Aspects of the population genetics and ecology of herbicide resistant annual ryegrass

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TABLE OF CONTENTS

Chapter One
Introduction to herbicide resistance in *Lolium rigidum* (Gaudin)

Chapter Four Mechanisms of resistance in a herbicide cross-resistant *Lolium rigidum* population developed from a susceptible population

4.2 Materials and methods

4.3 Results

Chapter Five Comparative fitness of herbicide resistant *Lolium rigidum* biotypes

5.2 Materials and methods

Chapter Six Removal of *Lolium rigidum* seed during crop harvesting operations and the effect of seed removal on the soil seedbank

Chapter Seven Summary and discussion

ABSTRACT

Although the grass *Lolium rigidum* was exotic to Australia it is now a widespread, well adapted species. It has become a weed of crops and the focus of intensive management efforts to reduce its presence in cropped areas. A limited range of selective herbicides have been used to suppress *L. rigidum* populations and many populations have evolved resistance to the commonly used herbicides.

A number of populations of *L. rigidum* that had never been exposed to herbicides were sampled and treated with the cereal selective herbicide diclofop-methyl. Populations that were collected from sites on farmland showed significant proportions of survivors. Populations from other sites that were subject to occasional waterlogging also displayed significantly higher levels of survival compared to populations from adjacent drier sites. Most of the survivors from only one herbicide application displayed heritable resistance which was observed in the F_1 as a dominant factor. The conclusion is made that resistance to herbicides is almost inevitable when diclofop-methyl is repeatedly applied to *L. rigidum* populations in the field.

Successive annual selections with diclofop-methyl were imposed on surviving plants from a previously susceptible population. By the third generation accessions were extremely resistant to the herbicide diclofop-methyl, with over 95% surviving the normal application rate. However, the accessions were also resistant to other selective herbicides as well. The ACCase inhibitor sethoxydim showed reduced efficacy on the diclofop-methyl selected accession. The sulfonylurea herbicide, chlorsulfuron and the photo-system II inhibitor simazine both showed reduced efficacy on the *L. rigidum* selected accessions.

An altered herbicide target site was the major mechanism of resistance to diclofop-methyl and to sethoxydim, but other mechanisms were involved in resistance to the other herbicides. Resistance to chlorsulfuron and to simazine was due to metabolism. Metabolism of diclofop-methyl was also identified. The accessions selection with diclofop-methyl displayed target site resistance to diclofop-methyl, target site cross resistance to sethoxydim, non-target site resistance to diclofop-methyl and non-target site cross resistance to chlorsufuron and simazine.

There was no identifiable pleiotropic influence on fitness in each of three resistance biotypes each with different resistance conferring mechanisms. Specific herbicide resistance mechanisms have caused substantial costs to whole plant fitness *viz.* the triazine herbicides. F_1 crosses between a resistant biotype and a susceptible parent were made and the F_1 compared with the parents for fitness. No fitness differences were observed for the F_1 when compared to each of the parents, an important finding in an allogamous species. Resistant individuals from a susceptible population that had never been exposed to herbicides also showed no fitness differences when compared to the whole population. The fitness of resistant plants appears to be not different from susceptible populations.

The widspread and persistent nature of resistance conferring alleles has the potential to cause serious management problems. An approach to weed control is required that does not rely on herbicide application. A novel method of reducing weed seed return to the field at the time of crop harvest is reported. Catching *L. rigidum* seed during the harvesting process and preventing the contamination of the field has shown to be successful in reducing *L. rigidum* populations in cropped areas.

This study shows the widespread nature of resistance in *L. rigidum* populations, the tendency to genetic dominance even in the early generations of selection and the propensity of resistant populations to display resistance to many herbicides. The fitness of resistant plants and the likely failure of herbicide based approach to weed management suggests that Integrated Weed Management will be the best approach to manage herbicide resistant *L. rigidum* populations.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Chapter One

Introduction to herbicide resistance in *Lolium rigidum* (Gaudin)

1.1 History of the genus *Lolium* in Australia

The genus *Lolium* is exotic to Australia, originating in temperate Europe, North Africa, and the Eastern Mediterranean. Several species have been recorded as widespread and naturalised in southern Australia (Western Australia, South Australia, Victoria, New South Wales and Tasmania) and occasional herbarium specimens were noted from the other states by Kloot (1983). The genus was introduced to Australia with agricultural settlement. *Lolium temulentum* L. or "Drake" was mentioned in the literature in 1798, (Collins, 1798; cited in Micheal, 1972). The botanist Robert Brown in his papers from National Herbarium, London 1802 described *L. temulentum* as a weed of crops "*In agris frequens vitium*" and *Lolium perenne* L. as rare specimens only; "*Rarius ad vias,*" in the agricultural development around Sydney N.S.W., (Britten, 1906).

Many botanists and historians of agricultural development have mentioned members of the genus during the 19th and early 20th century. Behr (1847) mentions *L. temulentum* as present in South Australia, 11 years after South Australia was officially established. Muller in 1873 and Bentham in 1878 are reported as recording the presence of *L. perenne* and *L. temulentum* as abundant in Victoria and New South Wales (Kloot, 1983). Mullet (1919) gave a glowing description of a species of ryegrass established in central Victoria by 1887 which was provisionally identified as *Lolium subulatum* Vis. Kloot (1983) writes that *Lolium italicum* (syn. *Lolium multiflorum* L.) was recorded in Tasmania by Rodway in 1903 and in South Australia by Black in 1909. Thus four species of the genus *Lolium* had been recorded as widespread in Australia within almost 100 years of European settlement.

There was confusion in Australia regarding the identification of *L. rigidum* Gaud. and it may have been placed with *L. temulentum*, as both Black and Bentham apparently had difficulty discriminating among the annual species (Kloot, 1983). Interestingly, *L. temulentum* is considered a summer annual and an obligate weed of crops in the Northern Hemisphere and *L. rigidum* a winter annual (Cooper, 1959). The ryegrass established in central Victoria by 1877, which was provisionally identified as *L. subulatum* (Mullet, 1919) was apparently the major introduction to the area known as the "Wimmera" region, which was to give its name to the annual ryegrass ecotype. The sample of ryegrass described by Mullett was provisionally identified as *L. subulatum* Heldr. ex Boiss, first described in 1884. However, Terrell (1968) suggests that *L. subulatum* may be synonymous with *L. loliaceum* Hand.-Mazz, and *L. loliaceum* and *L. rigidum*. ssp. *rottbolliodes* Heldr. may be ecotypes. Other systematic treatments do not include *L. subulatum*, (Kloot 1983). It is possible that the early descriptions of annual *Lolium* species in Australia were ecotypes of the annual *L. rigidum* or hybrids, and it is likely that *L. rigidum* was more widespread than was indicated by the early literature.

There may have been many introductions of *Lolium* species to Australia from diverse sources such as contaminants of crop seed or as seed for planting. Recent authors agree with Barnard (1972) that the predominant annual can be best described as *L. rigidum*. Both Barnard (1972) and Oram (1990) regard the "Wimmera" type of ryegrass and, therefore, most of the weedy ryegrass of southern Australia, as a variable ecotype of the winter annual *L. rigidum* Gaud.

1.1.1 Species within the genus *Lolium*

All the species and sub-species of the genus *Lolium* including important life history traits and the 6 species recorded in Australia are listed in Table 1.1. There have been various treatments of the genus *Lolium*, and the species structure in Table 1.1, taken from Terrell (1968), is simplified from previous treatments. Terrell (1968) suggested a general structure for the genus based on morphological characteristics which are usually distinguishable in most environments when ecologically separated, however confusion can occur with hybrids or when ecotypes are not distinct. The species *L. perenne*, *L. multiflorum* and *L. rigidum* are freely inter fertile and with extensive crossing in the wild can form a continuum of morphological characters between the extremes of the associated species (Terrell, 1968). It is possible that the many hybrids have confused identification for more than a century. Confusion about the species structure has occurred particularly with the annual types, (*L. rigidum* var. *rigidum* Gaud.; *L. rigidum* var. *rottboelliodes* Heldr. ex Boiss and *L. subulatum* Vis.).

Authors such as Malik and Thomas (1966), Naylor (1960) and Turesson (1929) had already suggested the outcrossing species (*L. perenne*, *L. multiflorum* and *L. rigidum*) were "not sufficiently differentiated to be regarded as distinct species" (Naylor, 1960). Bulinska-Raomska and Lester (1985) used polyacrylamide protein electrophoresis on caryopsis proteins and concluded that *L. perenne*, *L. multiflorum* and *L. rigidum* have "little genetic differentiation due to similarities between the protein profiles". Although caryopsis proteins may not provide optimum discrimination there is little doubt that the inter-fertility of the species and the morphological similarities does not fit the classical definition of species. Bulinska-Raomska and Lester reiterate the case for considering the three inter-fertile species as one "cenospecies" or polymorphic species, as discussed by Terrell (1968) or, as Naylor suggested, three subspecies. According to Bulinska-Raomska and Lester (1985), *L. rigidum* is "the ancestral form from which the other groups diverged or the bridge from which the annual self-pollinated species arose". *L. remotum* and *L. temulentum* are not inter-fertile in the wild and although hybridisation can occur with each other and the other *Lolium* species under laboratory conditions, they are considered to be defined enough to be classified as species (Terrel, 1968).

The term "species" will be used in this dissertation, although it is obvious that all three species groups encompass substantial phenotypic and genotypic diversity.

Table 1.1. Species of the genus *Lolium* (adapted from Terrell, 1968).

The genus *Lolium*	Life cycle	Pollination	Recorded in Australia
L. perenne L.	perennial	allogamous	*
L. multiflorum Lam.	bi-annual	allogamous	*
L rigidum Gaudin var. *rigidum*	annual	allogamous	*
L. loliaceum Hand-Maz.	annual	allogamous	*
L. temulentum L.	annual	autogamous	*
L. remotum Schrank	annual	autogamous	*
L. persicum Boiss & Hohen	annual	autogamous	
L. canariensis Steud.	annual	unknown	

L. subulatum Vis. was ascribed by Bor to *L. rigidum* var. *rottboelliodes* to form *L. Loliaceum* Hand.-Mazz, and by Humphries (1980) to *L rigidum* ssp. *lepturoides* (Boiss.) Sennen and Maur. *L. rigidum* var. *rottboelliodes* Heldr. appears to be synonymous with *L rigidum*. *L. canariensis* has been disregarded by all other authors (cited by Terrel, 1968).

1.1.2 Ploidy and self-fertility

All naturally occurring *Lolium* species are diploid (2n = 14) (Terrell, 1968). Tetraploids have been created to improve growth rates and the commercial register in Australia has many ecotypes and selections with both diploid and tetraploid types from the important species.

The common *L. perenne*, *L. multiflorum* and *L. rigidum* species are recorded as being outcrossing species, the other species are all self pollinated (Kloot 1983). There are reports of the annual *L. rigidum* populations displaying both allogamy and autogamy (Bullita *et al.*, 1993; Terrell, 1968; Jenkin and Thomas 1939), although all authors agree that *L. rigidum* is basically an allogamous species. A low proportion of autogamy is consistent with its reputation as a weed and with its genetic adaptability. Annual weed species could derive evolutionary benefit from autogamy, particularly the ability to produce seed under difficult environmental conditions and also the ability to produce some seed in isolation as founder populations. *L. rigidum* does not seed prolifically under autogamous conditions and is better adapted as a weed of crops where crop seed contamination and annual seedfall establish sufficient population density to allow allogamy.

1.1.3 Distribution of *L. rigidum* in Australia

In Australia *L. rigidum* is frequently referred to as "Wimmera ryegrass". Barnard (1972) reports that the cultivar named as "Wimmera ryegrass" is a collection of ecotypes selected for high growth rate from the area around Horsham, (36.43S, 142.13E) Victoria, Australia called the "Wimmera". It was first recorded in the Wimmera area in about 1887 (Mullet, 1919). Due to the combination of a number of factors such as the mediterranean climate, the fertile clay soils and the (historically) early establishment of cereal crop production on the fertile loess plains, ryegrass became abundant in that area. Mullett (1919) describes the rapid dispersion of the annual ryegrass ecotype to many other farms over a large area with the principle method of spread by deliberate plantings. A certified ecotype was selected in Western Australia called "Merriden Early Ryegrass" which had earlier maturity than the "Wimmera" type (Oram, 1990). The "Wimmera" ecotype is not certified due to its morphological variability, however, it has spread across southern Australia both by planting as pasture and as a seed contaminant of crop seeds and hay. The distribution and persistence of *L. rigidum* and its success as a pasture species in southern Australia are related to its adaptability and it was well suited to become a weed of cereals and other crops (Forcella, 1984).

1.2 *L. rigidum* as a pasture species.

"*L. perenne* is the most important pasture grass in Northern Europe," (Kloot, 1983) and was introduced to Australia early in its European settlement for the same purpose. *L. perenne* and *L. multiflorum* are still important in Australian as pasture species in the higher rainfall areas. The annual *L. rigidum* is important in lower rainfall areas as it can be found across most of southern Australia.

L. rigidum is an easily established pasture species. Good seedling vigour and rapid tillering are a feature of the species and enable it to compete with other plant species present in pastures (Cocks, 1969; McGowan, 1970). For many years it was deliberately sown as a pasture species across much of southern Australia because it was productive and palatable to livestock. The vigorous autumn and winter growth of *L. rigidum* make it a productive component of grazed pastures (Kemp, 1988). Tolerance to cold and wet conditions are a feature of the species and the contribution of *L. rigidum* to productive pastures in critical winter periods where pasture growth is often limited is well recognised. The lack of sharp awns or barbed seed structures ensures that pastures of *L. rigidum* are suitable for direct grazing by sheep and cattle and for production of conserved forage without contaminating wool or causing discomfort or mechanical injury to grazing stock.

Pastures in southern Australia depend on seed reserves in the soil to regenerate annually and following periods of crop production. The ability to set seed under a range of environmental conditions has contributed to the success of *L. rigidum* as a pasture species. Because of the plasticity of growth form, grass species are adaptable to variations of density and also to rapid changes in environmental conditions. Many annual grass species are tolerant of drought conditions during growth and seed set, which contributes to persistence in pure or mixed stands (Donald, 1951). *L. rigidum* possesses all of these characteristics.

The practice of sowing *L. rigidum* as a pasture species has decreased in recent years due to a number of factors. A general decline in the profitability of grazed livestock is one reason; the proportion of farmland committed to livestock production has fallen by 3.5% from 1980 to 1990 (Australian Bureau of Census and Statistics, Adelaide, South Australia). Favourable economic returns from crop production relative to livestock grazing have accounted for this change. Crop production in the mediterranean regions of southern Australia is now characterised by longer phases of crop production and relatively short periods of pasture. The length of the crop production phase varies with the local environmental conditions, but two to six years of crop production and one or two years of pasture for direct grazing are common. *L. rigidum* has often persisted from plantings in the pasture phase to become a weed of crops. Farmers do not plant *L. rigidum* in the intervening pasture phase because of the likelihood of the seed persisting to become a weed in the subsequent crop production phase. Another reason for the decline of plantings of *L. rigidum* for direct grazing as a pasture is the occurrence of a bacterial infection in ryegrass which can rapidly kill grazing animals.

