-2}	Seeds $plant^{-1}$	Seeds m^{-2}	Tillers $plant^{-1}$	Spikelets $head^{-1}$	Seeds $spikelet^{-1}$	Heads $plant^{-1}$	Head length
Heads m^{-2}	0.86	-0.28	0.97	-0.22	0.06	-0.37	-0.26	-0.12
Head length	-0.34	0.60	0.04	0.41	0.49	0.58	0.49	
Heads $plant^{-1}$	-0.71	0.97	-0.14	0.90	0.15	0.58		
Seeds $spikelet^{-1}$	-0.57	0.71	-0.17	0.49	0.18			
Spikelets $head^{-1}$	-0.04	0.31	0.23	0.21				
Tillers $plant^{-1}$	-0.63	0.88	-0.11					
Seeds m^{-2}	0.78	-0.12						
Seeds $plant^{-1}$	-0.71							

The number of heads m^{-2} and seeds m^{-2} were highly positively correlated to plant density with a positive correlation of 0.86 and 0.78, respectively. As the number of plant m^{-2} increases, the number of heads m^{-2} and consequently seeds m^{-2} also increases (Figure 4.5 and Figure 4.6). The number of tillers, heads and seeds $plant^{-1}$ all had a relatively high negative correlation with plant density with values -0.63, -0.71 and -0.71, respectively. This means that when the number of plants m^{-2} increases the number of tillers, heads and seeds $plant^{-1}$ decreases (Figure 4.5 and Figure 4.6).

The number of seeds $spikelet^{-1}$ was also affected by the plant density (Figure 4.5) although to a lesser degree, with a correlation coefficient of -0.57. However, the correlation coefficients for head length and the number of spikelets $head^{-1}$ were -0.34 and -0.04 respectively. This means that there is little or no correlation between these variables and the plant density, and therefore, changes in plant density have little effect on head length or spikelets $head^{-1}$ (Figure 4.5).

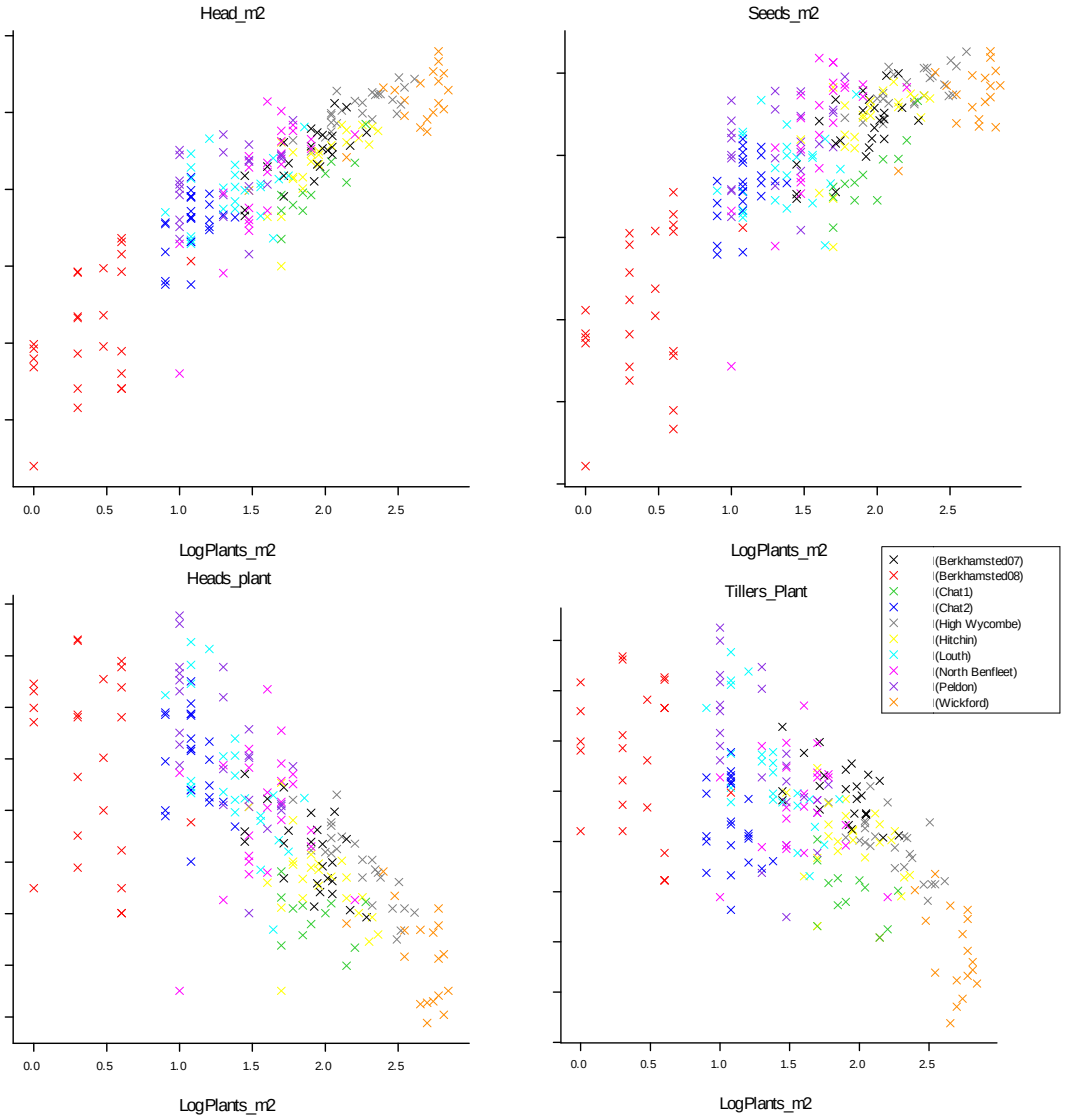


Figure 4.6. Correlation plots between the log_{10} transformed values of plants m^{-2} and the log_{10} transformed values of heads m^{-2}, seeds m^{-2}, heads $plant^{-1}$ and tillers $plant^{-1}$. In the first two plots the correlation is obviously positive while it is negative for heads and tillers $plant^{-1}$.

Since ANOVA is a univariate technique it allows comparisons to be made between populations based only on a single variate, and it cannot give an insight into how the populations are grouped or which is the most important element in defining groups. However, canonical variate analysis (CVA) allows these types of comparisons to be made. Therefore, to study these within-field differences between plant traits a canonical variate analysis was carried out using the variables initially measured; head

length, heads plant^{-1}, seeds spikelet^{-1}, spikelets head^{-1}, tillers plant^{-1} and plants m^{-2}. The results are illustrated in a canonical variates bi-plot in Figure 4.7.

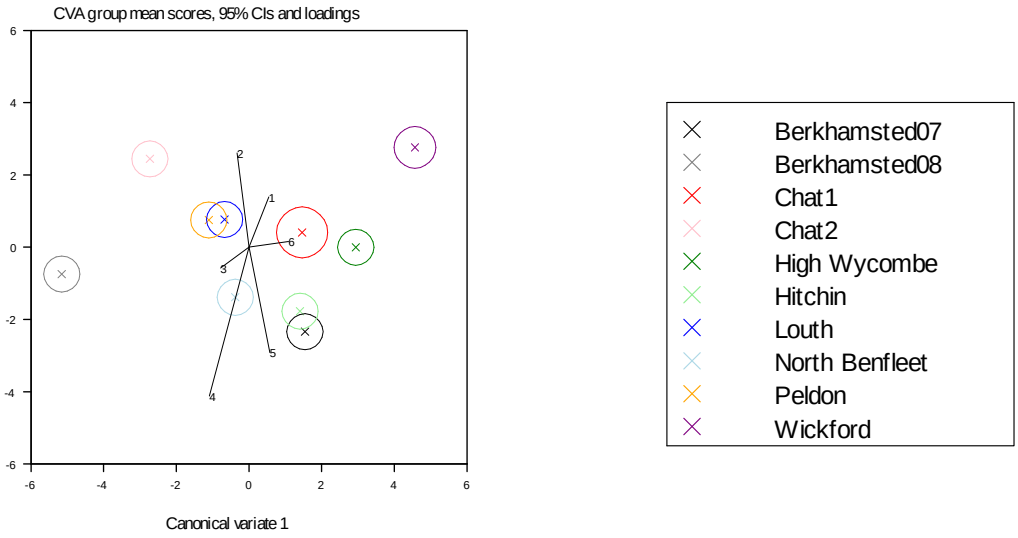


Figure 4.7. Canonical variates bi-plot of plant traits (plants m^{-2}, heads m^{-2}, seeds m^{-2}, heads plant^{-1}, tillers plant^{-1} and seeds plant^{-1} in *Lolium multiflorum* populations from ten winter wheat fields. The crosses in the plot represent the population means. The circles surrounding the dots represent the 95% confidence interval for each population. The lines represent vectors derived from trait 1 (head length), trait 2 (heads plant^{-1}), trait 3 (seeds spikelet^{-1}), trait 4 (spikelets head^{-1}), trait 5 (tillers plant^{-1}) and trait 6 (plants m^{-2}) that spatially separate the populations. The intersection of the vectors represents the mean response for all populations.

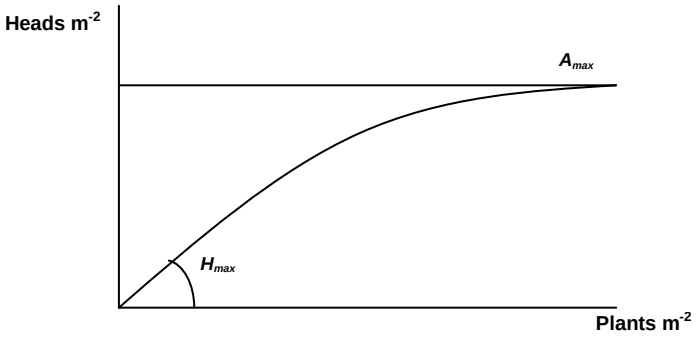
After data transformation, the first two dimensions accounted for 82.5% of the variability. 60.4% was explained by the first canonical variate while 22.1% was explained by the second canonical variate. The vectors on the plot show how the variables relate to the sites and the axes of the plot. The first canonical variate was most closely associated with vectors 6 and 3, so consequently, these two variables, plants m^{-2} and seeds spikelet^{-1}, are important in the discrimination between populations. The second dimension is more difficult to interpret as it accounts for just 22.13% of the variability. The separation between the sites to the top and bottom of the graph appears to be associated with the pairs of variables 1 and 2 (head length and heads plant^{-1}) versus 4 and 5 (spikelets head^{-1} and tillers plant^{-1}).

The first dimension appears to contrast the populations Wickford, Chat1, High Wycombe, Hitchin and Berkhamsted07, which are those with highest plant densities, from the rest. The variables associated with these five sites appear to be 1, 5 and 6 (head length, tillers plant^{-1} and plants m^{-2}), whilst 2, 3 and 4 (heads plant^{-1}, seeds spikelet^{-1} and spikelets head^{-1}) are more closely associated with the other sites on the left. These five populations also appear to have the lowest values for heads plant^{-1} and seeds spikelet^{-1} and most of the lowest values for tillers plant^{-1} and head length. We can see from the proximity of these populations in the plot that Peldon and Louth are quite similar to each other as are Hitchin and Berkhamsted07. Wickford and Berkhamsted08, the populations with the highest and lowest plant density respectively, are distinct from the rest of the populations, as can be seen from their position on the plot.

In summary, after comparing all the variables studied and analysing the similarities between all populations, they can be divided in to two groups with similar population dynamics, the five populations with highest plant densities (Berkhamsted07, Chat1, Hitchin, High Wycombe and Wickford) and the five with lowest plant densities (Berkhamsted08, Chat2, Peldon, Louth and North Benfleet).

A regression analysis was also performed to relate *Lolium multiflorum* plant density with heads m^{-2} and heads plant^{-1}. The relationship between *L. multiflorum* plant and head densities (Figure 4.8A) was calculated using a variation of a hyperbolic yield loss equation described by Cousens (1985) and shown below.

$$y = \frac{H_{max}d}{1+(H_{max}d/A_{max})}$$



Where y is the number of weed heads m^{-2}, *d* is the weed density in plants m^{-2}, H_{max} is the number of heads as plant density approaches zero (or head production by a single plant with no intra specific competition) and A_{max} is the number of heads as weed density approaches infinity.

The equation was fitted using Genstat. Estimates of H_{max} (11.834) and A_{max} (1,786) were obtained. The percentage of variance accounted for 71.3, which means that 71.3% of the variance within these data is explained by the model. The data used to fit the equation were the mean values for plants and heads m^{-2} of the 222 quadrats measured in the ten sites. The relationship between the plant density of *Lolium multiflorum* and the number of heads per plant can be obtained derived from this equation (Figure 4.8B).

The results in Table 4.8 show that as the number of *L. multiflorum* plants decreases, thus reducing intra-specific competition, the numbers of heads per plant increases considerably. They also indicate that very little intra-specific competition occurs at plant densities below about 10 plants m^{-2} but becomes increasingly influential at plant densities higher than 25 plants m^{-2}.

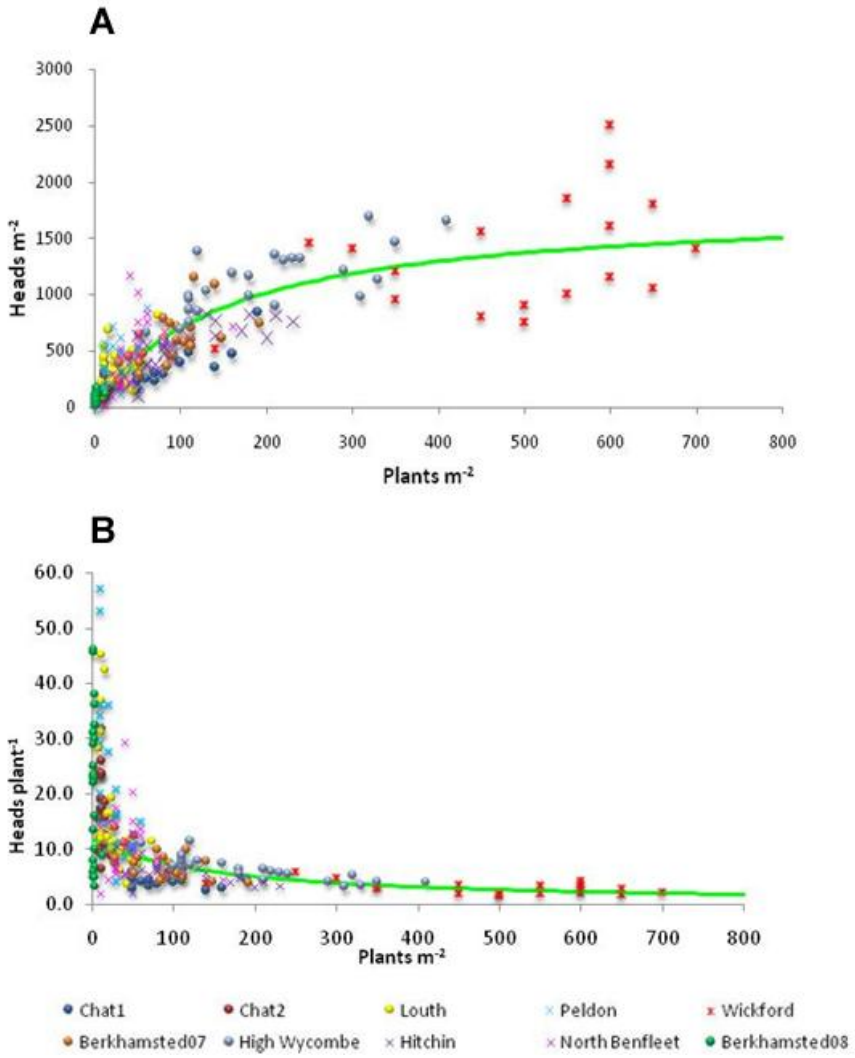


Figure 4.8. Relationship between *Lolium multiflorum* plant and head density and plant density and heads plant^{-1} (A & B, respectively) using the mean values of these variables from 222 quadrats placed in untreated plots of ten winter wheat fields. Each dot represents a different quadrat within a field.

However, this estimate of the number of heads per plant seems quite unrealistic when looking at real values from the populations studied, and it does not seem to be very accurate at densities below 50 plants m^{-2}. As it was shown after performing the canonical variate analysis, all the populations seem to be clearly separated into two groups. Thus, the predictions for the number of heads per plant for fields with high plant density and fields with low plant density could be obtained separately. This way we can have two different models to predict the number of heads per plant in high and low plant density fields and we can thus obtain more realistic and sensible estimates

for both groups. Therefore, the relationship between *Lolium multiflorum* plant and head densities was again calculated for fields with a plant density below 50 plants m^{-2} (Berkhamsted08, Chat2, Peldon, Louth and North Benfleet) and fields with a plant density greater than 80 plants m^{-2} (Berkhamsted07, Chat1, Hitchin, High Wycombe and Wickford). The estimates were H_{max}=23.75 and A_{max}=1,010 for low plant density fields and H_{max}=9.024 and A_{max}=2,098 for high plant density fields. The percentage of variance accounted for 54.0 and 65.5 respectively.

Table 4.8. Influence of *L. multiflorum* plant density on heads m^{-2} and heads $plant^{-1}$ based on all data from the ten winter wheat fields studied.

Plants m^{-2}	Heads m^{-2}	Heads $plant^{-1}$
1	12	11.8
5	57	11.5
10	111	11.1
25	254	10.2
50	444	8.9
75	593	7.9
100	712	7.1
250	1,114	4.5
500	1,372	2.7
1000	1,552	1.6

The predicted values for the number of *Lolium multiflorum* heads per plant and per m^2 depending on the weed plant density were calculated using these new variables (Table 4.9). The model on the left of Table 4.9 will predictably be more accurate with densities up to approximately 100 plants m^{-2}, while with density values over that density the model on the right should be used.

Table 4.9. Influence of *L. multiflorum* plant density on heads m^{-2} and heads $plant^{-1}$ based on two sets of data from quadrats from populations with low (Berkhamsted08, Chat2, Peldon, Louth and North Benfleet) and high (Berkhamsted07, Chat1, Hitchin, High Wycombe and Wickford) plant density. Up to 134 plants m^{-2}, predictions should be made with the model on the left while over this value the model of the right should be used. The value where predictions change from one model to the other was chosen to be 134 plants m^{-2} because this was the point at which both models had the same prediction.

Plants m^{-2}	Below 134 plants m^{-2}		Over 134 plants m^{-2}	
	Heads m^{-2}	Heads $plant^{-1}$	Heads m^{-2}	Heads $plant^{-1}$
1	23	23.2	*9*	*9.0*
5	106	21.3	*44*	*8.8*
10	192	19.2	*87*	*8.7*
15	263	17.6	*127*	*8.5*
20	323	16.2	*166*	*8.3*
25	374	15.0	*204*	*8.1*
30	418	13.9	*240*	*8.0*
35	456	13.0	*275*	*7.8*
40	490	12.2	*308*	*7.7*
45	519	11.5	*340*	*7.6*
50	546	10.9	*371*	*7.4*
75	645	8.6	*512*	*6.8*
100	709	7.1	*631*	*6.3*
134	767	5.7	767	5.7
200	*833*	*4.2*	970	4.9
250	*863*	*3.5*	1087	4.3
500	*931*	*1.9*	1432	2.9
1000	*969*	*1.0*	1702	1.7

If the mean values of number of heads $plant^{-1}$ for all the populations studied are compared to those predicted for the models presented here, it seems that the model from Table 4.9 is more accurate than the model from Table 4.8. We also have to take into account that there may be some differences from the predicted values, as crop type and density will also influence the weed's development. Again these results show clearly that as the number of *Lolium multiflorum* plants decreases the number of heads per plant, and consequently seeds per plant, increases considerably.

An average of 295 seeds $head^{-1}$ was estimated, with values from individual sites ranging from 187 seeds $head^{-1}$ at Wickford to 374 seeds $head^{-1}$ at North Benfleet

(Table 4.10). The number of seeds m^{-2} ranged from 18,262 seeds m^{-2} at Berkhamsted08 to 262,131 seeds m^{-2} at High Wycombe, showing that this weed could potentially have a very high seed production.

Table 4.10. Number of seeds head^{-1}, seeds plant^{-1} and seeds m^{-2} for the ten *L. multiflorum* populations and average value, ordered from low to high plant density.

Site	Seeds head^{-1}	Seeds plant^{-1}	Seeds m^{-2}	Plants m^{-2}
Berkhamsted08	313	6,816	18,262	3
Chat2	340	5,620	69,799	13
Peldon	328	7,220	136,006	25
Louth	266	4,467	93,958	27
North Benfleet	374	4,209	183,935	45
Berkhamsted07	276	2,050	159,587	87
Chat1	235	909	83,582	94
Hitchin	332	1,961	173,500	103
High Wycombe	241	1,568	262,131	195
Wickford	187	546	246,735	488
Average	295	3,759	143,137	

These results highlight the ability of *Lolium multiflorum* plants to produce a high number of heads per plant, and consequently a large number of seeds per unit area, even at low plant densities.

4.4 Discussion

The results from these emergence pattern studies show that regardless of site and weed density, the vast majority of *Lolium multiflorum* plants emerge in the autumn. There was no evidence of a 'spring flush' of emergence, which many farmers and advisors believe commonly occurs. To predict weed emergence some simulation models have tried to describe and understand the process, breaking it down into three main components; dormancy loss, germination and pre-emergence growth (Grundy, 2003). Therefore, as part of the process involved in the emergence pattern, such a

high proportion of early-emerging plants would indicate that there is little or no innate dormancy in *L. multiflorum* seeds by the time winter cereal crops are sown, which is typically in September or early October in the UK. This is supported by the dormancy studies described in chapter 3. Knowing the proportion of plants emerging in autumn and in spring can help to decide when to apply herbicides. Spraying herbicides in autumn has been recommended for a number of reasons (Clarke, 2002). In spring, weeds will be bigger and more difficult to kill, so higher doses of herbicide might be needed. At this time crop yield may already be compromised by the competition for nutrients, light and water with the weed, and the more advanced crop growth stage in spring may shield rye-grass and restrict spray coverage. Many farmers believe that *L. multiflorum* plants emerge over a long time period and consequently delay herbicide treatments until spring to ensure that all weeds have emerged prior to spraying. This study indicates that this is unnecessary, since most of the *L. multiflorum* plants emerge in the autumn.

The results from the seed production potential studies showed that *L. multiflorum* plants have the ability to produce a large number of seeds per unit area even at low plant densities, given that the number of heads plant^{-1} increases as the number of plants per unit area decreases. Even a small number of surviving plants may thus produce a considerable number of heads, and consequently a high seed return. This justifies the management of even small populations because a high seed return could lead to an increase in population and subsequent yield reductions. This could indicate that the small proportion of plants that do emerge in spring could still pose a substantial threat, at least in terms of seed production. However, these studies clearly indicate that those plants emerging in spring would not represent such a big threat. Comparisons between spring and autumn emerging plants showed that spring emerging plants will develop much less and will produce a much lower number of seeds than those emerging in autumn. The mean numbers of seeds per plant were 85

and 1,973, for spring and autumn emerging cohorts respectively, a 23 fold greater seed production from autumn emergers. The seed return to the seed bank for these spring emergers will be minimal because of the low proportion of plants that emerge in spring and survive to produce seeds, the low number of seeds produced, and the pre- and post-dispersal losses. These post-dispersal losses can account for 35% of the seed bank, owing to factors like predation or seeds being buried below the depth of emergence (Marshall *et al.*, 2007).

However, when control measures fail or are not applied, just a few surviving plants could potentially produce a high seed return. In the studies carried out in ten sites with winter wheat; seed return from *L. multiflorum* plants ranged from 546 to 7,220 seeds plant^{-1} and from 18,262 to 262,131 seeds m^{-2}, with an average value of 143,137 seeds m^{-2}. This means there will be a huge increase in population density for the next season. The density of wheat plants at all these sites was similar ranging from 210 to 283 plants m^{-2}, indicating that inter-specific competition was typical for winter wheat crops growing in UK conditions.

These results are very different from those of studies carried out with *Lolium rigidum* plants in Spain in competition with barley (Fernandez-Quintanilla *et al.*, 2000), with sowing rates between 170 and 480 seeds m^{-2} for barley and between 120 and 400 seeds m^{-2} for *L. rigidum*. The seed production ranged from 7 to 237 seeds plant^{-1}, while the average estimated in the studies described in this chapter was 3,759 seeds plant^{-1} (Table 4.10). In studies in Australia with glyphosate resistant and susceptible biotypes of *L. rigidum* in competition with wheat the mean number of seeds per plant ranged between 595 and 2,581 with overall means of 1,401 and 1,506 seeds plant^{-1} for resistant and susceptible plants, respectively (Pedersen *et al.*, 2007). This was carried out in wheat fields with wheat densities between 0 and 600 plants m^{-2} and a weed density of 100 plants m^{-2}. The maximum seed production estimated for *Lolium*

persicum in monoculture and in competition with spring wheat was 53,000 and 39,290 seeds m^{-2}, respectively (Holman *et al.*, 2006). The wheat density here ranged from 48 to 552 plants m^{-2}. Seeding rates for the weed were ranged between 581 to 7,180 seeds m^{-2} with seedling establishment between 34 and 39% in both monoculture and spring wheat. These values of seed production were still smaller than the average value obtained in the studies described in this chapter, which was 143,137 seeds m^{-2}. These differences in seed production can potentially be explained by the different *Lolium* spp, weather conditions, crop type, soil types, moisture availability and sowing density. In any case, these studies indicate that in wheat growing under UK conditions, seed production per *L. multiflorum* plant at low weed densities can be 2.5 fold greater than for *L. rigidum* growing in Australian cropping systems and also much higher levels of seed production may occur. This means that even if there is much known about other *Lolium* spp in other countries, studies regarding the agro-ecology of *L. multiflorum* were needed as no generalisation could be made because there are a number of individual factors involved and the growing conditions for each country differ.

Through this work, we are able to confirm that the high competitiveness of *L. multiflorum* at low infestation levels is due to its high tillering capacity. The impact that time of sowing and time of emergence on *L. multiflorum* plant numbers on winter wheat fields has been investigated (Orson, 2007a). The number of *L. multiflorum* plants was lower from plots with later sowing dates. This strategy of sowing late was traditionally used for controlling black-grass in winter wheat. Thus, more weeds can be killed by cultivation or by using a non-selective herbicide. On the other hand, early sowings can bring more problems with pest and diseases, although delaying sowings too much has the risk of yield loss as the length of growing season is reduced (Attwood, 1985). Considering this and the low percentage of plants emerging in spring, sensibly

delaying the sowing date and having an effective chemical control in autumn could potentially greatly reduce *L. multiflorum* infestations.

4.5 Chapter summary

- The majority of *Lolium multiflorum* plants in winter wheat fields in the UK emerge in autumn. On average, 94% of plants emerge in October, November and December and only 6% in spring.
- Plants emerged in autumn were much larger and produced on average 23 times more seeds per plant than spring emerging cohorts.
- The main determinant of seed production was the number of heads per unit area.
- The number of seeds per spikelet, spikelets per head and head length vary relatively little with plant density or between sites, and had much less influence on seed production. The mean number of seeds per head varied little between populations having an average value of 295 seeds $head^{-1}$.
- *Lolium multiflorum* plants are highly adaptable to different weed densities and very high seed production is possible from low density populations. Populations with just 3 plants m^{-2} produced an average of 6,816 seeds $plant^{-1}$.
- The success of *Lolium multiflorum* as a weed of winter cereals appears to be linked to its ability to produce a high number of heads, having an average of 20 heads per plant at low weed densities.
- Delaying sowing date, good crop competition and effectively controlling autumn emerging plants with herbicides should reduce *Lolium multiflorum* infestations.

Chapter 5 Effects of crop and weed densities on the interactions between winter wheat and *L. multiflorum* and *A. myosuroides*

5.1 Introduction

With the number of herbicide resistant weeds increasing each year (Heap, 2009) an integrated management approach incorporating more cultural control measures should be adopted. This would reduce weed populations without imposing a selection pressure for herbicide resistance. Some cultural methods have been proposed elsewhere including crop rotation, delayed planting, timely tillage, the use of competitive crops and enhancing crop competitiveness through increased seed rates (Godel, 1935, Weiner *et al.*, 2001, Beckie, 2006). Moss *et al*., (2007) also proposed the adoption of integrated cultural methods for the management of herbicide resistant *Alopecurus myosuroides*. Elsewhere, the control of a herbicide resistant population of *A. myosuroides* was shown to be most effective when combined with non-chemical practices (Chauvel *et al.*, 2001). It has been shown that both crop density and choice of cultivar are two of the most easily adopted strategies and that these have a significant role in the crop's ability to compete with weeds. Crop density tends to be the more reliable factor although sometimes it might also reduce crop yield if carried to extremes (Korres & Froud-Williams, 2002). Studies carried out by Grundy *et al.,* (1999) showed that the majority of the crops investigated reduced weed biomass although, as expected, there was no effect on seedling emergence. Lemerle *et al.,* (1996) studied the competitive abilities of a range of genotypes of wheat and durum wheat concluding that traditional standard varieties and hybrids were more weed suppressive than current varieties. Studies of weed seed production and crop yield loss carried out with winter wheat in competition with *Lolium multiflorum* showed that yield reduction tended to be greater for short-strawed wheat cultivars and with higher amounts of N fertilizer

(Appleby *et al.*, 1976). Nitrogen levels and the application method have also been shown to affect weed biomass (Togay *et al.*, 2009). In other studies carried out with winter wheat in competition with *Bromus secalinus* (Koscelny *et al.*, 1990) and *L. multiflorum* (Alshallash & Drennan, 1993) it was shown that increasing the wheat seeding rate increased wheat yield and decreased the weed seed production. Olsen (2006) showed that a combination of increased crop density and a uniform spatial pattern can reduce weed biomass and yield loss. The weed suppression ability of three winter wheat varieties at different row spacing has also been studied and results showed that more competitive varieties and narrower row spacing reduced weed growth (Drews *et al.*, 2009). It has also been shown that variety differences in the competitive ability of wheat genotypes are significant at early crop stages up to 100 plants m^{-2} of *L. multiflorum*, with a wheat density of 280 plants m^{-2} (Acciaresi *et al.*, 2001). At early stages and with weed densities up to 100 plants m^{-2}, aggressivity was higher in wheat, while with a weed density of 150 plants m^{-2} and in older stages, *L. multiflorum* was more aggressive. Champion *et al.* (1998) suggested that height differences and earlier development were the main factors responsible for weed suppression by different wheat cultivars. In this same study row spacing had little effect on crop yield or weed suppression while crop density had a very significant effect. In studies carried out with winter wheat and the weeds *Avena sterilis* ssp *ludoviciana* and *Phalaris paradoxa*, Walker (2002) found that similar levels of weed control can be achieved by increasing crop density and reducing herbicide rates instead of using standard crop densities and 100% herbicide recommended rate. Increasing crop density reduced the number of tillers $plant^{-1}$, seeds $tiller^{-1}$ and weight $seed^{-1}$ in *L. persicum* plants (Holman *et al.*, 2006).