In many areas of southern Australia the disease "Annual Ryegrass Toxicity" (ARGT) has caused livestock losses and generated concern among farmers. ARGT is caused by heads of *L. rigidum* becoming infected with a nematode, *Anguina funesta*, which forms a visible gall in the maturing seed. The gall is infected by a bacteria of the genus *Corynebacterium*. When the bacteria contain a specific bacteriophage, production of a neurotoxin results (McKay *et al.*, 1982). If sufficient galls are ingested by grazing animals, believed to about 10,000 for cattle and 1-2000 for sheep, the animals rapidly become affected and usually die under extreme stress. The period of time for a field to develop toxicity was from 5-15 years from first infection depending on the spread within the field. During that time the symptoms are rarely seen and the disease can build up without warning.

ARGT is controlled by reducing or preventing seed production in *L. rigidum* in fields with high levels of infected ryegrass. Heavy grazing by livestock in the winter and spring to reduce seed set was recommended (Pearce and Holmes, 1976). Treatment of maturing ryegrass with paraquat to rapidly desiccate seed heads is also successful in reducing nematode densities to below damage threshold levels (McKay *et al.*, 1982). With the threat of serious stock losses and with the control measures actively targeted against seed production in this annual species, ryegrass densities declined and have not been replanted.

1.3 *L. rigidum* as a weed of crops

1.3.1 Competition with annual crops

L. rigidum has been a serious weed of crop production in southern Australia for many years and continues to be so especially where the duration of crop rotations has increased. If it is present in annual crops at a sufficient density severe competition can result. Reeves (1976) suggested that the percent yield loss in wheat is directly proportional to the square root of the ryegrass density per unit area. Thus 100 plants per square metre could result in a 10% yield loss. At high densities that competitive relationship with wheat crops changes due to intraspecific competition (Pearce and Holmes, 1976). Ryegrass seedling densities can be high and may often exceed 1000 seedlings per square metre early in the season.

Weed control thresholds for infestations in wheat crops have been developed in South Australia and a simple model called "Weed Decide" for herbicide application costs and crop yield responses to weed removal in a "typical" environment have been produced, (Black and Dyson 1993). A sample calculation from the "Weed Decide" model for an infestation of *L. rigidum* indicate that in a 2 tonne per hectare wheat (*Triticum aestivum*) crop, 70-75 plants per square metre of ryegrass would be about the threshold density to justify the expenditure for the application of 540 g a.i. per hectare of the cereal selective graminicide diclofop-methyl (see "Chemical control of *L. rigidum*", page 13 for a description of diclofop-methyl), at the 1990-1993 average price of wheat.

The threshold ryegrass density for barley (*Hordeum vulgaris*) would be higher than for wheat because of the competitive nature of barley. For the many legume crop species grown in rotations with cereals the threshold ryegrass density is much lower as *L. rigidum* competes very strongly with most legume crop species. In addition to its competitive nature, much effort is expended to control infestations of *L. rigidum* because of the potential to set large numbers of seeds. This is especially the case when the weed is present in disturbed soil conditions with high fertility and adequate moisture such as land given to crop production. The potential for a high seed set which may reinfest crop land in subsequent crops is an important consideration in assessing the economics of ryegrass control.

Seed production from *L. rigidum* in the absence of crops is relatively constant at densities ranging from 1 to 10,000 plants per square metre, with seed production averaging 122,000 per square metre. Tiller number per plant under these conditions ranged from 172 to 1 with no discernible plant mortality (Matthews unpub.). Ryegrass seed yields in a wheat crop were unaffected by ryegrass densities ranging from 33 to 900 plants per square metre, and seed output was about 30,000 seeds per square metre (Rerkasem *et al.*, 1980). The morphological plasticity of *L. rigidum* accounts for the competitive and reproductive ability under a variety of conditions. When fertility is high and the densities of ryegrass and crops are low, ryegrass can tiller profusely resulting in high levels of set seed. When either crop or weed densities are high, tillering and seed production is restricted, but complete mortality due to density is unlikely to occur except under extreme conditions.

1.3.2 *L. rigidum* as a host for cereal diseases

The presence of grass weeds in the pasture phase is believed to host fungal and nematode pathogens of some cereal crop varieties. In particular, *L. rigidum* is considered to be an alternative host of the root disease *Rhizoctonia solani* (Roget *et al.*, 1987). Such root diseases reduce crop yields especially in the lighter loam and sandy soils. The beneficial effects of a pasture phase on subsequent cereal crop yields can be reduced by yield-limiting levels of root diseases if ryegrass and other grassy weeds contaminate a final year pasture. To achieve reduced levels of root diseases the removal of grasses during the final winter of a pasture phase is recommended ("Right Rotations"; South Australian Department of Primary Industry, Adelaide, South Australia). Selective graminicides are recommended for use to remove unwanted grasses from the legume components of the pasture. Other recommended methods of removal of annual grasses from pastures are the use of non selective herbicides when the seeds of the grass weeds are at the soft to mid dough stage of grain filling (Zadocks growth stage 77-83) to prevent the formation of viable seed.

1.3.3 Control in crop

Control of *L. rigidum* in areas used for annual crop production can be achieved by either herbicide application or cultural methods. Cultural methods were used for many years prior to the availability of appropriate herbicides for use on *L. rigidum* in both monocotyledonous and dicotyledonous crops.

1.3.4 Cultural control

Repeated cultivation was the traditional method of controlling ryegrass seedling emergence prior to crop establishment. Cultivation can only be effective when the germination characteristics of a population permit emergence of a substantial proportion of the germinable propagules within the time available for soil preparation and weed control prior to planting. In a mediterranean climate the period of time between the onset of rainfall in the autumn and the optimum time for planting may be brief. *L. rigidum* tends to germinate following substantial rainfall events assuming a period of after ripening has occurred. Cocks (1969) found that 10 days of conditions suitable for germination were adequate for 60-70% germination. Gramshaw and Stern (1977a) found more than 50% emergence following the first substantial rainfall event in autumn and Heap (1988) observed about 50% germination in autumn followed by 2-3% germination per calendar month thereafter until conditions ceased to be suitable for germination.

If the period of time between adequate autumn rainfall events and crop establishment is three weeks or more then according to the data of Gramshaw and Stern (1977a), propagule densities could be reduced by 60-70% by removal of germinated ryegrass prior to sowing. The success of cultivation is dependent upon receiving adequate rainfall to initiate germination of a substantial proportion of germinable ryegrass seeds. The potential for continuous emergence of *L. rigidum* seedlings through the winter and spring is a survival strategy that sustains populations even if pre-planting weed control has been excellent. It is this characteristic that made selective herbicides agronomically desirable for the control of *L. rigidum* in crop.

The practice of early shallow cultivation prior to the occurrence of autumn rainfall to stimulate ryegrass germination following rainfall events can be effective, Pearce and Holmes (1976) reported a 30-43% increase in emergence following such a practice. Davidson (1992) did not find a similar response, and suggested that different soil types or different rainfall patterns to those in the Pearce and Holmes study may account for those discrepancies. Gramshaw and Stern (1977b) found that deep burial tended to cause enforced dormancy, probably by oxygen deprivation. However, Reeves and Smith (1975) had shown that deep burial by mouldboard ploughing permanently reduced emergence whereas shallow cultivation stimulated germination. Deep burial of *L. rigidum* seeds is impractical in much of southern Australian agriculture as shallow soils and limestone accumulation prevent deep tillage.

All broad scale cultural techniques have the disadvantage of being unable to influence the reproduction of a population once crop establishment has taken place. Burning seed that has matured either in pasture or among crop residues can reduce the density of seed that has been produced in the current season. Burning has been shown to be effective by Davidson (1992) and Pearce and Holmes (1976) only when the seeds are not shed from the rachis of the plant. Pearce and Holmes reported greater than 95% reduction in viable seed per square metre following burning when adequate fuel was present and burning was done before seed shedding. The effectiveness of burning is influenced by the quantity of fuel, which affects the temperature and the duration of the fire. The fuel is limited to *in situ* crop or pasture residues. Davidson (1992) showed that burning treatments reduced surface ryegrass seed numbers to less than 8% of the pre-burn density in undisturbed soil. This work by Davidson in a three year trial under different cultivation regimes also indicated that an average of 72% of ryegrass seed was rendered unviable by burning crop residues across a range of cultivation treatments.

In the drier parts of southern Australia the lack of crop residues may limit the effectiveness of burning residues to control seed density in some seasons. Also stubble burning is a practice that is discouraged because of the potential for soil erosion following burning and the reduction in soil carbon levels. The concept of reducing seed numbers even after seedset has occurred is, however, extremely valid and an extension of that concept is discussed in Chapter 6, where *L. rigidum* seed is removed from the field during the harvesting process.

Reducing or preventing the seed set of *L. rigidum* in pastures would be effective in reducing seedling emergence and ryegrass density in a following crop. Also mechanical cutting prior to anthesis should be effective. It is extremely important to limit the seed production from any regrowth of *L. rigidum* following cutting for hay or silage. If seasonal conditions are favourable, ryegrass regrowth can set many seeds and render this valuable cultural method ineffective.

Direct grazing by livestock has shown substantial reductions in seed set. Gramshaw and Stern (1977a) suggested that with sheep, sufficient grazing intensity is of most importance and sheep were more effective than cattle as sheep graze closer to the ground. However, the eruption of ARGT (see section 1.2), has diminished the use of grazing by livestock as a long term, effective control measure in cropping systems.

1.3.5 Chemical control of *L. rigidum*

The availability of selective herbicides for control of *L. rigidum* in cereal and other crops changed the prospects for suppression of ryegrass populations during cropping phases. Effective herbicides for the selective removal of *L. rigidum* from some commonly grown crops became available with the release in 1973 of the dinitroaniline herbicide trifluralin. Diclofop-methyl was released for widespread use in 1978 and other aryloxyphenoxypropionate herbicides such as fluazifop, haloxyfop and quizalofop followed. Sethoxydim, a cyclohexanedione herbicide, is also an ACCase inhibitor with high efficacy on ryegrass and was released in 1986. Other herbicides from the same chemical group, tralkoxydim and clethodim have also been released and are recommended for control of *L. rigidum*. Diclofop-methyl, tralkoxydim and trifluralin are recommended for application in wheat, barley and dicotyledonous crops to control ryegrass and are widely used.

Trifluralin, 2,6-dinitro-*N,N*-dipropyl-4-(trifluromethyl)benzenamine, is a soil incorporated herbicide which can be applied either pre-sowing or post-sowing and pre-emergence. It is incorporated into soil to reduce volatilisation, degradation by ultra-violet light and to maximise contact with emerging weeds. Trifluralin is registered for application in a range of crops including wheat, barley and most grain legumes and oilseed crops and is effective in reducing the emergence of several weed species including *L. rigidum*, (Parka and Soper, 1977; Australian Weed Control Handbook, 9th edition, Ed. J. M. Parsons, Inkata Press, Melbourne and Sydney). In cereal crops, seed is sown beneath a layer of trifluralin and an insensitive coleoptile sheath protects the emerging shoot. Precise depth of sowing is important to protect crop roots and crowns as roots of sensitive species should not contact the herbicide. Tolerant crops such as legumes and oilseeds are relatively insensitive to trifluralin. The weed seeds germinating in the trifluralin band absorb the trifluralin and growth is inhibited because of trifluralin interference with mitotic spindle polymerisation which inhibits the formation of tubulin in mitotic cells leading to arrested cell division (Parka and Soper, 1977). Trifluralin is not readily metabolised by either weeds or crop plants.

Diclofop-methyl, (±)-2-(-[2,4-dichlorophenoxy]phenoxy)propanoic acid and the other aryloxyphenoxypropionate herbicides, fluazifop-*p*-butyl, haloxyfop-methyl and quizalofop-ethyl inhibit the plastidic enzyme acetyl Co enzyme A carboxylase (ACCase)

(EC., 6.4.1.2), in sensitive plant species. ACCase is the enzyme which catalyses the carboxylation of pyruvate to malonyl Co-A as a precursor for fatty acid synthesis (Kobek *et al.*, 1987). The post emergent herbicide diclofop-methyl is selective in wheat and barley as it is more rapidly metabolised in the cereals than in ryegrass (Shimabukuro *et al.*, 1979). Other aryloxyphenoxypropionate herbicides are not selective in cereal crop species but are widely used in legume and oilseed crops where an insensitive ACCase enzyme confers crop tolerance. Sethoxydim, 2-(1-[ethoxyimino]butyl)-5-(2-[ethylio]propyl)-3-hydroxy-2-cyclohexen-1-one, is also an ACCase inhibitor with high efficacy on ryegrass and is not selective in cereal crops (Gealy and Slife, 1983). Other herbicides from the same chemical group, tralkoxydim and clethodim are available and are recommended for control of *L. rigidum*.

Chlorsulfuron, 2-chloro-N-([{4-methoxy-6-methyl-1,3,5-triazin-2-yl}amino]-carbonyl)bezenesulfonamide, from the sulfonylurea group is a herbicide that inhibits the enzyme acetolactate synthase (ALS) (EC., 4.1.3.18) in susceptible species (Ray, 1984). The ALS enzyme is the first enzyme common to the biosynthesis of the branched chain amino acids valine, leucine and isoleucine in plant species (Ray, 1984). Chlorsulfuron and another herbicide from the same group, triasulfuron, are widely used to suppress ryegrass in wheat and other cereals. Selectivity is gained by rapid metabolism of the herbicide in the crop plants. Oxidative metabolism of the sulfonylurea herbicides in wheat has been demonstrated by Frear *et al.*, (1991), Sweetser, (1985) and Sweetser *et al.*, (1982). Crop species can be affected by these herbicides as a result of stress related inhibition of herbicide metabolism (Beyer *et al.*, 1988) or by herbicide induced reduction in micronutrient uptake (Osborne, *et al.*, 1993; Altman and Rovira, 1989).

Other herbicides, particularly from the chemical groups known collectively as the photo-system II (PSII) inhibitors, including atrazine, simazine, diuron, cyanazine and metribuzin have all been available for some time in Australia. The PSII inhibitors are effective in suppressing electron transfer from PSII thereby causing photo-oxidative stress to the chloroplast reaction centre and chloroplast lipids (Gronwald, 1994). The use of PSII inhibitors is limited by crop selectivity and these herbicides are largely limited to the legume phase of the crop rotation. (Australian Weed Control Handbook, 9th edition, Ed. J. M. Parsons, Inkata Press, Melbourne and Sydney).

Simazine, 6-chloro-*N*, *N*'-diethyl-1,3,5-triazine-2,4-diamine, is the most widely used of the PSII inhibitors for control of *L. rigidum*. Lupins (*Lupinus angustifolius* L) and beans (*Vicia faba* L) are tolerant of simazine at rates that can prevent ryegrass seedling development and suppress ryegrass growth. Tolerance is vested in increased metabolism by the crop species and some degree of avoidance by rapid tap-root development away from the simazine layer (Esser *et al.,* 1975).

1.4 Conclusion

L. rigidum has been distributed widely across southern Australia because of its valuable properties as a grass species suitable for grazing. It displays many of the characteristics of an adaptable and persistent grass species. However, the desirability of ryegrass as a component of a grazed pasture changed with the advent of ARGT and the shift in land use to more sustained cropping. Various management treatments have been developed to reduce ryegrass densities to minimise the risk of ARGT and to reduce ryegrass numbers prior to crop establishment. When present in crops, yields can be substantially reduced and ryegrass seed numbers tend to increase, thereby multiplying the risk of yield limitations in the future.

As a result of plasticity and genetic diversity, ryegrass survival and seed production under extremes of density or environmental conditions has been shown to be more than adequate to sustain the presence of the species (Donald, 1951). Control of ryegrass infestations can be difficult due to extended germination and persistent seed set within crops. Although various techniques are available to reduce seed numbers in the soil in pasture phases prior to crop establishment, these require a change of land use from frequent crop production.

The availability of selective chemicals for control of ryegrass in cereal and non-cereal crops has proved to be a boon to farmers and weed control specialists. Since their release herbicides have been widely adopted for control of *L. rigidum* in both cereal crops and legume crops (Gill, 1995). Although the increase in crop yields due to reduction of ryegrass densities has been adequate, suppression of the weed populations has in some cases been less than anticipated. Less than 100% mortality has allowed many populations to eventually increase and these populations often prove to be more difficult to kill than their predecessors.

1.5 Herbicide Resistance

1.5.1 Herbicide resistance - definitions

The definition of herbicide resistance is evolving to describe the patterns of resistance that have been reported (Hall *et al.*, 1994). With many species displaying resistant biotypes and biotypes displaying different combinations of resistance-conferring mechanisms, descriptive terms have arisen with experience. The scope of resistance found within *L. rigidum* resistant populations has been complex, hence the need to differentiate between simple and complex resistance patterns. The following terminology has emerged to describe resistance as well as the different types of cross resistance and to indicate the mechanistic basis of resistance and therefore, the likely complexity of the responses to other herbicides.

Herbicide resistance covers cases of resistance within a population to only one herbicide or is used in the collective adjectival sense.

Multiple resistance; expression of more than one resistance mechanism within a population.

Target site cross resistance; resistance due to target site modification which confers resistance to other herbicide classes which inhibit the same site of action.

Non-target site cross resistance; resistance conferred by a mechanism other than the target site(s) of the selective herbicide.

Tolerance to a herbicide is defined here as the innate ability of a species to be unaffected by a herbicide and not due to a process of selection from a larger population by differential mortality.

Over 100 species of weeds world wide have biotypes with developed resistance following exposure to herbicides (Holt, 1992). Some of these cases of resistance are resistance to only one herbicide group. In many cases the resistance can extent to other groups of herbicides, usually those related by herbicide mode of action, such as the PS II inhibitors. In a few species, resistance occurs to many unrelated herbicides following exposure to one herbicide or herbicide group.

Target site resistance occurs when an altered site of action of a herbicide confers resistance to that herbicide. This is the most common form of resistance and is often termed "herbicide resistance" without any adjective. The best examples are resistance to the triazine herbicides due to a single amino acid modification at the target site which prevents the herbicides from binding to the site of electron transfer from the D1 protein (Fuerst *et al.*, 1986; Pfister and Arntzen, 1979). Target site cross resistance is also common in the cases of triazine resistance where selection with one herbicide has conferred resistance to another class of herbicides with the same site of action in the plant. There are many other cases of altered target sites conferring resistance to other herbicides within the herbicide group that was used during the selection process and to other herbicide groups with the same herbicide site of action. Target site ACCase resistance and cross resistance has been described in *L. rigidum* by Tardif *et al.*, (1993). Also target site resistance to the ALS inhibitors has been described in *L. rigidum* by Christopher *et al.*, (1991) and reviewed by Saari *et al.*, (1994).

Target site cross resistance is common as resistance frequently develops to members of the same chemical group or chemical groups with the same site of action in the plant. Non-target site cross resistance develops when alternative herbicides from chemical groups other than were used in the selection process are not successful on the resistant weed biotype and resistance is not associated with the herbicide target site. There are considered to be thousands of non-target site cross resistant *L. rigidum* populations in southern Australia. Other weed species to develop non-target site cross resistance other than *L. rigidum* are biotypes of *Alopecurus myosuroides* (Hall *et al.*, 1994; Kemp *et al.*, 1990) and in *Phalaris paradoxa* (Yaacoby *et al.*, 1986).

1.5.2 Mechanisms of resistance to major herbicide groups

Resistance to herbicides within individual plants or within plant populations can be further discussed by the physiological processes conferring resistance or cross-resistance. Metabolism of a herbicide or active component of a herbicide refers to the inactivation of a usually potent molecule by oxidation, and often, conjugation to glutathione an amino acid, or a sugar molecule (Jones, 1991; Shimabukuro, 1985). All plants and weedy species have general xenobiotic detoxification mechanisms, however, when herbicide resistance within a population is conferred by enhanced metabolism it is because of an increased frequency of individuals with increased metabolism when compared to a normal susceptible population.

Reduced translocation or adsorption of active herbicidal molecule(s), may confer resistance to herbicides when compared to susceptible plants. Preston and Powles (1995) report that reduced translocation was observed in paraquat resistant biotypes of *Hordeum glaucum, H. leporinum* and *Arctotheca calendula.* Apart from these examples, reports of resistance due to reduced translocation or adsorption are rare.

Sequestration or compartmentation have also been postulated as mechanisms that may be implicated in resistance. No examples of vacuolar or intra cellular sequestration have been cited for whole plants, although increased sequestration of diclofop-methyl in protoplasts has been observed in tissue from resistant plants as compared to susceptible tissue (Devine *et. al.*, 1993). Recovery from toxic symptoms with repair or regeneration of plant function following exposure of coleoptile tissue *in vitro* to diclofop-methyl and other ACCase herbicide has been proposed for some *L. rigidum* biotypes (Häusler *et al.*, 1991) and *Avena fatua* (Devine and Shimabukuro, 1994).

The major classes of herbicides to which resistance has developed and the physiological processes conferring resistance both in Australia and in other countries which are relevant to development of resistance in *L. rigidum* are briefly discussed below.

1.5.3 Resistance to PSII inhibitors

In 1992, 57 weed species worldwide were listed with resistance to the PSII inhibitors (Holt, 1992). It was estimated that 3 million hectares were infested with PSII inhibiting herbicides (LeBaron, 1991). This herbicide group includes atrazine and simazine from the s-triazine group, diuron, chlorotoluron and linuron from the phenylureas and metribuzin, a triazinone herbicide. The PSII inhibitor group of herbicides has been available for many years and has been used extensively in North America, Canada and Europe in corn and soybean cropping systems (Gronwald, 1994). Plants resistant to this group include the first confirmed report of resistance to herbicides, *Senecio vulgaris* L., reported by Ryan (1970).

In nearly all cases of resistance to the PSII inhibitors, resistance is due to a modification of the PSII inhibitor target site, the plastoquinone (Q_b) binding site on the 32-kDa protein of photosystem 2. The 32-kDa protein is termed the D1 protein and is coded for by the chloroplast *psb*A gene, the herbicide target site is the plastoquinone (Q_b) binding site on D1 and herbicide binding prevents Q_b transfer of electrons at the binding site (Fuerst and Norman, 1991). The details of the modification are well known, with a glycine substitution the most common alteration (Pfister and Arntzen, 1979; Mazur and Falco, 1989). Although other amino acid substitutions conferring different patterns of resistance are known, this list of species does not include weedy plants (Trebst, 1991). High levels of resistance to PSII herbicides (up to a 100 fold) are conferred by the target site alteration in weedy species (Fuerst *et al.*, 1986; Pfister and Arntzen, 1979).

Increased metabolism of the triazine herbicides atrazine and simazine has been demonstrated in a resistant biotype of the weed *Abutilon theophrasti* (Anderson and Gronwald, 1991). This biotype displayed about a 10 fold resistance which is due to over-expression of the detoxifying enzyme glutathione transferase within the resistant biotypes compared to susceptibles biotypes. Increased metabolism of atrazine has also been shown in some forage grasses by Weimer *et al.* (1988). In spite of these reports, increased metabolism has been rarely identified as a mechanism of resistance to the triazine herbicides in weeds from the herbicide intensive corn rotations. This may be because the target site alteration provides a higher level of resistance and hence an advantage over increased metabolism in populations where genes for both mechanisms existed, which could lead to the exclusion from populations of the plants carrying low level resistance.

Another weed species reported with resistance to PSII inhibitors conferred by increased metabolism is the grass weed *A. myosuroides* in which many populations from continuous wheat fields in Europe have become resistant. Resistance to a range of substituted urea herbicides, chlorotoluron, isoproturon and linuron and some triazines, by N-demethylation has been shown to be the principle mechanism of resistance (Kemp *et al.,* 1990).

In Australia non-target site cross resistance to PSII inhibiting herbicides conferred by increased metabolism has been reported in *L. rigidum* by Burnet *et al.* (1991). Of the biotypes described by Burnet *et al.*, one was selected by a mixture of atrazine and amitrole and displayed 3-9 times resistance to a range of PSII inhibitors when compared to susceptible biotypes. Another biotype was subjected to 17 years of selection by a phenylurea herbicide diuron and showed about a six fold increase in resistance (Burnet *et al.*, 1993). Resistant biotypes similar to the latter types abound due to the extensive industry in which the resistance developed and distribution of resistant seed into other markets. Both resistant *L. rigidum* biotypes have resistance conferred by metabolism of the herbicide molecule by N- demethylation which has been shown indirectly to be a cytochrome P-450 mediated reaction (Burnet *et al.*, 1993).

1.5.4 Resistance to ALS inhibitors

While the PSII herbicides have been used for many years, the ALS inhibitors became available in 1982 in Australia and since then these herbicides have been used extensively. Many chemical groups are available within the ALS inhibitor spectrum and many individual herbicides have been developed for use in the major crop species. The sulfonylurea group has many members however, chlorsulfuron, metsulfuron methyl and triasulfuron are used in cereal production. Other groups of ALS inhibitors include the triazolopyrimidine, the sulfonanilides (Gerwick *et al.*, 1990) and the imidazolinones (Shaner *et al.*, 1984). Resistance has developed to many of the ALS inhibitor herbicides in approximately 14 weed species, reviewed by Saari *et al.* (1994).

Target site resistance is the most common expression of insensitivity to ALS herbicides and usually involves amino acid substitutions at a proline residue in the ALS sequence (Saari, *et al.*, 1994). Other amino acid substitutions have been identified which result in some variation in the whole plant response to herbicides. In the case of *L. rigidum,* the altered ALS conferred about 80 fold insensitivity to the herbicide chlorsulfuron, which was the selective agent (Christopher *et al.*, 1994). Resistance to ALS inhibiting herbicides developed rapidly after the introduction of chlorsulfuron, within five years extensive infestations of resistant *Kochia scoparia* was observed in the U.S. (Saari, *et al.*, 1994) and resistance in *L. rigidum* was observed after only 3 applications (Gill, 1995).

Of the 14 weed species reported with biotypes resistant to the ALS inhibitors only *L. rigidum* has been identified as possessing the capacity for increased metabolism. A biotype of *L. rigidum* investigated by Christopher *et al.* (1992) displayed non target site cross resistance conferred by increased metabolism of ALS inhibiting herbicides. Other biotypes of *L. rigidum* have emerged with both target site resistance and non-target site cross resistance to the ALS inhibitors (Gill, 1995) depending on the selective regime. In *L. rigidum* biotypes, non-target site cross resistance to the ALS inhibitors has been the result of an increased capacity to metabolise the herbicides. Indirect evidence of cytochrome P-450 metabolism of the herbicide by aryl hydroxylation and glucose conjugation has been demonstrated (Christopher *et al.*, 1994; Christopher *et al.*, 1992; Cotterman *et al.*, 1992).

1.5.5 Resistance to ACCase inhibitors

The aryloxyphenoxypropionate herbicides diclofop-methyl, fluazifop, haloxyfop and quizalofop and the cyclohexanedione herbicides sethoxydim, tralkoxydim and clethodim inhibit the plastidic enzyme ACCase. All show acceptable herbicidal activity on normal populations of *L. rigidum* and some on other grass weeds.

Resistance to the ACCase inhibitors has developed in approximately 10 species (reviewed by Devine and Shimabukuro, 1994), including *A. myosuroides* (L. Hall pers. comm). Target site insensitivity is the most frequently reported resistance mechanism to ACCase inhibiting herbicides (Devine and Shimabukuro, 1994). Resistance conferred by target site alterations has been confirmed in two *L. rigidum* biotypes (Tardif and Powles, 1994; Tardif *et al.*, 1993), and in *L. mutilflorum* (Gronwald *et al.*, 1989), *Setaria viridis* (Marles *et al.,* 1993), *A. sterilis* (Maneechote *et al.*, 1994) and *Sorghum halepense* (Devine and Shimabukuro, 1994).

Diclofop-methyl is a cereal selective herbicide and is used to control *L. rigidum* and *Avena* spp. in wheat, triticale and barley crops. Most of the other ACCase inhibitors are not selective in cereals and are used for *L. rigidum* and *Avena* spp. control only in broadleaf crops. Tralkoxydim is also a cereal selective herbicide whereas sethoxydim and clethodim are not. Dicotyledonous species have ACCase enzyme that are unaffected by these herbicides.

Metabolism and detoxification of the cereal selective ACCase inhibiting herbicides is the mechanism of selectivity of diclofop-methyl and tralkoxydim in wheat and barley. Weed species also have the capacity to metabolise these herbicides (Maneechote *et al.*, 1994; Matthews *et al.*, 1990), but as yet, differential metabolism of the herbicide between resistant and susceptible biotypes has not been confirmed as a widespread mechanism conferring resistance to ACCase inhibiting herbicides.

Some resistant biotypes of *L. rigidum* and *A. fatua* which do not posses an altered ACCase and yet are strongly resistant to ACCase inhibitors have been described (Matthews *et al.*, 1990). Investigation of possible mechanisms uncovered a phenomenon which was not present in susceptible biotypes. Recovery from diclofop-induced depolarisation of the plasma membrane potential was correlated with resistant *L. rigidum* biotypes (Häusler *et al.*, 1991) and *A. fatua* biotypes (Devine *et al.*, 1992). Further work is required to reveal if these observations represent a widespread, uncharacterised mechanism of resistance.