For autumn-sown crops, an optimal crop density of 260 wheat plants m^{-2} surviving the winter has been recommended (Sylvester-Bradley *et al.*, 2008). In studies carried out on wheat in the cropping years 1996/97, 1997/98 and 1998/99 it was shown that the

average economic optimum plant density was 62, 93 and 139 plants m^{-2} for late-September, mid-October and mid-November sowings respectively (Spink *et al.*, 2000). Considering only grain yield response and using the same costs and crop values as used by Spink *et al*., (2000), Gooding *et al.*, (2002) calculated that economic optimum seed rates of winter wheat were between 175 and 375 seeds m^{-2}. Considering grain quality in terms of increased protein concentration and reduced blackpoint severity, Gooding *et al.*, (2002) recommended reducing target plant populations to 100-150 plant m^{-2}. The range of recommended values for wheat plant density or seeding rate seems quite wide. In addition, all these studies were carried out without considering the effect of a potential weed infestation on crop yield or the effect of crop density on weed seed production. In the work described in this chapter the effects of both crop and weed densities on weed seed production and on crop yield were examined. Both *A. myosuroides* and *L. multiflorum* were included in the studies since both are major weeds in the UK. As there is an extensive literature on the competitive interactions between wheat and *A. myosuroides* and relatively little on *L. multiflorum, A. myosuroides* was included in the study as a comparative measure to validate results.

5.2 Materials and methods

5.2.1 Experimental design

The experiment was carried out in the 2007/08 cropping year to study the seed production potential of *L. multiflorum* and *A. myosuroides* when influenced by different weed and crop plant densities to determine the weed's effects on head and seed production and on crop yield. The field experiment was carried out in Sawyers field at Rothamsted Research (Hertfordshire). The soil at this site is a flinty silty clay loam with a clay with flints sub soil of the Batcombe-Carstens Series, a Typic Paleudalf (USDA-NRCS. Soil Survey Staff). A split-plot design was used with two blocks with two winter

wheat densities in the main plots and eight *L. multiflorum* and *A. myosuroides* densities in the subplots. Two areas of 1 m^2 were marked within each subplot, one for assessment of plant and head populations, to assess weed seed production (S) and the other for crop yield assessment (Y) (Figure 5.1). This was done so the area would always include eight rows of wheat.

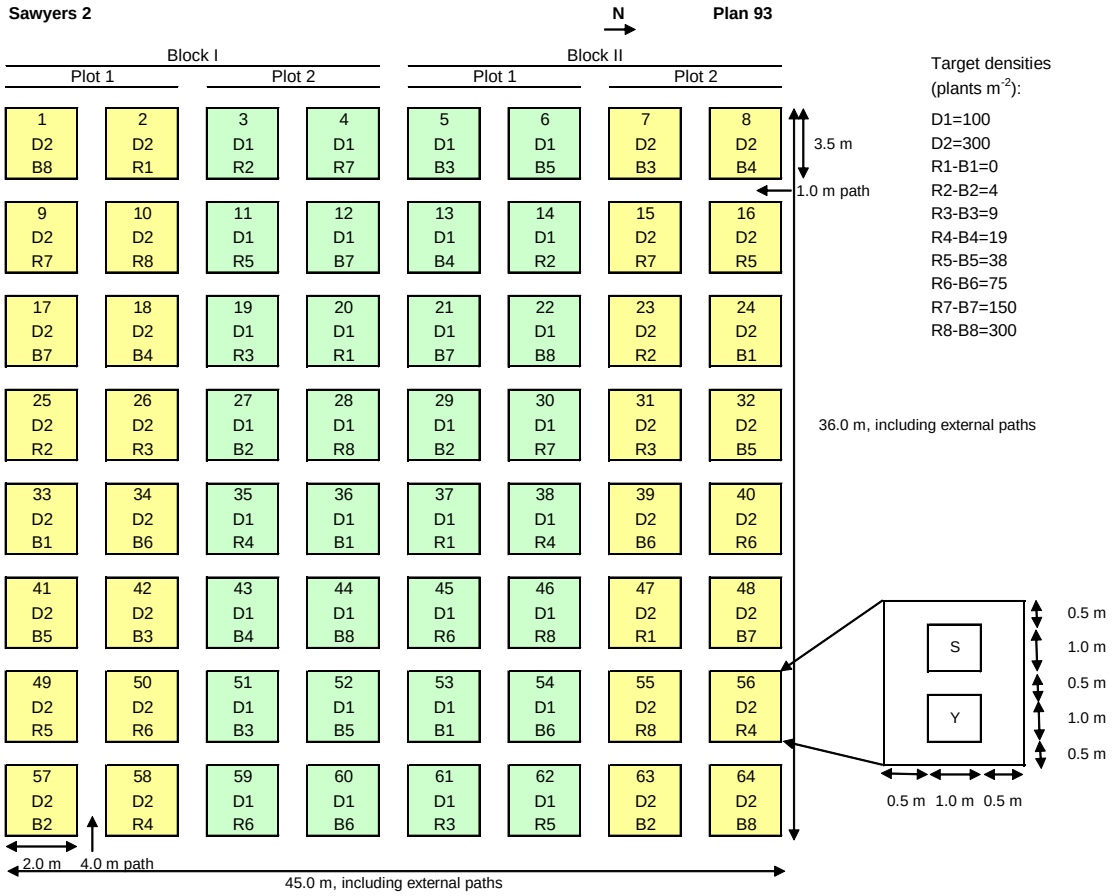


Figure 5.1. Split-plot design layout of the experiment plan. The experiment was divided in two blocks and each block was divided in two plots, each of them with a different wheat density (D1-D2) and subplots with eight different *L. multiflorum* (R1-R8) and *A. myosuroides* (B1-B8) densities. Each subplot included two 1m^2 quadrats for weed seed production (S) and crop yield (Y) assessments.

A commercial *L. multiflorum* cultivar (Trajan) was used in this experiment together with a susceptible *A. myosuroides* population supplied by Herbiseed (Twyford, UK). The winter wheat cultivar used was Hereward, a non-competitive variety of medium height

(84 cm without PGR) (HGCA, 2009). Target plant densities for crop and weeds are listed in Figure 5.1. Germination rate in Petri dishes and 1000 seed weight were determined to quantify the number of weed seeds necessary to achieve the target densities. Germination rates in the field of 25% for *A. myosuroides* and 50% for *L. mutilflorum* of the germination rate in Petri dishes were assumed. In subplots R2-B2 and R3-B3 the amount of seeds sown was that corresponding to subplots R4-B4 to make sure enough seeds would germinate. Afterwards, seedlings in these subplots would be thinned down to achieve the corresponding plant densities.

The field was ploughed on 20th September, cultipressed on 25th September and a glyphosate based herbicide applied on 13th October. On 15th October the field was spring-tine cultivated, grass-weed seeds were hand broadcast and the field was accordingly drilled with wheat at seed rates of 133 and 400 seeds m^{-2} and subsequently rolled. A methiocarb-based insecticide and molluscicide at a rate of 7 kg ha^{-1} was applied on 17th October.

Plots were marked with canes on 23rd October and quadrats were marked with pegs on 6th November. Nitrogen fertiliser was applied at rates 40 Kg ha^{-1} and 160 Kg ha^{-1} on 18th March and 25th April, respectively. A total of 200 kg N ha^{-1} is very typical of winter wheat in UK. Broadleaved weeds controlled with mecoprop plus ioxynil/bromoxynil on 25th April. A fungicide programme was used on May. Plant density assessments were carried out in January and March and low density plots of *L. multiflorum* and *A. myosuroides* were thinned down to achieve the target densities. The average wheat densities obtained were 102 plants m^{-2} (s.e. ±2.45) and 298 plants m^{-2} (s.e. ±5.46), very close to the target densities (100 and 300 plants m^{-2}). The average weed plant densities achieved for each subplot are listed in Table 5.1.

Table 5.1 Mean numbers of *A. myosuroides* and *L. multiflorum* plants m^{-2} and size and number of quadrats used for the assessment in the different subplots within the low and high wheat densities plots. Values within brackets are the standard error of the mean.

Weed density/Wheat density	*A. myosuroides*			*L. multiflorum*		
	Low	High	Quadrat	Low	High	Quadrat
1	0	0	-----	0	0	-----
2	10.0 (1)	13.0 (1)	1x 1mx1m	6.0 (1)	8.5 (4.5)	1x 1mx1m
3	15.5 (3.5)	21.5 (3.5)	1x 1mx1m	10.0 (1)	13.0 (1)	1x 1mx1m
4	40.0 (10)	40.0 (5)	2x 0.1mx0.1m	21.5 (1.5)	18.5 (2.5)	1x 1mx1m
5	65.0 (0)	55.0 (15)	2x 0.1mx0.1m	32.5 (7.5)	37.5 (2.5)	2x 0.1mx0.1m
6	135.0 (0)	112.5 (17.5)	2x 0.1mx0.1m	57.5 (7.5)	67.5 (12.5)	2x 0.1mx0.1m
7	237.5 (87.5)	287.5 (37.5)	2x 0.02mx0.02m	212.5 (12.5)	212.5 (12.5)	2x 0.02mx0.02m
8	362.5 (37.5)	412.5 (12.5)	2x 0.02mx0.02m	325.0 (75)	312.5 (37.5)	2x 0.02mx0.02m

5.2.2 **Assessment**

Weed plants with intact inflorescences were collected in June from each sampling subplot. Sampling was carried out using one to two quadrats (range of sizes within 0.02mx0.02m and 1mx1m, depending on weed density (Table 5.1)) per subplot and all plants within each quadrat were collected. The numbers of plants m^{-2}, tillers and heads $plant^{-1}$, head length, spikelets $head^{-1}$ and seeds $spikelet^{-1}$ were measured in subplots with *L. multiflorum* plants as described in Chapter 2. Only the numbers of plants m^{-2}, tillers and heads $plant^{-1}$ were measured in subplots with *A. myosuroides* plants. The dry weight of all *L. multiflorum* plants collected was also determined. Wheat plants within the 1 m^2 area marked within each subplot for crop yield assessment were harvested by hand in August. Grain was removed for the ears using a static thresher and then sieved, dried and weighed. Yields were corrected to 85% dry matter and wheat yield in t ha^{-1} and 1000 seeds weight in g determined.

5.2.3 **Statistical analysis**

A general ANOVA was carried out with the data from every subplot. The structure used was 'blocks/plots/subplots' and the treatment 'Wheat*(Grass/BG+IRG))'; with 'Wheat' having two levels, 'high' and 'low' wheat density, 'Grass' having two levels, 'black-grass' and 'rye-grass', and BG and IRG having nine levels each, one for each density and the ninth one for the subplots with the other weed on it. This means that in the table with all the data there will be two columns with level numbers, one for *A. myosuroides* and another one for *L. multiflorum*. Those rows with data from *A. myosuroides* will have their corresponding level number in the '*A. myosuroides* level column' (values from 1 to 8) whilst all the rows with data from *L. multiflorum* will have a number 9 in the '*A. myosuroides* level column'. In the '*L. multiflorum* level column' will happen the same and the rows with data from *A. myosuroides* will all have a number 9 in that column.

For the assessment of the effect of wheat and weed densities on weed seed production the variates analysed were those measured; plants m^{-2}, tillers and heads $plant^{-1}$, head length, spikelets $head^{-1}$, seeds $spikelet^{-1}$ and dry weight $plant^{-1}$, and those calculated from them, seeds $head^{-1}$, seeds $plant^{-1}$, seeds m^{-2} and heads m^{-2}. As no measure of the number of seeds $spikelet^{-1}$ and spikelets $head^{-1}$ was made for *A. myosuroides* plants, to calculate seeds $plant^{-1}$ and seeds m^{-2}, an average value of 104 seeds $head^{-1}$ was used (Moss, 1990b). Correlation coefficients between variates were also calculated to determine relations between the different traits. For the assessment of the effect on the crop, the variates analysed were crop yield (t ha^{-1}) and 1000 grain weight (g).

The effect of the weed on the crop was also evaluated by analysing the relationship between the weed density and % yield loss for each group of plants separately. The

model selected to fit the data was the rectangular hyperbolic equation proposed by Cousens (1985):

$$Y_L = \frac{id}{1+(id/A)} \qquad \textbf{(Equation 1)}$$

Where Y_L is the % yield loss, *d* the weed density in plants m^{-2}, *i* % yield loss per unit weed density as weed density approaches zero and *A* % yield loss as weed density approaches infinity.

The effect of the crop and weed densities on the weed was analysed evaluating the relationship between weed density and weed heads m^{-2}, using a variation of the previous equation (Equation 1) already used in chapter 4, section 4.3.2:

$$y = \frac{H_{max}d}{1+(H_{max}d/A_{max})} \qquad \textbf{(Equation 2)}$$

Where y is the number of weed heads m^{-2}, *d* is the weed density in plants m^{-2}, H_{max} is the number of heads as plant density approaches zero (or head production by a single plant with no intra-specific competition) and A_{max} is the number of heads as weed density approaches infinity.

5.3 Results

Crop and weed density significantly affected some vegetative characteristics in both weed species. There were significant differences in the number of heads $plant^{-1}$ between wheat levels ($F_{6, 26}$=5.18, p=0.001 for *A. myosuroides* and $F_{6, 26}$=3.02, p=0.022 for *L. multiflorum*) and between weed levels ($F_{6, 26}$=8.70, p<0.001 for *A. myosuroides* and $F_{6, 26}$=3.63, p=0.010 for *L. multiflorum*).

Significant differences were also obtained in the number of tillers $plant^{-1}$ between wheat levels ($F_{6, 26}$=6.65, p<0.001 for *A. myosuroides* and $F_{6, 26}$=8.43, p<0.001 for *L. multiflorum*) and between weed levels ($F_{6, 26}$=9.42, p<0.001 for *A. myosuroides* and

$F_{6, 26}$=9.16, p<0.001 for *L. multiflorum*). The statistical analysis also showed that the number of seeds plant^{-1} was significantly different between wheat levels ($F_{6, 26}$=3.10, p<0.020 for *A. myosuroides* and $F_{6, 26}$=9.91, p<0.001 for *L. multiflorum*) and between weed levels ($F_{6, 26}$=5.21, p=0.001 for *A. myosuroides* and $F_{6, 26}$=15.38, p<0.001 for *L. multiflorum*).

Table 5.2. Mean number of heads plant^{-1}, tillers plant^{-1} and seeds plant^{-1} in *A. myosuroides* plants for each weed (2 to 8) and wheat (low and high) density levels. The number of plants m^{-2} given for each weed density level is an average of those shown for both wheat density levels in Table 5.1.

Weed densities	Mean plants m^{-2}	Tillers plant^{-1}		Heads plant^{-1}		Seeds plant^{-1}	
		Low	High	Low	High	Low	High
2	11.5	16.3	7.7	12.8	6.4	1,333	662
3	18.5	17.1	8.7	16.3	6.9	1,690	718
4	40.0	15.9	6.6	12.8	5.7	1,335	590
5	60.0	10.8	10.8	8.4	8.0	872	836
6	123.8	7.9	6.8	6.4	5.5	662	572
7	262.5	8.5	6.1	6.7	4.4	693	462
8	387.5	6.7	6.7	5.2	5.0	541	516
Mean	129.1	11.9	7.6	9.8	6.0	1,018	622
s.e.d.	26.2	1.61		1.6		215	
l.s.d	53.7	3.31		3.28		441.5	
d.f.	26	26.3		26.36		26.45	

Both *A. myosuroides* and *L. multiflorum* responded in a broadly similar way to increasing weed and wheat density. At high wheat density the number of tillers, heads and seeds per plant was rather constant independently of weed density. At low wheat density there were fewer tillers, heads and seeds per plant with increasing weed density (Table 5.2, Table 5.3). In *A. myosuroides* this effect mainly occurs up to approximately 50 plants m^{-2}. Above this, there is a much smaller reduction in these traits with increasing density (Table 5.2). These differences in the number of tillers, heads and seeds per plant between low and high wheat density that persist in *A.*

myosuroides up to about 50 plants m^{-2}, in *L. multiflorum* persist to higher plant densities (Table 5.2,Table 5.3).

Table 5.3. Mean number of heads $plant^{-1}$, tillers $plant^{-1}$, seeds $plant^{-1}$ and dry weight $plant^{-1}$ in *L. multiflorum* plants for each weed (2 to 8) and wheat (low and high) density levels. The number of plants m^{-2} given for each weed density level is an average of those shown for both wheat density levels in Table 5.1.

Weed densities	Mean plants m^{-2}	Tillers $plant^{-1}$		Heads $plant^{-1}$		Seeds $plant^{-1}$		Dry weight (g)	
		Low	High	Low	High	Low	High	Low	High
2	7.3	21.0	7.1	14.0	4.6	3,063	1,042	12.4	4.0
3	11.5	20.6	8.3	13.8	5.4	3,052	1,109	13.6	4.6
4	20.0	13.4	7.5	10.5	4.7	2,332	931	8.9	4.2
5	35.0	14.2	10.1	9.0	5.0	1,718	1,015	8.4	5.0
6	62.5	14.4	9.8	10.1	5.6	1,969	1,186	8.2	5.1
7	212.5	9.2	8.1	7.1	5.2	1,296	937	5.0	3.9
8	318.8	9.2	6.5	6.4	4.2	1,143	732	4.7	3.2
Mean	95.4	14.6	8.2	10.1	4.9	2,082	993	8.7	4.3
s.e.d.	26.2	1.61		1.6		215		1.16	
l.s.d	53.7	3.31		3.28		441.5		2.54	
d.f.	26	26.3		26.36		26.45		11.51	

Increasing crop density also had a significant effect on dry weight $plant^{-1}$ between wheat levels and between weed levels ($F_{6, 26}$=8.16, p=0.001 and $F_{6, 26}$=11.35, p<0.001). Dry weight $plant^{-1}$ had a similar trend to the previous traits and was reduced by a range of between 22% and 68% (depending on weed density) in high crop density plots.

The number of heads m^{-2} was significantly different between weed levels ($F_{6, 26}$=30.61, p<0.001 for *A. myosuroides* and $F_{6, 26}$=28.10, p<0.001 for *L. multiflorum*) but not between wheat levels ($F_{6, 26}$=0.41, p=0.868 for *A. myosuroides* and $F_{6, 26}$=1.01, p=0.439 for *L. multiflorum*), although the overall mean values were greater for the low wheat density plots (Table 5.4). This means that the effect of reducing the number of heads m^{-2} produced by increasing weed density was higher than the effect on this same trait produced by a higher crop density. Results were similar for the number of seeds m^{-2}.

Table 5.4. Mean number of heads m^{-2} and seeds m^{-2} in *A. myosuroides* and *L. multiflorum* plants for each weed (2 to 8) and wheat (low and high) density levels.

Weed densities	Heads m^{-2}				Seeds m^{-2}			
	A. myosuroides		*L. multiflorum*		*A. myosuroides*		*L. multiflorum*	
	Low	High	Low	High	Low	High	Low	High
2	128	83	82	33	13,260	8,580	18,310	7,703
3	261	151	140	70	27,105	15,683	30,686	14,337
4	480	228	229	86	49,920	23,660	50,394	16,944
5	545	420	302	185	56,680	43,680	58,195	37,609
6	859	613	581	366	89,375	63,787	113,979	76,954
7	1,538	1,288	1,501	1,105	159,900	133,900	273,157	197,738
8	1,863	2,050	2,086	1,335	193,700	213,200	369,827	233,017
Mean	810	690	703	454	84,277	71,784	130,650	83,472
s.e.d.		258.4				33,983		
l.s.d		537.9				70,080		
d.f.		20.68				24.37		

These traits, heads and seeds m^{-2}, showed opposite trends to the number of tillers, heads and seeds $plant^{-1}$, and were found to increase as weed density increased. Independent of weed or wheat density, both weeds showed a very high seed production potential. This showed how adaptable *A. myosuroides* and *L. multiflorum* plants are to different plant densities and that they both have a great capacity for producing many seeds even at low plant density. The head and seed production per m^2 were quite similar for both weeds although the number of seeds m^{-2} was higher for *L. multiflorum.*

The number of seeds $spikelet^{-1}$ and seeds $head^{-1}$ were significantly different between weed levels ($F_{6, 27}$=3.54, p=0.030 and $F_{6, 26}$=6.21, p<0.001) but not between wheat levels ($F_{6, 27}$=0.58, p=0.740 and $F_{6, 26}$=1.19, p=0.343). The ANOVA carried out for the last two variables (head length and spikelets $head^{-1}$) showed that neither displayed significant differences between wheat levels and weed levels (Table 5.5). There was much less difference in these traits between weed and wheat densities. These results

at the weed density level agree with the results in Chapter 4, where the variables head length and spikelets head^{-1} were not correlated to weed density and the number of seeds spikelet^{-1} was affected by the plant density but to a lesser extent. This shows again that the main trait influenced by plant density and determinant of seed production is the number of heads plant^{-1} and not head length, spikelets head^{-1}, seeds spikelet^{-1} or seeds head^{-1}.

Table 5.5. Mean head length and mean number of spikelets head^{-1}, seeds spikelet^{-1} and seeds head^{-1} in *L. multiflorum* plants for each weed (2 to 8) and wheat (low and high) density levels.

Weed densities	Head length (cm)		Spikelets head^{-1}		Seeds spikelet^{-1}		Seeds head^{-1}	
	Low	High	Low	High	Low	High	Low	High
2	23.4	27.4	20.3	20.4	10.9	11.2	221	228
3	24.9	25.4	20.7	20.0	10.7	10.3	222	206
4	25.0	25.5	21.3	20.1	10.5	9.9	223	199
5	22.4	25.7	19.7	20.7	9.6	9.8	189	202
6	22.5	25.2	20.9	20.5	9.4	10.3	196	211
7	25.2	25.0	20.6	18.8	8.8	9.6	182	181
8	25.4	23.1	20.4	19.3	8.8	9.3	179	180
Mean	24.1	25.3	20.6	20.0	9.8	10.1	202	201
s.e.d.	2.8		1.18		0.73		13.7	
l.s.d	6.06		3.86		1.59		28.2	
d.f.	12.91		2.86		12		24.99	

The correlation coefficients between all these variables and plant density were calculated for the *L. multiflorum* data for low and high crop density plots. The correlation coefficients for the low crop density plots agreed with those from Chapter 4. The number of heads m^{-2} and seeds m^{-2} were highly positively correlated to weed plant density with a positive correlation of 0.99 for both variables. The number of heads plant^{-1}, tillers plant^{-1}, seeds plant^{-1}, seeds spikelet^{-1} and dry weight plant^{-1} had a high negative correlation with weed plant density. Head length and spikelets head^{-1} showed no correlation with weed plant density (Table 5.6).

Table 5.6. Correlation coefficients between plant density (plants m^{-2}) and all the traits determined for *L. multiflorum* (dry weight $plant^{-1}$, tillers $plant^{-1}$, spikelets $head^{-1}$, seeds $spikelet^{-1}$, seeds $plant^{-1}$, seeds m^{-2}, heads $plant^{-1}$, heads m^{-2} and head length) for the low and high wheat density plots.

Traits	Low wheat density	High wheat density
Dry weight $plant^{-1}$	-0.79	-0.45
Head length	0.19	-0.51
Heads m^{-2}	0.99	0.97
Heads $plant^{-1}$	-0.73	-0.19
Seeds m^{-2}	0.99	0.97
Seeds $plant^{-1}$	-0.77	-0.48
Seeds $spikelet^{-1}$	-0.70	-0.53
Spikelets $head^{-1}$	-0.13	-0.61
Tillers $plant^{-1}$	-0.74	-0.32

However, the correlation coefficients for the high crop density plots were different. The number of heads m^{-2} and seeds m^{-2} were still highly positively correlated with weed plant density with a positive correlation of 0.97 for both variables. The rest of the variables showed a lower correlation with weed plant density with values between -0.19 and -0.63 (Table 5.6). Thus, at high wheat density, weed density had a smaller effect on most of these traits probably due to the high inter-specific competition.

For the low crop density plots, as weed plant density increased, the number of tillers $plant^{-1}$, heads $plant^{-1}$, seeds $plant^{-1}$, seeds $spikelet^{-1}$ and dry weight $plant^{-1}$ decreased; while there was no general trend for the high crop density plots, for both *A. myosuroides* and *L. multiflorum* (Figure 5.2, Figure 5.3). The most likely reason was that at low wheat density there was mainly intra-specific competition affecting both weeds while at high wheat density weeds were affected more by inter-specific competition giving a constant and rather low tiller, head and seed production at any weed density.

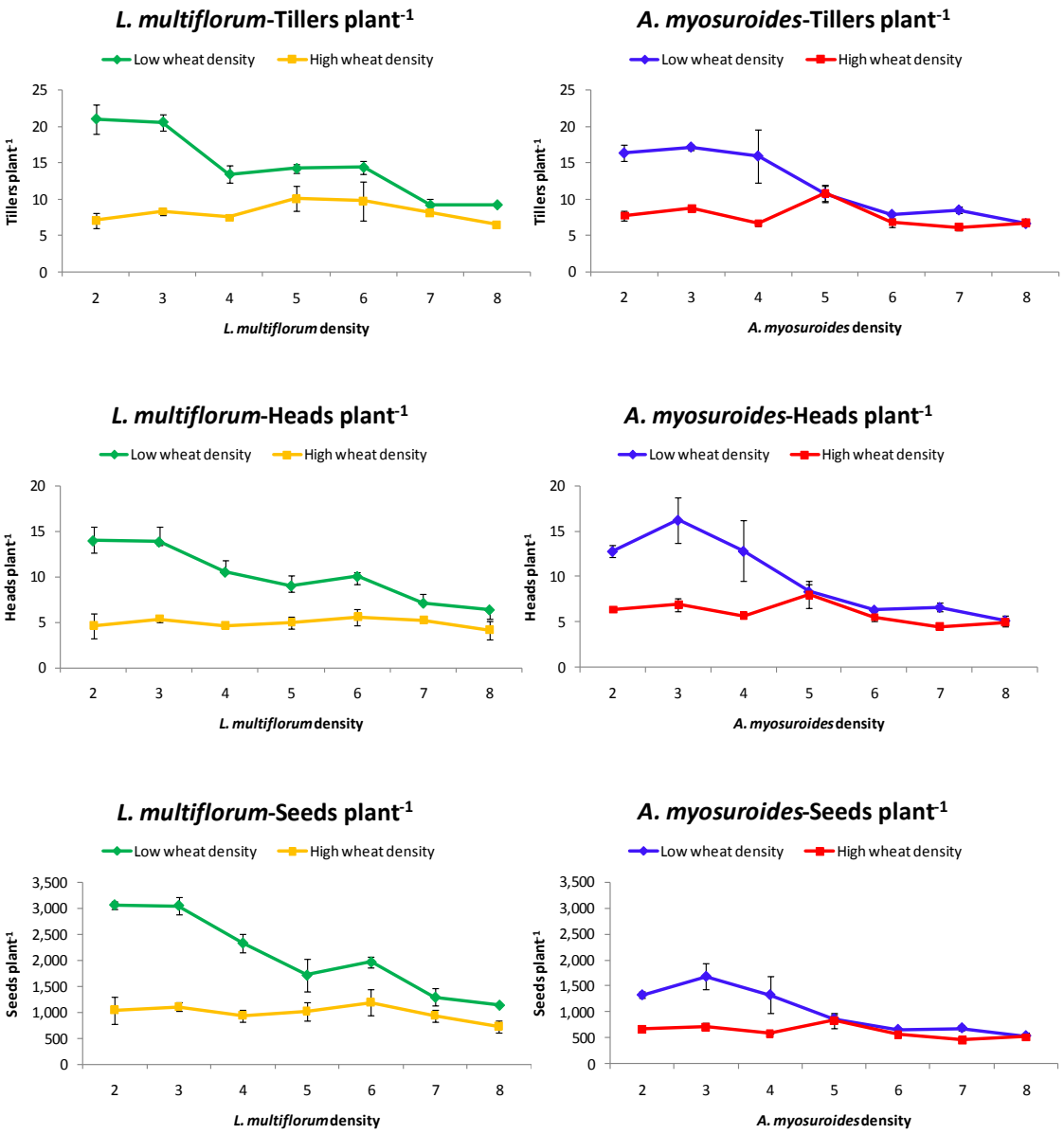


Figure 5.2. Effect of two different wheat densities on the number of tillers plant^{-1}, heads plant^{-1}, seeds plant^{-1}, in *L. multiflorum* and *A. myosuroides* plants.

The reduction in number of tillers, heads and seeds per weed plant obtained with the high wheat density at any weed density was probably due to inter-specific competition, which occurred at low wheat density only when the weed infestation was over 50 plants m^{-2} for *A. myosuroides* and over 250 plants m^{-2} for *L. multiflorum*. Thus, increasing crop plant density seemed to reduce weed seed production and subsequently reduced weed seed return for the next season. At low wheat density *L.*

multiflorum dry weight plant^{-1} decreased as weed density increased. At high wheat density *L. multiflorum* dry weight plant^{-1} was strongly suppressed and was insensitive to changes in weed density (Figure 5.3). The head length and the number of spikelets head^{-1} showed no clear trend at any crop density (Figure 5.3).

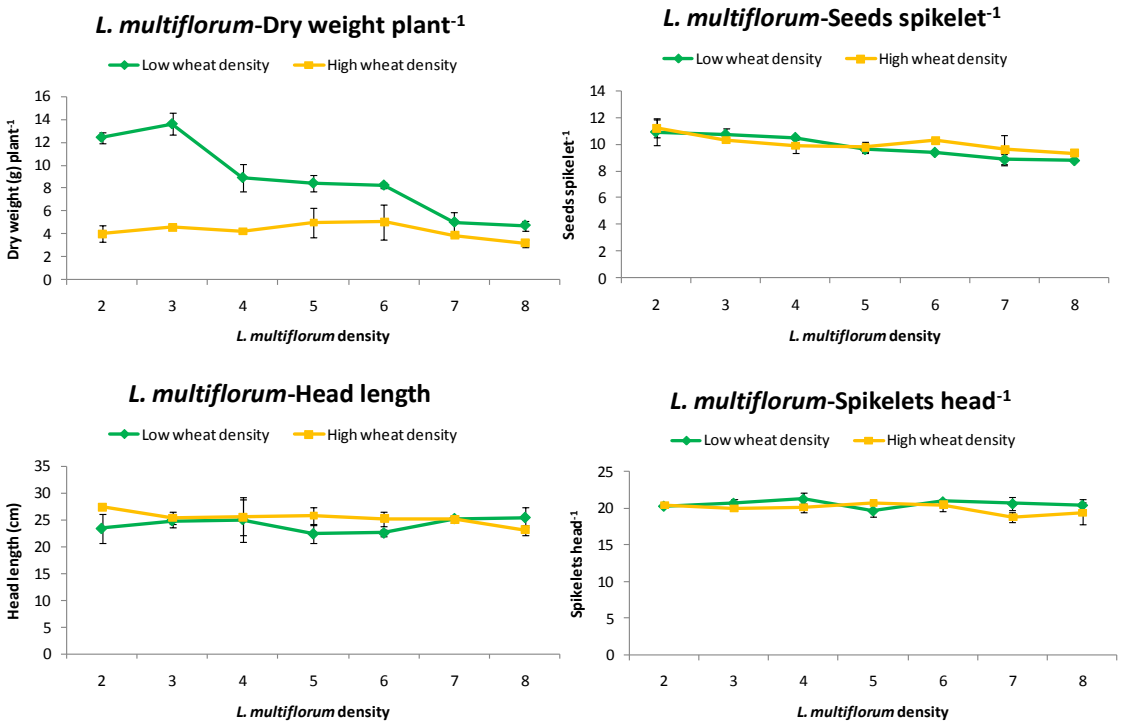


Figure 5.3. Effect of two different wheat densities on the number of seeds spikelet^{-1}, head length, spikelets head^{-1} and dry weight plant^{-1} in *L. multiflorum* plants.