1.6 Herbicide resistance in Australia

Sixteen weed species have been documented in Australia with biotypes that display resistance to herbicides, as listed in Table 1.2.

Table 1.2. Weed species in Australia with positively identified resistant biotypes.

Weed species with resistance	Herbicide groups to which resistance has developed	Refs *
Arctotheca calendula (L) Levyns.	Bipyridyls	1
Avena fatua L.	ACCase inhibitors	2
Avena sterilis Malzew.	ACCase inhibitors	2
Brassica tournefortii Gouan.	ALS inhibitors	3
Cyperus difformis L.	ALS inhibitors	4
Damasonium minus	ALS inhibitors	4
Digitaria sanguinalis (L.) Scop.	ACCase inhibitors	5
Hordeum glaucum Steud.	Bipyridyls	6
Hordeum leporinum Link.	Bipyridyls	7
Lactuca serriola L.	ALS inhibitors	8
Lolium rigidum Gaud.	ACCase, ALS, PSII inhibitors and others.	9
Polygonum convolvulus L.	ALS inhibitors	8
Sagittaria montevidensis	ALS inhibitors	4
Sonchus oleraceus L.	ALS inhibitors	3
Sysimbrium orientale Torn.	ALS inhibitors	3
Vulpia bromoides (L). S. F. Gray	Bipyridyls	10

* References: 1) Powles *et al.*, (1989); 2) Mansooji *et al.*, (1992); 3) Boutsalis and Powles, (1995); 4) Pratley, (pers. comm); 5) C. Preston, (unpublished); 6) Powles, (1986); 7) Tucker and Powles, (1991); 8) P. Boutsalis, (unpublished); 9) Burnet *et al.*, (1994); 10) Purba *et al.*, (1993).

1.6.1 Herbicide resistance in *L. rigidum*

Resistance to herbicides from different chemical groups which are normally effective on *L. rigidum* has been extensively documented (Burnet *et al.*, 1994; Hall *et al.*. 1994; Powles and Matthews, 1991; Matthews *et al.*, 1990; Heap and Knight, 1986). The most troublesome aspect of herbicide resistance in populations of *L. rigidum* is the extent to which cross resistance is apparent to herbicides that were not used in the selection process. Cross resistance can cause many difficulties for selective weed management on infested sites.

Resistant populations of *L. rigidum* are extremely widespread in southern Australia, with an estimated 5000 resistant populations in 1994. This has occurred since the first report in 1982 (Heap and Knight, 1982). The results of a random survey undertaken in 1993 and covering about 5,000 square kilometres of an intensively cropped region of South Australia, showed that 46% of cropped sites in the area surveyed have diclofop-methyl resistant ryegrass populations present (Neitschke *et al.*, 1996).

Diclofop-methyl has been widely used because it is selective for both *L. rigidum* and *Avena* spp. in cereal crops and very few other suitable selective herbicides (either for pre-emergent or post-emergent application) were available until the release of tralkoxydim in 1987. Both ryegrass and wild oats are often present in the same paddock. The survey of Llewellyn *et al.* (1994) showed both ryegrass and wild oats were present in the same paddock on over 90% of sites surveyed. This explains why diclofop-methyl applications have been widespread. In addition there has been a shift to post-emergence herbicide applications for weed control in southern Australian crop production systems to facilitate earlier crop sowing ("Right Rotations"; Department of Primary Industry, Adelaide, South Australia).

Given the above factors and the widespread use of diclofop-methyl the emergence of resistant populations was not surprising. Resistance to diclofop-methyl in the field has occurred after as few as 4-5 applications (Gill, 1995; Matthews and Powles, 1992). There are many factors influencing the time taken to observe resistance in the field and these are discussed in sections 1.7 and 1.8. Resistance to the other ACCase inhibitors can occur concurrently with the onset of diclofop-methyl resistance or within a similar period of time if other ACCase inhibitors are used exclusively (Tardif *et al.*, 1993).

Resistance to the ALS inhibitors is increasing as selection pressure is sustained over time. Many *L. rigidum* biotypes have been recorded with resistance to the sulfonylureas and to the other ALS inhibitors (Gill, 1995; Christopher *et al.*, 1992). Resistance to the ALS inhibitors is endowed by both target site alteration and enhanced metabolism of herbicides. Populations where an altered target site is the principle mechanism of resistance have been selected by ALS usage, usually a sulfonylurea herbicide applied to a cereal crop (Gill, 1995; Christopher *et al.*, 1992). However, the effectiveness of the cereal selective ALS inhibitors on ryegrass has been reduced by non-target site cross resistance induced by diclofop-methyl selection in large areas of the southern Australian cropping region (Gill, 1995; Hall *et al.*, 1994; Christopher *et al.*, 1992).

Although resistance to PSII inhibiting herbicides conferred by increased metabolism has been reported in *L. rigidum*, as yet, target site resistance to the PSII inhibiting herbicides in *L. rigidum* has not been identified (Burnet, 1991). The extent of resistance to PSII inhibiting herbicides due to PSII herbicide use is probably limited or confused by the application of other herbicides within a selection regime, however cross resistance to PSII inhibiting herbicides due to selection by ACCase inhibiting herbicides has been documented and may be widespread (Matthews and Powles, 1992).

1.6.2 Summary of herbicide resistance

Herbicide resistance has developed in many weed species world wide and the spectrum of resistance covers all the major herbicide groups with the exception of the non selective herbicide glyphosate. In Australia 16 weed species have been reported to display resistance, *L. rigidum* being the most widespread and important of these.

Within *L. rigidum*, widespread resistance has been identified to the ALS inhibiting herbicides and two mechanisms have been identified. One, an altered target site is a common phenomenon in other species of ALS resistant weeds; but the second, the metabolism of the herbicide as a resistance mechanism has not been reported, except among *L. rigidum* populations. Resistance to the ACCase inhibitors is widespread in Australia and many *L. rigidum* populations have resistance to some or all of the aryloxyphenoxypropionate herbicides and some to the cyclohexanediones. The mechanisms of resistance to the ACCase inhibitors are not yet fully understood, but of the few resistant populations studied in detail about half display target site resistance due to an altered ACCase. *L. rigidum* biotypes resistance to the PS II inhibitors are common, some biotypes have developed resistance by selection with a PS II inhibiting herbicide and some by developing non target site cross resistance.

It is common for resistant *L. rigidum* biotypes to display resistance to some or all of these herbicide groups, either from varied herbicide use or due to non target site cross resistance induced by the use of an ACCase inhibitor herbicide.

1.7 Other studies of resistance to xenobiotics

The onset of herbicide resistance has provided an example of rapid evolutionary change in response to a changing environment. The problem of resistance covers a range of disciplines as herbicide action is a physiological property and resistance is usually a physiological or biochemical response in plants. Resistance is genetic and when applied to populations the development of resistance is observed in an evolutionary context. The evolution of resistance depends on ecological factors that may vary with species, population and location. When the object of study are sessile weeds the local management system of crop protection is also relevant. The farmer or weed control specialist may also be involved if the focus of studies on resistance is to control weed populations that have become intractable to normal herbicide use. There are other examples of resistance to xenobiotics, namely insecticide resistance and tolerance of plants to heavy metals. Both fields have contributed to the study of evolution of populations to sustained and usually lethal selection pressures. Aspects from each of these areas of study relevant to the onset of herbicide resistance are presented below.

1.7.1 Insecticide resistance

Resistance to effective chemicals for the control of pest populations was not new when resistance to herbicides was first observed in 1968. Pesticide resistance is a common occurrence and the experience of evolutionary entomologists has provided valuable lessons for the phenomenon of resistance in plants. Since the first case of insecticide resistance in 1908 reported by Melander (1914), resistance has increased to cover at least 500 species and all current chemical insecticide groups (Georghiou and Lagunes, 1988).

The rapidity with which insecticide resistance has developed, following the use of highly effective pesticides, surprised many. Substantial resistance often developed within 2 years of an initial application following the introduction of organochlorine or organophosphate insecticides (Georghiou 1972). The issue of the frequency of resistant alleles in a population has been of considerable importance in understanding pesticide resistance. Georghiou and Taylor (1977) suggested an initial gene frequency of 10^{-2} - 10^{-4}, others such as Whitten and McKenzie (1982) suggest 10^{-3} - 10^{-13}, whereas Wood (1981) suggests 1-2 x 10^{-5}. The issue was resolved with the observation that the initial frequency of resistant individuals can be expected to vary with the species and the location. Populations within and between species may differ in this regard (Wood and Bishop, 1981).

Importantly, the argument regarding the frequency of resistance genes enabled the question of pre-adapted or post-adapted alleles within a population to be addressed. Genes for resistance have been shown to be pre-existing in populations prior to exposure to insecticides. Also, Bennett (1960) and Crow (1957) presented evidence to support pre-adaptation to insecticides in wild populations. The "post-adaptiveness" of inherited high levels of inducible oxidase enzymes does not exclude the presence of pre-adapted genes for inducibility.

Georghiou (1972) wrote, "The time taken for resistance to develop will depend on factors such as the frequency of resistance genes, their dominance, the selection pressure and history of previous exposure." The issue of dominance is well covered by Georghiou, who quotes several examples of rapid development under conditions of partial or complete dominance. The outcome of selection pressure is straightforward, although with some laboratory populations of *Drosophila melanogaster,* populations achieved a faster rate of development of resistance with selection at 50% mortality than at 95% (King, 1954). This was due to limited population size and reduced genetic diversity at the high selection pressure. Whitten and McKenzie (1982) suggested that laboratory populations may contain less genetic diversity than field populations as the exposure to pesticides is likely to be more homogenous in a laboratory than in the field. Given that, Georghiou writes "that the more intense the selection pressure, the more rapid is the progress of development of resistance, provided that the number of survivors is large enough to maintain genetic variability" (Georghiou, 1972).

Importance was given to the effects on fitness of resistance genes which can markedly affect the rate of development of resistance and the likely regression from resistance when insecticide application is relaxed. If resistant types are less productive, then the onset of resistance will be delayed, assuming that susceptible genotypes are still present. Some examples of progressive acquisition of normal fecundity associated with the onset of resistance to DDT (dichloro-diphenyl-trichloro-ethane) in *Aedes aegypti* indicate that resistance can often confer lower fecundity. However, another population of this species with different selection history and other species, for example, *Tetranychus urticae*, showed no fitness differential. Roush and Croft (1986) quote many studies of fitness and stability of resistant strains of insects and while many homozygote resistant genotypes appear less fit than comparable susceptibles, the heterozygotes were, for the most part, equally productive. Again, the issue of fitness of resistant types is dependent upon many factors and species and populations may vary due to many factors.

Regression of resistance following relaxation of insecticide pressure has been quoted as an indication of stability of resistance and fixation of resistance genes in populations. Populations appear variable for this characteristic and Forgash and Hansens (1962) report that a population of cross resistant *Musca domestica* was extremely stable for all but one insecticide when selection was relaxed for 35 generations. Georghiou (1972) reports similar stability also with *Musca domestica* and concluded that "resistance appears more stable towards certain compounds than towards others." Wood and Bishop (1981) cite many examples of stability in a range of species over many generations. The fitness of homozygous resistant genotypes governed the persistence of novel mechanisms appeared to be the determining aspect.

The potential for regression of herbicide resistance in *L. rigidum* has not been canvassed in the literature. It is likely to be very low given the annual generation interval of field grown plants. Also relevant aspects of the fitness of herbicide resistant biotypes have not been investigated, some studies of the fitness of herbicide resistant *L. rigidum* biotypes and at various times during the development of herbicide resistance are presented in Chapter 5.

There are frequent observations and reports of genetic dominance associated with insecticide resistance. Georghiou (1972) rates dominance as an "obviously important factor" in the development of resistance. An important aspect of dominance when resistance genes are rare is to preserve the heterozygote resistant gene and maintain genetic diversity within a treated population. Also, the rate of onset of resistance is increased when resistance genes are completely or partially dominant. Roush and Croft (1986) also consider dominance and discuss the relationship of dominance to dose rate. Where dose rates can be adjusted to influence the mortality of heterozygotes, dominance can be manipulated. Nevertheless, genetic dominance is an important consideration in the onset of resistance in a population especially where application rates are not variable, such as in weed crop associations where crop tolerances limit the herbicide application rate.

Like *L. rigidum*, many insect populations have displayed cross resistance following selection by a single insecticide. Cross resistance has been documented for *Musca domestica* following selection with the organophosphorus insecticide diazinon (Forgash and Hansens, 1962). The population was cross resistant to nine other classes of insecticides. Specific insecticide detoxification enzymes were identified for most of the products to which resistance was displayed (Forgash *et al.*, 1962). Georghiou (1986) documents that more than 25% of all insect and mite species displaying resistance were cross resistant to one other class of insecticide and 12% were cross resistant to two other classes.

The management difficulties associated with insect cross resistance presage the poor prospects of herbicides for field management of herbicide cross-resistant *L. rigidum* and other herbicide cross-resistant weed species.

1.7.2 Tolerance of plants to heavy metals

The appearance of plant populations with biotypes tolerant of toxic levels of heavy metals is another example of adaptation to xenobiotics in a selective environment. The frequency of resistance genes in plant species occupying sites contaminated by heavy metals has been investigated. Wu *et al.,* (1975) found a frequency of 5×10^{-4} survivors from non-tolerant populations of *Agrostis stolonifera* when grown on a contaminated site. A similar experiment by Walley *et al.*, (1974) uncovered a frequency of 3×10^{-3} survivors in *Agrostis tenuis*. Gartside and McNeilly (1974b) showed a high level of initial survivorship in *A. tenuis* and in *Dactylis glomerata*, however, not all species examined by Gartside and McNeilly were successful in producing tolerant survivors. Ingram (1984) cited by McNair (1987) found a high correlation with species that were present on mine sites and a detectable level of tolerance to contaminated sites in unselected plants. Whereas, those species adjacent to but not inhabiting mine sites did not display tolerance to contaminated sites. Both Gartside and McNeilly and Ingram's observation point to the specificity of tolerance mechanisms within plant species for heavy metal toxicity.

The level of cross tolerance to other heavy metals displayed by already tolerant populations is low but detectable (Symeonidis *et al.*, 1985). They showed that low levels of cross tolerance existed for intra-specific comparisons on a range of heavy metal contaminants. Walley *et al.* (1974) made the point that copper tolerant plants were more tolerant of zinc than plants taken from a non-selective environment and zinc tolerant plants were more tolerant of copper than non tolerant plants.

The fitness of plants inhabiting contaminated mine sites has been studied by comparing plants from mine sites with non-tolerant plants under normal conditions. Most of these studies have shown that plants from mine sites are competitively inferior to pasture plants (Hickey and McNeilly, 1975). However it can be argued that mine sites have many edaphic differences from pasture soil and the adaptations of tolerant plants may be more than tolerance to metal contaminants alone. McNair and Watkins (1983) used backcrossed copper tolerant *Mimulus guttatus* to identify any fitness penalty from the tolerance gene, and found no difference in any fitness related character. *M. guttatus* had been previously shown to be tolerant because of a single gene which may have reduced the genetic adaptation required for tolerance. Fitness is an important character in adapted plants or populations especially when considering persistence as a competitive weed. This is discussed in Chapter 5 with relevant experimental data.