As different plant densities were obtained for *A. myosuroides* and *L. multiflorum* at the same weed density levels (1 to 8 in Table 5.1) comparisons between both weeds could not be carried out using an ANOVA. For this reason a regression analysis was carried out. The effect of wheat density on both weeds was evaluated by analysing the relationship between the weed density and number of weed heads m^{-2} (Equation 2). Both weeds had a very similar response up to a weed density of 100 plants m^{-2}, with *A. myosuroides* having a slightly higher head production at the lowest wheat density (Figure 5.4). The parameter H_{max}, or estimate of head production by a single plant with

no intra-specific competition, was higher in *A. myosuroides* than in *L. multiflorum* (Table 5.7). However, at the highest wheat density both weeds produced fewer heads m^{-2} than at the lowest weed density. With wheat densities of 100 plants m^{-2} and weed densities more than 100 plants m^{-2} *L. multiflorum* produced more heads m^{-2} than *A. myosuroides*, while the opposite was true with wheat densities of 300 plants m^{-2} (Figure 5.4).

Table 5.7. Estimates of parameters for the hyperbolic regressions (Equation 2) of weed heads m^{-2} against weed density (plant m^{-2}). B1: *A. myosuroides* and 100 wheat plants m^{-2}; B2: *A. myosuroides* and 300 wheat plants m^{-2}; R1: *L. multiflorum* and 100 wheat plants m^{-2}; R2: *L. multiflorum* and 300 wheat plants m^{-2}.

	Hmax	Amax	r^2
B1	10.00	4,095 (1,007)	0.87
B2	7.23	5,053 (1,754)	0.91
R1	9.00	8,415 (2,144)	0.97
R2	7.60	2,990 (879)	0.88

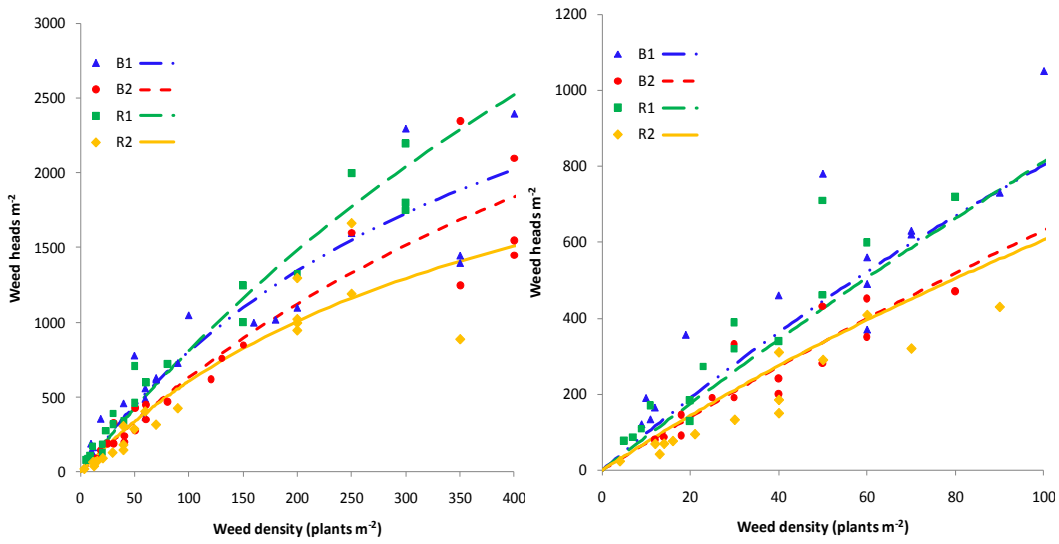


Figure 5.4. Relationships between the number of head m^{-2} and the density (plants m^{-2}) of *L. multiflorum* and *A. myosuroides* at different wheat densities: *A. myosuroides* and 100 wheat plants m^{-2} (B1), *A. myosuroides* and 300 wheat plants m^{-2} (B2), *L. multiflorum* and 100 wheat plants m^{-2} (R1) and *L. multiflorum* and 300 wheat plants m^{-2} (R2). Response curves are those for the parameters given in Table 5.7.

Despite both weeds having similar responses to differences in wheat density, at a weed density of 10 plants m^{-2} there was less difference in the number of weed heads $plant^{-1}$ for *L. multiflorum* than for *A. myosuroides*. At a weed density of 10 plants m^{-2} the number of heads $plant^{-1}$ in *L. multiflorum* ranged from 8.9 to 7.4 whereas for *A. myosuroides* plants the number ranged from 9.8 to 7.1 heads $plant^{-1}$, showing than *A. myosuroides* is more affected by competition with wheat than *L. multiflorum*. However, when the density of weeds was 100 plants m^{-2} the difference between low and high wheat density plots in head production per plant in *L. multiflorum* plants seemed to increase while this difference decreased in *A. myosuroides* plants. Both *A. myosuroides* and *L. multiflorum* seemed to be affected by competition with wheat and had a lower head production, and subsequently a lower seed production, when wheat density was 300 plants m^{-2}.

There were big differences in crop yield between the different weed densities; with a range of values for *A. myosuroides* subplots from 1.0 to 8.9 and 2.4 to 7.9 t ha^{-1}, for low and high wheat density respectively, and for *L. multiflorum* subplots from 3.7 to 9.0 and 2.7 to 8.4 t ha^{-1}, for low and high wheat density respectively (Table 5.8).

There were also differences in crop yield between low and high wheat density, but these differences were not significant. The ANOVA carried out showed that there were significant differences in crop yield between weed levels ($F_{7, 30}$=34.32, p<0.001 for *A. myosuroides* and $F_{7, 30}$=26.88, p<0.001 for *L. multiflorum*) but not between wheat levels ($F_{7, 30}$=1.19, p=0.340 for *A. myosuroides* and $F_{7, 30}$=1.53, p=0.196 for *L. multiflorum*).

These results from crop yield data indicate that low wheat density plots with a low weed infestation will have a higher yield than plots with a higher wheat density and a low weed infestation. It seems that with a low weed infestation the main competition affecting wheat plants is intra-specific competition, therefore, at a higher wheat density the intra-specific competition will be greater than in wheat plots with lower crop density,

therefore reducing intra-specific competition and resulting in a higher yield. As the weed density increases so does the inter-specific competition reaching a peak when it has a greater effect than the intra-specific competition. At this point the yield will be higher for the high crop density plots. With both weeds, results indicate that at a weed density of 11.5 plants m^{-2} (weed levels 3 and 2 for *L. multiflorum* and *A. myosuroides* respectively) the inter-specific competition already has a greater effect on crop yield than intra-specific competition does (Table 5.8, Figure 5.5).

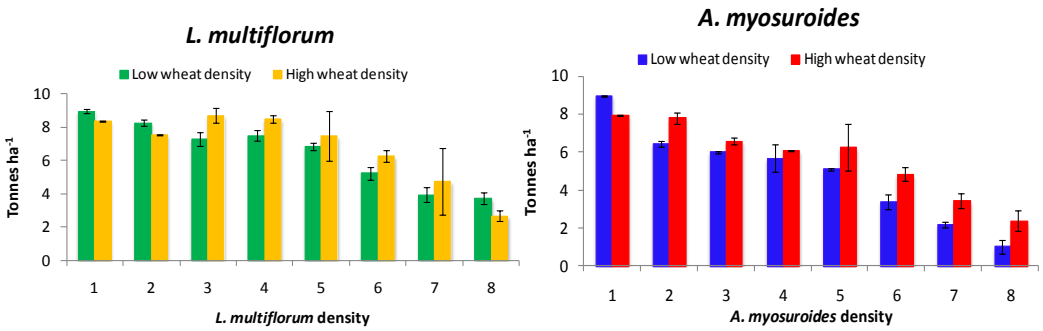


Figure 5.5. Mean values for wheat yield (t ha^{-1}) as influenced by different weed (*L. multiflorum* and *A. myosuroides*) and wheat densities (low and high).

Table 5.8. Mean values for wheat yield (t ha^{-1}) and 1000 grain weight (g) as influenced by different weed (*L. multiflorum* and *A. myosuroides*) and different weed (1 to 8) and wheat (low and high) densities.

Weed densities	Yield (t ha^{-1})				1000 grain weight (g)			
	A. myosuroides		*L. multiflorum*		*A. myosuroides*		*L. multiflorum*	
	Low	High	Low	High	Low	High	Low	High
1	8.9	7.9	9.0	8.4	47.6	42.2	47.3	43.2
2	6.4	7.8	8.3	7.6	45.1	45.1	45.8	42.4
3	6.0	6.6	7.3	8.7	46.0	41.7	45.1	42.8
4	5.7	6.1	7.5	8.5	45.4	38.1	44.8	44.9
5	5.1	6.2	6.8	7.5	43.7	42.0	41.2	40.4
6	3.4	4.8	5.2	6.3	46.4	42.5	43.0	42.1
7	2.2	3.4	3.9	4.8	43.7	40.1	40.9	36.3
8	1.0	2.4	3.7	2.7	40.1	38.6	45.1	31.9
Mean	4.8	5.7	6.5	6.8	44.7	41.3	44.1	40.5
s.e.d.		0.82				2.86		
l.s.d		1.73				5.86		
d.f.		17.66				28.23		

The ANOVA carried out with the 1000 grain weight data showed no significant differences between weed levels and wheat levels for *A. myosuroides* ($F_{7,30}$=1.93, p=0.099 and $F_{7,30}$=0.69, p=0.680) but there were significant differences between weed levels for *L. multiflorum* ($F_{7,30}$=3.71, p=0.005) and there is some evidence of differences between wheat levels ($F_{7,30}$=2.24, p=0.059) although it is not significant at the 5% level (Table 5.8).

The effect of both weeds on the crop was evaluated by analysing the relationship between the weed density and the % yield loss (Equation 1). Comparisons between the effects produced for both weeds could then be carried out. Both weeds produce a greater yield loss at lower wheat density, with *A. myosuroides* producing a higher yield loss than *L. multiflorum*. At a higher wheat density both weeds produce similar % yield losses (Figure 5.6).

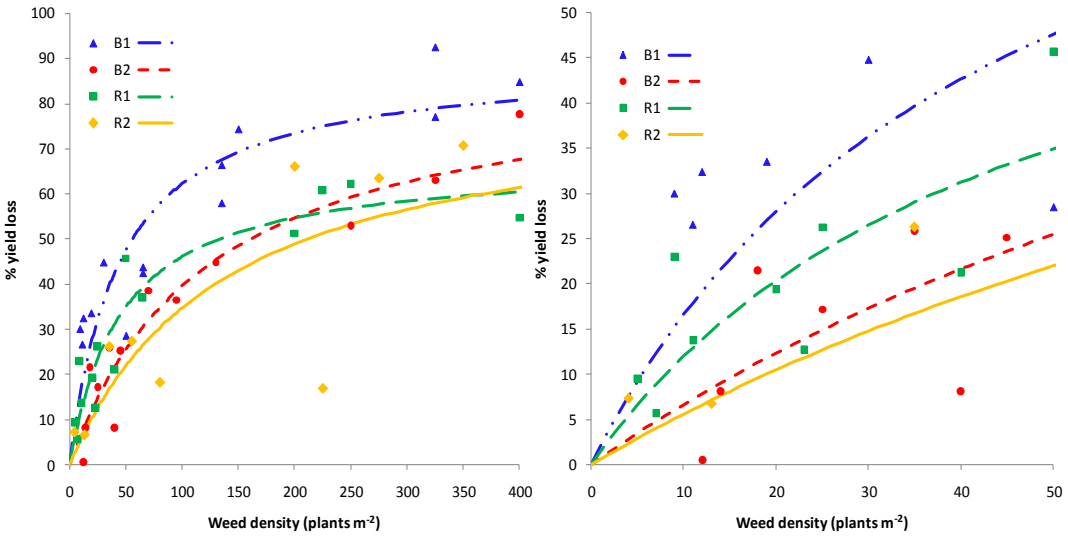


Figure 5.6. Relationships between % yield loss in wheat and weed density (plants m^{-2}) at different wheat densities: *A. myosuroides* weed and 100 wheat plants m^{-2} (B1), *A. myosuroides* weed and 300 wheat plants m^{-2} (B2), *L. multiflorum* weed and 100 wheat plants m^{-2} (R1) and *L. multiflorum* weed and 300 wheat plants m^{-2} (R2). Response curves are those for the parameters given in Table 5.9.

Table 5.9. Estimates of parameters for the hyperbolic regressions (Equation 1) of % yield loss against weed density (plants m^{-2}).

	i	A	r^2
B1	2.034	89.6	0.76
B2	0.717	88.41	0.91
R1	1.456	67.31	0.88
R2	0.6	82.39	0.63

According to the model, ten *A. myosuroides* plants would produce a 17% yield loss with a wheat density of 100 plants m^{-2} while at a wheat density of 300 plants m^{-2} the yield loss would be 7% (Table 5.10).

Table 5.10. Relationship between *A. myosuroides* and *L. multiflorum* plant density and % yield loss of wheat, at crop densities of 100 and 300 plants m^{-2}.

Weed plants m^{-2}	*A. myosuroides*		*L. multiflorum*	
	100 wheat plants $^{-2}$	300 wheat plants m^{-2}	100 wheat plants $^{-2}$	300 wheat plants m^{-2}
1	2	1	1	1
5	9	3	7	3
10	17	7	12	6
15	23	10	16	8
20	28	12	20	10
25	32	15	24	13
50	48	26	35	22
75	56	33	42	29
100	62	40	46	35
200	73	55	55	49
300	78	63	58	57
400	81	68	60	61
500	82	71	62	65
600	83	73	62	67
700	84	75	63	69
800	85	77	64	70
900	85	78	64	71
1000	86	79	64	72

With *L. multiflorum* plants the trend would be similar but would produce a lesser effect. At a crop density of 100 plants m^{-2} ten *L. multiflorum* plants would produce 12% yield loss but only a 6% yield loss when the wheat density is 300 plants m^{-2}. In terms of an

economic threshold, a figure for weed density that causes a 5% yield loss is often used. According to this model, a density of only 3 and 4 plants for *A. myosuroides* and *L. multiflorum* respectively is likely to cause a 5% yield loss for a wheat density of 100 plants m^{-2}. With a wheat density of 300 plants m^{-2} this 5% yield loss is caused by 7 and 8 plants of *A. myosuroides* and *L. multiflorum* respectively. These results highlight the detrimental effect that even low plant densities of *A. myosuroides* and *L. multiflorum* can have on wheat.

5.4 Discussion

The results of these studies demonstrate that, as recommended in previous studies (Beckie, 2006, Moss *et al.*, 2007), the use of cultural control methods, in this case increasing crop seeding rate, can help in the control of weeds. The weed seed production potential results obtained at low crop density also agree with the results presented in Chapter 4; as the number of *L. multiflorum* plants decreases, the number of spikes per plant, and consequently seeds per plant, increases considerably.

At high crop density, because of the high inter-specific competition, the number of weed heads, tillers and seeds $plant^{-1}$ as well as dry weight $plant^{-1}$ were low at any weed density. The range of values for these variables was similar to that obtained for low crop density plots when the weed density was more than 60 plants m^{-2}, as a consequence of the high intra-specific competition. Increasing crop seeding rate gave significant reductions in all these variables even at the highest weed densities. The number of seeds $plant^{-1}$ in *L. multiflorum* was reduced between 28% and 60% when using a higher crop seeding rate. Thus, with higher crop densities there will be a reduction in weed biomass and a lower weed seed return as has been shown previously (Alshallash & Drennan, 1993, Lemerle *et al.*, 1996, Champion *et al.*, 1998, Korres & Froud-Williams, 2002, Walker *et al.*, 2002, Izquierdo *et al.*, 2003).

The regression analysis carried out to compare the effect of wheat density on both *L. multiflorum* and *A. myosuroides* showed that the increase in crop density reduced the number of heads m^{-2} in both weeds, with both weeds behaving in a similar way up to 100 plants m^{-2} (Figure 5.4). These estimates of head production for both weeds at different weed and wheat densities support the argument for increasing crop seeding rates as a complementary weed control measure.

Weed density had a highly significant effect on crop yield with reductions of up to 89% and 68% for *A. myosuroides* and *L. multiflorum* subplots, respectively. This happened with weed densities of 388 and 319 plants m^{-2} for *A. myosuroides* and *L. multiflorum* respectively, and 100 and 300 wheat plants m^{-2}, respectively. The value for the yield loss caused by *A. myosuroides* is higher than that reported by Moss (1987), where densities of 250-500 *A. myosuroides* plants m^{-2} reduced yield by 45%. In another study on the effects of different densities of *Avena* spp. and *A. myosuroides* on two wheat cultivars at a crop density of 325 plants m^{-2} indicated that *Avena* spp. appear to be more competitive than *A. myosuroides* causing up to 21.7 and 37.9% yield losses at densities of 20 and 40 plants m^{-2} respectively, while *A. myosuroides* gave yield losses up to 11.1 and 16.8% at these same densities (Mennan & Isik, 2004). These yield losses for *A. myosuroides* are similar to those predicted for a wheat density of 300 plants m^{-2} by equation 1 shown in Table 5.10, with 20 and 50 plants m^{-2} resulting in 12 and 26% yield loss, respectively.

In the studies presented in this chapter the yield loss caused by *A. myosuroides* was higher than that caused by *L. multiflorum* and the average values of yield loss for both weeds in high crop density plots were smaller than for low crop density plots, although this difference was not significant. Stone *et al.,* (1999) studied wheat yield loss in response to *L. multiflorum* infestations and found that the most accurate equation describing the effect of *L. multiflorum* interference in wheat was a simple linear

regression: % wheat yield loss = 5.7 + (1.5 x % rye-grass plants in the total plant population), total plant population being the sum of crop and weed density. The results obtained for *L. multiflorum* using equation 1 and the parameters from Table 5.9, are similar to those obtained by Stone *et al.,*(1999), especially at a wheat density of 300 plants m^{-2}. Yield losses of 10 and 22% were estimated for a wheat density of 300 plants m^{-2} and *L. multiflorum* densities of 20 and 50 plants m^{-2}, respectively, while the equation above predicts losses of 13 and 22%. For a wheat density of 100 plants m^{-2}, the estimated yield losses were 20 and 35% for weed densities of 20 and 50 plants m^{-2}, respectively, while the equation given by Stone *et al.,* (1999) gave losses of 25 and 44%. At low plant densities, values from both equations differ, most likely because the equation given by Stone *et al.,* (1999) is unreliable at low weed densities as it predicts a yield loss of 5.7% at zero weed density. However, this contrasts with other studies that showed that *L. multiflorum* was highly competitive against winter wheat and 20 plants m^{-2} reduced grain yield by an average of 38% (Appleby & Brewster, 1992). Studies of the interference of *L. multiflorum* on a semi-dwarf variety of winter wheat gave lower yield losses of 24% at a weed density of 39 plants m^{-2} (Appleby *et al.*, 1976). Yield losses were higher in studies carried out by Hashem *et al.* (1998) where 25 *L. multiflorum* plants in 100 winter wheat plants m^{-2} reduced crop yield by 39-42%, depending on the rectangularity. Ingle *et al.,* (1997) studied the interference between winter wheat and three weed species, *Stellaria media*, *A. myosuroides* and *Galium aparine*. The weed density at which a yield loss of 5% was recorded varied greatly between species, site and season. In *A. myosuroides* an average of 24 plants m^{-2} caused a 5% yield loss, although the weed density causing a 5% yield loss had a very wide range, between 4 and 82 plants m^{-2}. The range of wheat density was 64-464 plant m^{-2}. Taken together, these studies show that results are highly variable and that more factors may need to be considered. Ingle *et al.,*(1997) concluded that the use of weed density alone to predict yield loss was unrealistic and other factors should be introduced.

To provide an estimate of yield loss at any weed density and to have a better comparison of the effect of both *L. multiflorum* and *A. myosuroides* on crop yield, a regression analysis was carried out and the relationship between weed density and yield loss obtained. The values obtained from the regression analysis clearly indicate that *A. myosuroides* is more competitive than *L. multiflorum* and that both weeds are less competitive at increased crop densities. The economic threshold value gives the number of weed plants at which the cost of the treatment balances the economic damage caused by the weed. In integrated weed management the use of herbicides would be justified only when this economic damage is greater than the cost of the treatment. A figure for weed density that causes a 5% yield loss has often been used as this equates approximately to the cost of treating an infestation with herbicide. Applying this to the curves previously obtained, with a crop density of 100 plants m^{-2}, a 5% yield loss would be caused by just two *A. myosuroides* plants while this same yield loss would be caused by four *L. multiflorum* plants. For a crop density of 300 plants m^{-2} the number of weed plants predicted to cause a 5% yield loss would be higher; eight *A. myosuroides* plants and nine *L. multiflorum* plants. These values are very different from those obtained by Zanin *et al.* (1993) where the economic threshold for *A. myosuroides* and *L. multiflorum* varied between 21 and 42 plants m^{-2} and 20 and 34 plants m^{-2}, respectively, depending on the herbicide treatment used. These differences could perhaps be explained by the many different factors involved in their calculation.

Thus, it appears that an accurate prediction of yield loss based on weed density alone is difficult because of the many additional factors involved which produce variable results between species. It should be taken into consideration that the experiment described here presents certain limitations. There was only one replicate hence results may vary between sites and years. More *L. multiflorum* and *A. myosuroides* populations and wheat cultivars should be included as differences in competitiveness between populations and cultivars may exist. To gather more information, a wider

range of wheat densities should also be used. In addition, this experiment should also be repeated across more sites and over different years and other factors might be taken into consideration such as residual nitrogen or time of emergence as suggested by Ingle *et al.,* (1997).

Nonetheless, the experiments described here show one clear trend; higher crop densities have a significant effect on weed suppression and there is a benefit in terms of crop yield compared to lower crop densities. This is supported by studies of the effects of different wheat and *Avena* spp. densities on wheat grain yield where it was shown that grain yields were reduced by the weed and that this competitive effect could be reduced by increasing wheat density (Martin *et al.*, 1987, Armin *et al.*, 2007). Medd *et al.,* (1985) studied the influence of wheat density and spatial arrangement on *Lolium rigidum* competition and showed that geometrical arrangement of the crop had no significant effect on weed competition or improving wheat yield, but increasing crop density from 40 or 75 to 200 plants m^{-2} seemed beneficial as it did not affect wheat yields but noticeably reduced *L. rigidum* growth. In studies with *L. rigidum* in Australia Lemerle *et al.,* (2004) showed that increasing wheat density suppressed weed growth and to a lesser extent reduced losses in grain yield from competition. Average crop yield loss from weed competition declined from 23% at 100 wheat plants m^{-2} to 17% at 200 wheat plants m^{-2}. Yield of weed-free plots declined only slightly by 4-5% at densities up to 425 plants m^{-2}.

In summary, both *A. myosuroides* and *L. multiflorum* have a highly detrimental effect on winter wheat yield, with *A. myosuroides* being slightly more competitive. Increasing crop seeding rate would have a beneficial effect on weed suppression and to a lesser degree, reduce yield losses.

5.5 Chapter summary

- Both *Lolium multiflorum* and *Alopecurus myosuroides* have a highly detrimental effect on winter wheat yield causing losses of up to 89%.
- *A. myosuroides* and *L. multiflorum* are highly competitive with *A. myosuroides* being slightly more competitive.
- A yield loss of 5% can result from just two and four *A. myosuroides* and *L. multiflorum* plants m^{-2}, respectively at a crop density of 100 winter wheat plants m^{-2}.
- Increasing crop seeding rate has a beneficial effect on weed suppression with reductions of head numbers and seeds $plant^{-1}$ of up to 58 and 67% on *A. myosuroides* and *L. multiflorum* plants, respectively.
- Increasing crop density can be used as a weed control measure as winter wheat densities of 300 plants m^{-2} have a significant effect on weed suppression, compared to that of 100 plants m^{-2}, while there are no significant differences in crop yield.
- The use of higher crop seeding rates as a weed control measure could complement the use of herbicides and thus decrease the selection pressure on the weed imposed by herbicides.

Chapter 6 Herbicide resistance assays

6.1 Introduction

Resistance to herbicides in *Lolium* species has evolved in 17 countries to date. Herbicide-resistant *L. rigidum* populations have been reported in eleven countries worldwide, *L. multiflorum* in eight countries, *L. perenne* in three countries and *L. persicum* in two countries. In Europe, resistant *Lolium* spp. populations have been identified in six countries, France, Spain, Italy, UK, Greece and Germany. However, resistant *L. multiflorum* populations have been reported in only four of them, France, Spain, Italy and UK (Heap, 2009).

ACCase-inhibiting herbicides are the most frequently used herbicides for the control of *L. multiflorum* in the UK. The repeated use of ACCase inhibitors has led to an increase in the number of grass weed species with resistant individuals, including *Alopecurus myosuroides, L. multiflorum* and *Avena* spp. The first cases worldwide of populations resistant to ACCase inhibitors were reported in *L. rigidum* in 1982 in Australia (Heap & Knight, 1982) and the first case of resistance in *L. multiflorum* was reported in 1987 in Oregon, USA (Stanger & Appleby, 1989). Currently, a total of 37 weeds worldwide have evolved resistance to ACCase-inhibiting herbicides.

The first case in the UK of resistance to ACCase-inhibiting herbicides was reported in *A. myosuroides* in 1990 (Moss, 1990a). Resistance in the UK to these herbicides was later detected in *Avena fatua, Avena sterilis* ssp. *ludoviciana* and *L. multiflorum*. *L. multiflorum* populations resistant to diclofop-methyl were first detected in the UK in 1991 and reported by Moss *et al.* (1993). These were detected in five farms in four counties in England. At the time, the resistance to other aryloxyphenoxypropionate ('fops') herbicides was also demonstrated but there were also indications of resistance

to the cyclohexanedione ('dim') herbicide, tralkoxydim. Resistance to diclofop-methyl was also found in *L. multiflorum* populations in other countries in Europe (De Prado *et al.*, 2000).

By 2004, a total of 324 farms in England had been shown to possess herbicide-resistant *L. multiflorum* populations. Populations resistant to at least one herbicide were identified in a total of 28 counties in England, but had not been identified in Wales or Scotland (Moss *et al.*, 2005). A total of 13 different herbicides were included in this study, with diclofop-methyl, cycloxydim and tralkoxydim being the most commonly used. To date, only cross resistance to ureas and ACCase inhibitors has been found in UK *L. multiflorum* populations. Worldwide, *L. multiflorum* has been found to be resistant to many other ACCase inhibitors and to have cross resistance to four chemical groups including, ureas, glycines, ALS inhibitors and chloroacetamides (Heap, 2009). In a relatively short period of time, herbicide resistance in *L. multiflorum* could develop along similar lines to that of *L. rigidum* today, which exhibits cross resistance to ten chemical groups and has resistant populations in 11 countries. Other grasses in the UK resistant to ACCase inhibitors, including *A. myosuroides* and *Avena* spp., have also evolved cross-resistance to other chemical groups; ureas, ALS inhibitors and dinitroanilines in the case of *A. myosuroides* and ALS inhibitors and arylaminopropionic acids in the case of *Avena* spp. Adequate control measures should be developed to prevent the evolution of resistance to more ACCase inhibitors and other chemical groups in UK *L. multiflorum* populations and it is important to gain an insight into the evolution of resistance in UK *L. multiflorum* populations. Moss *et al.,* (1993) found resistance to diclofop-methyl in all the six UK *L. multiflorum* populations studied, including Essex A1 (1991), Essex B1 (1992), Lincs A1 (1991), Oxford A1 (1991), Oxford A2 (1991) and Wilts A1 (1992), but only in the population Lincs A1 was clear evidence found of resistance to fluazifop-P-butyl, fenoxaprop-ethyl and tralkoxydim and partial resistance to isoproturon. Further studies with the populations

Essex A1 (1995), Lincs A1 (1991), Wilts B1 (1997) and Yorks A2 (1998) showed that resistance had evolved a few years later (Cocker *et al.*, 2001). All these populations were resistant to diclofop-methyl, fluazifop-P-butyl and tralkoxydim and partially resistant to isoproturon, and the population Yorks A2 also showed resistance to cycloxydim. In the Yorks A2 population an insensitive ACCase conferring target site resistance was detected while in the other three populations biochemical studies indicated that resistance was due to an enhanced rate of herbicide metabolism. In 2005, White *et al.,* (2005) reported resistance to diclofop-methyl, tralkoxydim, cycloxydim and sethoxydim in the populations Yorks A2 (1998) and Pyl (1999). The resistance in Yorks A2 was found to be conferred by an Ile-1781-Leu substitution in the *L. multiflorum* chloroplastic ACCase. However, no amino acid substitutions were found to be associated with the resistant phenotypes in Pyl and the molecular basis of resistance in this population remained unsolved.

The main objectives of the studies described here were to provide a better understanding of the extent of resistance to ACCase-inhibiting herbicides and the cross-resistance pattern within UK *L. multiflorum* populations and to determine what the main mechanisms of resistance are. Consequently, a series of experiments were carried out at the whole-plant level under a glasshouse environment. These included a dose response experiment with some known *L. multiflorum* populations to validate the methodology and to determine adequate herbicide rates in further experiments, single dose response experiments to determine the cross-resistance pattern of a number of UK *L. multiflorum* populations and a single dose response experiment to find populations highly resistant to fluazifop-P-butyl in order to be analysed at the molecular level. A rapid test in Petri dishes was also carried out with the herbicide pinoxaden in order to validate the methodology.

6.2 Materials and methods

6.2.1 Whole plant response assays

Whole plant pot assays are the most widely used assays to test for resistance (Moss, 1999). They involve growing plants from seeds and spraying them with either a single herbicide dose or a range of doses. They are conducted in a glasshouse and the assessments carried out usually involve a visual estimation of mortality or plant vigour, followed by measurements of fresh or dry foliage weight. They should always include an appropriate susceptible reference population. In initial studies it is better to use a range of doses to obtain a response curve and thus calculate the ratio of doses required to produce the same effect in resistant and susceptible populations. Once this information has been obtained, a single dose can be used in future screening assays.

6.2.1.1 Response of five Lolium multiflorum populations to different doses of the ACCase-inhibiting herbicides diclofop-methyl, tralkoxydim, cycloxydim and pinoxaden

This assay was carried out to validate the methodology and to determine adequate herbicide rates in further experiments. The *Lolium multiflorum* populations used were Wilts B1, Yorks A2, Pyl, G. Rising01 and Trajan and are listed in Chapter 2, section 2.1. All the populations, except G. Rising01, had been previously studied for resistance to herbicides. Wilts B1 was known to be resistant to diclofop-methyl, fluazifop-P-butyl and tralkoxydim and Yorks A2 and Pyl were resistant to these same herbicides as well as cycloxydim and sethoxydim (Cocker et al., 2001, White et al., 2005). The susceptible standard used was Trajan, a commercial cultivar used previously (Cocker et al., 2001, White et al., 2005), which is sensitive to all the herbicides studied. The population G. Rising01 seemed to be a good reference population as it belonged to a

field from a farm in Essex where resistance has been confirmed. The five populations were tested for response to different rates of diclofop-methyl, tralkoxydim, cycloxydim and pinoxaden in a pot experiment comprising a fully randomized design with four replicate pots per herbicide dose, and eight untreated pots per population. Seeds were sown one cm deep into nine cm pots containing a peat-based growing medium. After emergence, seedlings were thinned to six plants per pot. Commercial formulations of the four herbicides were applied at a staggered range of six doses between 1.37 and 1,400g a.i. tralkoxydim ha^{-1}, 0.18 and 180g a.i. pinoxaden ha^{-1}, 2.3 and 2,400g a.i. cycloxydim ha^{-1} and 4.5 and 4,536g a.i. diclofop-methyl ha^{-1}. Each dose was four times the preceding dose in the range. Recommended adjuvants were used with tralkoxydim, pinoxaden and cycloxydim (see Chapter 2, section 2.6.2). Pots were sprayed at the two to four leaf stage and then placed in the glasshouse as described in Chapter 2.

Plants were rated using a 1-4 injury scale compared to control plants and harvested 22 days after treatment with herbicides as described in Chapter 2. Data were analysed using the Maximum Likelihood Program (MLP) (Ross, 1980) and $\log_{10}$ ED_{50} values and R/S ratios calculated. Detransformed ED_{50} values represent the herbicide dose required to reduce foliage fresh weight by 50%, relative to the untreated.

6.2.1.2 Response of 55 Lolium multiflorun populations to diclofop-methyl, fluazifop-P-butyl, tralkoxydim, cycloxydim and pinoxaden

In this assay fifty-five *Lolium multiflorum* populations collected on a semi-random basis in 2006 and 2007 from winter wheat fields from 50 farms in 22 counties of England were used. The populations were collected principally for evaluating seed dormancy and were not chosen because herbicide control had been poor. The objective of the experiment was to determine the extent of resistance to ACCase inhibiting-herbicides in England and the cross-resistance pattern.

Two single dose assays were conducted, each comprising a randomised block design with five replicate pots per herbicide and five untreated pots per population. In the first assay the response of 20 *L. multiflorum* populations collected in 2006 to the ACCase inhibiting herbicides diclofop-methyl, fluazifop-P-butyl, tralkoxydim, cycloxydim and pinoxaden was studied. In the second assay 38 populations collected in 2007 were used (Chapter 2, Table 2.1). In both assays the three *L. multiflorum* populations Yorks A2, Wilts B1 and Trajan were used as standard populations (Chapter 2, Table 2.2). The Yorks A2 population was used as target site resistant standard as it was known to carry the Ile-1781-leu point mutation (White et al., 2005). Wilts B1 was used as an enhanced metabolism standard since no key point mutation had been found so far in this population (Cocker *et al.*, 2001). Trajan was used as susceptible standard.

In both assays pre-germinated seeds were sown into nine cm pots containing peat-based compost and thinned to six plants per pot. A total of thirty pots were prepared for each population and were divided into six treatments (including nils) with five replicates. Commercial formulations of the five herbicides were applied at 1/2x field rate: 567g a.i diclofop-methyl ha^{-1}, 93.75g a.i. fluazifop-P-butyl ha^{-1}, 175g a.i. tralkoxydim ha^{-1}, 75g a.i. cycloxydim ha^{-1} and 22.5g a.i. pinoxaden ha^{-1}. Recommended adjuvants were used as described in Chapter 2, section 2.6.2. All the herbicides were used at half of the field rate as herbicides are more active under glasshouse conditions and the herbicides previously studied (see section 6.3.1.1), diclofop-methyl, tralkoxydim, cycloxydim and pinoxaden were shown to be effective against the susceptible standard Trajan at 1/4x field rate. After spraying, pots were returned to the glasshouse and randomised within replicates. Twenty-two days after treatment leaf samples from each surviving plant were collected and plants rated using a 1-4 injury scale prior to harvest as described in Chapter 2, sections 2.6.4, 2.6.5 and 2.6.6.

In both assays, the percentage reduction in fresh weights relative to the mean weight of untreated plants was calculated for each herbicide and a one-way ANOVA test carried out using Genstat. A principal component analysis was also carried out using the percent reduction in fresh weight data and results illustrated in a principal component bi-plot.

6.2.1.3 Response of ten Lolium multiflorum populations to fluazifop-P-butyl

The response of ten *L. multiflorum* populations to fluazifop-P-butyl was studied in a single dose assay. The populations used were those that in previous assays had shown resistance to diclofop-methyl, fluazifop-P-butyl and tralkoxydim but not to pinoxaden and cycloxydim. The resistance to diclofop-methyl and tralkoxydim are known to be mediated by both target site and enhanced metabolism mechanisms. In contrast, pinoxaden and cycloxydim resistance is thought to be conferred by a target site mutation. However, in fluazifop-P-butyl the mechanism conferring resistance is still unclear. The aim of this experiment was to rate and select populations highly resistant to fluazifop-P-butyl but not to cycloxydim and pinoxaden for further molecular studies to characterise the mechanism conferring resistance. The *L. multiflorum* populations Trajan and Rothamsted were used as susceptible standards. Two pre-germinated seeds were sown per pot in five cm pots containing peat-based compost and thinned to one plant per pot. A total of 50 pots were prepared for each of the ten populations used in the assay. Five pots per population were kept as untreated and the rest of the plants were sprayed with 1/2x field rate of fluazifop-P-butyl (93.75g a.i. ha^{-1}). Because differences exist in the germination rate between populations, the range of treated plants for each population ranged from 38 to 44. Twenty pots were prepared for each susceptible standard, five of which were untreated. After spraying, pots were returned to the glasshouse and randomised. Plants were rated using a 1-4 injury scale 22 days

after treatment as described in Chapter 2, section 2.6.4, transferred to the glasshouse and some of them used for subsequent molecular studies (Chapter 7, section 7.2.2).

6.2.1.4 Response of six Lolium multiflorum populations from the same farm to diclofop-methyl, fluazifop-P-butyl, tralkoxydim, cycloxydim and pinoxaden

Six *L. multiflorum* populations from different fields within the same farm at Peldon, in Essex, were tested for resistance to diclofop-methyl, fluazifop-P-butyl, tralkoxydim, cycloxydim and pinoxaden. This farm was one of the first sites in England where herbicide resistance was detected and *L. multiflorum* occurs in many fields. The populations used were G.Rising (2006), Geedon (2007), Wallraven (2007), Bald (2007), Barnfield (2007) and Lovatt (2007). Trajan was used as a susceptible standard. Distances between fields ranged between 500m and 3km (Figure 6.1).

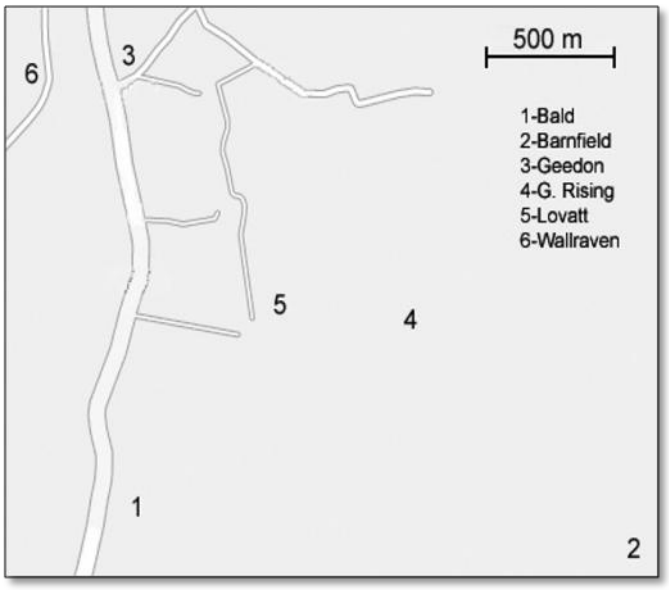


Figure 6.1. Map showing the position of the six fields from the farm in Peldon: Bald, Barnfield, Geedon, G. Rising, Lovatt and Wallraven.

Pre-germinated seeds were sown into nine cm pots containing peat-based compost and thinned to six plants per pot. A total of thirty pots were prepared for each

population and were divided into six treatments (including nils) and five replicates. Commercial formulations of the five herbicides were applied at 1/2x the field rate: 567g a.i diclofop-methyl ha^{-1}, 93.75g a.i. fluazifop-P-butyl ha^{-1}, 175g a.i. tralkoxydim ha^{-1}, 75g a.i. cycloxydim ha^{-1} and 22.5g a.i. pinoxaden ha^{-1}. Recommended adjuvants were used as described in Chapter 2, section 2.6.2. After spraying, pots were returned to the glasshouse and randomised within replicates.

Leaf samples were collected from each surviving plant 22 days after treatment and plants were rated using a 1-4 injury scale prior to harvest as described in Chapter 2, sections 2.6.4, 2.6.5 and 2.6.6. The percentage of reduction in fresh weights relative to mean weight of untreated plants was calculated for each herbicide and a one-way ANOVA test carried out using Genstat in both assays.

6.2.2 Rapid resistance test in Petri dishes

The rapid resistance test in Petri dishes is a faster and simpler method than whole plant pot assays, but has some limitations. It cannot be used with every herbicide and the results must be interpreted carefully, since herbicides are applied in a completely different way to the field. Also innate dormancy may reduce the potential advantage of this technique when fresh seed samples need to be tested (Moss, 1995). This test has been developed for both *A. myosuroides* and *L. multiflorum.* It would be useful and relevant to validate the methodology for the new herbicide pinoxaden as this would make resistance tests quicker, simpler and cheaper.

6.2.2.1 Resistance of five Lolium multiflorum populations to pinoxaden

The five *L. multiflorum* populations Yorks A2, Pyl, Wilts B1, G. Rising01 and Trajan (susceptible standard) were tested for resistance to pinoxaden using a dose response experiment in Petri dishes. Seeds were exposed to 0.001, 0.01, 0.1, 1, 5, 10 and 100 mg L^{-1} pinoxaden.

Fifty seeds per population were placed in Petri dishes containing three Whatman cellulose and one glass microfibre filter papers soaked with the specific concentrations of herbicide in a KNO_3 solution (2g L^{-1}) (Moss, 2000). Control dishes contained potassium nitrate solution only. Seven millilitres of the appropriate solutions were added to each dish which were then placed in sealed transparent polythene bags and stored in an incubator with a 17°C 14h light and an 11°C 10h dark phase. After two weeks the number of emerged shoots ≥1 cm in length in each dish was determined. The percent reduction in number of shoots >1 cm relative to the untreated controls for each population were calculated and the degree of resistance to pinoxaden estimated. Data were analysed using the Maximum Likelihood Program (MLP) and log_{10} ED_{50} values and R/S ratios calculated.

6.3 Results

6.3.1 Whole plant response assays

6.3.1.1 Response of five Lolium multiflorum populations to different doses of the ACCase inhibiting herbicides diclofop-methyl, tralkoxydim, cycloxydim and pinoxaden

The dose range used in this assay included doses both below and above the field recommended rate. The field recommended rates for each herbicide were 350g a.i. tralkoxydim ha^{-1}, 45g a.i. pinoxaden ha^{-1}, 150g a.i. cycloxydim ha^{-1} and 1,134g a.i. diclofop-methyl ha^{-1}. At both field rate and 1/4x field rate, the susceptible standard Trajan was well controlled for all herbicides and for tralkoxydim, was controlled at 1/8x field rate (Figure 6.2).

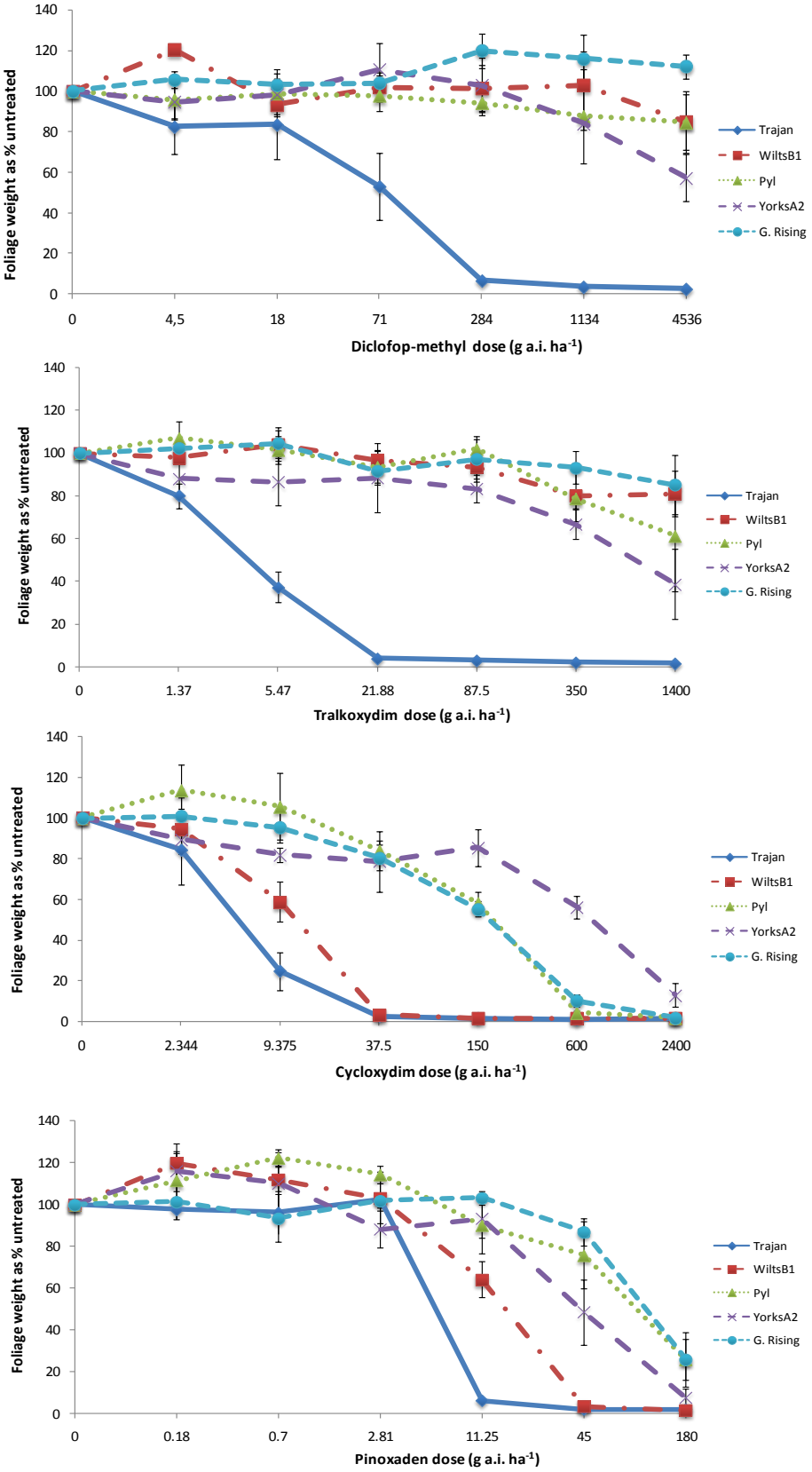


Figure 6.2. Mean foliage weight as % untreated plotted against diclofop-methyl, tralkoxydim, cycloxydim and pinoxaden doses for the *L. multiflorum* populations, Trajan, Wilts B1, Pyl, Yorks A2 and G. Rising01.

Table 6.1. Summary of ED_{50} values of diclofop-methyl, tralkoxydim, cycloxydim and pinoxaden calculated using MLP. The resistance index (RI) is the resistant ED_{50} value for each population divided by the ED_{50} value for the susceptible standard. (*) No ED_{50} values could be obtained for diclofop-methyl with the population G. Rising01 because the level of resistance to this herbicide was so high for that population.

Herbicide	Population	$Log_{10}ED_{50}$	ED_{50} g a.i. ha^{-1}	Resistance index (RI)
Diclofop-methyl	Trajan	1.847	70.3	1.0
	WiltsB1	>3.657	>4536	>64.5
	Pyl	>3.657	>4536	>64.5
	YorksA2	>3.657	>4536	>64.5
	G. Rising01	(*)		
	S.E.	0.590		
	L.S.D. (P≤0.05)	1.668		
Tralkoxydim	Trajan	0.577	3.8	1.0
	WiltsB1	>3.146	>1400	>370.8
	Pyl	3.111	1292.4	342.3
	YorksA2	>3.146	>1400	>370.8
	G. Rising01	>3.146	>1400	>370.8
	S.E.	0.815		
	L.S.D. (P≤0.05)	2.306		
Cycloxydim	Trajan	0.683	4.8	1.0
	WiltsB1	1.024	10.6	2.2
	Pyl	2.224	167.5	34.8
	YorksA2	2.905	802.7	166.7
	G. Rising01	2.218	165.2	34.3
	S.E.	0.068		
	L.S.D. (P≤0.05)	0.191		
Pinoxaden	Trajan	0.923	8.4	1.0
	WiltsB1	1.128	13.4	1.6
	Pyl	1.836	68.6	8.2
	YorksA2	1.562	36.5	4.4
	G. Rising01	2.028	106.7	12.8
	S.E.	0.189		
	L.S.D. (P≤0.05)	0.535		

Very high resistance was found to tralkoxydim and diclofop-methyl in all four populations. Populations Yorks A2, Pyl and G. Rising01 showed clear resistance to cycloxydim, but resistance in Wilts B1 was marginal with RI of 2.2. Wilts B1 had a lower resistance to pinoxaden (RI of 1.6) compared with cycloxydim.

Populations Yorks A2, G. Rising01 and Pyl showed very high resistance to all four herbicides at field rate but had a greatly reduced growth with pinoxaden at 4x the recommended rate. Cycloxydim was effective against all populations except Yorks A2 at 4x field rate while tralkoxydim and diclofop-methyl did not control any of the

populations at any rate tested. Wilts B1, a population known to have enhanced metabolism, was controlled by pinoxaden at field rate and by cycloxydim at 1/4x field rate (Figure 6.2).

The resistance indexes in Table 6.1 indicate that all populations were highly resistant to tralkoxydim and diclofop-methyl. Because of the very high level of resistance in G. Rising, the ED_{50} value was impossible to obtain and, consequently, a resistance index could not be calculated. Populations Yorks A2, Pyl and G. Rising01 showed clear resistance to cycloxydim, but resistance with Wilts B1 was marginal with RI of 2.2.
Wilts B1 had a lower resistance to pinoxaden (RI of 1.6) compared with cycloxydim. Yorks A2 was highly resistant to cycloxydim and partially resistant to pinoxaden, with RI of 166.7 and 4.4, respectively. Populations Pyl and G. Rising01 were highly resistant to cycloxydim with RI >34 and showed evidence of resistance to pinoxaden, although the RI values were lower (8.2 and 12.8, respectively). In summary, these results show that resistance to tralkoxydim and diclofop-methyl is similar in all the populations studied. Resistance to pinoxaden and cycloxydim are somewhat similar, but generally there is less resistance to pinoxaden.

6.3.1.2 Response of 55 Lolium multiflorum populations to fluazifop-P-butyl, pinoxaden, cycloxydim, tralkoxydim and diclofop-methyl

Resistance 'R' ratings were calculated from the percent reduction values as described in Chapter 2, section 2.6.7. Results were consistent across the standards in both experiments. As expected, the susceptible standard Trajan was well controlled by all five herbicides. Yorks A2 showed resistance to all herbicides although it showed a lower degree of resistance to pinoxaden. Wilts B1 showed resistance only to diclofop-methyl, tralkoxydim and fluazifop-P-butyl. The range of mean untreated weight varied between 6.6 and 19 g in the first experiment and between 9.8 and 25.9 g in the second

experiment, with an average value for both experiments of 17.8 g (Table 6.2, Table 6.3). Trajan had a rather low mean weight in both experiments with 9.7 and 9.8 g in the first and second experiment, respectively. However, mean weight did not seem to have an effect on the degree of resistance as the mean untreated weight values for most susceptible populations were near the average or above it. The population Rothamsted had a mean weight much higher than the average with a value of 25.9 g.

Table 6.2. Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants within herbicide treatments for 20 *L. multiflorum* populations collected in 2006. Colour code = resistance 'R' ratings indicating degree of resistance: RED = RRR; ORANGE = RR; YELLOW = R?; GREEN = Susceptible.

Population	Mean untreated weight (g)	Mean % reduction relative to mean weight of untreated plants				
		Cycloxydim 75g a.i. ha^{-1} 1/2x field rate	Diclofop-methyl 567g a.i. ha^{-1} 1/2x field rate	Fluazifop-P-butyl 93.8g a.i. ha^{-1} 1/2x field rate	Pinoxaden 22.5g a.i. ha^{-1} 1/2x field rate	Tralkoxydim 175g a.i. ha^{-1} 1/2x field rate
Trajan (Susc.)	9.7	96	91	94	94	93
Yorks A2 (T.S.R.)	15.6	33	19	45	58	31
Wilts B1 (E.M.R)	14.7	97	28	49	94	11
20AC	17.6	95	68	92	94	91
Hitchin	15.1	95	86	93	92	88
Chat1	19.0	98	39	88	95	58
Chat2	15.2	97	68	94	95	82
Cott	14.6	94	51	90	92	82
Fish	17.5	96	92	96	95	92
Geedon	15.5	61	22	12	48	21
G. Rising	17.8	60	15	14	47	1
Grum	16.3	97	60	94	94	88
Hill	12.3	97	13	55	90	-7
High Wycombe	17.1	96	5	43	92	-2
Long	18.9	96	15	43	93	24
Louth	6.6	95	80	96	91	89
Maid	19.0	96	37	91	94	67
Port	14.6	95	87	91	92	91
Raven	16.7	96	83	92	94	84
School	15.9	97	48	74	93	64
Wallraven	18.8	82	2	25	66	1
Wickford	16.8	21	7	27	18	7
Wood	13.3	96	77	88	94	90
S.E. ±	0.94	4.65	6.08	4.18	4.00	6.17
LSD (P<0.05)	2.65	13.08	17.09	11.74	11.24	17.33

Table 6.3. Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants within herbicide treatments for 38 L. multiflorum populations collected in 2007. Colour code = resistance 'R' ratings indicating degree of resistance: RED = RRR; ORANGE = RR; YELLOW = R?; GREEN = Susceptible.

Population	Mean untreated weight (g)	Mean % reduction relative to mean weight of untreated plants				
		Cycloxydim 75g a.i. ha^{-1} 1/2x field rate	Diclofop-methyl 567g a.i. ha^{-1} 1/2x field rate	Fluazifop-P-butyl 93.8g a.i. ha^{-1} 1/2x field rate	Pinoxaden 22.5g a.i. ha^{-1} 1/2x field rate	Tralkoxydim 175g a.i. ha^{-1} 1/2x field rate
Trajan (Susc.)	9.8	96	93	94	96	95
Yorks A2 (T.S.R.)	18.2	19	30	36	80	37
Wilts B1 (E.M.R.)	22.0	94	28	51	93	13
Adams	19.3	32	-2	22	35	9
Aviary	20.3	96	65	89	96	35
B. LandsL2	22.3	71	24	50	79	28
Belaugh	22.2	95	26	93	95	19
Berkhamsted07	17.8	93	54	90	92	75
Chapel	17.7	94	35	94	94	30
Charleston	25.4	37	31	38	68	27
Cinder	22.0	94	92	94	95	93
Clements	16.0	26	-15	15	31	3
Corner	17.5	93	-18	59	92	-17
Court	21.3	52	14	33	69	10
Dairy	20.1	55	34	51	44	32
Eppleton	20.7	93	89	92	93	86
Galons	17.1	95	70	89	93	74
Green Ball	18.9	92	67	91	92	90
Hay Shed	16.2	98	10	68	98	26
Heacham	17.5	95	82	93	95	91
Ken	19.0	92	85	90	91	66
Lewis	18.0	96	94	96	96	95
Low	18.3	96	94	94	95	89
Manor	19.7	92	83	91	95	90
North Benfleet	20.6	43	-1	34	65	17
Oaktree	18.4	34	2	-4	18	13
Paddock	17.9	94	79	88	93	76
Parson	19.3	95	31	59	94	21
Pidley	21.7	95	20	74	92	5
Pub	21.1	96	77	96	96	53
3in1	12.9	95	88	94	95	94
Roedowns	19.0	96	90	95	95	95
Bridgnorth	20.5	97	49	93	97	28
Ripple	17.1	95	89	93	94	90
Rothamsted	25.9	94	92	93	94	92
Smiths	17.0	94	88	94	95	88
Thornham	21.0	94	55	80	94	78
Walette	17.6	95	84	93	94	70
S.E. ±	1.54	3.67	6.27	4.11	3.24	5.54
LSD (P<0.05)	4.30	10.25	17.53	11.49	9.04	15.49

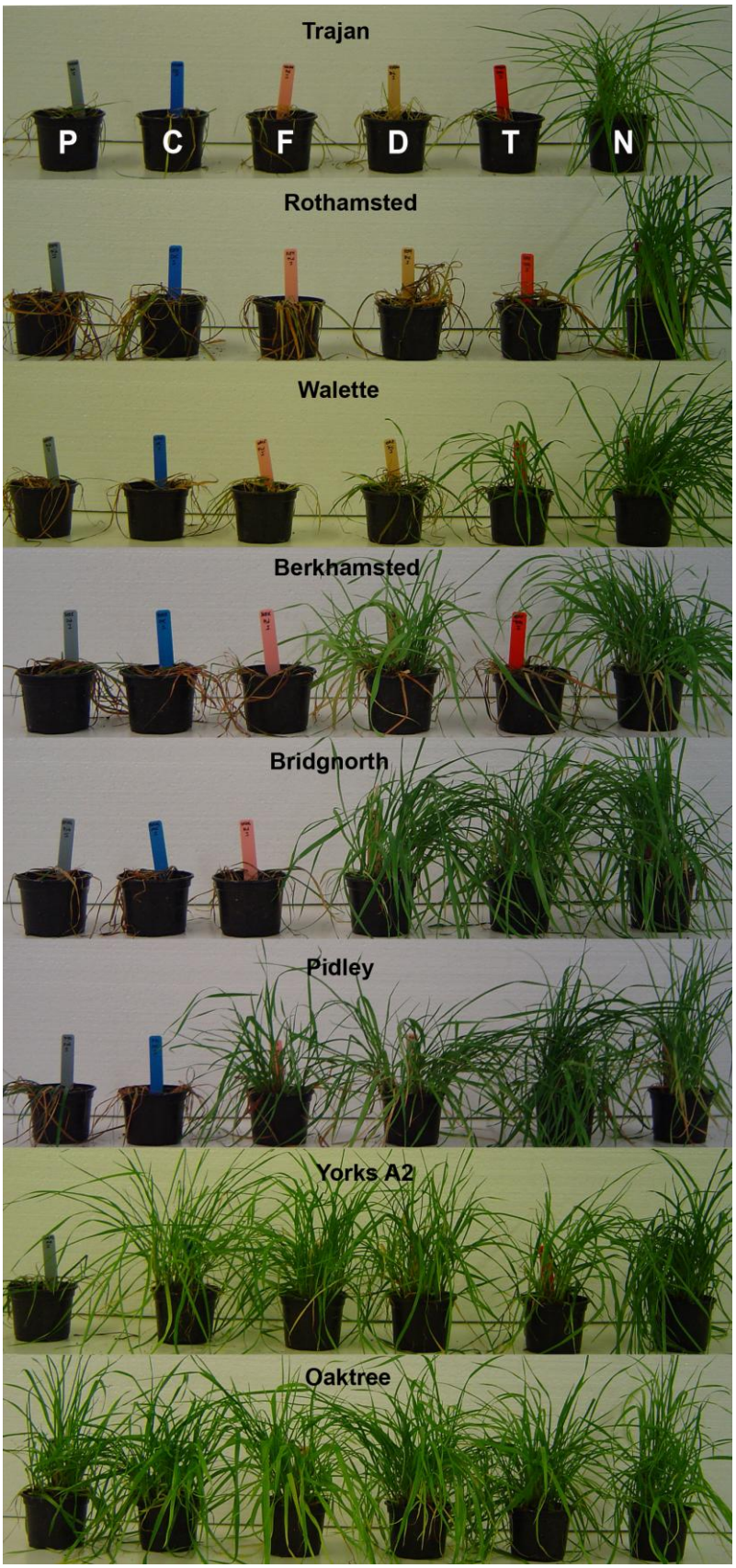


Figure 6.3. Examples of susceptible and resistant plants from different *L. multiflorum* populations to the ACCase-inhibiting herbicides pinoxaden (P), cycloxydim (C), fluazifop-P-butyl (F), tralkoxydim (T) and diclofop-methyl (D) compared to untreated plants (N).

Within the 55 populations analysed, 34 (62%) showed confirmed resistance (RR or RRR) to diclofop-methyl and 32 (58%) resistance to tralkoxydim (Table 6.2, Table 6.3). In most cases, resistance to diclofop-methyl was associated with resistance to tralkoxydim with a correlation coefficient of 0.93. Seven (13%) populations showed a lesser degree of resistance (R?) to diclofop-methyl and three (5%) to tralkoxydim. The resistance to these two herbicides was associated in some cases to resistance to fluazifop-P-butyl with a correlation coefficient of 0.83 for diclofop-methyl and 0.80 for tralkoxydim. Twenty (36%) populations showed confirmed resistance to fluazifop-P-butyl while only one had a degree of resistance rated R?. Eleven populations (20%) showed resistance (RRR, RR) to cycloxydim and pinoxaden, three of which were from fields in the same farm. Only the population Wallraven, with cycloxydim, and B.LandsL2, with pinoxaden, had the lowest degree of resistance (R?), indicating that resistance to these herbicides may be developing in these populations. This also indicates how highly correlated these two herbicides are, with a correlation coefficient of 0.88. However, the correlation coefficients between these two herbicides, pinoxaden and cycloxydim, and diclofop methyl and tralkoxydim were much lower with values between 0.50 and 0.60.

A principal component analysis was carried out using the percent reduction in fresh weight data from these two experiments and results illustrated in a principal component bi-plot (Figure 6.4). This analysis agrees with the correlation coefficients obtained previously. Herbicides in the bi-plot are divided into three groups with tralkoxydim and diclofop-methyl in one extreme, cycloxydim and pinoxaden in another and fluazifop-P-butyl in between these two groups. The results from the ANOVA carried out with these same data also confirmed the results obtained previously. The herbicides were also divided into three groups as diclofop-methyl and tralkoxydim were significantly different to the other herbicides, although they were not significantly different from each other. There were no significant differences between pinoxaden and cycloxydim, but these

two herbicides were significantly different to the others. Fluazifop-P-butyl was significantly different to all of them. In summary, the responses to these five herbicides are significantly different. Resistance to diclofop-methyl, tralkoxydim and, to a slightly lesser extent, fluazifop-P-butyl appears to be widespread, but resistance to both cycloxydim and pinoxaden is much less common.