1.8 Review of the factors leading to herbicide resistance

1.8.1 Frequency of herbicide resistance genes

The frequency of pre-existing herbicide resistance genes in weed populations that have not previously been exposed to herbicides is not well known. Early studies by Price *et al.* (1983), Somody *et al.*, (1984) and Thai *et al.*, (1985) showed that populations of *Avena barbata* and *Avena fatua* were variable in their reactions to the wild oat herbicides. In a later study Price *et al.*, 1985, showed a relationship of the herbicide response variability with the presence of enzyme polymorphisms within discrete populations.

Initial levels of triazine resistance in *Poa annua* were established by Darmency and Gasquez (1990) and in *Alopecurus myosuroides* for the herbicide fenoxaprop-*p*-ethyl (Chauvel *et al.*, 1992). The proportion of survivors in each case were higher than anticipated from theoretical mutation rates and selection on that mutation. A similar condition was discussed by Saari *et al.* and presumed to apply to the onset of ALS resistance in *K. scoparia* documented in Saari *et al.* (1994), although due to the mode and rate of seed spread from surviving plants accurate assessments cannot be made.

The frequency of novel genes in a population is considered to be due to either mutation, migration, selection or genetic drift. If herbicide resistance genes are present, some degree of genetic dominance if the gene or genes is present as a heterozygote or homozygosity for a recessive allele is required for selection by herbicide applications to result in evolution towards resistance. The high initial frequencies of genes conferring resistance both in weed and insect populations is of great interest. If a novel gene is present at higher than mutation frequency such that it is not maintained by mutation alone, either it has bestowed no disadvantage to the phenotype and may have been of adaptive significance under some environmental conditions.

Emphasis is often given to mutation selection theory to place an initial figure on the frequency of resistance genes in unselected populations for the purpose of modelling the onset of resistance (Gressel and Segal, 1990; 1991). The frequency of pre-existing alleles has been deduced from the mutation balance theory of Wright (1937). Briefly, the mutation frequency is balanced by the selective disadvantage of the new allele, as in m/d, where m, is the mutation rate, and d, the selective disadvantage. However, population size affects this relationship due to genetic drift and exclusion of alleles in small populations. The frequency of mutations conferring resistance to ALS inhibiting herbicides was found to be 1.2×10^{-4} in mutagenised *Arabidopsis thaliana*, (Haughn and Somerville, 1990) when about one million seed were treated. The frequency of immediate novel mutations conferring resistance is a factor of the population size and the mutation rate alone in populations exposed to herbicides. For fecund weed species this means a greater probability of encountering a mutation than for a species with lower seed output.

Brown (1979) has drawn together reports of the considerable amount of enzyme variation existing in plant populations by isoenzyme studies. Quoting many sources, Brown, reviewed the level of polymorphism in outcrossing and selfing species and found that more polymorphisms were identified in selfing species and less in outcrossing species than were predicted. As a consequence the inter-population variation was greater in the selfing species and intra-population variation greater in the outcrossing species. The presence of genetic variation provide the basis the selection of resistant alleles by herbicide application in weeds. Although, as is the case with tolerance to heavy metals, the genetic variation has to be appropriate and capable of conferring resistance in particular species to particular herbicidal molecules. The initial frequency of genes bestowing resistance is an important factor in the observation of the rapid onset of herbicide resistance in *L. rigidum* and this is examined in Chapter 2.

1.8.2 Genetic aspects of the rate of appearance of resistance

The rate of onset of resistance in weed populations is of considerable interest. In particular the high rate of onset of resistance in *L. rigidum* populations (Gill, 1995; Powles and Matthews, 1991) was quite surprising, with high levels of resistance following 3-5 years of herbicide application.

The rate of appearance of an allele in a population under constant selection is influenced by the inheritance of that trait. If resistance to a toxicant is conferred by many genes each with a small contribution to the resistance phenotype, resistance is likely to progress at a slower rate and reach a lower phenotypic level than if a major gene is involved (McNair, 1991). In evolution by natural selection, adaptation to a changing environment by minor genes or polygenes is considered to be more probable (Lande, 1983). This is likely to be the case when selection is not severe and does not select at the extreme of the phenotypic range of the population. However, when selection occurs at the extreme of the phenotypic range then a (rare) major gene is likely to be selected, (Lee and Parson, 1968; McNair, 1991). The many cases of resistance to the PSII inhibtors (section 1.5) differ in that although resistance to the PSII inhibitors is endowed by a single gene alteration, the *psb*A chloroplast gene has been shown to be maternally inherited in the majority of cases (Darmency, 1994).

Jasieniuk *et al.*, (1996) discuss the trends in herbicides to which resistance has evolved and suggest that as recent herbicides such as the ACCase and ALS inhibitors are both site specific to a major enzyme in sensitive biochemical pathways, a single amino acid substitution would be likely to confer high levels of resistance. The selection pressure of these products can be high and therefore selection for an altered single gene is likely to be favoured. The experience from many cases of herbicide resistance shows that this is the case. Of 21 studies of the mode of inheritance of resistance to herbicides, excluding resistance to the triazine herbicides, 17 were conferred by one or two major genes, (Darmency, 1994 and Saari *et al.*, 1994). Fourteen of those cases were reported to display either complete or semi dominance, four examples were given of quantitative inheritance of which two had heritabilities greater than 0.5. Darmency also lists 14 cases of maternal or chloroplastic inheritance of triazine resistance. All the above inheritance studies were of established resistant biotypes that had been exposed to selection for long periods of time. Similarly, the pattern of dominant inheritance of a major gene or genes was found to apply in most cases of adaptation of plants to toxic levels of heavy metals (McNair, 1981). Dominance in an allogamous population undergoing selection is necessary for the survival of resistance conferring allele(s) where the resistant allele is rare and occurs as a heterozygote. This is not the case for an autogamous species (Jasieniuk *et al.*, 1996)

During the critical early period of selection, the heritability of resistance may be different to that encountered following several phases of selection. As a consequence the rate of onset of herbicide resistance in the first cycle of exposure to a herbicide may vary from that postulated by studies on mature resistant material. This issue is investigated in Chapter 2.

1.8.3 Fitness of herbicide resistance populations

The role of fitness studies in determining the relative success of a resistant biotype has featured in many studies of herbicide resistant biotypes, particularly the fitness of weed species that have developed resistance to the triazine herbicides. Populations resistant to the PSII inhibitors where resistance is conferred by an altered Q_b protein have been shown to be less fit than susceptible counterparts (reviewed by Holt, 1990). The altered Q_b protein decreases the affinity of the herbicide at the active site but also reduces the rate of electron flow. This reduces photosynthetic rates and dry matter accumulation leading to reduced seed production and competitiveness when compared to a susceptible biotype (Holt, 1990).

Following the observation of reduced fitness in triazine resistant biotypes, many resistant species have been investigated for comparative fitness. The role of fitness studies are three-fold. Firstly, to assess the adaptation of the gene conferring resistance and identify any physiological disadvantage that may have occurred with the enrichment of the resistance gene in the population. Secondly, to identify any environment or management routine in which the resistant biotypes may be at a disadvantage and to exploit any substantial difference between resistant and susceptible biotypes. Thirdly, to help to predict the onset or decline of resistance in the field in either the presence or absence of herbicides.

The relative success of one biotype when compared to another may be measured in many ways. Relative dry matter at some stage of growth or age or final seed or propagule yield are commonly used. However, for most annual weed species, fitness in its fullest sense is best measured as the relative performance in seed or propagule yield and measuring dry matter accumulation or dry matter yield may not be an accurate assessment of fecundity. Other factors such as relative germination rates and seedling survival are also important to the overall persistence of plants carrying a resistance gene.

Fitness studies have been most meaningful when resistant and susceptible biotypes differing only in the resistance trait were compared. Such studies have given meaningful physiological information also (Ducruet and Lemoine, 1985; Holt and Radosevich, 1983). This is easier to obtain with maternally inherited triazine resistance than in outcrossing species. As a compromise, fitness studies other than those conducted on PSII inhibitor resistant biotypes have mainly relied upon comparative material from a common environment.

Information is becoming available on the relative fitness of species with biotypes resistant to herbicides other than the triazines. Paraquat resistant biotypes have not displayed substantial differences in relative dry matter accumulation (Tucker, 1989; Purba, 1993). Biotypes of *Elusine indica* resistant to ACCase inhibitors produced less biomass than susceptible plants (Holt and Thill, 1994), but the concise relative fitness of ACCase resistant *L. multiflorum* is not known. Holt and Thill (1994) have reviewed the biotypes with ALS inhibitor resistance and none except *Lactuca serriola* appear to be substantially different in growth, competitiveness or seed production. Resistance to the dinitroaniline herbicide trifluralin, has been shown to reduce fitness in *E. indica* (Murphy *et al.*, 1986).

Fitness studies in resistant biotypes with extensive cross resistance have not been reported upon. Mortimer *et al.* (1992) studied the relative competitiveness and productivity of *A. myosuroides* in the presence and absence of the herbicide chlorotoluron. Some biotypes of *A. myosuroides* have been shown by Moss and Hall, (pers. comm.) to be cross resistant, however, no mention was made of the cross resistance status of the biotype used by Mortimer *et al.* The fitness of three *L. rigidum* biotypes resistant each resistant to a range of herbicides and each selected under a different herbicide regime is reported in Chapter 5.

1.8.4 Resistance and selection pressure

The mortality of, or selection pressure applied to, a weed population undergoing selection for resistance is considered to have an influence of the rate of onset of resistance (Gressel and Segel, 1990). Gressel and Segel suggested that selection pressure should be considered as a season long influence on the relative productivity of embryos of comparative types. However, Mortimer (1992) inferred from a simulation of the time required to reach visible resistance (20%) that the duration of a viable seed bank and the annual per capita rate of seed production are the major determinants of the rate of onset of resistance rather than selection pressure.

Reducing selection pressure when the frequency of resistance genes is high in an outcrossing species will favour survival of more heterozygotes only if dominance is less than neutral or is variable. If that is the case then maintaining high selection pressure when resistance genes are infrequent will keep them at a low frequency and reduce the number of heterozygotes surviving. This point is often made with insecticide resistance

(Curtis *et al.*, 1978). In the case of insect species with cross resistance a high selection can reduce genetic variability and may preclude some resistance mechanisms.

In practice, herbicides often have upper limits to doses due to crop tolerance, and the full extent of dose rate adjustments cannot be made. Where herbicides have no crop tolerance considerations higher rates have been used with limited success in reducing the scope of cross resistance, (Matthews 1994). When resistance is inherited as a dominant character then all the heterozygotes will display resistance and the use of high dose rates will exclude only the susceptible portion of the population. In addition, the use of lower rates to maintain a level of susceptible alleles in a population will also select for polygenic resistance, (Mortimer 1992) and there will be the added weed burden from poor control. It appears there is little scope within the practical limits of crop herbicide tolerance to alter application rates such that the level of resistance or the time taken to reach an observable level of resistance will be influenced.

1.9 Conclusion

Resistance to insecticides is a common occurrence and many of the factors involved in the onset of insecticide resistance have relevance to herbicide resistance. Of particular interest is the high level of initial survivors found in untreated insect populations. There are substantial intra- and inter- species differences for initial survivorship and although very little experimental data is available for weed populations, the few examples quoted show little difference to those quoted for insect species that have developed resistance.

Studies performed on pesticide resistant insect and weed species indicate the importance of an understanding of fitness to evaluate the potential for increasing the proportion of susceptible individuals and for persistence of the resistant individuals in the absence of the pesticide. Many weed populations resistant to the triazine herbicides have been studied and the resistant biotypes have been shown to be less fit. Other resistant weed biotypes have variable fitness when compared to susceptible counterparts. The relative fitness of resistant *L. rigidum* populations is important to understanding the dynamics of resistant populations.

The role of genetic dominance in the rate of onset of resistance has been shown to be critical for insect species. Most insect species that have rapidly acquired resistance display dominance of the resistance mechanisms. This may be an outcome of the selection procedure where the susceptible and non-dominant heterozygous component of the population is removed following pesticide application. However the heritability of resistance with the remaining, or immigrant susceptible, part of a population will affect the rate of onset of resistance.

Cross resistance poses the biggest threat to management of both resistant insect and weed populations. The similarities between the insects and weeds are pertinent. Where insects display extensive cross resistance, control is extremely complicated. The same situation applies to herbicide cross resistant *L. rigidum* except that control with herbicides is further limited by crop tolerance considerations. Metal tolerant plants display some tendency to cross resistance. For both plants and insects, the variability for response to xenobiotics has not been constant between species which illustrates the requirement for species specific information. There is considerable genetic variation in plant species, both in the allogamous and also the autogamous species. It is therefore, not surprising that weedy species when challenged with effective toxicants should respond by developing resistant populations in the face of extreme selection pressure.

1.10 Objectives of this study

The content of this thesis is concerned with aspects of the development of herbicide resistance in *L. rigidum.* There has been an extremely rapid increase in the number of populations of *L. rigidum* displaying both resistance and cross-resistance. An understanding of the factors leading to the development of resistance may be useful for designing control measures and procedures to manage herbicide resistance.

The frequency of genes conferring resistance in untreated populations of *L. rigidum* and the heritability of resistance in the early generations of selection for resistance is considered. The response to herbicides of a population undergoing selection by repeated applications of diclofop-methyl and the onset of target site resistance and non-target site cross resistance in that population is presented. The mechanistic basis of resistance and cross-resistance and the inheritance of the mechanisms is reported upon in the diclofop-methyl selected population. Also, the fitness of various resistant populations of *L. rigidum* each with different resistance mechanisms is examined. Finally, with the inevitable onset of resistance and cross-resistance in populations of *L. rigidum* a novel method of managing *L. rigidum* in the field is discussed.

Chapter Two

The incidence and ecology of herbicide resistant plants in natural populations of *L. rigidum*

2.1 Introduction

The onset of resistance to herbicides in plant populations subjected to herbicide application is due to a number of factors as referred to in Section 1.8. A major determinant in the onset of resistance is the initial frequency of plants within the original population under selection that have a resistant phenotype and survive the initial herbicide application. In the absence of resistant individuals in a population there is no likelihood of resistance developing, however, if plants displaying a resistant phenotype are present within a treated population and if the surviving plants are able to produce seed then selection for resistance can be initiated.