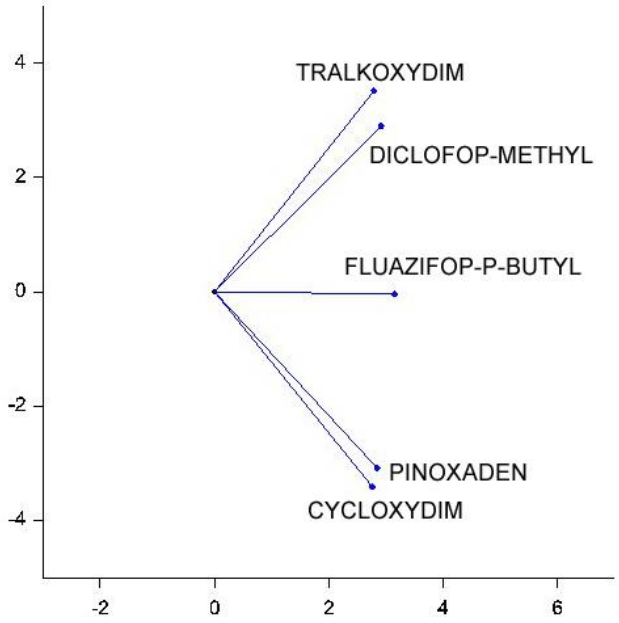


Figure 6.4. Bi-plot of the results of principal component analysis after centering and normalising the percent reduction in fresh weight data from a matrix of 5 herbicides (tralkoxydim, diclofop-methyl, fluazifop-P-butyl, pinoxaden and cycloxydim) and 61 *L. multiflorum* populations (55 populations plus 3 standard populations) . First component (y) accounts for 78% of the variability while second component (x) only accounts for 16%.

6.3.1.3 Response of ten Lolium multiflorum populations to fluazifop-P-butyl

All populations were organised according to the proportion of resistant plants and plants were kept in the glasshouse for further molecular studies (See chapter 7). The populations Hill and Hay Shed showed greatest levels of resistance with 100% of plants surviving (Category 1) and with none or minimal symptoms of herbicide damage (Table 6.4). Corner, Parson and High Wycombe had 89-95% of plants in category 1, while Long, Pidley and Wilts B1 had resistance levels of 80%, 80% and 81%,

respectively. Thornham and School were the populations that showed lower resistance to diclofop-methyl, tralkoxydim and fluazifop-P-butyl in previous studies (section 6.3.1.2). As expected, these two populations, Thornham and School, also showed lower resistance to fluazifop-P-butyl in this single dose assay, with 7% and 34% of plants in category 1, respectively. The two susceptible standards, Trajan and Rothamsted, were well controlled by fluazifop-P-butyl. As fluazifop-P-butyl seems to be affected by enhanced metabolism resistance to a lesser degree than diclofop-methyl and tralkoxydim, the resistance to fluazifop-P-butyl in these highly resistant populations might be due to another mechanism such as target site resistance and a 'fop' specific mutation might be involved.

Table 6.4. Percentage of plants per injury category (see Chapter 2, section 2.6.4) from each population following treatment with fluazifop-P-butyl.

Populations	% plants			
	1	2	3	4
Rothamsted	0	0	0	100
Trajan	0	0	0	100
Thornham	7	2	7	83
School	34	0	21	45
Long	80	0	0	20
Pidley	80	0	0	20
Wilts B1	81	2	0	17
Corner	89	0	0	11
Parson	90	2	0	7
High Wycombe	95	0	2	2
Hill	100	0	0	0
Hay Shed	100	0	0	0

6.3.1.4 Response of six Lolium multiflorum populations from the same farm to fluazifop-P-butyl, pinoxaden, cycloxydim, tralkoxydim and diclofop-methyl

Results were analysed by comparing mean percent reduction in plant weight relative to untreated controls. The susceptible standard Trajan was well controlled by all herbicides. Three of the six populations, Bald, Barnfield and G.Rising, were highly

resistant to all the herbicides (RRR), and some even had an increase in fresh weight compared to the untreated plants (Table 6.5). The other three populations, Geedon, Lovatt and Wallraven were highly resistant (RRR) to diclofop-methyl, tralkoxydim and fluazifop-P-butyl, but showed different degrees of resistance to cycloxydim and pinoxaden. The most resistant population seemed to be Bald (Figure 6.5). The three populations closest to Bald (see Figure 6.1) are Barnfield, G. Rising and Lovatt which are the populations that showed the highest degree of resistance after Bald. Populations Geedon and Wallraven located in further fields had the lowest degree of resistance within this farm. If we also consider that the *L. multiflorum* population first sampled on this farm was Essex A1 which was collected from Bald and it was one of the first *L. multiflorum* populations in England with confirmed resistance (Moss *et al.*, 1993), this might indicate that resistance started in the field Bald and was later spread to the other fields.

Table 6.5. Analysis of variance comparing mean percent reduction in plant weight relative to untreated plants within herbicide treatments for six *L. multiflorum* populations from different fields in the same farm in Essex. Colour code = resistance 'R' ratings indicating degree of resistance: RED = RRR; ORANGE = RR; YELLOW = R?; GREEN = Susceptible.

Population	Mean untreated weight (g)	Mean % reduction relative to mean weight of untreated plants				
		Cycloxydim 75g a.i. ha^{-1} 1/2x field rate	Diclofop-methyl 567g a.i. ha^{-1} 1/2x field rate	Fluazifop-P-butyl 93.8g a.i. ha^{-1} 1/2x field rate	Pinoxaden 22.5g a.i. ha^{-1} 1/2x field rate	Tralkoxydim 175g a.i. ha^{-1} 1/2x field rate
Trajan (Susc.)	16.7	95	92	93	95	92
Bald	18.9	7	-8	-5	11	3
Barnfield	21.9	30	9	12	13	25
G. Rising	22.5	28	13	12	26	3
Geedon	23.0	89	11	10	50	14
Lovatt	21.6	42	3	5	31	8
Wallraven	22.1	77	5	20	81	3
S.E. ±	1.06	5.43	4.05	5.34	5.74	5.09
LSD (P<0.05)	3.09	15.85	11.81	15.60	16.75	14.86

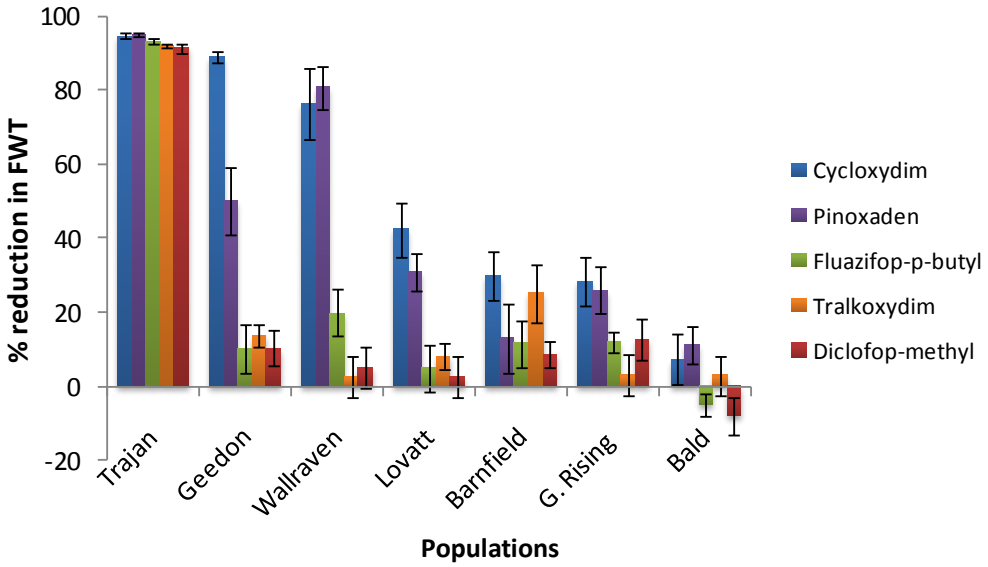


Figure 6.5. Response to cycloxydim, pinoxaden, fluazifop-P-butyl, tralkoxydim and diclofop-methyl of the *L. multiflorum* populations Trajan, Geedon, Wallraven, Lovatt, Barnfield, G. Rising and Bald expressed as percent reduction in fresh weight relative to untreated plants.

Correlation coefficients were also calculated with the mean percent reduction in fresh weight data. The population Barnfield, located in a field isolated from all the other fields, showed no correlation with any of the other populations (values between 0.28 and 0.62). However, the other isolated population Bald showed a relatively high correlation with Lovatt (0.84) and Wallraven (0.81). The population from the field located in the middle of the farm, Lovatt, was highly correlated to all the populations except Barnfield with correlation coefficients 0.88, 0.98 and 0.94, with G. Rising, Geedon and Wallraven, respectively. G. Rising was also highly correlated to the populations Wallraven, Geedon and Lovatt but not to Bald or Barnfield.

A thorough study of the resistance at the molecular level would help to understand how resistance has evolved and spread within this farm. Only three of these six populations could be screened for mutations (See Chapter 7), Geedon, G. Rising and Wallraven. Populations Geedon and G. Rising had two of the seven point mutations known to confer resistance to ACCase-inhibiting herbicides, while Wallraven had only one and no mutations were found in half of the plants that were screened. Most of the plants

without any mutation were resistant to diclofop-methyl, fluazifop-P-butyl or tralkoxydim, which could be explained by an enhanced metabolic resistance mechanism. The lower proportion of plants with mutations could explain the lower level of resistance found in this population. No molecular study could be carried out with the populations Bald, Barnfield and Lovatt, leaving unsolved the molecular basis of resistance for these populations. However, a higher proportion than in Wallraven of plants carrying the two mutations found in the other fields in this farm would be expected in all of them.

6.3.2 Rapid resistance test in Petri dishes

6.3.2.1 Resistance of five Lolium multiflorum populations to pinoxaden

At a herbicide dose of 0.1mg L^{-1}, the proportion of germinated seeds was visibly reduced for both Trajan and Wilts B1, while the other three populations seemed not to be affected by the herbicide (Figure 6.6). At a dose of 1mg L^{-1} the percentage of shoots >1cm for Wilts B1 and Trajan was zero or close to zero, while for Yorks A2 it was still 17% and for G. Rising and Pyl around 45% (Figure 6.7).The resistance index for Wilts B1 was higher than 1 (RI of 2.8) but still very low which indicated that it was essentially sensitive to pinoxaden (Table 6.6).

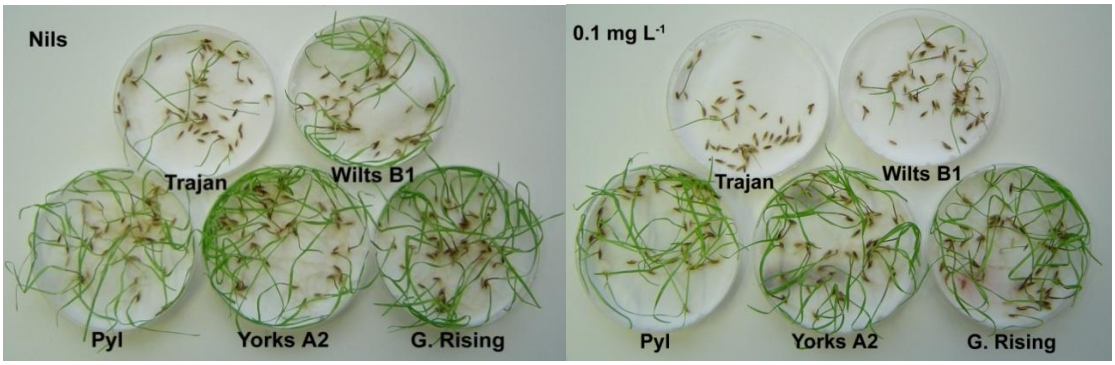


Figure 6.6. Response to pinoxaden in the Petri dish assay. At the dose 0.1mg L^{-1}, the percentage of seeds with shoots >1cm is visibly reduced for the susceptible standard Trajan and for Wilts B1, while hardly any reduction in number of shoots is apparent for the other three populations.

The other three populations were highly resistant to pinoxaden with degrees of resistance similar to those shown in a previous glasshouse assay (section 6.3.1.1), Pyl and G. Rising having a larger RI than Yorks A2.

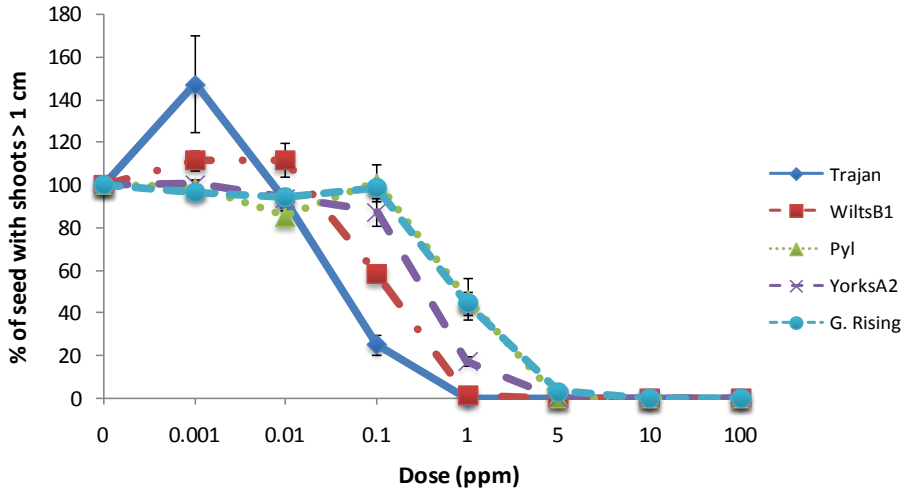


Figure 6.7. Percentage of seeds with shoots >1cm relative to the nils plotted against pinoxaden dose for all the populations.

Table 6.6. Summary of ED_{50} values of every population calculated using MLP. The resistance index (RI) is the resistant ED_{50} value for each population divided by the ED_{50} value for the susceptible standard.

Herbicide	Population	$Log_{10}ED_{50}$ (S.E.)	ED_{50}	Resistance index
Pinoxaden	Trajan	-1.405	0.039	1.0
	WiltsB1	-0.952	0.111	2.8
	Pyl	-0.010	0.977	24.9
	YorksA2	-0.388	0.409	10.4
	G. Rising	-0.039	0.913	23.2
	Combined S.E.	0.064		
	L.S.D. (P≤0.05)	0.181		

These results from the Petri dish assay showed a very good correlation with the results shown in Table 6.2, where Wilts B1 showed no resistance to pinoxaden and G. Rising showed a higher level of resistance than Yorks A2. These results also support the finding of the whole plant assay in section 6.3.1.1, that G. Rising and Pyl are more resistant to pinoxaden than Yorks A2 is, although Yorks A2 is more resistant to cycloxydim in pots. Yorks A2 is known to have a target site mutation, while until now

(see Chapter 7) nothing was known about the mechanism conferring resistance in the populations G. Rising and Pyl. The differences in the degree of resistance to different herbicides might be explained by these populations having different target site mutations. This kind of test cannot be used for every herbicide and for every grass species. However, results showed here demonstrate that different degrees of resistance to pinoxaden can be detected using this test and with *L. multiflorum*.

6.4 Discussion

The results from the herbicide resistance assays show how widespread the resistance to ACCase-inhibiting herbicides is in England (Figure 6.8). Seed samples were semi-randomly collected from a total of 50 individual farms in 22 counties of England giving a good geographical distribution. Samples were not collected because of a poor herbicide control but for dormancy tests. However, some bias may have occurred as collectors were likely to visit fields where they knew *L. multiflorum* would be present. Resistance (RRR/RR) to at least one herbicide was detected in *Lolium multiflorum* samples from 35 (70%) of the 50 farms semi-randomly sampled. Interestingly, this result is the same as that obtained by Moss *et al.,* (2005) where samples from 70% of non-randomly sampled farms showed resistance to one or more herbicides. They also found that the incidences of resistance to fenoxaprop (83%) and sethoxydim/cycloxydim (41%) in a non-random compilation exercise with *A. mysosuroides* were very similar to the results (80% and 40% respectively) from a smaller random study (Moss *et al.*, 2005). These results, for both *L. multiflorum* and *A. myosuroides,* indicate that non-random compilation exercises, which tend to be much easier and cheaper to conduct, are not as biased as might be expected, and can provide an accurate indication of the current status of herbicide resistance. This has important logistical and financial implications for the detection and monitoring of

resistance, but would benefit from additional validation with other resistant weeds in other countries.

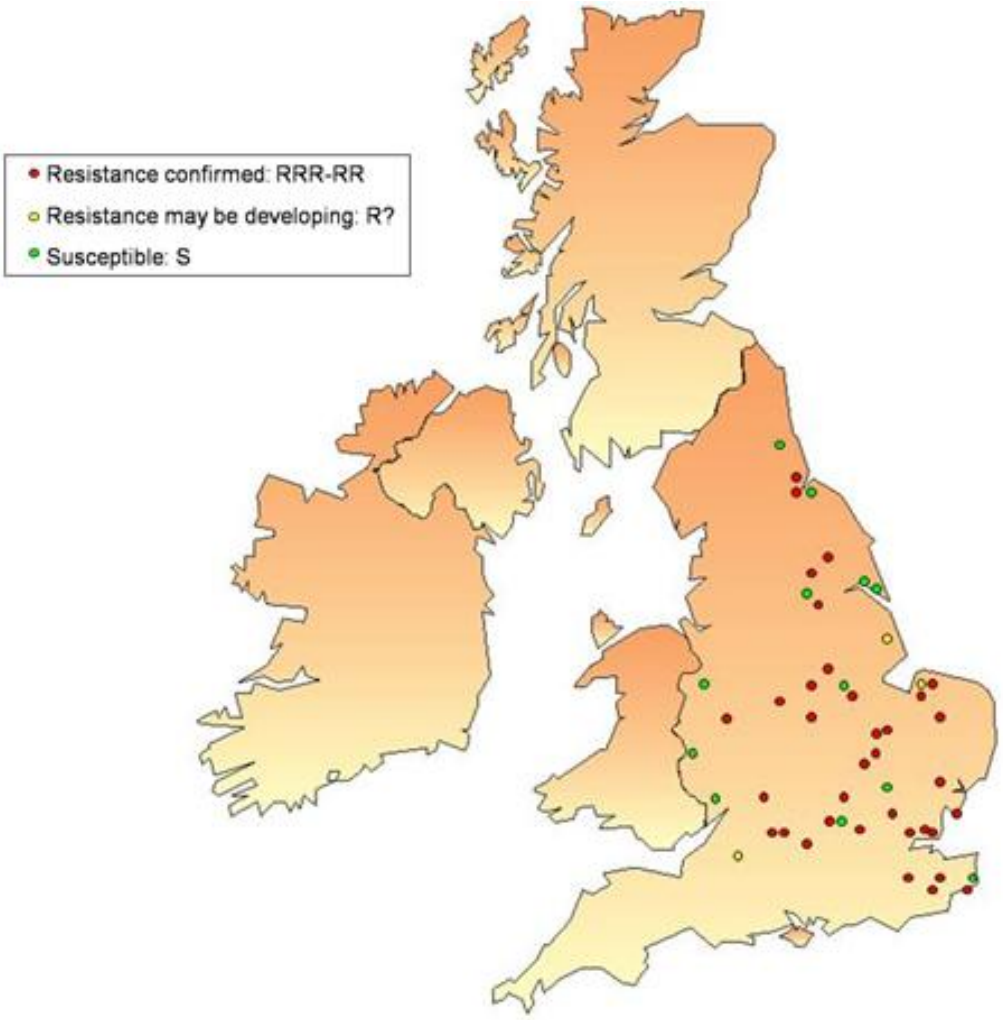


Figure 6.8. Frequency of resistance to at least one ACCase-inhibiting herbicide in *Lolium multiflorum* populations from 50 farms in England. Resistance rates were calculated as described in Chapter 2, section 2.6.7. and correspond to the highest resistance rates obtained for each farm which are showed in Table 6.2 and Table 6.3.

In the study presented here, herbicide resistance in *L. multiflorum* was discovered in 16 out of the 22 counties sampled. Resistance to diclofop-methyl and tralkoxydim was detected on 31 (62%) and 30 (60%) of farms respectively from 16 counties. Resistance to fluazifop-P-butyl was detected on 18 farms (36%) from 12 counties, and to cycloxydim and pinoxaden on 10 (20%) and 9 (18%) farms, respectively, from 6

counties. The only counties sampled where no resistance was detected were Gloucestershire, Tyne and Wear, East Riding of Yorkshire, Herefordshire and Stock-on-Tees. This contrasts with data from Moss *et al.,* (2005) where resistance to cycloxydim was detected on 9% of the farms, showing that target site resistance to ACCase-inhibiting herbicides might have increased, as cycloxydim seems not to be affected by enhanced metabolism.

Resistance to diclofop-methyl in other *L. multiflorum* populations from Oregon has also been associated to haloxyfop but not to sethoxydim and clethodim, and was also found to be conferred by an insensitive ACCase (Gronwald *et al.*, 1992). In studies on *Lolium* spp. in Italy with diclofop-methyl, there was evidence of a possible involvement of herbicide sequestration or immobilization (Dinelli *et al.*, 2005). It has also been found in one *L. rigidum* population resistant to diclofop-methyl that a possible mechanism to explain resistance to this herbicide could be the ability of membranes to recover from depolarization following exposure to diclofop-methyl (Haüsler *et al.*, 1991, Holtum *et al.*, 1991).

In more recent studies carried out on *L. multiflorum* populations in Oregon, resistance to diclofop-methyl was found in 81% of the populations and seemed to be associated to some extent with resistance to clodinafop but not to pinoxaden (Kuk *et al.*, 2008). 80% of diclofop-resistant populations were clodinafop-resistant while only 20% of diclofop-resistant populations were pinoxaden-resistant. All populations were susceptible to clethodim and sethoxydim. *L. rigidum* and *A. myosuroides* populations resistant to diclofop-methyl have been shown to be cross-resistant to herbicides with different modes of action (Heap & Knight, 1986, Moss, 1990a). This is most likely to be associated with an enhanced metabolic detoxification as this resistance mechanism has been shown to confer or contribute to resistance to several herbicides in *A. myosuroides* populations, including chlorotoluron, diclofop-methyl and fenoxaprop-P-

ethyl (Hall *et al.*, 1997). The primary resistance mechanism in the majority of some European fenoxaprop-P-ethyl resistant *A. myosuroides* populations, was shown to be an enhanced metabolic detoxification. However in one of the populations resistance was explained by multiple mechanisms, enhanced metabolic detoxification and an insensitive ACCase, while in another population it could be explained by none of them (Cocker *et al.*, 1999). These studies indicated that resistance to fenoxaprop-P-ethyl in *A. myosuroides* can be conferred by at least three mechanisms: enhanced metabolic resistance, target site resistance and at least one more uncharacterized mechanism. More recent studies in France showed that in *A. myosuroides* populations the point mutation Asp-2078-Gly confers cross-resistance to fenoxaprop, clodinafop, haloxyfop, cycloxydim and clethodim, while Ile-1781-Leu also confers resistance to the fops and cycloxydim but only moderate resistance to clethodim (Délye *et al.*, 2008). Populations with the point mutation Gly-2096-Ala appear to be resistant to fops and moderately resistant to clethodim but are sensitive to cycloxydim. The point mutations Trp-2027-Cys and Ile-2041-Asn confer resistance only to the three fops assayed. However, despite finding mutant alleles in 56.8% of assayed *A. myosuroides* plants, with the Ile-1781-Leu mutation being predominant, Délye *et al.*, (2007) found that more than 75% of the plants resistant to ACCase-inhibiting herbicides in France are resistant via increased herbicide metabolism.

In studies on *A. myosuroides* using pinoxaden it was found that the point mutations Ile-1781-Leu, Trp-2027-Cys and Asp-2078-Gly alone can confer resistance to this herbicide although more data are needed to prove that this is also the case for the Trp-2027-Cys mutation (Petit *et al.*, 2009). The point mutations Ile-2041-Asn and Gly-2096-Ala appear not to be sufficient to confer resistance to pinoxaden at field rates and additional resistance mechanisms present in some plants would enable them to survive pinoxaden. In this study, plants containing no known mutations also survived pinoxaden treatments suggesting that other resistance mechanisms were present.

In recent studies with *Lolium* spp. (Yu *et al.*, 2007) plants homozygous for the mutations Ile-1781-Leu, Asp-2078-Gly and Cys-2088-Arg were found to be resistant to clethodim and cross-resistant to clodinafop, diclofop-methyl, fluazifop-P-butyl, haloxyfop, butroxydim, sethoxydim, tralkoxydim and pinoxaden.

On studies on *Avena* spp. in Australia, 71% of the populations studied showed resistance to diclofop-methyl and 16% of them showed cross resistance to fenoxaprop or sethoxydim (Owen & Powles, 2009). However, no cross-resistance was found with the herbicides clodinafop, pinoxaden, clethodim and tralkoxydim. In five US *Avena fatua* populations studied, all the populations were found to be cross-resistant to the herbicides fenoxaprop, diclofop-methyl and quizalofop, while only one population was also found to be resistant to pinoxaden and tralkoxydim and none of them were found to be resistant to sethoxydim and clethodim (Uludag *et al.*, 2008). These different levels of resistance and cross-resistance patterns between populations indicated the presence of either more than one mechanism of resistance or different resistant mutations in each population.

Resistant patterns to ACCase inhibitors seem to be very variable depending on species and location, thus indicating that different weed control strategies will lead to differences in the evolution of resistance. The mechanisms conferring resistance seem to be different depending on populations although it appears that in all species studied the most common resistant mechanism would be a non target site resistance mechanism. While some of the seven known mutations seem to confer resistance to the majority of ACCase-inhibiting herbicides, non-target site mechanisms seem not to affect most ACCase-inhibiting herbicides, although they can confer resistance to herbicides with other modes of action.

The efficacy of diclofop-methyl and tralkoxydim is affected by both ACCase target site and enhanced metabolic resistance (Cocker et al., 2001). On the other hand, fluazifop-P-butyl seemed to be affected by a non-target site resistance mechanism but to a lesser degree than diclofop-methyl and tralkoxydim. All the populations that were resistant to cycloxydim and pinoxaden were also resistant to fluazifop-P-butyl, diclofop-methyl and tralkoxydim. Resistance here could be explained by a target site resistance mechanism. However, all the populations resistant to fluazifop-P-butyl but not resistant to cycloxydim and pinoxaden showed a high degree of resistance (RRR) to both diclofop-methyl and tralkoxydim, except for one, School, that was rated RR for these three herbicides. This could mean that a non-target site resistance mechanism was responsible for the resistance to fluazifop-P-butyl in these populations. Consequently, target site resistance would be responsible for the resistance in just 12 (22%) out of the 55 populations evaluated from 50 separate farms. This would indicate that diclofop-methyl, fluazifop-P-butyl and tralkoxydim are affected by both enhanced metabolism and target site resistance and probably additional resistance mechanisms, while cycloxydim seems to be affected only by target site resistance. Pinoxaden seems to be affected mainly by target site resistance. This was confirmed by results from the molecular studies carried out with 384 surviving plants from 52 of the 55 *L. multiflorum* populations studied here (see Chapter 7). None of the seven known target point mutations conferring resistance to ACCase-inhibiting herbicides were found in 37 populations. Except in a few cases, those plants where no mutation was found were shown to be resistant to diclofop-methyl, fluazifop-P-butyl or tralkoxydim, and most of the plants resistant to pinoxaden and cycloxydim had at least one of the seven known point mutations conferring resistance to ACCase-inhibiting herbicides. Resistance in a few plants that survived pinoxaden seemed to be caused by a non-target site resistance mechanism, although, with such a low proportion of plants, this resistance mechanism appears not to be widespread.

These results were also consistent with those shown in section 6.3.1.1. The *L. multiflorum* population Wilts B1, together with the populations Essex A1 and Lincs A1, were shown to be able to metabolise phytotoxic diclofop-acid into polar metabolites at a rate twice that of susceptible populations, this being the mechanism conferring resistance to diclofop-methyl in these populations (Cocker *et al.*, 2001). This population, Wilts B1, was used in the experiment described in section 6.2.1.1, and showed high resistance to diclofop-methyl (RI > 370.8) and tralkoxydim (RI > 64.5) but only marginal resistance to cycloxydim (RI = 2.2) and even lower resistance to pinoxaden (RI = 1.6) (Table 6.1). The other populations used in this experiment showed high levels of resistance to all herbicides which is now known to be conferred by an insensitive ACCase (See Chapter 7). This confirmed that cycloxydim and pinoxaden appear to be unaffected by the enhanced metabolic resistance mechanism that confers resistance in Wilts B1 while resistance to diclofop-methyl and tralkoxydim were affected by this mechanism. The resistance test in Petri dishes with pinoxaden supported these results previously obtained at the whole plant level as Wilts B1 had a RI of 2.8, showing marginal resistance, while all the other populations had higher RI (Table 6.6). These results also confirm that resistance to pinoxaden can be detected using a rapid resistance test in Petri dishes.

Thus, 13 (24%) of the 55 populations (from 50 separate farms) were found to be largely sensitive to all the herbicides assayed (with none or very few plants surviving), 12 populations (22%) were found to be resistant to all the herbicides and 30 populations (55%) were found to be resistant only to diclofop-methyl, fluazifop-P-butyl and tralkoxydim. These results indicate that the most common mechanism of resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations appears to be a non-target site resistance mechanism, most probably an enhanced herbicide metabolic detoxification.

6.5 Chapter summary

- Resistance in *Lolium multiflorum* to at least one ACCase-inhibiting herbicide is widespread in England as it has been detected in 35 (70%) out of 50 individual semi-randomly sampled farms included in the survey.
- Resistance to the herbicides diclofop-methyl and tralkoxydim is the most widespread as it was detected on 31 (62%) and 30 (60%) farms, respectively. Resistance to fluazifop-P-butyl was detected on 18 farms (36%) and resistance to cycloxydim and pinoxaden was less common and was detected only on 10 (20%) and 9 (18%) farms, respectively.
- The ACCase-inhibiting herbicides diclofop-methyl, fluazifop-P-butyl and tralkoxydim seem to be affected by both non-target site (probably enhanced metabolism) and ACCase target site resistance mechanisms, and possibly additional mechanisms too. Cycloxydim seems to be affected only by ACCase target site resistance.
- The ACCase-inhibiting herbicide pinoxaden seems to be affected mainly by ACCase target site resistance. It is possible that non-target site resistance mechanisms exist, but that they are not very widespread in *L. multiflorum* in the UK at present.
- 24% of the populations tested were found to be sensitive to all the herbicides assayed, 22% were found to be resistant to all the herbicides and 55% were found to be resistant only to diclofop-methyl, fluazifop-P-butyl and tralkoxydim.
- The most common mechanism of resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations appears not to be target site resistance but, most probably, enhanced metabolic resistance.
- Differences in the degree of resistance to the herbicides cycloxydim and pinoxaden were found between populations known to possess ACCase target

site resistance, thus indicating that not all the target site point mutations confer the same levels of resistance to the same herbicides.