Variants within a population may be due to *de novo* mutation of genes coding for herbicide target sites as is evident with triazine resistance conferred by *psb*A mutants (Darmency and Gasquez, 1990). Where genes such as the *psb*A mutants also confer a fitness penalty in the absence of the herbicide (Holt, 1990) they may be less persistent in a wild population. Where no fitness penalty is conferred, altered genes could be expected to persist or perhaps accumulate within the population over time Mutation of herbicide target sites are not dissimilar from other examples of mutations involving already existing mechanisms of plant defences and normal constitutive function. Mutations endowing herbicide resistance that have been documented include both target site mutation or over-expression of detoxification mechanisms (Dyer, 1994; Christopher, 1994; Leah *et al.*, 1994; Gronwald *et al.,* 1989).

There have been many studies that show the considerable genetic variation in plant populations (Brown, 1979). Other studies have linked polymorphisms to ecological conditions (Hamrick *et al.*, 1979). However, there have been few studies which establish the initial frequency of pre-existing resistance to herbicides in a population of a single species of plants that are usually controlled by that herbicide. Many authors assume that alleles conferring resistance are present in populations at frequencies ranging from 10^{-5} to 10^{-12} (Gressel and Segel, 1990; Putwain and Mortimer, 1989; Gressel, 1986) but a limited number of studies have shown that higher frequencies do occur. Darmency and Gasquez, (1990) documented populations of *Chenopodium album* in which the frequency of plants conferring triazine resistance in the next generation occurred at about 8% in the original population. Chauvel *et al.*, (1992) reported a mean survival of 0.46% in *A. myosuroides* in the first generation of 4 untreated populations when treated under laboratory conditions with the herbicide fenoxaprop-*p*-ethyl.

Pre-existing resistance to herbicides in wild type populations of *A. fatua* has been noted by Price *et al.* (1983), who found significant levels of genetic variation in the response to several herbicides. In that study, substantial inter-population variation and some intra-population variation to the herbicide barban (4-chloro-2-butynyl-3-chlorophenylcarbamate) was detected. Somody *et al.* (1984) reported variability in response to seven herbicides in samples from over 1000 populations of *A. fatua*. Thai *et al.* (1985) noted some limited variability to the herbicide triallate [S-(2,3,3-trichloro-2-propenyl) bis(1-methylethyl)carbamothioate] and reported a 1% "tolerance" to diclofop-methyl. Price *et al.* (1985) also found significant correlations between enzyme polymorphisms, morphological variation and response to herbicide application in *A. fatua*. Other examples of pre-existing genetic diversity have been for tolerance to the herbicide chlorotoluron in the progenitors of current wheat species (Snape *et al.*, 1991) and polymorphisms allowing crop mimicry in wild rice (Mortimer, 1984). These studies indicate that genetic diversity exists to a greater extent than has been appreciated for many herbicides and weed control measures.

Ecological factors either alone or in combination may also serve to vary the frequency of alleles conferring resistance. Many cases of selection by pre-existing ecological factors have been reported, such as heavy metal contamination leading to tolerance in plant populations (Antonovics, 1984; Antonovics *et al.,* 1971). There are also numerous reports of genetic variability within stable populations of plants that are frequently weedy when present in crop producing areas, *viz*; Marshall and Allard (1970) with *A. fatua* and Darmency and Gasquez (1983) with *Poa annua*. From these studies it can be concluded that there is sufficient variation within most plant populations to respond to one or a series of selection events.

Although the application of a herbicide, may appear to be a variable component of the selection process, the production of crops in monocultures or regular rotations frequently involves repeated use of the same or similar herbicides. Many of the effective grass herbicides implicated in resistance in *L. rigidum* are from the same chemical group. Herbicides are very reliable when applied correctly and usually cause high levels of mortality. Therefore, herbicide usage can provide an intense and consistent selection regime.

The rate of increase of the resistant proportion of a population undergoing selection is strongly influenced by the level of dominance of the resistance trait and the fitness of plants displaying the trait. Thus, the mode of inheritance of the traits conferring resistance to individuals within a population affects the rate of selection of resistance. *L. rigidum* is an allogamous species and resistance will only develop in a field population under selection if the allele(s) conferring resistance have some level of dominance. Undoubtedly, the mode of inheritance of traits conferring resistance are important in determining the rate of onset of herbicide resistance in the early generations of selection. Inheritance of resistance has been documented for many weedy species and is reviewed by Darmency (1994). Darmency reviews 14 cases of resistance and notes that where resistance was examined using Mendelian ratios, all but two were inherited in a dominant or semi-dominant fashion. Where resistance is inherited quantitatively the heritability is greater than 0.5 in three cases out of four. These populations were all from established resistant populations with long histories of exposure to herbicides which does not give an accurate assessment of heritability in the early generations of selection. Chauvel *et al.*, (1992) has reported on the early generations of selection in *A. myosuroides* and although the mode of inheritance was not reported, the survival of successive generations shows that resistance is highly heritable.

In this chapter, studies were conducted with previously unselected populations of *L. rigidum* which were treated with the grass herbicide diclofop-methyl to establish the frequency of resistant individuals in the populations. The mode of inheritance of the resistance in the survivors of an initial herbicide application is also presented. Some investigations into the ecological aspects of the frequency of resistant phenotypes from the previously unsprayed populations is also discussed.

2.2 Population sampling and experimental methods

Populations of *L. rigidum* were collected from a range of sites across southern and central South Australia and Victoria, a distance of approximately 900 kilometres in an East-West direction. The collections were made in the summer of 1991 and 1992 and the locations of the sites are given in Table 2.1. Collections were made from both farmland and woodland sites.

The farmland collections were from pastured areas or within cropped areas and all of the farmland samples were personally collected by the author and were from farms with no herbicide applied to the area of collection. Herbicides may have been applied to adjacent farms but with the exception of sample 3, other farms were at least 100m away from the collection site. Sample 3 was collected about 30 metres from an adjacent farm. Collections from woodland sites were made from among wooded areas on roadsides or from bushland sites with no herbicide history as they were inaccessible for herbicide application. Sampling was as extensive as possible in each site as the density within sites were variable. Approximately 1000 seed heads were collected at each site from mature plants, placed in paper bags with location and site details noted. Seeds were subsequently threshed by hand and stored in a cool, dry environment.

Table 2.1. Geographic origins and site details of *L. rigidum* populations.

Population Identification	Location (State and map refs.)	Land use	Site rainfall (approx) mm.
1	Unknown *		
2	S.A. 36. 18° S: 140.40° E	farmland	500
3	S.A. 36.19° S: 140.38° E	farmland	500
4	S.A. 36.45° S: 140.55° E	farmland	475
5	S.A. 36.47° S: 140.44° E	farmland	450
6	S.A. 37.03° S: 140.54° E	farmland	500
7	S.A. 36.47° S: 140.53° E	farmland	500
8	S.A. 35.24° S: 138.57° E	farmland	450
9	S.A. 33.08° S: 138.41° E	farmland	425
10	Vic. 37.09° S: 143.08° E	farmland	600
11	Vic. 36.02° S: 146.51° E	farmland	600
12	S.A. 36.15° S: 140.56° E	farmland	450
13	S.A. 34.16° S: 138.51° E	farmland	475
14	S.A. 34.16° S: 138.51° E	farmland	475
15	S.A. 36.18° S: 140.45° E	farmland	475
16	S.A. 35.01° S: 137.40° E	woodland	450
17	S.A. 34.10° S: 135.40° E	woodland	380
18	S.A. 33.40° S: 135.42° E	woodland	380
19	Vic. 36.47° S: 140.03° E	woodland	475
20	Vic. 36.56° S: 141.57° E	woodland	475
21	Vic. 36.56° S: 141.57° E	woodland	400
22	Vic. 35.46° S: 143.48° E	woodland	425
23	Vic. 36.06° S: 145.16° E	woodland	475
24	Vic. 37.42° S: 142.55° E	woodland	575
25	Vic. 37.19° S: 142.50° E	woodland	590
26	Vic. 37.09° S: 143.08° E	woodland	580
27	Vic. 37.38° S: 142.54° E	woodland	575

* Population 1 is a commercial sample acquired by the University of Adelaide from an unknown source in 1975, before the release of selective graminicides.

Methods used for growing seedlings and applying herbicides

Seeds were sown as required into sterile recycled potting soil during the winter and allowed to germinate and grow to the 2-3 leaf stage. Winter is the normal season for germination and growth of *L. rigidum.* Where seedlings were transplanted, seeds were placed on 0.6% agar and chilled at $4^{o}C$ under dark conditions for 7 days to improve germination. Seeds were then germinated on 0.6% agar in a growth cabinet with $22^{o}C$ and 14hr light and $15^{o}C$ 10hr dark period, and transplanted at the one leaf stage to pots. Plants were grown outside during the normal winter growing season.

Herbicides were applied in a laboratory spray cabinet. Herbicide was delivered in water carrier at a total volume of 113L ha^{-1} at a pressure of 250 kPa, pressurised by compressed air. Commercial herbicide was used and wetting agent was used as recommended by the manufacturer. The plants were maintained outside after herbicide treatment. Plants were counted before treatment, and surviving plants counted 3-4 weeks after herbicide application. Plants which were green and growing were classified as surviving plants.

Experimental details; frequency of resistant plants in natural populations.

Two experiments with 2 replicates each of about 500 plants were performed Data was analysed with anova and t-tests and transformed $(X+0.5)^{1/2}$ where indicated.

Conditions for inheritance studies

Plants selected for crossing were established in 30 cm diameter 10 litre capacity polythene tubs filled with recycled potting soil. The plants were allowed to grow and prior to anthesis were surrounded with a clear polythene shield of 35 cm diameter and about 2m in height to exclude external pollen. Cross pollination was therefore limited to those plants within the plastic shield. The pots were watered frequently until seed filling was complete and seed harvested when the plants were mature. The seed was threshed by hand and stored under cool dry conditions.

2.3 Frequency of resistant plants in natural populations.

2.3.1 Results

The frequency of survivors from the *L. rigidum* populations listed in Table 2.1 following an application of the recommended field rate of the herbicide diclofop-methyl was assessed and the percentage that survived is listed in Table 2.2. The response of these *L. rigidum* populations to treatment with 360 g a.i. ha^{-1} diclofop-methyl are also presented as a graph in Figure 2.1 to show the natural division of the populations into distinct groups.

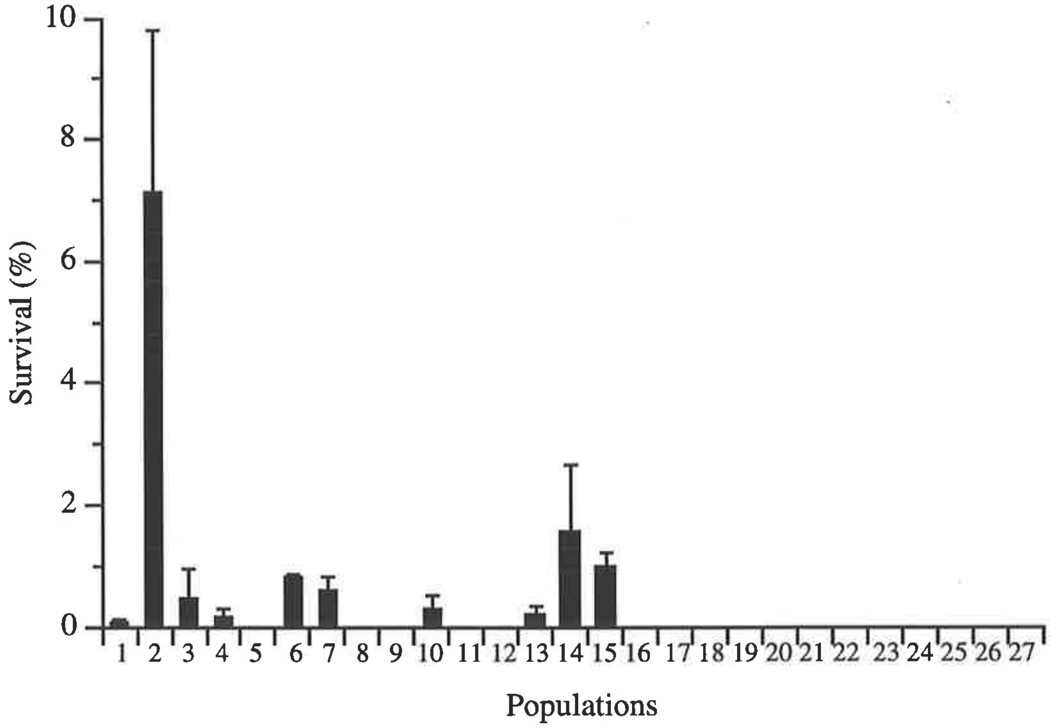
It is emphasised that all populations from farmlands (1-15) were collected from field sites (Table 2.1) from farms with a history of no herbicide use. They will be referred to as farmland populations. Similarly, the collections from woodland sites (16-27) were from areas in natural parklands that were inaccessible to herbicide application equipment and also had no herbicide history and will be referred to as woodland populations.

Table 2.2. Per cent survival of *L. rigidum* populations (Table 2.1) following treatment with diclofop-methyl; SE of the mean in parenthesis.

	% survival		
	Diclofop-methyl		
Population ID	360 g a.i. ha^{-1} (SE)	270 g a.i. ha^{-1} (SE)	180 g a.i. ha^{-1} (SE)
1	0.08 (0.025)	1.40 (0.04)	0.91 (0.14)
2	7.13 (2.675)	nt*	nt
3	0.47 (0.47)	nt	nt
4	0.14 (0.14)	2.6 (0.44)	9.13 (1.7)
5	0	0.86 (0.29)	5.5 (0.5)
6	0.8 (0.05)	5.37 (0.32)	11.9 (0.74)
7	0.61 (0.19)	2.38 (0.56)	13.25 (3.28)
8	0	0.83 (0.03)	2.75 (0.19)
9	0	0.26 (0.18)	1.03 (0.5)
10	0.3 (0.21)	1.86 (0.91)	2.48 (0.54)
11	0	0.35 (0.25)	0.25 (0.18)
12	0	2.08 (0.36)	2.63 (0.67)
13	0.20 (0.14)	0	0.33 (0.24)
14	1.50 (1.1)	1.24 (0.17)	1.98 (0.32)
15	1.0 (0.2)	1.75 (0.45)	4.8 (1.3)
16	0	0	0
17	0	0.6 (0.02)	0.57 (0.4)
18	0	0	1.91 (0.09)
19	0	0	2.04
20	0	0.45 (0.32)	0.417 (0.29)
21	0	nt	nt
22	0	0.35 (0.24)	0.75 (0.53)
23	0	0.35 (0.24)	0
24	0	0.35 (0.25)	0.65 (0.45)
25	0	1.11 (0.25)	1.21 (0.85)
26	0	nt	nt
27	0	0	0.32 (0.23)

* nt; not treated due to insufficient seed.

Figure 2.1. Survival of the ryegrass populations listed in Table 2.1 after treatment with diclofop-methyl (360 g a.i. ha^{-1}).



Legend: Individual ryegrass populations listed as in Table 2.1. Error bars indicate SE of mean. The data is the mean of 2 replicates.

The data in Table 2.2 shows that some populations had a low percentage of survivors when treated with 360 g a.i. ha^{-1} of diclofop-methyl. Figure 2.1 shows that the farmland populations (1-15) as a group had some survivors and by contrast, none of the woodland populations (16-27) had any survivors at the field rate of diclofop-methyl.

The mean survival percentage and S.E. to diclofop-methyl applied at 360 g a.i. ha^{-1} in the populations tested is presented in Table 2.3. The frequency of resistant plants identified from farm populations (populations 1-15) was about 1% compared to zero in the samples from woodland sites. The mean of all the collected samples was

0.472% (SE 0.27). It is clear from these results that collections of *L. rigidum* from farmland sites (populations 1-15) with no herbicide history showed a high initial percentage of individuals resistant to the herbicide diclofop-methyl. The frequency of diclofop resistant individuals in previously untreated ryegrass populations from farmlands (populations 1-15) is different from the frequency of resistant survivors from woodland sites (populations 16-27) from within the same geographic region.

Table 2.3. Mean survival of all *L. rigidum* populations and the mean of selected groups of ryegrass populations following exposure to 360 g a.i. ha^{-1} of diclofop-methyl.

Populations	Mean survival % (SE)
1-15 (farm sites)	0.937 (0.54)^{a*}
16-27 (non-farm sites)	0^{b}

* Rows followed by different letters are different, anova P<0.05, transformed data.

These results establish that there was a substantial level of resistant plants within unsprayed populations of *L. rigidum* originating from farmland and indicates that resistance to the herbicide diclofop-methyl when applied at the usual field application rate was present in populations with no herbicide application histories.

Populations 1, 4 - 21 and 23 - 26 were also treated at 270 g a.i. ha^{-1} and 187 g a.i. ha^{-1} of diclofop-methyl and the proportion surviving is shown in Table 2.2. Most of the populations tested showed increased survival when treated at lower rates of herbicide. At lower rates of herbicides the grouping was similar. Of the populations treated at either the field rate or with lower rates of diclofop-methyl the survival of populations 1-15 were different at each rate of herbicide applied (anova, p<0.02) and populations 1-15 were different from populations 16-26 at each rate of herbicide (anova, $p<0.05$).

2.3.2 Discussion

There have been very few studies that establish the frequency of herbicide resistant individuals in weed populations not previously treated with herbicides. In the absence of data, most authors of population dynamics models simulating the development of herbicide resistant populations have assumed the initial frequency of herbicide resistant individuals to be in the range of 10^{-5} to 10^{-12} (Maxwell and Mortimer, 1994; Gressel and Segel 1991).

In this study the frequency of resistant individuals of *L. rigidum* sampled from woodland populations was not able to be established due to the absence of survivors in the number treated. Of approximately 1000 plants from the 12 woodland populations screened at the field rate of diclofop-methyl there were no survivors (Table 2.2 and Figure 2.1). Thus, the frequency of survivors is less than 1 in 12000 or less than 8.3×10^{-5}. This is not inconsistent with the assumptions from mutation theory or from the literature. In contrast, the frequency of resistant individuals of *L. rigidum* from farmland populations was found to average about 1% (Table 2.3). This value is much higher than expected from theoretical considerations. The frequency of resistant plants described here is similar to studies on four previously untreated populations of *A. myosuroides* by Chauvel (1992). Although about half of the populations treated did not contain any resistant survivors at the 360 g a.i. ha^{-1} rate of diclofop-methyl, the response to lower rates of herbicides (Table 2.2) indicates that substantial genetic diversity exists within those populations also.

L. rigidum is an extremely adaptable species and as is evident from the geographic range over which it thrives, is able to adapt to environmental variations (Forcella, 1984). In this case the high frequency of herbicide resistant *L. rigidum* collected within populations across such an extensive geographic range (900 kms) indicates that the species has the genetic variability to generate phenotypic diversity and for that diversity to persist in certain environments. As an allogamous species it has the capacity to generate genetic diversity at each generation and maintain heterozygosity for a novel allele(s) not undergoing selection.

The observed frequency of resistance to herbicides (1%) in the farmland populations is very high. This may be due to there being more than one resistance mechanism capable of conferring resistance in *L. rigidum* populations. Previous work has shown there is a substantial diversity of resistance mechanisms to diclofop-methyl and to other herbicides in resistant populations of *L. rigidum*. Mechanisms of resistance to herbicides that have been identified include target site alteration (Tardif and Powles, 1993; Christopher *et al.*, 1992), enhanced metabolism of herbicides (Burnet *et al.*, 1993; Christopher *et al.*, 1992) and a postulated sequestration mechanism (Häusler *et al.*, 1991). The observed frequency of the alleles bestowing resistance may be due to either mutations or over-expression of beneficial genes, for example, diclofop is known to be metabolised by a cytochrome P450 hydroxylase (Zimmerlin and Durst, 1990).

Even with the possibility of several different mechanisms conferring resistance being present in a population the finding of close to 1% resistance in farmland populations is striking. This level of resistance is much higher than expected and the possibility that gene flow from resistant populations has enriched the populations from farmland sites (populations 1-15) cannot be disregarded. However, gene flow by pollen drift was investigated in *L. perenne* by Copeland and Hardin (1970) and they report that pollen was undetected at 12m from the source. Maxwell (1992) simulating the hybridisation of resistant and susceptible *L. multiflorum* calculated that a 1% likelihood of effective gene flow may occur within 7m of a source depending on the size of both the pollen source and receptor populations. All farmland populations were separated by about 100 metres from potential source of herbicide resistant pollen, except population 3 which was about 30 metres from another farm. Thus, the potential of pollen dispersal for gene flow was considered to be very limited. Because of the disturbed nature of some of the farmland sites the potential for gene flow by resistant seed introduction in grains or hay or by water movement is possible. Evidence from more stable sites is presented in Section 2.3.5.

Weedy plants that occur across a wide range of environments could be expected to contain, within the species genotype, a range of mechanisms conferring adaptive benefit. For example, the ability to rapidly metabolise a range of xenobiotic molecules would confer an adaptive advantage to a weedy species that thrives in fertile disturbed habitats or the ability to metabolise naturally occurring solutes contained in soil water or surface water could also confer an advantage. A diversity of resistance mechanisms can be expected to provide an advantage for a weedy species.

The level of resistant individuals in stable populations is a function of the mutation frequency and the persistence of the novel allele or alleles over time. Fitness and the genetic dominance of the allele or alleles contribute to the persistence. Experimental data considering the genetic dominance of resistance conferring alleles is presented in Section 2.4.1. Evaluation of the data presented in Table 2.2 discussing the persistence of resistance individuals in a population is shown in Section 2.5.1.

In conclusion, it is evident that farmland populations of *L. rigidum* which have not been exposed to herbicides exhibit a high frequency of resistance to diclofop-methyl. Resistant populations of *L. rigidum* are now numerous and widespread; in 1991 there were at least 5000 populations of *L. rigidum* populations resistant to herbicides in southern Australia (unpublished telephone survey by the Australian Agricultural and Veterinary Chemicals Association 1991). These resistant populations have occurred in widely diverse environments separated by thousands of kilometres at the extremes of the range, which illustrates the wide geographic spread of the alleles conferring resistance. The high initial frequency of resistance genes that have been shown to be present in populations of *L. rigidum* from untreated farmland as shown in Table 2.2 explains how these populations have rapidly evolved resistance following a limited number of applications of the herbicide diclofop-methyl.

2.4 Mode of inheritance of resistance in the first generation of selection

2.4.1 Results

There have been many studies of the inheritance of herbicide resistance, (reviewed by Darmency, 1994) however, all the studies have focussed on the inheritance of resistance in established resistant biotypes. The inheritance of resistance in the first generation of selection in an unselected population is unknown. The initial events in the early selection of resistant plants from an untreated population are important to understand the dynamics of the onset of resistance. To establish the genetic basis of resistance in the first generation of selection, the survivors of populations 1, 4, 6, 13, 14 and 15 (Table 2.1) were allowed to hybridise as paired crosses with cohort survivors. Four pairs of plants were crossed for populations 4 and 6, and 2 each for the remainder of the populations. The seed within a cohort was bulked. The resulting progeny are described as F_1 with the population identification in parenthesis. Seedlings from each F_1 accession were treated with 360 g a.i. ha^{-1} of diclofop-methyl and the segregation ratio of dead to live plants was compared to the expected ratio for single factor segregation (Table 2.4).

Most of the survivors of the initial application of diclofop-methyl herbicide at 360 g a.i. ha^{-1} showed inheritance of resistance to this herbicide consistent with resistance occurring as a dominant allele in a heterozygous state in the farmland populations of *L. rigidum*. As shown in Table 2.4, the product of crosses of these initial survivors, the F_1 accessions, inherited herbicide resistance which segregated in a 3:1 ratio. A cross between heterozygous plants with alleles conferring resistance to herbicides displaying genetic dominance can account for such a segregation ratio. Of the populations that were tested for inheritance of resistance, two crosses did not show survival or mortality of plants consistent with segregation in a 3:1 ratio due to a surplus of susceptible plants in the crosses (data not shown).

Table 2.4. Segregation of the F_1 generation of survivors of selected populations crossed with cohorts and treated with 360 g a.i. ha^{-1} diclofop-methyl.

Population ID	Live plants	Dead plants	Total	χ^2
F_1. (4 X 4)	181	67	248	0.49 *
F_1. (6 X 6)	105	23	128	3.37 *
F_1. (13 X 13)	16	9	25	1.37 *
F_1. (14 X 14)	10	6	16	1.08 *

* Indicates significant χ^2 goodness of fit to 3:1 ratio ($p < 0.05$).

Backcrossing of the survivors of the initial application of diclofop-methyl was done to plants grown from susceptible populations to verify the observation (Table 2.4) that initial survivors were occurring as heterozygotes. A backcross between homozygous susceptible and heterozygous resistant individuals should result in 1:1

segregation of resistant (live) and susceptible (dead) plants when treated with an appropriate rate of herbicide. The data in Table 2.5 shows that backcrosses between the F_1 survivors and susceptible plants segregated consistent with a heterozygote resistant plant being crossed with a homozygous susceptible when treated with diclofop-methyl herbicide at 360 g a.i. ha^{-1}. Two populations that were backcrossed to a susceptible population did not show a 1:1 ratio of dead to live plants following the screening of the backcrosses. These populations displayed a surplus of susceptible plants in the backcross generation (data not shown).

Table 2.5. Segregation of survivors of selected populations crossed with susceptible plants and treated with 360 g a.i. ha^{-1} diclofop-methyl.

Population ID	Live plants	Dead plants	Total	χ^2
F_1 6 X Sus	11	7	18	0.94*
F_1 13 X Sus	10	19	29	2.82*
F_1 14 X Sus	5	10	15	1.73*
F_1 29 X Sus	32	26	58	0.39*

* Indicates significant χ^2 goodness of fit to 1:1 ratio ($p < 0.05$).

2.4.2 Discussion

The presence of resistant plants within a population is a necessary condition for the onset of herbicide resistance in herbicide selected populations. However, the rate at which herbicide resistance develops when exposed to a constant selection pressure, is determined by the heritability of the factor(s) endowing resistance. As there is a substantial level of resistance within unselected *L. rigidum* populations (Table 2.1), the mode of inheritance in the initial phase of selection is of great interest as the level of dominance governs the rate of resistance development. There can be difficulties in assessing inheritance with a variable outcrossing species as the genetic variability can confound analysis of phenotypic classes, however in the present case the herbicide effects were clear and the assessment could be made by differential mortality. Observations on the heritability of survival or resistance can therefore be made on the basis of Mendelian ratios rather than by quantitative assessment. The technique of using survivors in a test for dominance is compromised by the lack of survival of a substantial proportion of the population which may contain alleles conferring resistance. However, understanding and predicting the rate of evolution of herbicide resistance in the field requires the use of survivors as no visible markers of the resistant phenotype are available in *L. rigidum.* It also has the added advantage of considering the phenomenon that occurs under frequent herbicide application in the field.

The occurrence of a high frequency of alleles conferring resistance (Table 2.1) and displaying dominance (Table 2.4) in the samples of the farmland *L. rigidum* populations is of great practical importance for the rate of onset of resistance in a population when selection with herbicides is initiated. The segregation of the F_1 plants, representing the progeny generated from the initial survivors selected from the farmland populations shown in Table 2.4, is consistent with expected segregation ratios for a dominant single factor conferring the observed phenotype. As the observations were based on survival only and survival may be conferred by more than mechanism it is not possible to state that a single gene is involved, only that the resistance phenotype is dominant. The segregation of the F_1 progeny also shows the survivors of the initial herbicide application to be heterozygous for the resistance conferring allele(s). This is consistent with the allogamous mating behaviour of this species. These observations are supported by the data from backcrossing F_1 survivors and plants from susceptible populations (Table 2.5). The results presented in Table 2.4 and Table 2.5 shows that under herbicide selection most of the survivors from an initial herbicide application behaved as heterozygotes with resistance inherited as a dominant factor. There were some of the crosses and backcrosses that did not segregate consistent with the inheritance of dominance probably due to a surplus of susceptible plants surviving the initial herbicide application.

Genetic theory predicts that in an allogamous species such as *L. rigidum* which is not undergoing selection for a trait, an allele(s) conferring resistance to a herbicide would be expected to occur mostly in the heterozygous state. A population genetics simulation model based on the Hardy-Weinberg principle, "Pop-Gen" (Dept of Zoology, Wellington University, New Zealand) was used to simulate the relative proportion of alleles over 50 generations. The simulation was performed beginning with an initial frequency of resistant plants of about 1%, as encountered in Table 2.4. Assumptions were that the resistance alleles were present as heterozygotes and that there was no fitness differences for the resistance conferring factor(s) (see Chapter 5). The population simulation, with neutral selection, where the frequency of an allele changes only with random drift shows that the gene would occur as a homozygote only at a mean frequency of 0.0023% (SE 0.037%) or 2.3 in a population of 100,000.

In conclusion, the data presented in this section establishes that the genes conferring resistance in field populations of *L. rigidum* with no history of previous herbicide application occur mostly in a heterozygous state. There would be a very low frequency of homozygous resistant plants occurring in unselected populations. The dominance of the resistance conferring allele was confirmed by the data from Tables 2.4 and 2.5. As there are no other reports in the literature, this study is the first to detail the heritability of the early survivors of a herbicide application in a natural population. Such data is important because the early generations of selection are the most critical for the development of resistance in the field. This section establishes that another of the conditions for rapid development of herbicide resistance in the field has been shown to be present in *L. rigidum* populations, that of genetic dominance of the factors conferring resistance to the herbicide.