- The rapid resistance test in Petri dishes could potentially be used to detect different degrees of resistance to pinoxaden in *L. multiflorum*.

Chapter 7 Molecular studies

7.1 Introduction

The selection pressure imposed by the intensive use of herbicides over many years has resulted in weed populations that have evolved resistance and that can no longer be controlled by herbicides at practical field rates. With the fast evolution of resistance in weeds, studies of resistance at the whole plant level are not sufficient to understand the mechanisms underlying this resistance. Molecular biology techniques have been widely used in weed science most notably for the development of crop plants resistant to certain herbicides, but also to gain a better understanding of how weeds compete and interact with other plants, survive harsh environmental conditions and evolve resistance to herbicides (Tranel & Horvath, 2009). Molecular techniques can therefore be used to gain a better understanding of the mechanisms conferring resistance to herbicides and ultimately help in the development of new control strategies.

This chapter details work carried out to investigate the molecular basis of resistance to acetyl CoA carboxylase inhibiting herbicides in *Lolium multiflorum* field populations. There are two main mechanisms conferring resistance to herbicides: target site based resistance (TSR) and non-target site based resistance (NTSR). TSR usually arises because of mutations in the gene encoding the herbicide target protein which result in a change in protein conformation, thus decreasing its affinity for herbicides. Conversely, NTSR can be caused by diverse mechanisms, the most common being an enhanced metabolic degradation of herbicide molecules, preventing the action of the herbicide on the target protein (Eerd *et al.*, 2003, Yuan *et al.*, 2007). Enhanced metabolic resistance can occur across several herbicide modes of action (Cummins *et al.*, 1999) and can affect other herbicide groups such as PSII inhibitors, AHAS inhibitors, dinitroanilines, thiocarbamates and synthetic auxins (Powles & Yu, in press).

In contrast, TSR only confers cross-resistance to herbicides targeting the same protein, although two different resistance mechanisms may co-exist in the same plant allowing it to present cross-resistance to herbicides with different modes of action (Letouzé & Gasquez, 2001).

In the work presented here only TSR was studied. Currently, a total of seven target site mutations have been reported to confer resistance to ACCase-inhibiting herbicides in several grass species (Table 7.1). All of them are within the CT domain of the chloroplastic ACCase gene. The first mutation reported was Ile-1781-Leu. The isoleucine to leucine substitution at the amino acid position 1781 is due to a nucleotide change from adenine (A) to either thymine (T) or cytosine (C) at position 5341 in the chloroplastic ACCase gene (Zagnitko *et al.*, 2001). The Ile-1781-Leu is the only one of these seven mutations that has been reported so far in *L. multiflorum* plants (White et al., 2005).

Different methods have been developed to detect mutations in the chloroplastic ACCase gene. Initially, mutations can be detected using 'direct sequencing' or 'cloning and sequencing' of DNA from resistant and susceptible plants. When the resistance mechanism is known and results from an insensitive ACCase caused by a single nucleotide polymorphism (SNP), DNA from resistant plants can be screened for some of the previously characterised SNPs. Several methods have been used elsewhere to detect known SNPs. A PCR-based allele-specific assay (ASA) was developed to detect the Ile-1781-Leu mutation in *L. rigidum*, *A. myosuroides* and *A. sterilis* (Délye *et al.*, 2002a, Délye *et al.*, 2002b, Tal & Rubin, 2004, Zhang & Powles, 2006a, Liu *et al.*, 2007), the Asp-2078-Gly and Ile-2041-Asn mutations in *A. myosuroides* and *A. sterilis* (Délye *et al.*, 2003, Délye *et al.*, 2005, Liu *et al.*, 2007), the Trp-2027-Cys and Trp-1999-Cys mutations in *A. sterilis* (Liu *et al.*, 2007) and the Gly-2096-Ala mutation in *A. myosuroides* (Délye *et al.*, 2005).

Cleaved amplified polymorphic sequence (CAPS) markers as well as derived cleaved amplified polymorphic sequence (dCAPS) markers, have been developed to screen for several SNPs, including Ile-1781-Leu, Asp-2078-Gly, Ile-2041-Asn and Cys-2088-Arg in *Lolium* spp, *A. myosuroides*, *A. fatua, P. paradoxa* and *S. viridis* (Kaundun & Windass, 2006, Zhang & Powles, 2006b, Yu *et al.*, 2007, Hochberg *et al.*, 2009). Real-time quantitative PCR assays for quantification of the Leu-1781 resistance allele in *Lolium* spp. have also been used (Kaundun *et al.*, 2006). This technique is suitable for the analysis of large numbers of samples to determine the frequency of a mutation rather than to detect mutations in specific samples, as the other methods mentioned previously.

In the studies described here, four different methodologies were carried out. DNA extracted from resistant plants from 54 *L. multiflorum* populations was screened for the presence of seven SNPs shown to confer resistance to ACCase-inhibiting herbicides using the CAPS, dCAPS and SNaPshot multiplex methods. In addition, RNA from plants from one resistant and one susceptible population was also extracted, in order to clone and sequence the CT domain of the chloroplastic ACCase gene.

There are several advantages to using CAPS and dCAPS markers as opposed to other methods. The CAPS and dCAPS methodology is very simple as it is carried out in three steps: PCR, selective restriction digestion and gel electrophoresis. The ASA method is also a very simple method that requires only two steps: PCR followed by gel electrophoresis. Both methods are fast and inexpensive as they use minimal equipment and reagents common in any molecular biology laboratory. However, the ASA method has some limitations as a single PCR assay cannot distinguish between homozygous and heterozygous mutant plants if there are two possible nucleotide substitutions, as in the case of the Ile-1781-Leu mutation. It can also generate false positives due to non-specific amplification. CAPS and dCAPS markers provide a more

reliable and accurate method than the ASA method as it can distinguish between homozygous and heterozygous individuals. However, this method also has its own limitations. It cannot discriminate between different nucleotide changes, thus risking the generation of false positive or negative samples (See sections 6.2.1.2 and 6.2.1.3). With these methods, ASA, CAPS and dCAPS, DNA can be screened for only one mutation in each assay. Using the SNaPshot multiplex method it is possible to discriminate between nucleotides and it also allows the screening of many samples for up to ten mutations in a single assay, which makes it faster and reduces the amount of DNA used. On the other hand this method is more expensive and more specific equipment is needed. As is the case with the ASA, CAPS and dCAPS methods, the SNaPshot multiplex assay will only detect previously known SNPs which have been set up on the analysis to be found. With the cloning and sequencing assays every mutation (previoulsy known or not) can be revealed but it is a more expensive and time-consuming technique that can be carried out practically in just a few samples.

The primary goal of the studies described in this chapter was to determine the mechanisms of resistance to ACCase-inhibiting herbicides in populations of *Lolium multiflorum* from England and to find the most common mutations responsible for target site resistance. A further aim was to discover more regarding the molecular basis of resistance in some *L. multiflorum* populations previously studied, including Wilts B1 and Pyl (Cocker *et al.*, 2001, White *et al.*, 2005). To achieve this, a series of experiments were carried out to screen field populations of *Lolium multiflorum* for herbicide resistance and to investigate the molecular basis of this resistance. The experiments at the whole plant level are described in Chapter 6 while the molecular work is described here.

Table 7.1. Seven SNPs known to confer resistance to ACCase-inhibiting herbicides. (*) Degree of resistance varies between grass species and herbicides. Not all herbicides have been assayed for all the mutations. Herbicides in the list are those that each author included in their own studies.

Amino Acid Substitution	Nucleotide change	Confers resistance to(*)	Grass species	References
Ile-1781-Leu	A to T/C	Diclofop-methyl Fenoxaprop Fluazifop-P-butyl Clodinafop Haloxyfop Butroxydim Sethoxydim Tralkoxydim Cycloxydim Clethodim Pinoxaden	*Lolium rigidum*	Zagnitko *et al*., (2001), Delye *et al*., (2002b) Tal & Rubin, (2004), Zhang & Powles, (2006a)
			Setaria viridis	Delye *et al*., (2002c)
			Alopecurus myosuroides	Delye *et al.,* (2002a), Delye *et al*.,(2002b), Delye *et al*.,(2008), Brown *et al*.,(2002), Petit *et al.,* (2009)
			Avena fatua	Christoffers *et al.,* (2002)
			L. multiflorum	White *et al.,* (2005)
			Avena sterilis	Liu *et al*., (2007)
			Lolium spp.	Yu *et al.,* (2007)
Trp-1999-Cys	G to T/C	Fenoxaprop	*A. sterilis*	Liu *et al*., (2007)
Trp-2027-Cys	G to T/C	Fenoxaprop Clodinafop Haloxyfop	*A. myosuroides*	Delye *et al.,* (2005), Delye *et al.,*(2008), Petit *et al.,* (2009)
			A. sterilis	Liu *et al*., (2007)
			Lolium spp.	Yu *et al.,* (2007)
Ile-2041-Asn	T to A	Fenoxaprop Clodinafop Haloxyfop	*A. myosuroides*	Delye *et al.,* (2003), Delye *et al*.,(2008), Petit *et al.,* (2009)
			L. rigidum	Delye *et al.,* (2003) Zhang & Powles, (2006b)
			A. sterilis	Liu *et al*., (2007)
			Lolium spp.	Yu *et al.,* (2007)
			Phalaris paradoxa	Hochberg *et al.,* (2009)
Asp-2078-Gly	A to G	Diclofop-methyl Fenoxaprop Fluazifop-P-butyl Clodinafop Haloxyfop Tralkoxydim Butroxydim Sethoxydim Clethodim Cycloxydim Pinoxaden	*A. myosuroides*	Delye *et al.,* (2005) Delye *et al*.,(2008) Petit *et al.,* (2009)
			A. sterilis	Liu *et al*., (2007)
			Lolium spp.	Yu *et al.,* (2007)
			Phalaris paradoxa	Hochberg *et al.,* (2009)
Cys-2088-Arg	T to C	Diclofop-methyl Fluazifop-P-butyl Clodinafop Haloxyfop Butroxydim Sethoxydim Tralkoxydim Clethodim Pinoxaden	*Lolium* spp.	Yu *et al.,* (2007)
Gly-2096-Ala	G to C	Diclofop-methyl Fenoxaprop Clodinafop Haloxyfop Clethodim	*A. myosuroides*	Delye *et al.,* (2005) Delye *et al*.,(2008)

7.2 Materials and methods

7.2.1 Ploidy analysis

In order to interpret the results of the genetic studies it is important to establish the ploidy levels of the populations. To determine the number of different ACCase copies present in each population, leaf samples from each *L. multiflorum* population used in the molecular studies were sent to the company "Plant Cytometry Services" (Postbus 299, 5480 AG Schijndel, the Netherlands). Samples were analysed as described in Chapter 2, section 2.7.13 and ploidy levels determined.

7.2.2 Screening for SNPs

7.2.2.1 Plant material

Two different groups of plants were screened for SNPs. DNA was extracted as described in Chapter 2, section 2.7.2. In the first group of plants, known populations were chosen to validate the methodology; DNA was extracted from 16 shoots of germinated seeds from each of the *L. multiflorum* populations Yorks A2, Pyl, G. Rising01, Wilts B1 and Trajan and the *Alopecurus myosuroides* population Notts. The *L. multiflorum* populations Yorks A2, Wilts B1 and Pyl were used as resistant standards as they had been previously studied for resistance and had been shown to be resistant to several herbicides. Wilts B1 was found to be resistant to the ACCase-inhibiting herbicides diclofop-methyl, fluazifop-P-butyl and tralkoxydim and Yorks A2 and Pyl were resistant to these same herbicides as well as cycloxydim and sethoxydim (Cocker et al., 2001, White et al., 2005). Yorks A2 had also been shown to posses the Ile-1781-Leu mutation. G. Rising01 is a resistant *L. multiflorum* population from Peldon farm known to possess resistance to ACCase-inhibiting herbicides. The susceptible

standard used was Trajan, a commercial cultivar used previously (Cocker et al., 2001, White et al., 2005), which is sensitive to all the herbicides studied. The *A. myosuroides* population Notts was also included in these studies as a resistant standard known to possess the Ile-1781-Leu mutation (Brown *et al.*, 2002, Moss *et al.*, 2003). In this assay plants were screened for only two SNPs, Ile-1781-Leu and Asp-2078-Gly, using dCAPS markers (Neff *et al.*, 1998, Kaundun & Windass, 2006). This first work to validate the methodology was carried out at Jealott's Hill, Syngenta.

The second group of plants screened were a selection of the resistant plants that resulted from the glasshouse assay described in Chapter 6, section 6.2.1.4 and listed in Table 2.1 in Chapter 2. In this second group DNA was extracted from dry leaves of a total of 384 resistant plants from 54 *L. multiflorum* populations. The number of samples assayed per population varied depending on the number of plants surviving treatments and ranged between one and sixteen samples. Populations Yorks A2 and Wilts B1 were used as target site and enhanced metabolism resistance standards, respectively. CAPS or dCAPS markers were used to screen for three SNPs, Ile-1781-Leu, Asp-2078-Gly and Cys-2088-Arg and then a high throughput SNaPshot multiplex method (Hurst *et al.*, 2009) was used to screen for these three SNPs as well as four other SNPs known to confer resistance to ACCase inhibitors. Two additional primers were included to screen for two SNPs found in the *L. multiflorum* population Hay Shed in the experiment described in section 7.2.2. This second study was carried out at Rothamsted Research.

7.2.2.2 dCAPS analysis

The procedure followed to carry out the dCAPS analysis is described in Chapter 2, section 2.7.4. To detect the Ile-1781-Leu point mutation a dCAPS marker was provided by Kaundun & Windass (2006) (Table 7.2). The reverse primer introduces an A to G

mismatch that creates a restriction site for *Nsi*I in the susceptible sequence (Figure 7.1).

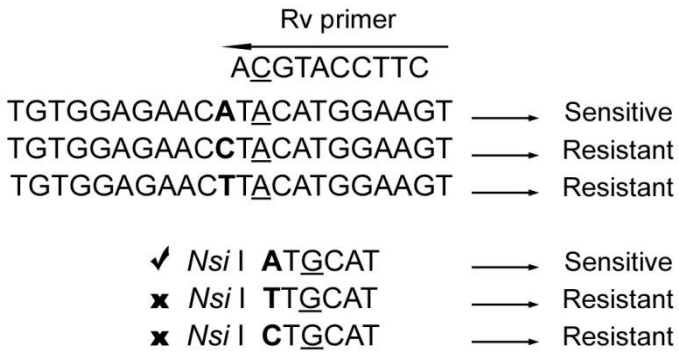


Figure 7.1. Sequence polymorphisms between wild type and mutant individuals for the Ile-1781-Leu point mutation. The position of the SNP is marked in bold and the position where the mismatch to create a restriction site for the enzyme is introduced is underlined.

The PCR will amplify fragments of 165 bp. After the digestion with the enzyme *Nsi*I, the homozygous-resistant individuals will have an undigested band of 165 bp. The homozygous-sensitive individuals will have a digested band of 130 bp and heterozygous individuals will have both bands. In the case of Ile-1781-Leu mutation this assay presents some limitations. This assay identifies the susceptible Ile allele at this position but does not positively identify the resistant allele. This means that the enzyme will digest the sequence when the codon ATA will be present but will not digest it when TTA, CTA or GTA will be present. TTA and CTA are coding sequences for leucine, which changes the protein conferring resistance. However, GTA encodes for valine which would be identified as resistant while this change in the protein in this position has not been reported to confer resistance.

The dCAPS marker used to detect the Asp-2078-Gly point mutation was provided by Syngenta (Table 7.2). The reverse primer introduces a G to C mismatch that creates a restriction site for the enzyme *Rsa*I in the resistant sequence (Figure 7.2). The primers will amplify a 181 bp fragment. Following *Rsa*I digestion, individuals with homozygous-

sensitive alleles will have an undigested band of 181 bp, while homozygous-resistant individuals will have a digested band of 146 bp. Individuals with both susceptible and resistant alleles will have two bands. This assay positively identifies the resistant allele but the possibility of different substitutions at this position exists which would test as false negatives if they conferred resistance.

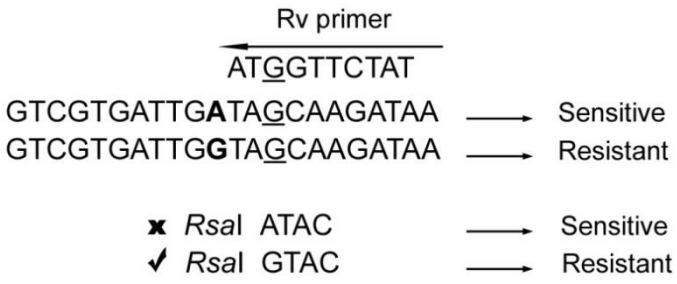


Figure 7.2. Sequence polymorphism between wild type and mutant individuals for the Asp-2078-Gly point mutation. The position of the SNP is marked in bold and the position where the mismatch to create a restriction site for the enzyme is introduced is underlined.

7.2.2.3 CAPS analysis

The procedure to carry out the CAPS analysis is described in Chapter 2, section 2.7.4. The marker used to detect the Cys-2088-Arg point mutation was also provided by Syngenta (Table 7.2). The nucleotide change from T to C, which causes a Cys to Arg amino acid change, creates a restriction site for the enzyme *Hha*I in the resistant sequence (Figure 7.3).

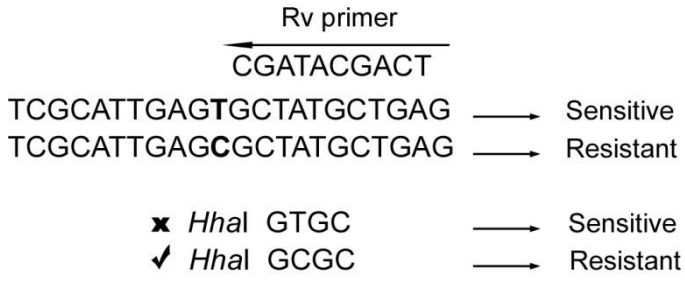


Figure 7.3. Sequence polymorphism between wild type and mutant individuals for the Cys-2088-Arg point mutation. The position of the SNP is marked in bold.

Thus, there is no need to introduce a mismatch in one of the primers and a CAPS analysis can be carried out instead of a dCAPS. The PCR will amplify fragments of 161 bp. Following *Hha*I digestion, individuals with homozygous-sensitive alleles will have an undigested band of 161 bp while homozygous-resistant individuals will have a digested band of 126 bp. Heterozygous individuals will have both bands. This assay positively identifies the resistant allele but there is also the possibility of having different substitutions at this position which would be identified as false negatives if they conferred resistance. The change from TGC to AGC/GGC would induce an amino acid change from cysteine to serine/glycine that would be detected as susceptible when it is not known if these changes confer resistance.

Table 7.2. Primer sequences for three dCAPs and CAPS markers to detect three of the seven mutations known to confer resistance to ACCase inhibitors.

SNP	Species	Primer	Sequence
Ile-1781-Leu	*Lolium* spp.	Forward	5’-CTGTCTGAAGAAGACTATGGCCG-3’
	A. myosuroides	Forward	5'-ATGACTGACGAAGACCATGATCG-3'
	A. myosuroides and *Lolium* spp.	Reverse	5’-AGAATACGCACTGGCAATAGCAGCACTTCCATGCA-3’
Asp-2078-Gly	*A. myosuroides and L. multiflorum*	Forward	5’-TTCTCTGGTGGGCAAAGAGACCTTTTTGAAGG-3’
	A. myosuroides and L. multiflorum	Reverse	5’-CATAGCACTCAATGCGATCTGGATTTATCTTGGTA-3’
Cys-2088-Arg	*L. multiflorum*	Forward	5’-TCAACAATTGTTGAGAACCTTAGG-3’
	L. multiflorum	Reverse	5’-AGAACATTCCCTTTTGCAGTTGTCTCAGCATAGC-3’

7.2.2.4 SNaPshot multiplex method

The SNaPshot multiplex method (Applied Biosystems) is a primer extension-based method that enables multiplexing of up to ten SNPs at known locations on multiple DNA templates in a single tube. This is the first time this technique has been applied on ACCase resistance mutation detection. DNA templates were amplified using the primers CT-Domain Fw1 and CT-Domain Rv1 (Table 7.4) and the conditions and

components given in Chapter 2, section 2.7.8.1. An EXOSAP treatment was carried out to purify the PCR product as described in Chapter 2, section 2.7.8.2. The SNaPshot multiplex reaction was carried out using pooled primers with the concentrations given in Table 7.3 and the components and conditions given in Chapter 2, section 2.7.8.3. SNaPshot multiplex primers were designed to detect the seven SNPs known to confer resistance to ACCase-inhibiting herbicides as well as two SNPs found after cloning and sequencing individuals from one resistant *L. multiflorum* population (See section 6.3.3). Primers were designed so that their 3´ends were positioned one base pair upstream of the target SNP. The primers varied in length from 20 to 46 nucleotides to allow separation by capillary electrophoresis. Prior to loading, SNaPshot reactions were treated with calf intestinal phosphatase to prevent comigration of unincorporated labelled nucleotides. Samples were electrophoresed as described in Chapter 2, sections 2.7.8.4 and 2.7.8.5. Genotypes were scored using GeneMapper 4.0 software (Applied Biosystems).

Table 7.3. List of SNaPshot primers and concentration for each primer in the pool. Polymorphisms for the 2041, 2078 and 2088 markers are given on the reverse strand. Nucleotide translation: r=A/G; y=C/T; w=A/T; s=C/G.

SNP	Primer	Sequence 5'-3'	Change	Concentration (μM)
2096	*Forward*	TrCTGAGACAACTGCAAAAG	G/C	2.5
1781	*Forward*	GGATGGACTAGGTGTGGAGAAC	A/T/C	2.5
2078	*Reverse*	CAATGCGATCTGGATTTATCTTGCTA	T/C	2.5
2088	*Reverse*	CCCTTTTGCAGTTGTCTCAGyATAGC	A/G	5
695	*Forward*	TTGACCrTCTGACTGTTCsAGATGACCTTG	A/C	2.5
706	*Forward*	TTTTACTGTTCSAGATGACCTTGMAGGTGTTTCT	A/C	2.5
1999	*Forward*	TTTTTTTAGCGGTCyGwTCCTCsTGCTGGGCAAGTCTG	T/C/G	2
2027	*Forward*	TTTTTTTTTTGAAGGGTTACCTCTrTTCATCCTTGCTAACTG	T/C/G	1
2041	*Reverse*	TTTTTTTTTTTTTTTTTTTTTAACAAwTGTTGATCCAGCCTGCAGA	A/T	5

7.2.2.5 Sequencing

To confirm genotypes, the CT domain of the chloroplastic ACCase gene from a selection of samples was sequenced using the primers CT-Domain Fw1, CT-Domain Rv1, CT400F, CT800F and CT1600R (Table 7.4), and analysed as described in Chapter 2, sections 2.7.9 and 2.7.11.

Table 7.4. List of primers used to clone and sequence the ACCase CT-Domain in *L. multiflorum* plants.

Primer	Sequence
CT-Domain Fw1	5′-GGTATGGTAGCCTGGATCTTGGAC-3′
CT-Domain Rv1	5′-ATGGAAAGACCCTGCGGCAAG-3′
M13F-20	5′-GTAAAACGACGGCCAGT-3′
M13R-24	5′-GGAAACAGCTATGACCATG-3′
CT400F	5′-GTGGGCAAGGAGGATGGACT-3′
CT800F	5′- AGACAGACCTGTTGCATACA-3′
CT1600R	5′-CTTGTAGAAGAAAGACCGAG-3′

7.2.3 Cloning and sequencing of the CT-domain of the chloroplastic ACCase gene from a selection of resistant and susceptible *Lolium multiflorum* plants

7.2.3.1 Plant material

Seven resistant and five susceptible plants from the glasshouse assay described in section 6.3.1.3 were selected for sequencing. Plants from the population most resistant to fluazifop-P-butyl, Hay Shed, and from the susceptible standard Trajan were used. Populations highly resistant to fluazifop-P-butyl but not to cycloxydim and pinoxaden were selected and, within them, Hay Shed was the most resistant to fluazifop-P-butyl. The mechanism conferring resistance to fluazifop-P-butyl remains unclear. It is thought to be affected to some degree by enhanced metabolism but a fop-specific mutation

could also be responsible for the resistance to this herbicide in some populations. The aim of this experiment was to elucidate this mechanism, so DNA from the population most likely to present a fop-specific mutation was extracted and the CT domain of the chloroplastic ACCase gene was cloned and sequenced and compared with sequences from a susceptible population.

In the work carried out at Jealott's Hill (Syngenta) two plants from the population Hay Shed were used. In the work carried out at Rothamsted Research five plants from each population, Hay Shed and Trajan, were used.

7.2.3.2 Sequencing procedure

RNA was extracted as described in Chapter 2, section 2.7.2. and was reverse transcribed into cDNA followed by a standard PCR amplification using primers CT-Domain Fw1 and CT-Domain Rv1 (Table 7.4). Conditions are described in Chapter 2, section 2.7.5. PCR products were run on an agarose gel and the PCR product of interest was extracted and purified as described in Chapter 2, section 2.7.6. Purified *taq*-polymerase-amplified fragments were ligated into the Strataclone PCR cloning vector pSC-A-amp/kan (Figure 7.4) and transformed into Strataclone SoloPack competent *E. coli* cells from Stratagene, as described in Chapter 2, section 2.7.7.

Transformants were selected using blue-white screening and positive colonies confirmed by PCR using primers CT-Domain Fw1 and CT-Domain Rv1 (Table 7.4). Single, positive transformants were cultured overnight and plasmid DNA purified. Positive cloned inserts were again confirmed by *Eco*RI digestion followed by agarose gel electrophoresis. Reaction components and PCR conditions are given in Chapter 2. Purified plasmid DNA samples were sent to be sequenced with primers M13F-20,

M13R-24 and the custom-designed sequencing primers CT400F, CT800F and CT1600R (Table 7.4).

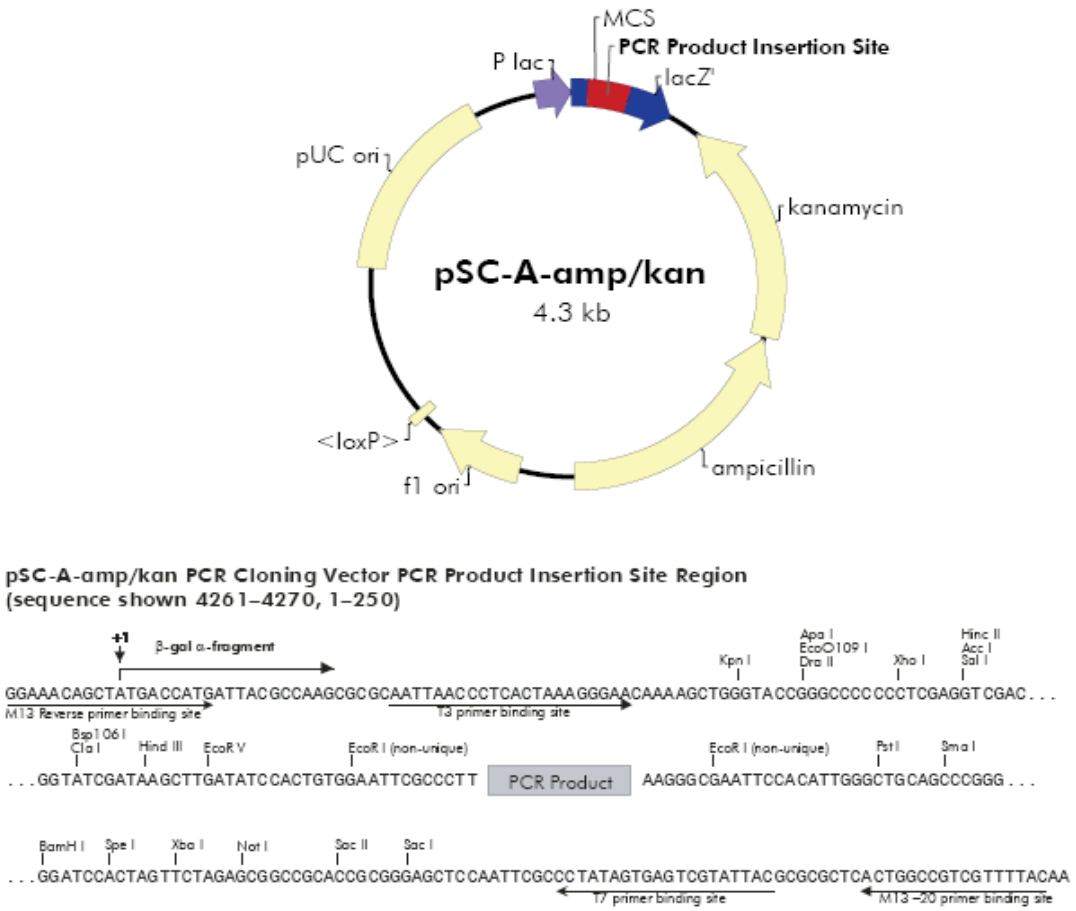


Figure 7.4. Map for the StrataClone PCR Cloning Vector pSC-A-amp/kan. *Eco*RI has two restriction sites on both sides of the insert, which is useful to confirm the presence of the insert.

7.3 Results

7.3.1 Ploidy analysis

Most of the *L. multiflorum* populations used for these studies were diploid with one population being tetraploid (Rothamsted) and another one (Manor) having a mix of diploid (2n=2x=14 chromosomes), tetraploid (2n=4x=28 chromosomes) and aneuploid (2n=26 chromosomes) plants. This had no implications as none of these two populations showed resistance to any of the herbicides.

7.3.2 **Screening for SNPs**

7.3.2.1 Allele frequency for the Ile-1781-Leu and Asp-2078-Gly point mutations in the L. multiflorum populations Yorks A2, Wilts B1, Pyl and G. Rising, and the A. myosuroides population Notts.

As expected, the Ile-1781-Leu mutation was identified in the *A. myosuroides* population Notts and the *L. multiflorum* population Yorks A2. After digestion with the enzyme *Nsi*I, two different restriction digest patterns were found.

Heterozygous individuals with both digested and undigested bands and homozygous-resistant individuals with an undigested band of 165 bp were found. No homozygous-sensitive individuals with a digested band of 130 bp were found (Figure 7.5).

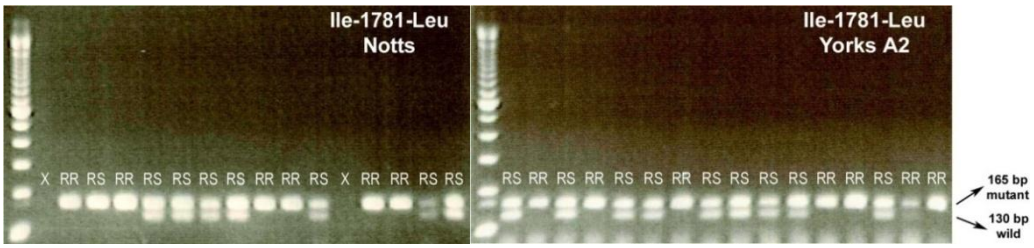


Figure 7.5. dCAPS Ile-1781-Leu: The PCR generated a product of 165 bp for all samples. After digestion with *Nsi*I the alleles carrying an adenine residue at nucleotide position 5341 generated a fragment of 130 bp.

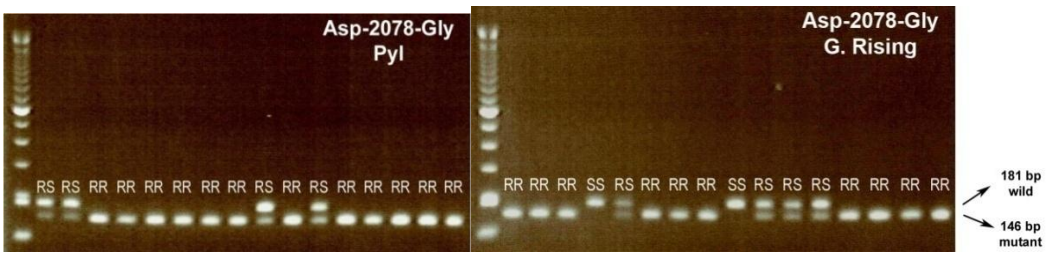


Figure 7.6. dCAPS Asp-2078-Gly: The PCR generated a product of 181 bp for all samples. After digestion with *Rsa*I the alleles carrying a guanine residue at nucleotide position 6233 generated a fragment of 146 bp.

The Asp-2078-Gly mutation was identified in the *L. multiflorum* populations Pyl and G. Rising01. Three different restriction digest patterns were found after digestion with *Rsa*I: Homozygous-resistant individuals with a digested band of 146 bp, heterozygous individuals with both digested and undigested bands and homozygous-sensitive individuals with an undigested band of 181 bp (Figure 7.6).

Table 7.5. Genotypic distribution and allelic frequency of the Ile-1781-Leu mutation. Number in parenthesis is the number of plants analysed for each population.

	Genotypic distribution			Allelic frequency	
Populations	Ile/Ile	Ile/Leu	Leu/Leu	Ile	Leu
Wilts B1 (16)	1	0	0	1	0
Yorks A2 (16)	0	0.560	0.440	0.280	0.720
G. Rising (16)	1	0	0	1	0
Pyl (16)	1	0	0	1	0
Notts (14)	0	0.500	0.500	0.250	0.750
Trajan (16)	1	0	0	1	0

Table 7.6. Genotypic distribution and allelic frequency of the Asp-2078-Gly mutation. Number in parenthesis is the number of plants analysed for each population.

	Genotypic distribution			Allelic frequency	
Populations	Asp/Asp	Asp/Gly	Gly/Gly	Asp	Gly
Wilts B1 (16)	1	0	0	1	0
Yorks A2 (16)	1	0	0	1	0
G. Rising (16)	0.125	0.250	0.625	0.250	0.750
Pyl (16)	0	0.250	0.750	0.125	0.875
Notts (16)	1	0	0	1	0
Trajan (16)	1	0	0	1	0

Neither of these two mutations was found in the population Wilts B1, which was also the case in Trajan, as expected.

Within the populations Yorks A2 and Nott, 100% of plants were homozygous-resistant (Leu/Leu) or heterozygous (Ile/Leu) for the Ile-1781-Leu mutation although there were differences between these populations in genotypic distribution and the allelic frequency (Table 7.5). The genotypic distribution and the allelic frequency of the Asp-

2078-Gly mutation were also different for the two populations G. Rising and Pyl (Table 7.6). In the population Pyl 100% of plants were homozygous-resistant (Gly/Gly) or heterozygous (Asp/Gly) while in the population G Rising01 87.5% of plants (14 plants out of 16) were homozygous-resistant (Gly/Gly) or heterozygous (Asp/Gly).

7.3.2.2 Detection of three target site mutations, Ile-1781-Leu, Asp-2078-Gly and Cys-2088-Arg in Lolium multiflorum populations from 50 farms using dCAPS markers

DNA was extracted from 384 resistant plants from the glasshouse assay described in Chapter 6, section 6.2.1.2. This assay was carried out with 55 *L. multiflorum* populations from 50 farms in England collected in 2006 and 2007. Three standard populations were also included: Yorks A2 as an ACCase target site resistant standard, Wilts B1 as an enhanced metabolism resistant standard and Trajan as a susceptible standard. Results from the glasshouse assay showed confirmed resistance to at least one herbicide in 38 populations from 35 farms. However, there was at least one surviving plant in 52 populations. The three populations with no survivors were Rothamsted, Cinder and Lewis. The susceptible standard Trajan was also completely controlled. Thus, plants from 52 populations and the two resistant standards were analysed. The number of plants analysed from each population ranged from 1 to 16 depending on the proportion of surviving plants. Among those populations analysed, the Ile-1781-Leu mutation was found in survivors from six populations, Wickford, Galons, B.LandsL2, Charleston, N. Benfleet and Adams (Figure 7.7).

The Ile-1781-Leu mutation was also found in one plant from the population G. Rising but it could not be confirmed in further analyses, which indicates that there may have been cross-contamination with this sample. This confirmation was important as only the Ile-1781 allele was positively identified. The change from adenine (A) to thymine

(T) or cytosine (C) was assumed in those samples that were not digested but there was also the possibility of a change for adenine (A) to guanine (G) that would result in a change from isoleucine to valine.

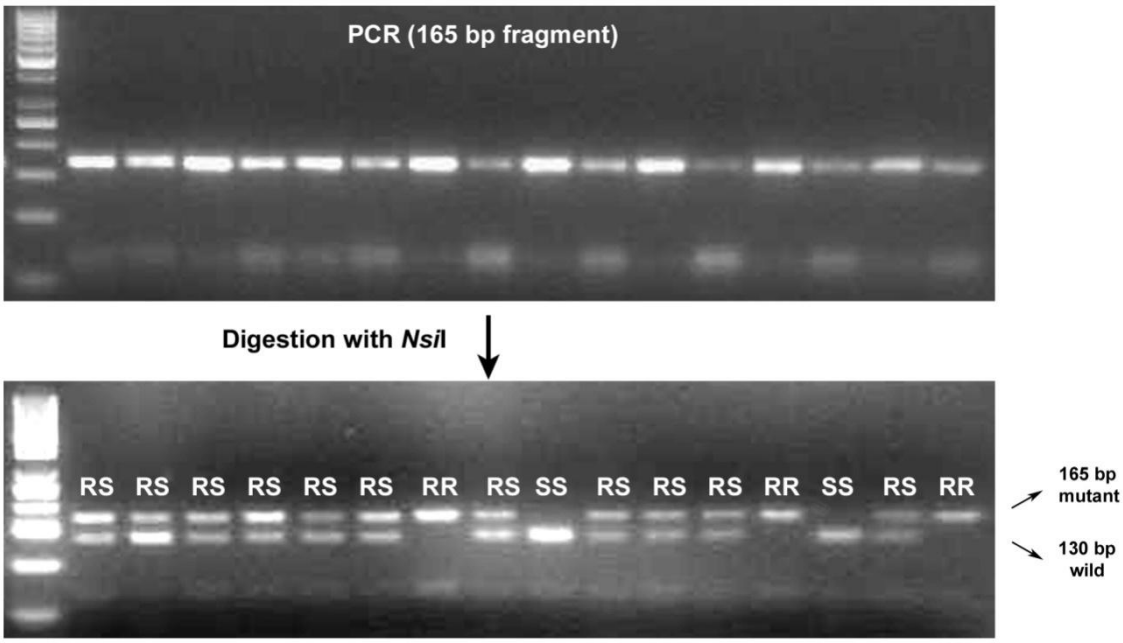


Figure 7.7. Representative result of dCAPS Ile-1781-Leu analysis of plants from the *L. multiflorum* population Adams: The PCR generated a product of 165 bp for all samples. After digestion with *Nsi*I the alleles carrying an adenine residue at nucleotide position 5341 generated a fragment of 130 bp.

The Asp-2078-Gly mutation was found in nine populations, Geedon, Wallraven, Wickford, B.LandsL2, Court, N. Benfleet, Clements, Oaktree and G. Rising (Figure 7.8). Some of these populations including Wickford, B.LandsL2 and N. Benfleet, also had the Ile-1781-Leu mutation, sometimes in the same plant. This seemed to be associated with higher levels of resistance to herbicides since Wickford, the only population with a high proportion of both mutations, was one of the most resistant populations. In this case, the resistant Gly-2078 allele was positively identified but nucleotide changes that would result in a change in amino acid other than aspartate or

glycine, would be identified as susceptible when it would not be known if they confer resistance or not.

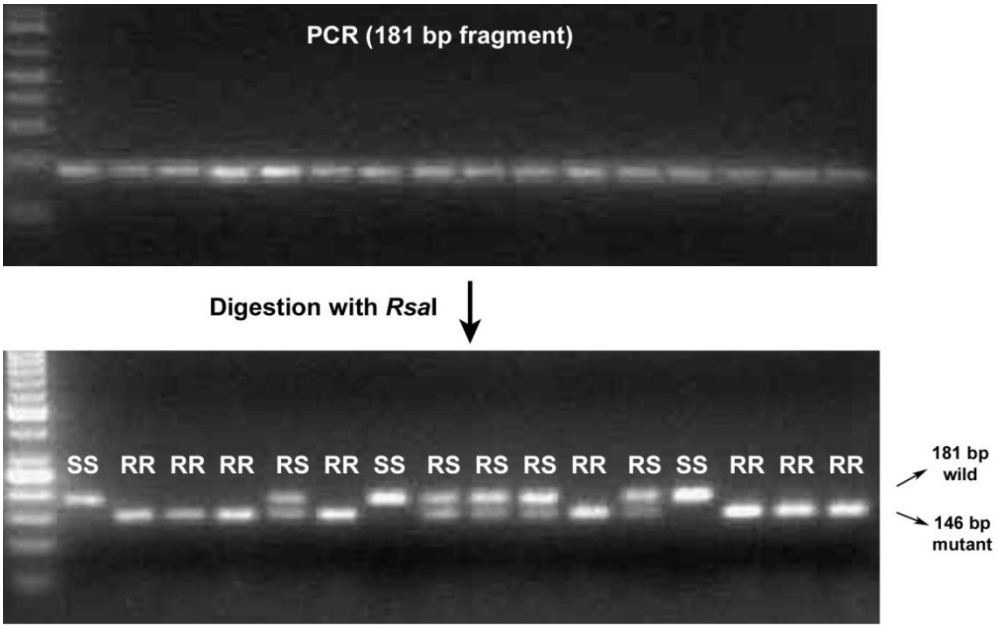


Figure 7.8. Representative result of dCAPS Asp-2078-Gly analysis of plants from the *L. multiflorum* population G. Rising (2006): The PCR generated a product of 181 bp for all samples. After the digestion with *Rsa*I the alleles carrying a guanine residue at nucleotide position 6233 generated a fragment of 146 bp.

The Cys-2088-Arg mutation was identified in just one population, Dairy (Figure 7.9). In this case the resistant Cys-2088 allele was also positively identified but there could be false negatives if different nucleotide changes were present and they conferred resistance.

As expected, the Ile-1781-Leu mutation was also found in survivors from the ACCase target site resistant standard population Yorks A2. None of these mutant alleles were found in the enhanced metabolism resistant standard Wilts B1.

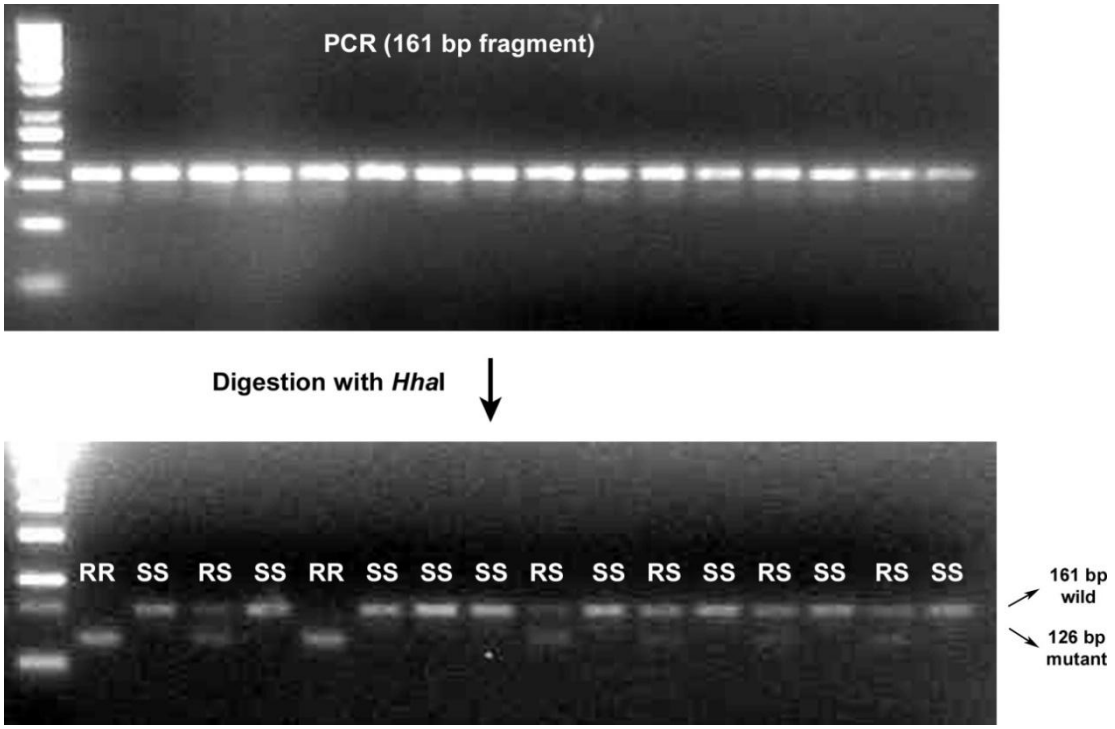


Figure 7.9. dCAPS Cys-2088-Arg of the *L. multiflorum* populations Dairy and Hay Shed: The PCR generated a product of 161 bp for all samples. After digestion with *Hha*I the alleles carrying a arginine residue at nucleotide position 6262 generated a fragment of 126 bp.

7.3.2.3 Detection of seven target site mutations in Lolium multiflorum populations from 50 farms using the SNaPshot multiplex method

The DNA extracted from 384 resistant plants from the glasshouse assay described in Chapter 6, 6.2.1.2 were re-analysed using SNaPshot multiplex to screen for the seven target site mutations known to confer resistance to ACCase-inhibiting herbicides (Figure 7.10). This study confirmed the results from the dCAPS and CAPs assays, except for one plant of the population G. Rising. This plant was found to have both Ile-1781-Leu and Asp-2078-Gly mutations with the dCAPS method, while only the Asp-2078-Gly mutation was found using the SNaPshot multiplex method. This plant was later sequenced and the absence of the Ile-1781-Leu allele was confirmed.

Within the 384 plants analysed all target site mutations, except Gly-2096-Ala, were found in at least one plant. The most common mutations were the Asp-2078-Gly

mutation that was found in 94 plants (24.5%) and nine populations, and the Ile-1781-Leu mutation that was found in 51 plants (13.3%) and seven populations. The Cys-2088-Arg mutation was found in seven plants (1.8%) all of which were within a single population, Dairy. The Ile-2041-Asn mutation was found in eight plants (2.1%) that belonged to three populations, Geedon, G. Rising and Dairy. The Trp-2027-Cys mutation was found in four plants (1.0%) from three populations Chat1, Pidley and the enhanced metabolism resistant standard Wilts B1. The Trp-1999-Cys mutation was found in just one plant (0.3%) from the population Court.

Using this method it was also possible to discriminate between the different nucleotide changes (Figure 7.10) which was not possible using dCAPS and CAPS markers. Within the populations that had the Ile-1781-Leu mutation, two of them, Wickford and B.LandsL2, had a nucleotide change from adenine (A) to cytosine (C), while the other five populations had a nucleotide change from adenine (A) to thymine (T). Within the populations with the Trp-2027-Cys mutation, Chat1 and Pidley had a nucleotide change from guanine (G) to thymine (T), while Wilts B1 had a nucleotide change from guanine (G) to cytosine (C). The only plant from the population Court that had the Trp-1999-Cys mutation had a nucleotide change from guanine (G) to thymine (T). Seven populations had two mutations, sometimes in the same plant in which case they were always heterozygous for both mutations.

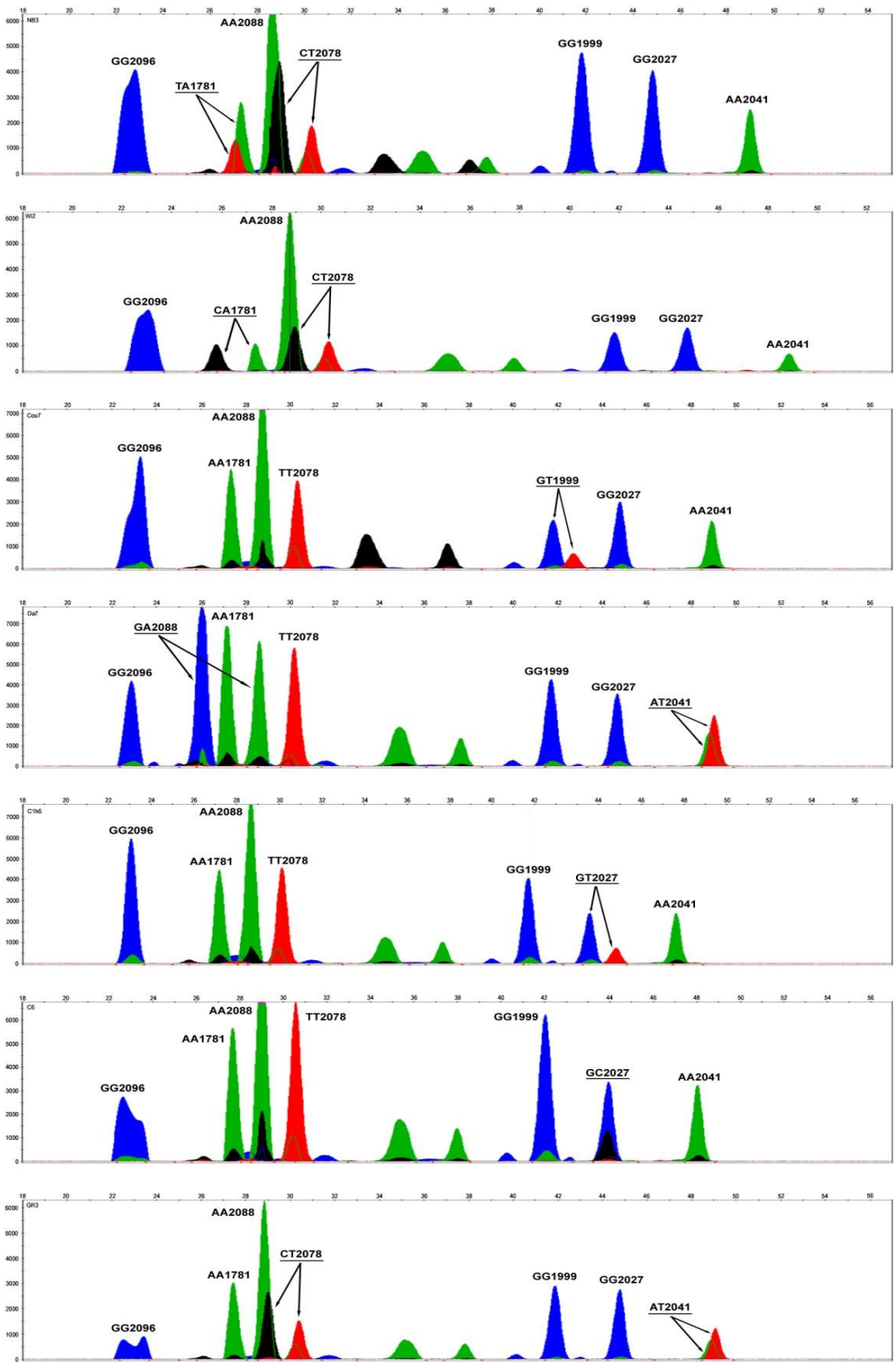


Figure 7.10. SNaPshot detection of the seven mutations known to confer resistance to ACCase-inhibiting herbicides in grass species. Mutant type SNPs are underlined. Polymorphisms for the 2041, 2078 and 2088 markers are given on the reverse strand. Examples from populations N. Benfleet (NB3), Wickford (Wi2), Court (Cou7), Dairy (Da7), Chat1 (C1h6), Wilts B1 (C6) and G. Rising (GR3).

These populations were Geedon and G. Rising with the Asp-2078-Gly and the Ile-2041-Asn mutations, Wickford, N. Benfleet and B.LandsL2 with the Asp-2078-Gly and the Ile-1781-Leu mutations, Court with the Asp-2078-Gly and the Trp-1999-Cys mutations and Dairy with the Cys-2088-Arg and the Ile-2041-Asn mutations.

In order to validate the results from the SNaPshot multiplex assay, direct sequencing of the CT domain of the ACCase gene was carried out on at least one sample from those carrying any of the mutations that were not screened previously with the dCAPS and CAPS assays. It was also carried out with some samples where no mutation had been found. These samples belonged to the populations G. Rising, Thornham, Wallraven, B.LandsL2, Chat1, Chat2, Wilts B1, Wood, Geedon, Yorks A2, Pidley, Cott, Low, Long and Court (Figure 7.11).

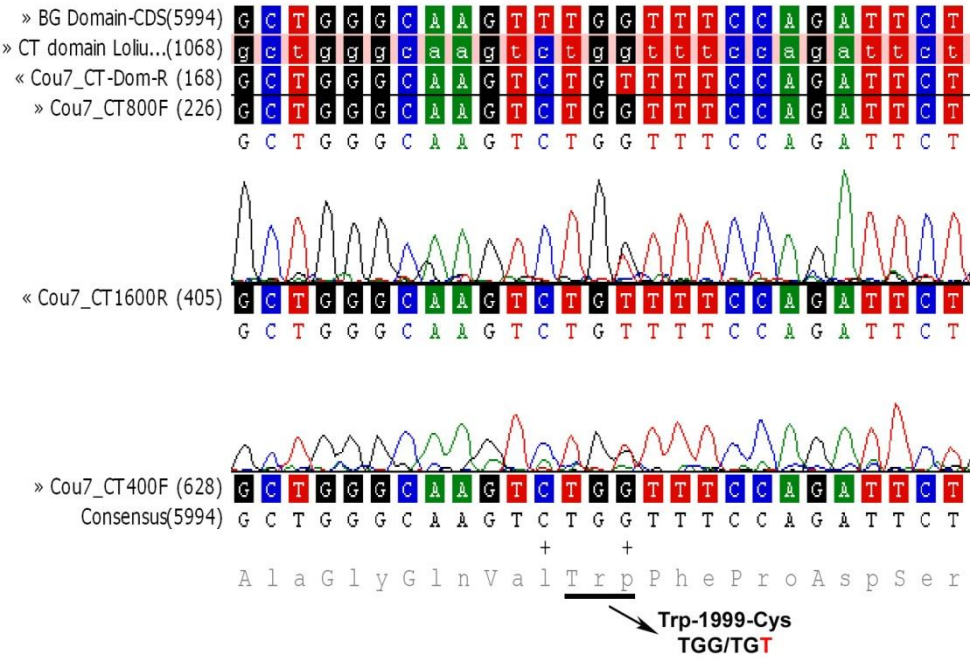


Figure 7.11. Alignment of acetyl CoA carboxylase partial nucleotide sequences centred on the 5997 nucleotide point mutation (equivalent to Trp-1999-Cys mutation) of a herbicide resistant *L. multiflorum* plant from the population Court and GeneBank sequences of *L. multiflorum* (AY710293) and *A. myosuroides* (AJ310767).

Of all the 384 resistant plants analysed 155 (40%) had at least one mutation while 229 (60%) plants carried none of these seven ACCase point mutations. In most of these plants resistance could be explained by a non-target site resistance mechanism, most likely enhanced metabolism, as 295 plants (76.8%) were resistant to diclofop-methyl, fluazifop-P-butyl or tralkoxydim, herbicides known to be affected at least partially by enhanced metabolic resistance.

Within the 89 plants resistant to pinoxaden or cycloxydim, four plants from the populations G. Rising, Court, Wilts B1 and Chat1 showed resistance to pinoxaden and had the resistant alleles TA2041, GT1999, GC2027 and GT2027, respectively. On the other hand, within this same group of plants resistant to one of these two herbicides, pinoxaden and cycloxydim, there were seven plants where none of these seven known mutations was found. These plants belonged to the populations Cott, Hill, Maid, School, Wallraven and Thornham. In most cases plants were resistant to pinoxaden but showed clear symptoms of herbicide damage and were given an injury score of 2 (See Chapter 2, section 2.6.4). However, in the population Wallraven one of the plants survived the treatment with pinoxaden and showed no symptoms of herbicide damage and in the population Thornham one of the plants survived the treatment with cycloxydim although showing some symptoms of herbicide damage.

The CT domain of the ACCase gene of plants from these populations was sequenced and a potentially novel point mutation was found in the population Thornham. This point mutation is a thymine (T) to adenine (A) change in the nucleotide position 6230 corresponding to a change from isoleucine (Ile) to asparagine (Asn) in amino acid position 2077 (Figure 7.12). This mutation in the ACCase gene is just before the Asp-2078-Gly mutation and according to the BLOSUM62 substitution matrix (http://www.uky.edu/Classes/BIO/520/BIO520WWW/blosum62.htm) this amino acid

substitution is likely to cause a change in protein structure. Further studies will be required to confirm the importance of this nucleotide change.

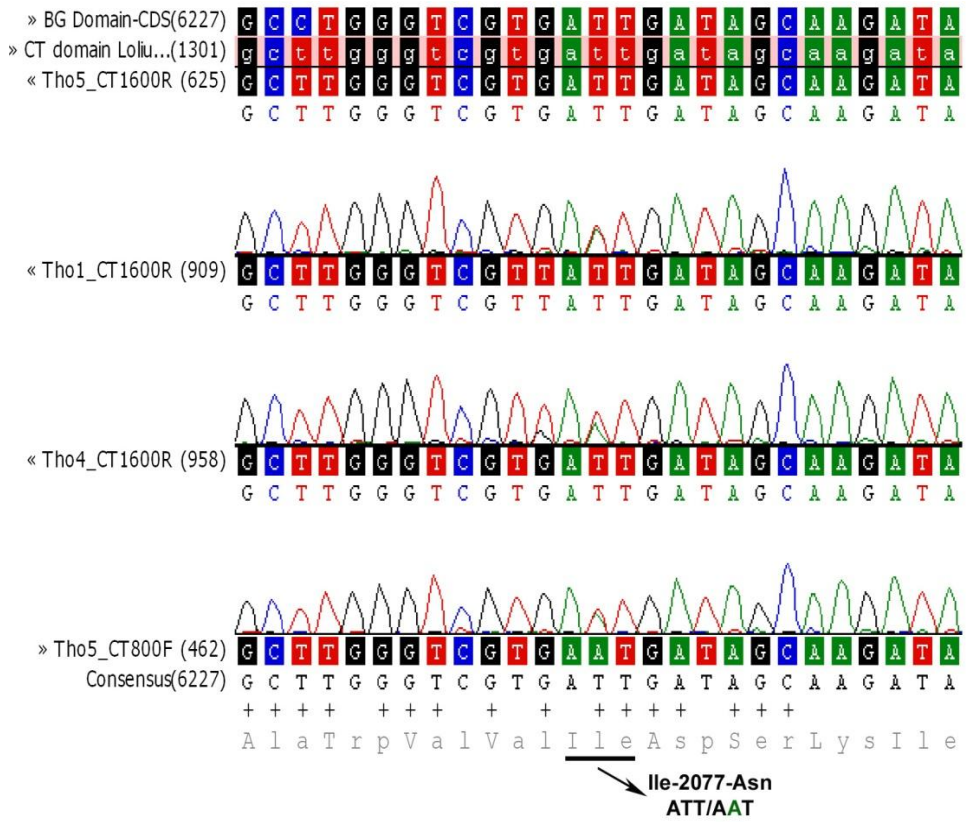


Figure 7.12. Alignment of acetyl CoA carboxylase partial nucleotide sequences centred around the 6230 nucleotide position, which corresponds with the amino acid position 2077, of several herbicide resistant *L. multiflorum* plants from the population Thornham and GeneBank sequences of *L. multiflorum* (AY710293) and *A. myosuroides* (AJ310767).

7.3.3 Cloning and sequencing of the CT-domain of the chloroplastic ACCase gene from a selection of resistant and susceptible *Lolium multiflorum* plants

Thirty-two clones from resistant plants (H) and 17 clones from sensitive plants (S) of the CT domain of the ACCase gene were sequenced and assembled using ContigExpress for comparison (Figure 7.13). The contig of DNA fragments amplified by the primers M13F-20, M13R-24, CT400F, CT800F and CT1600R (Table 7.4) resulted in a coding sequence 1973 bp in length. This partial *L. multiflorum* chloroplastic

ACCase sequence corresponds to nucleotide positions 4927 to 6899 of *A. myosuroides* full chloroplastic ACCase EMBL accession AJ310767 (Délye *et al.*, 2002a).

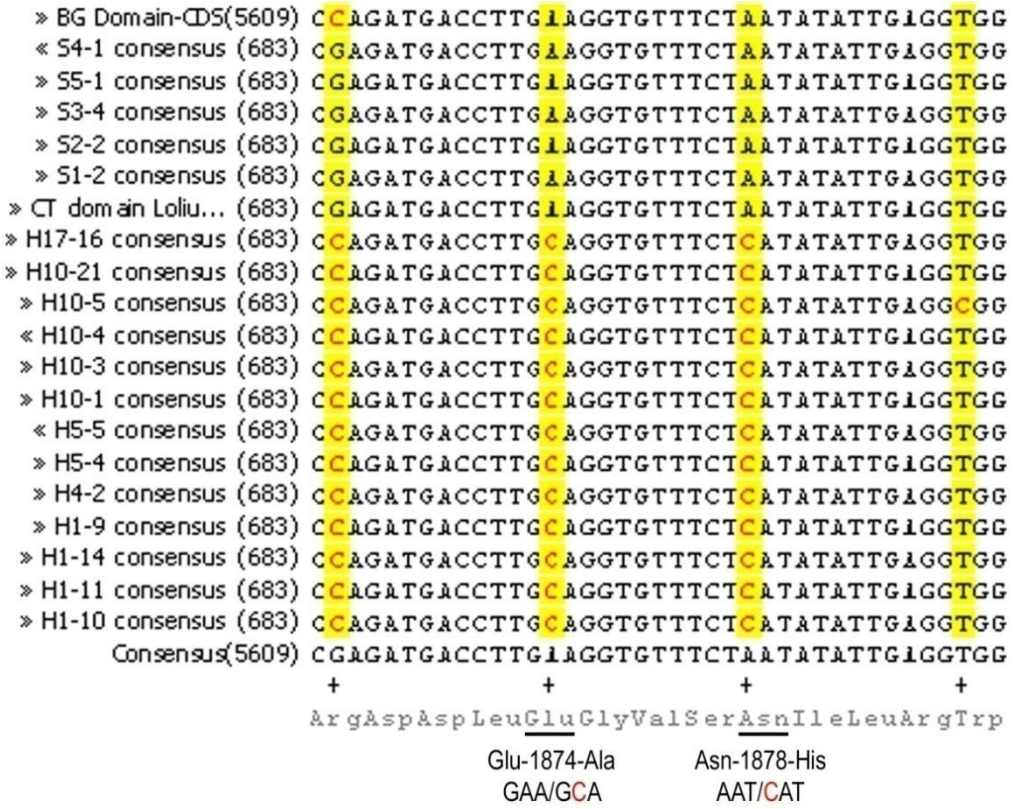


Figure 7.13. Alignment of nucleotide sequences of *L. multiflorum* clones from the population Hay Shed (H) showing the nucleotide changes in nucleotide positions 5621 (Glu-1874-Ala) and 5632 (Asn-1878-His) of the CT-Domain of the acetyl CoA carboxylase gene compared to susceptible clones from the standard Trajan (S) and GeneBank sequences of *L. multiflorum* (AY710293) and *A. myosuroides* (AJ310767).

A total of 90 polymorphic sites were found in the coding sequence. Because of the error-rate inherent in *taq* polymerase amplification, many of these polymorphisms are likely to represent PCR errors rather than genuine polymorphisms. In those instances where a polymorphism was found in a single clone, this was considered a PCR error. Only those mutations which were replicable in at least two clones were studied. Eliminating these PCR errors left 14 polymorphic sites, consisting of ten synonymous changes and four non-synonymous changes. These were a leucine/methionine

substitution in the *A. myosuroides* nucleotide position 5101, an arginine/proline substitution in position 5610, a glutamate/alanine substitution in position 5621 and an asparagine/histidine substitution in position 5632 (Figure 7.13). Only 13 clones had the first polymorphism and 12 of those clones plus a different one had the other three that were always associated with one another.

These sequences were also compared with published sequences from the GeneBank of *L. multiflorum* (AY710293) and the related species *A. myosuroides* (AJ310767), *L. rigidum* (AY995232, AF359515, AF359513, AF359514, AF359516), *Phalaris paradoxa* (AM745339), *Phalaris minor* (AY196481), *Setaria italica* (AF294805), *Setaria viridis* (AM408428), *Avena sativa* (FJ358607), *Avena fatua* (AF231335, AF231336), *Triticum aestivum* (AF029895) and *Zea mays* (U19183). Sequences AF359515, AF359513 and AF359514 belonged to resistant populations of *L. rigidum* (Zagnitko *et al.*, 2001). The first polymorphism was also shared by these three resistant *L. rigidum* and the *A. sativa* and *A. fatua* populations. The polymorphism in position 5610 was shared by most of the related species. However the polymorphisms in positions 5621 and 5632 were only shared by the three resistant populations of *L. rigidum*.

Primers to detect these two polymorphisms in positions 5621 and 5632 were also included in the SNaPshot multiplex assay carried out with 384 resistant plants (section 7.3.1.3.) but these were omitted from the analysis as they could not be reliably scored. To arrive to any robust conclusions, a test looking at segregation of the polymorphisms with resistance should be performed.

7.4 Discussion

In the experiment carried out with 55 *L. multiflorum* populations from 50 farms in England all the populations with confirmed resistance (RRR/RR) to cycloxydim and/or pinoxaden (experiment described in Chapter 6) were found to have at least one of the

seven known single point mutations in most of the plants analysed. Most of the resistant plants with none of the seven known mutations had been sprayed with diclofop-methyl, fluazifop-P-butyl or tralkoxydim. Resistance to these herbicides in these plants could thus be explained by a non-target site resistant mechanism. ACCase target site resistance would be the mechanism responsible for resistance in most of the plants that survived treatments with cycloxydim or pinoxaden, as the majority of them had a target site mutation. However, there were a few plants that showed resistance to these two herbicides and none of these known mutations was found. In the case of resistance to cycloxydim, only one resistant plant from the population Thornham was found while none of these mutations were present. Here, resistance could potentially be explained by the thymine (T) to adenine (A) change in the nucleotide position 6230 corresponding to a change from isoleucine (Ile) to asparagine (Asn) in amino acid position 2077 (Figure 7.12). However, this still needs to be proved. With pinoxaden it should be taken into consideration that resistance to this herbicide was also detected in plants carrying one of the Asn-2041, Cys-1999 and Cys-2027 alleles. In studies on *A. myosuroides,* plants containing no mutations or the Trp-2027-Cys and Ile-2041-Asn mutations survived pinoxaden treatments (Petit *et al.*, 2009). It was suggested that the Trp-2027-Cys mutation alone could confer resistance to pinoxaden but more data would be needed to prove it. However, the Ile-2041-Asn mutation appeared not to be sufficient to confer resistance to pinoxaden at field rates. To explain resistance in these plants and in those with no mutation, it is possible that an additional non-target resistance mechanism is present. Therefore, even if the main mechanism conferring resistance to pinoxaden appears to be target site resistance, an additional non-target site mechanism might be responsible for the resistance to this herbicide in some plants. However, this resistance does not appear to be widespread as it seems to be present in just a few plants.

Although six of the seven mutations known to confer resistance to ACCase inhibitors

were found in plants from UK *L. multiflorum* populations the frequency was quite low. Of the 384 resistant plants tested at the molecular level from a total of 54 populations, no ACCase mutation was found in 60% of the plants studied. However, within the 54 populations studied, only 38 populations from 35 farms had confirmed resistance to at least one of the five herbicides assayed. Within these 38 populations, 12 populations had confirmed resistance to the three herbicides diclofop-methyl, fluazifop-butyl, tralkoxydim, and to cycloxydim and/or pinoxaden. In these 12 populations (32% of the 38 populations with confirmed resistance to at least one herbicide) the main mechanism conferring resistance was TSR as at least one of the seven point mutations was found in most of the plants. Thus, in 26 populations (68% of the 38 populations with confirmed resistance to at least one herbicide) the main mechanism conferring resistance was NTSR, despite some populations, including Chat1, Pidley, Galons and the enhanced metabolic standard Wilts B1, having one or two plants with an ACCase mutation. In the case of the population Wilts B1, the Trp-2027-Cys mutation was found in just one plant out of eight analysed which suggests that the frequency of this mutation is very low in this population and confirms enhanced metabolic resistance as the main mechanism conferring resistance to ACCase-inhibiting herbicides in this population as shown in previous studies (Cocker *et al.*, 2001). However, the discovery of a target site resistant plant highlights a risk and, given further selection pressure, the frequency of this mutation within this population could increase to a level where this enhanced metabolism standard becomes compromised. Results in these and other studies indicate that enhanced metabolic detoxification seems to be the most common mechanism of resistance to ACCase-inhibiting herbicides in the UK populations of *L. multiflorum* (Cocker *et al.*, 2001) as well as in *A. myosuroides* (Cocker *et al.*, 1999, Moss *et al.*, 2003) and *Avena* spp. (Cocker *et al.*, 2000). This would agree with other studies performed in France on *A. myosuroides* that showed that despite finding mutant alleles in 56.8% of assayed plants, more than 75% of the plants resistant to ACCase-inhibiting herbicides in France

were resistant via increased herbicide metabolism (Délye *et al.*, 2007). In these studies the most common mutation found was the Ile-1781-Leu mutation which contrasts with the studies described here which show that the most frequent mutation in the UK appears to be the Asp-2078-Gly mutation. It was found in 94 plants or 24.5% of the 384 plants analysed, while the Ile-1781-Leu mutation was found in just 51 plants (13.3%). The remaining mutations were very scarce. The Ile-2041-Asn mutation was found in just eight plants which represents just 2.1% of the total number of plants analysed, although it was present in three populations. The Cys-2088-Arg mutation was found in just seven plants from a single population (1.8%), and the Trp-2027-Cys and Trp-1999-Cys mutations were found in just four (1.0%) and one (0.3%) plants, respectively. No plants with the Gly-2096-Ala mutation were found.

These results contrast with several studies that have been performed investigating potential fitness penalties associated with resistance to ACCase-inhibiting herbicides in grass weed species. There does not seem to be a fitness penalty associated with the Ile-1781-Leu mutation in the plastid ACCase in the grasses *A. myosuroides* (Menchari *et al.*, 2006, Menchari *et al.*, 2008) and *L. rigidum* (Vila-Aiub *et al.*, 2005). In *Setaria italica* the Ile-1781-Leu resistance gene has even been shown to correlate with an increase in some fitness components (Vila-Aiub *et al.*, 2009). However, a significant reduction was found in vegetative biomass, seed production and plant height in homozygous plants for the Asp-2078-Gly mutation compared to the wild type (Menchari *et al.*, 2008). In addition, the ACCase enzyme activity of the Leu-1781 ACCase was similar to that of the wild type enzyme (Yu *et al.*, 2007), while the activity of the Ala-2096 and Asn-2041 ACCase enzymes was slightly reduced and that of the Cys-2027, Arg-2088 and Gly-2078 ACCase enzymes was greatly reduced (Délye *et al.*, 2003, Délye *et al.*, 2005, Yu *et al.*, 2007). Therefore, as the Asp-2078-Gly mutation is detected less frequently in *A. myosuroides* populations in France, Menchari *et al.* (2008) arrived to the conclusion that there must be a fitness cost associated with the

Asp-2078-Gly mutation. However, the highest frequency of the Asp-2078-Gly mutation in UK *L. multiflorum* populations seems to contradict this.

In the first experiment carried out to validate the dCAPS method, the population Pyl, that had been studied previously and shown not to posses the Ile-1781-Leu mutation (White *et al.*, 2005), was found to possess the Asp-2078-Gly mutation in these studies with 100% of plants carrying the mutant Gly-2078 allele at a very high allelic frequency (0.875). The resistance of this population to ACCase-inhibiting herbicides can now be explained by this point mutation. The population G. Rising01 was also found to posses the Gly-2078 allele. These two populations G. Rising01 and Pyl had a similar response at the whole plant level although the proportion of resistant plants was higher in G. Rising01 (See chapter 6, section 6.3.1.1). This could seem contradictory as the allelic frequency of the Gly-2078 allele in the Pyl population is higher, meaning that a higher number of resistant plants would be expected in this population. This could be explained by the presence of a second mutation in the population G. Rising01. In the studies carried out with this same population from a different collection year (2006) a second mutation, the Ile-2041-Asn mutation, was found in some of the plants. The populations Yorks A2 had, as expected, the Ile-1781-Leu mutation. Despite having a very similar allelic frequency, Yorks A2 and Notts differed in terms of whole plant response (data not shown); with Notts *A. myosuroides* showing much higher levels of resistance than Yorks A2. This result suggests that additional resistance mechanisms may be involved. In *L. multiflorum* populations both these mutations appear to confer similar levels of resistance, although with slight differences to some herbicides. White *et al*. (2005) found that the *L. multiflorum* populations Yorks A2 and Pyl, now known to have the Ile-1781-Leu and Asp-2078-Gly mutations, respectively, responded very differently to increasing herbicides rates, with Yorks A2 having much greater resistance to the herbicides cycloxydim and sethoxydim. Both were resistant to both herbicides but Yorks A2 maintained a high degree of resistance at doses which killed all plants of

the Pyl population. In the glasshouse dose response assay described in Chapter 6, section 6.2.1.1, both populations Yorks A2 and Pyl showed high levels of resistance to all herbicides but Yorks A2 was more resistant to the dims cycloxydim and tralkoxydim, while Pyl was more resistant to pinoxaden. Both populations showed similar levels of resistance to diclofop-methyl. The allelic frequencies for each mutation in both populations were quite high with 100% resistant plants in both cases (Table 7.5, Table 7.6).

In the cloning and sequencing experiment carried out with one resistant and one susceptible *L. multiflorum* populations, no firm conclusions could be drawn. The two polymorphisms found (Figure 7.13) were only present in a few clones and results from the SNaPshot multiplex assay were not clear as no susceptible standard was present with which to compare and there was no detectable pattern in the presence of these polymorphisms in most of the populations. More experiments including sensitive and resistant individuals should be carried out to draw any robust conclusions about the potential importance of the polymorphisms in nucleotide positions 5621 and 5632.

7.5 Chapter summary

- Even though a point mutation was found in 40% of the 384 resistant plants from the 54 populations studied, the main mechanism conferring resistance in 26 out of the 38 populations (68%) with confirmed resistance to at least one of the herbicides assayed was a non target site resistance mechanism, most probably enhanced metabolism. At present, enhanced metabolic detoxification appears to be the most common mechanism of resistance to ACCase-inhibiting herbicides in UK populations of *L. multiflorum*.
- In only 12 out of 38 populations (32%) from 35 farms with confirmed resistance to at least one of the five herbicides studied was the main mechanism

conferring resistance target site resistance. However, 7 of these 12 populations also contained resistant individuals possessing no mutations, thus showing the importance of non-target site resistance mechanisms.

- In total, six of the seven known ACCase mutations were detected in resistant plants. The most common mutations found conferring resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations were Asp-2078-Gly and Ile-1781-Leu. The six mutations found, and their frequencies as a proportion of the total number of resistant plants assayed (384), were: Asp-2078-Gly (24.5%), Ile-1781-Leu (13.3%), Ile-2041-Asn (2.1%), Cys-2088-Arg (1.8%), Trp-2027-Cys (1.0%) and Trp-1999-Cys (0.3%). No plants with the Gly-2096-Ala mutation were found.
- Resistance to pinoxaden was found in a few plants carrying one of the Asn-2041, Cys-1999 and Cys-2027 alleles or carrying none of the seven known mutations. This might imply that a non-target site resistance mechanism and/or the presence of one of these three alleles could be responsible for the resistance to pinoxaden in some *L. multiflorum* plants.
- Resistance to ACCase-inhibiting herbicides in the UK *L. multiflorum* population Pyl is conferred by a change from Adenine (A) to Guanine (G) in nucleotide position 6233 of the plastidic ACCase gene, corresponding to a change from aspartate (Asp) to glycine (Gly) in amino acid position 2078 of the CT domain of the ACCase protein.
- A change from Thymine (T) to Adenine (A) at nucleotide position 6230 of the plastidic ACCase gene, corresponding to a change from isoleucine (Ile) to asparagine (Asn) in amino acid position 2077 of the CT domain of the ACCase, could be responsible for resistance to ACCase inhibitors in the *L. multiflorum* population Thornham. Further work should be carried out to confirm this.

- Two polymorphisms were found in nucleotide positions 5621 and 5632 that may confer resistance to fops. Further work should be carried out to confirm their importance.

Chapter 8 General discussion

8.1 General discussion

The aim of this project was to characterise better both the agroecology and the basis of resistance to acetyl CoA inhibiting herbicides in *L. multiflorum* populations occurring as weeds of arable crops. A better knowledge of the biology and herbicide resistance at the whole plant and molecular levels should help in the development of better control strategies for this weed.

8.1.1 Agro-ecology studies

In the series of experiments carried out to study and understand the agro-ecology of *L. multiflorum* in UK conditions, there were interesting results that could prove to be useful when deciding which weed control strategy to implement. It was shown that both *L. multiflorum* and *A. myosuroides* have a highly detrimental effect on wheat yield. At a crop density of 100 winter wheat plants m^{-2}, just two and four *A. myosuroides* and *L. multiflorum* plants m^{-2}, respectively, caused a mean yield loss of 5%, a value often taken as an economic threshold at which the cost of weed control approximately equals the return from increased yields. Much higher infestation levels were recorded on many fields, demonstrating the potential for much higher yield losses. In addition, apart from short term effects on yield, the results show that weed seed return should also be taken into consideration. This is important in achieving longer term control. *L. multiflorum* plants are highly adaptable to different weed densities and very high seed production is possible from low density populations. In the studies described here, just three *L. multiflorum* plants m^{-2} produced an average of 6,816 seeds $plant^{-1}$ in a winter wheat crop. This means that, if even a small number of plants survive a weed control treatment, they could cause significant longer term damage because of their high

potential seed return. Most seed shedding occurred in July or August, just prior to the harvest of winter wheat. When chemical control is inadequate, as may occur with increasing herbicide resistance, a more integrated approach should be considered, combining both chemical and non-chemical methods. Factors such as weed dormancy or emergence pattern should be taken into consideration in order to develop the optimum control strategies.

Results from dormancy studies with *L. multiflorum*, as previously shown by Swain *et al*., (2006) in *A. myosuroides*, showed that the most important factor affecting the initial dormancy in *L. multiflorum* was temperature during spike emergence and seed maturation. Higher temperatures during seed maturation were associated with lower dormancy, although the effect was much weaker than with *A.myosuroides*. However, in addition to this, there was evidence of an intrinsic genetic effect that could help explain the high levels of within-year variation between field samples despite similar weather conditions. Thus, different populations would have different degrees of genetic dormancy, with those genetically more non-dormant being less affected by weather conditions. However, dormancy in *L. multiflorum*, whether due to genetic or environmental factors, lasted for only a relatively short period in most cases, with most seeds germinating within two months of collection in laboratory assays. This agrees with the results of the emergence pattern studies conducted in the field.

These studies gave very consistent results and showed that, averaged over five field experiments, 88.1% of *L. multiflorum* seedlings emerge in autumn in winter wheat crops. This indicates that there is little or no innate dormancy in *L. multiflorum* seeds retained by the time winter cereal crops are sown, which is typically late September or October under UK conditions. All of this information has important implications for weed management.

A prediction of the dormancy status of *L. multiflorum* seeds as influenced by the weather conditions during seed development and maturation would help in the

development of control strategies. Thus, in a year when it is hot during the time of seed maturation (June and July in the UK) *L. multiflorum* seeds would be expected to have a high degree of non-dormancy and germinate as soon as soil and weather conditions are conducive for germination. In such years there is the potential to reduce the seed burden by encouraging as much germination as possible in early autumn and destroying the seedlings prior to sowing the next crop. In contrast, in a cool year, germination will be expected to be delayed slightly as a higher proportion of seeds will be dormant. Delaying drilling may give more opportunity for emergence or, alternatively, the application of a pre-emergence residual herbicide would be advisable to improve control of later emerging plants after sowing. However, regardless of weather conditions during the seed development and maturation period, the majority of *L. multiflorum* plants emerge in autumn. Therefore, spraying herbicides in the autumn would be recommended in any year in order to remove weeds early, and hence reduce crop competition. By spring, weed plants would be bigger and more difficult to kill, crop yield might already be compromised by the competition with the weed and the more advanced crop growth stage in spring might shield weed plants, restricting spray coverage (Clarke, 2002). Thus, delaying the sowing date of autumn sown crops, and having an effective chemical control in autumn could potentially greatly reduce *L. multiflorum* infestations. However, in the UK, there is a widely held perception by farmers that many *L. multiflorum* plants emerge in spring and these could still pose a big threat if weed control was confined to the autumn in winter wheat crops. Farmers are particularly concerned about seed production in plants they perceive to have emerged in spring. Results on seed production potential do indeed indicate that *L. multiflorum* plants can produce a large number of seeds per unit area even at low plant densities. This could mean that even a small number of plants emerging in spring could potentially produce a considerable number of heads and consequently a high seed return. In these studies, seed return ranged from 546 to 6,816 seeds plant^{-1}, with an average value of 3,759 seeds plant^{-1}, which, in terms of unit area ranged from

18,262 to 262,131 seeds m^{-2} and had an average value of 143,137 seeds m^{-2}. However, studies of the comparison of seed production potential between autumn and spring cohorts showed that spring emerging plants are much less productive with 23 times less seeds $plant^{-1}$ than autumn emerging plants. These plants were not fully developed due to the high competition exerted by other plants which had emerged previously and so produce very little seed return. Therefore, seed production from spring plants will not have a significant impact in terms of seed return since few plants emerge in spring and those that do produce very few seeds per plant. Many of the plants that farmers spot in spring are probably autumn emergers that were previously unnoticed because of the small size and are only seen in spring when they get bigger.

Small numbers of surviving plants should not be ignored, however, especially if they are potentially herbicide-resistant. In such cases, seed return from herbicide resistant *L. multiflorum* plants could be significant with just a few survivors. Additional non-chemical control measures should be taken to reduce seed return. In the studies described here it was shown that increasing crop seeding rate from 100 to 300 plants m^{-2} helped reduce weed seed return by up to 66% without significantly affecting crop yield.

In summary, more non-chemical, cultural control measures should be adopted to reduce dependence on herbicides. This is important both to reduce the risk of resistance developing and to manage it once it has evolved. These measures have been recommended elsewhere for other grasses and include those previously mentioned, such as increasing crop competition by the use of more competitive crop varieties and higher crop seed rates, delaying drilling date, crop rotation, cultivation and non-cropping (Moss *et al.*, 2007). However, more studies are still needed to determine the best management options for *L. multiflorum* in UK cropping systems.

The agro-ecology results presented here provide a starting point for the development of better control strategies for *L. multiflorum* populations.

8.1.2 Resistance at the whole plant and molecular levels

Resistance studies in *L. multiflorum* were focused on ACCase-inhibiting herbicides as it is the major class of herbicides used in the control of this weed in the UK and many other countries. Results from whole plant screening tests showed that resistance is widespread in England with resistant populations present on 70% of the 50 farms included in the survey. ACCase mutations did not fully account for the resistance detected at the whole plant level in all 38 populations from 35 farms that showed confirmed resistance to at least one herbicide. In only 12 populations was the main mechanism conferring resistance an insensitive ACCase as mutations were detected in almost every plant within these populations. Out of a total of 384 plants from 54 populations, 155 plants possessed at least one ACCase mutation. The most common mutations were the Asp-2078-Gly mutation that was found in 94 plants (24.5%) and nine populations, and the Ile-1781-Leu mutation that was found in 51 plants (13.3%) and seven populations. The Cys-2088-Arg mutation was found in seven plants (1.8%) all of which were within a single population. The Ile-2041-Asn mutation was found in eight plants (2.1%) that belonged to three populations. The Trp-2027-Cys mutation was found in four plants (1.0%) from three populations. The Trp-1999-Cys mutation was found in just one plant (0.3%) from one population. No plant was found possessing the Gly-2096-Ala mutation.

However, even though a point mutation was found in 40% of the resistant plants, the main mechanism conferring resistance in 26 out of the 38 populations (68%) with confirmed resistance to at least one of the herbicides assayed was a non target site resistance mechanism. This indicates that the most common resistance mechanism in

UK *L. multiflorum* populations is not target site resistance but a non-target site resistance mechanism. As other studies carried out previously in UK *L. multiflorum* populations show (Cocker *et al.*, 2001) this NTSR mechanism could be enhanced metabolic resistance. Knowing the proportion of resistant populations which have each of the different mechanisms responsible for resistance is important since this can help in the study of the evolution of resistance and in the development of future control strategies. TSR populations might be considered easier to manage as resistance only affects the specific target site and plants can be susceptible to herbicides with other modes of action, as long as these are available. However, NTSR plants can exhibit cross-resistance to herbicides with many other modes of action, which makes their control more complex. TSR is usually controlled by a single major gene while NTSR is more complicated and is generally considered to be due to the effects of several minor genes (Gressel, 2009). Weeds evolve both types of resistance due to the selection pressure exerted by herbicide application. However, successive high doses of herbicides with the same mode of action selects for monogenic resistance (TSR), while the weaker selection pressure of sub-lethal herbicide doses can result in an incremental increase of quantitative traits leading to increasingly higher levels of resistance (NTSR) (Gardner *et al.*, 1998). Recent studies on *L. rigidum* populations treated with sub-lethal doses of glyphosate showed that the use of low herbicide doses can result in the accumulation of minor genes that after a few generations conferred resistance to this herbicide (Busi & Powles, 2009). Therefore, the type of resistance has important implications for determining the dosages that will delay the appearance of resistant populations. As NTSR is the most common resistance mechanism in the UK, it is likely that this is due to the use of sub-lethal herbicide doses. To delay the appearance of this type of resistance in more UK *L. multiflorum* populations, herbicides should be used at the recommended doses to achieve high weed mortality. Using this strategy, the possibility of the accumulation of minor genes that after a few generations might confer cross-resistance to herbicides with different modes of actions would be

minimized. However, this control strategy may exert a high selection pressure for single major genes. To prevent this, integrated weed management strategies should be adopted, which means the use of cultural and mechanical control methods combined with chemical control. The work in this research project provides useful information on which to develop further integrated control strategies that are relevant, not only in the UK, but also in many other countries where this weed is an increasing threat to arable cropping.

8.2 Summary of major findings

1. Innate dormancy in *L. multiflorum* is relatively short and is determined by both genetics and weather conditions. Higher temperatures during seed maturation are associated with reduced seed dormancy, but this effect is relatively weak compared with some other grass species. However, genetically non-dormant populations seem to be less affected by weather conditions.
2. The majority of *L. multiflorum* plants in winter wheat fields in the UK emerge in autumn. On average, 94% of plants emerge in October, November and December and only 6% in spring. Plants which emerged in autumn were much larger and produced on average 23 times more seeds per plant than spring emerging cohorts.
3. *L. multiflorum* plants are highly adaptable to different weed densities and very high seed production is possible from low density populations. The success of *L. multiflorum* as a weed of winter cereals appears to be linked to its ability to produce a high number of heads and seeds, even at low weed densities.
4. *L. multiflorum* plants are highly competitive and have a highly detrimental effect on winter wheat yield with losses of up to 89%. Increasing crop seed rate can be used as a weed control measure with winter wheat crops. Winter wheat

plant densities of 300 plants m^{-2} reduced the number of *L. multiflorum* heads and seeds $plant^{-1}$ by up to 67% compared to wheat densities of 100 plants m^{-2}.

5. Resistance in *L. multiflorum* to at least one ACCase inhibiting herbicide is widespread in England and was detected on 35 out of 50 (70%) semi-randomly sampled farms included in a survey. Resistance to the herbicides diclofop-methyl and tralkoxydim was the most widespread as it was detected on 31 (62%) and 30 (60%) of sampled farms, respectively. Resistance to fluazifop-P-butyl was detected on 18 farms (36%) and resistance to cycloxydim and pinoxaden was less common, being detected on only 10 (20%) and 9 (18%) farms, respectively.
6. The most common mutations found conferring resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations were Asp-2078-Gly and Ile-1781-Leu. The six mutations found, and their frequencies as a proportion of the total number of resistant plants assayed (384), were: Asp-2078-Gly (24.5%), Ile-1781-Leu (13.3%), Ile-2041-Asn (2.1%), Cys-2088-Arg (1.8%), Trp-2027-Cys (1.0%) and Trp-1999-Cys (0.3%). No plants with the Gly-2096-Ala mutation were found.
7. ACCase target site resistance was the main mechanism conferring resistance in only 12 populations (32%) out of the 38 populations (from 35 farms) with confirmed resistance detected in whole plant assays. These involved five herbicides, diclofop-methyl, tralkoxydim, fluazifop-P-butyl, cycloxydim and pinoxaden. Thus, the most common mechanism of resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations appears, at present, to be a non-target site resistance mechanism, most likely, enhanced metabolic resistance.

8.3 Directions for future work

1. Surveys on resistance in *L. multiflorum* populations should be carried out regularly in order to monitor any changes in the status of resistance to ACCase inhibiting herbicides in the UK.
2. The status and frequency of resistance to ALS inhibiting herbicides and herbicides with other modes of action should be studied in UK *L. multiflorum* populations. The mechanisms responsible for any resistance should be determined.
3. A more thorough study of the frequency and importance of non-target site resistance mechanisms in the UK should be carried out and its implications for future management strategies determined.
4. Since it has been shown that, despite weather conditions affecting dormancy in *L. multiflorum* populations, there is a relative lack of dormancy in *L. multiflorum* seeds by the time crops are sown, studies to determine the optimum drilling date should be performed.
5. More studies on crop seed rates should be carried out using a wider range of rates to verify the robustness of this approach as a method of reducing seed return. Other means of increasing crop competition (e.g. use of more competitive varieties or crops) should also be investigated.
6. More emphasis should be put onto the use of integrated weed management strategies to try and maximize control from non-chemical methods to reduce dependence on herbicides.

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Appendix – List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
4-HPPD	4-hydroxyphenylpyruvate dioxygenase
a.i.	active ingredient
ABC	ATP-binding cassette
ACCase	acetyl coenzyme A carboxylase
ADs	aryl-diones
AHAS	acetohydroxyacid synthase
ALS	acetolactate synthase
ANOVA	analysis of variance
AOPP	aryloxyphenoxypropionate
APP	aryloxyphenoxypropionate
ASA	allele-specific assay
BC	biotin carboxylase
BCC	biotin carboxylase carrier
BG	black-grass
bp	base pair
CAPS	cleaved amplified polymorphic sequence
CAS	chemical abstracts service
CD	cool and dry
cDNA	complementary deoxyribonucleic acid
CHD	cyclohexanedione
CoA	coenzyme A
CT	carboxyltransferase
CVA	canonical variate analysis
CW	cool and wet
d.f.	degrees of freedom

dCAPS	derived cleaved amplified polymorphic sequence
DEFRA	Department of Environment, Food and Rural Affairs
Den	phenylpyrazoline
Dim	cyclohexanedione
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EB	elution buffer
ECPA	European Crop Protection Association
ED_{50}	effective dose 50
EDTA	ethylenediaminetetraacetic acid
EMR	enhanced metabolism resistance
EPSP	5-enoylpyruvyate shikimic acid 3-phosphate
EU	European Union
*Exo*I	exonuclease I
Fop	aryloxyphenoxypropionate
GM	genetic modified
GS	growth stage
GST	glutathione-S-transferase
GT	glucosyltransferase
HD	hot and dry
HGCA	Home-grown Cereals Authority
HRAC	Herbicide Resistance Action Committee
HSE	Healthy and Safety Executive
HW	hot and wet
IPM	integrated pest management
IRG	Italian rye-grass
IWM	integrated weed management

LB	lysogeny broth
LSD	least significant difference
MCPA	2-methyl-4-chlorophenoxyacetic acid
MLP	maximum likelihood program
NRCS	Natural Resources Conservation Service
NTSR	non-target site resistance
P450	cytochrome P450 mono-oxygenase
PCR	polymerase chain reaction
PGR	plant growth regulators
PSII	photosystem II
RI	resistance index
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse-transcriptase polymerase chain reaction
SAP	shrimp alkaline phosphatase
SDW	sterile distilled water
SED	standard error of the difference of the means
SEM	standard error of the mean
SNP	single nucleotide polymorphism
sp.	species
spp.	species
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TP	transit peptid
TSR	target site resistance
USDA	United States Department of Agriculture
X-Gal	D-galactopyranoside