2.5 Edaphic factors affecting the frequency of resistance plants

2.5.1 Results

The environment in which plants are growing can vary across an arable field or within non-arable wooded areas. However, in wooded areas and when growing in association with other species, *L. rigidum* populations do not usually occur as dense stands and are often restricted by soil fertility or other considerations, particularly competition from early emerging species such as *Bromus* and *Vulpia* species. It is likely that wooded and non-arable sites have high intra-site variability. In contrast, in arable areas the plant environment within a field is usually more homogenous due to cultivation and soil amelioration for crop production although there are differences between fields due to soil, rainfall and drainage characteristics.

The samples of *L. rigidum* populations (Table 2.1) were collected from across a large area and represent different soil and rainfall characteristics. For the farmland populations collection notes indicate that populations 2, 3, 4, 8, 9, 10, 12, 13 and 15 were collected from open farmland on arable areas or from within crops and the remaining farmland populations (populations 5, 6, 7, 10, 11 and 14) were from non-arable areas on farms. The mean survival of the populations from arable and non-arable sites on farmlands were compared by anova with $(X+0.5)^{1/2}$ transformation and were found to be not different at any of the rates of diclofop-methyl, $(P<0.5)$ (data not shown). Therefore there was no difference between the survival of populations from arable areas compared to populations from non-arable areas at any of the rates of diclofop-methyl used.

Another varying factor on farmland is that sites can be freely draining (dry) whereas other sites can be subject to waterlogging during the growing season (wet sites). A characteristic of some arable sites is that having been subject to cultivation, the soils are more compacted and have a higher potential to retain moisture or be subjected to occasional waterlogging than other sites. From the collection notes the populations were separated into samples representing wet and dry sites. Table 2.6 shows the mean survival percentage of populations from all farmland sites considered to be subject to waterlogging (populations 2, 3, 4, 6, 7, 10, and 11) and from drier sites not considered to be subjected to waterlogging (populations 5, 8, 9, 12, 13, 14 and 15).

Table 2.6. Mean survival and (S E of the mean) of ryegrass populations from farmland sites following exposure to diclofop-methyl at 360 g a.i. ha^{-1} and grouped into sites subject to waterlogging (wet) and freely draining (dry).

	% Survival (SE)
Site characteristic	Diclofop-methyl
Wet sites	1.558 (1.04)^{a}
Dry sites	0.406 (0.20)^{b}

* Treatments followed by the same letter are not different, ($P<0.5$) anova with $(X+0.5)^{1/2}$ transformation.

The separation of populations into "wet" and "dry" sites revealed a clear difference in the percentage of resistant individuals in these populations. The populations from wetter sites had a higher frequency of individuals resistant to 360 g a.i. ha^{-1} of diclofop-methyl than populations from drier sites (Table 2.6). The data from Table 2.2 indicates that *L. rigidum* populations collected from farmland have a level of resistant individuals considered to be high for a novel allele(s) conferring resistance to a herbicide and the data from Table 2.6 shows that resistant individuals are more frequent in populations collected from wet sites when compared to those from dry sites.

To further examine the hypothesis that plants resistant to diclofop-methyl within unsprayed populations were more frequent in populations from wet sites than from dry sites, further populations of ryegrass were collected from sites with no history of herbicide use (Collection 2, Table 2.7). These sites were chosen such that long established and undisturbed ryegrass populations could be sampled from both a wet and an adjacent dry site. Table 2.7 lists the sites from which the populations were collected. Collections are numbered following on from Collection 1 and are labelled "a" and "b" for wet and dry sampling sites respectively.

Table 2.7. Site details for ryegrass populations (Collection 2).

	Location	
Population	State and map reference	Site description
28 a & b	S.A. 36.47°S: 140.44°E	Non arable farmland, undisturbed for 26 years.
29 a & b	S.A.33.02°S: 137.95°E	Non arable farmland, undisturbed for 86 years.
30 a & b	S.A. 33.50°S: 138.36°E	National Park, undisturbed.
31 a & b	N.S.W. 35.55°S: 146.58°E	Wooded park area, undisturbed.

Edaphic details for Collection 2

Four populations were sampled from farms or park areas where herbicides had never been used. Sites were chosen where *L. rigidum* could be collected from wet and dry environments that were close to each other. The dry sites were elevated and hence drier because of the topography. The wet and dry sampling areas were separated by no more than 75m at each collection site. Samples were from areas where annual pasture species including *L. rigidum* were continuous over the area, even though the wet areas were subject to frequent waterlogging and occasional short-term inundation. All collections were made by the author from sites with a history of no herbicide applications and where the populations had been established for a long period of time. The seeds were collected and stored as previously described.

The samples from each site from each location were treated as described previously. The seedlings were sprayed with diclofop-methyl at 360 g a.i. ha^{-1} to establish if there was a difference in the frequency of surviving plants from the wet and dry sites in Collection 2. The results are presented in Table 2.8.

The mean survival after herbicide treatment was 2.16% for wet sites and 1.26% for dry sites (Table 2.8) and for three of the four specific collections the wetter sites had a significantly higher frequency of resistant individuals within the sample compared to the drier sites. Therefore, plants displaying resistant phenotypes were present at a higher relative frequency in wetter sites compared to closely adjacent drier sites. Because of the proximity of the wet and dry sites in each location and the long history of establishment and the lack of disturbance, the samples from each location were likely to be from similar original populations.

Table 2.8. Survival of *L. rigidum* samples collected from wet and dry environments from four locations (Collection 2) following application of 360 g a.i. ha^{-1} diclofop-methyl. Data is the mean of two experiments with the total number of seedlings treated as indicated in the square brackets.

	Survival % (SE) [n]	
Population	Wet site	Dry site
28	2.05 (0.11) [877]^{a*}	1.5 (0.07) [914]^{b}
29	1.1 (0.07) [728]^{a}	0.75 (0.11) [927]^{a}
30	2.95 (0.18) [740]^{a}	1.35 (0.71) [713]^{b}
31	2.55 (1.10) [529]^{a}	1.45 (0.18) [611]^{b}

* Means of populations followed by the same letter are not different, anova $(X+0.5)^{1/2}$ transformation ($P<0.05$). LSD within populations is 0.67.

2.5.2 Discussion

The unique observation that resistant individuals are more frequent in "wet" versus "dry" sites is interesting and of considerable ecological significance. There may be many (as yet unknown) reasons why the frequency of surviving plants and therefore of alleles conferring resistance in these populations is higher in wetter sites than in drier sites. *L. rigidum* and other members of the genus *Lolium* are pasture grasses suitable for a range of environments with growing season precipitation above about 350mm. Such environments can be characterised by occasional flooding or waterlogging. The more frequent presence of dissolved salts in the wet sites or decreased oxygen tension or organic residues in the root zone in such sites may be features of such environments. Adaptation to such environments either as a species or a genus and mutation or over expression of the genes conferring adaptation to wetter environments may account for the increased frequency of individuals displaying resistance to a common herbicide diclofop-methyl in populations of *L. rigidum*. For example, the complex physiological responses to waterlogging may also be associated with resistance to xenobiotics, including herbicides. Detailed examination of these populations from "wet" and "dry" sites should be made but are outside the scope of this thesis.

2.6 General conclusions

At least 5000 populations of *L. rigidum* resistant to herbicides infested southern Australia farms in 1991. These have occurred in widely diverse environments that are separated by thousands of kilometres at the extremes of the range. This indicates the wide geographic spread of the alleles conferring resistance. That resistance occurs rapidly in *L. rigidum* can now be understood from the studies reported here which show that the average of 1% survival in farmland populations with no history of herbicide use represents an extremely high risk of evolving resistance following a limited number of applications of the herbicide diclofop-methyl.

Further studies (Tables 2.4 and 2.5) established that resistance in the first generation is dominant and that resistance is present in a heterozygous state. The allogamous members of the genus *Lolium* display substantial genetic variation (Hayward and Nsowah, 1969). Due to the importance of *L. perenne* in temperate perennial pastures, much of the literature is focussed upon this species, however, extensive variability has been identified in *L. rigidum* (Bulinska-Radomska and Lester, 1984; Rees and Ahmad, 1963) and in *L. multiflorum* (Polans and Allard, 1989). The allogamous characteristic ensures that for gene(s) present in a population at a low frequency and in the absence of selection, that nearly all individuals are heterozygous for the novel gene(s).

As *L. rigidum* is a diploid plant and assuming the resistance is conferred as for the populations listed in Table 2.5 and 2.6, the frequency of resistant alleles can be deduced from Hardy-Weinberg (Table 2.9).

Table 2.9. Hardy-Weinberg frequency of resistant alleles in *L. rigidum* populations grouped according to site characteristics, from Tables 2.1, 2.6 and 2.8, following treatment with 360 g a.i. ha^{-1} diclofop-methyl.

Site characteristic	Frequency of resistant plants	Calculated frequency of resistant alleles
All sites	0.0084	0.0042
Wet sites	0.0165	0.0083
Dry sites	0.0070	0.0035

These results (Table 2.9) indicate that alleles conferring resistance to diclofop-methyl in *L. rigidum* occur at relatively high frequency in farmland populations of approximately 4.2×10^{-3}. However, the mean frequency of resistant alleles from all unsprayed wet sites was higher at 8.3×10^{-3}. These calculations do not account for the probability of the presence of homozygotes within the samples tested, which are calculated to occur at about 2.3×10^{-5}.

The inheritance of resistance as a dominant character in the first generation of selection (Table 2.5) is of considerable importance. Dominance is an important factor in the rate of onset of resistance once selection is initiated. The data presented in this chapter shows that resistance to diclofop-methyl is present in most untreated farm populations and when sprayed at the field rate of diclofop-methyl many of the selected resistant individuals display dominance for the characters bestowing resistance.

The increased frequency of resistant individuals evident in populations collected from wet sites is interesting. The comparisons of wet and dry sites shows conclusively (Table 2.7) the different frequencies. The mechanism(s) contributing to the increased frequency has not been investigated but there is little doubt that the species generally is adapted to such conditions (Kemp, 1988). These samples, (Collection 2), also reinforce the data from Collection 1 showing the high initial frequency of resistance in natural populations. The collections 28-31 were carefully selected for their long history of undisturbed sites and low probability of introduction of resistance genes.

Genes which endow herbicide resistance in *L. rigidum* may differ. It is possible that several different genes conferring different resistance mechanisms are present in populations and crossing between surviving plants can mix or accumulate mechanism of resistance in the progeny. This has the potential to create diverse patterns of resistance in the final population. This study suggests that many alleles conferring resistance are inherited in a dominant fashion in the early generations of selection. This observation, taken with the observed frequency of resistant plants in natural populations validates the experience in southern Australia that the selection for resistance to diclofop-methyl can be extremely rapid and occurs in separate populations of *L. rigidum*.

Chapter Three

Selection of herbicide cross-resistant *Lolium rigidum* from a susceptible population

3.1 Introduction.

As discussed in Chapter 1, *L. rigidum* is widely distributed across the southern Australian winter cropping regions. During cropping phases the frequent application of a limited range of selective herbicides has generated thousands of herbicide resistant populations (see section 1.6.1). Resistance to the graminicide diclofop-methyl is particularly widespread (Powles and Howat, 1990) due to its suitability and widespread adoption for *L. rigidum* control during the cereal cropping phase. Diclofop-methyl is an inhibitor of ACCase in *L. rigidum* and other sensitive species and is selective in wheat, barley and triticale crops. Resistance to sethoxydim, a cyclohexanedione herbicide which is also an ACCase inhibitor, is less common in *L. rigidum* populations (Tardif *et al.,* 1993). Resistance to the broad spectrum cereal-selective ALS-inhibiting herbicides (Christopher *et al.,* 1992) and to a mixture of amitrole and atrazine (an inhibitor of carotenoid synthesis and a PSII inhibitor, respectively) (Burnet *et al.,* 1991) have all been documented.

Cross resistance to herbicides from chemical groups not previously applied to a population was first reported by Heap and Knight (1986) and is now common in Australia in *L. rigidum* populations (Powles and Matthews, 1991). Cross resistance has reduced the efficacy of ACCase inhibitors, ALS inhibitors and other selective herbicides such as the PSII inhibiting herbicides in many areas of cereal and legume crop production. The actual loss of production from seed and grain crops due to herbicide resistance can be substantial but the unrecognised development of cross resistance in *L. rigidum* populations creates management difficulties and may cause additional losses.

Following the failure of a frequently used herbicide, the natural reaction of most farmers is to change to other available herbicides. There are many herbicides registered in Australia for control of *L. rigidum* in a variety of crop species (see Section 1.3.5). However, these herbicides represent a limited range of herbicide chemistries with limited sites of action in the plant. Target site cross resistance is common as resistance frequently develops to members of the same chemical group. Where non-target site cross resistance develops, alternative herbicides from other chemical groups may fail as a result of weed populations being wholly or partially resistant to these alternatives. In a population that is partially resistant to alternative herbicides, resistance will become evident with the continued use of such herbicides.

Reports of target site cross resistance to herbicides in weed species are common, particularly for the PSII (Le Baron, 1991; Holt and Le Baron, 1990), ALS (Saari *et al.*, 1994) and ACCase inhibiting herbicides (Devine and Shimabukuro, 1994). Reports of non target site cross resistance in weed species other than *L. rigidum* are limited to biotypes of *A. myosuroides* (Hall *et al.,* 1994; Kemp *et al.*, 1990) and *Phalaris paradoxa* (Yaacoby *et al.*, 1986).

Despite the great number of reports from field observations which indicate the propensity for *L. rigidum* populations to develop non-target site cross resistance, the lack of reliable field histories often makes it difficult to ascribe patterns of resistance and cross resistance to the herbicide or herbicides previously used. This chapter describes the onset of herbicide resistance in two accessions selected from an initially susceptible population of *L. rigidum* by repeated applications of diclofop-methyl only. The experiments describe the capacity of *L. rigidum* to develop resistance to the herbicide used for selection, to develop cross-resistance to herbicides with the same site of action and to develop resistance to herbicides with different modes of action.

3.2 Materials and Methods.

3.2.1 Selection from a field population

About half a hectare of a susceptible population of *L. rigidum* (VLR2) was sown in a field site free of ryegrass at Bordertown, South Australia (36.19°S:140.47°E). The *L. rigidum* (VLR2), obtained from a commercial source. Sixty kg ha^{-1} of 18% N and 20% P fertiliser was applied at sowing. Approximately 760,000 ryegrass plants were established with a mean density of 152 plants m^{-2}. The herbicide diclofop-methyl was applied to the *L. rigidum* with commercial equipment at the rate of 540 g a.i. ha^{-1} at the 2-4 leaf growth stage which is the appropriate developmental stage for application of this herbicide. The recommended application rate of diclofop-methyl for control of ryegrass ranges from 360 - 540 g a.i. ha^{-1}. *L. rigidum* is an obligate outcrossing species and therefore, some of the surviving plants were surrounded with a 2 m high barrier of hessian to exclude pollen from outside sources. Fifty-nine surviving plants in about 30 square metres were screened in this way and allowed to mature and set seed in the field. The seeds were harvested by hand and was classed as the first generation, Selection one (S_1) and used for subsequent selections.

3.2.2 Details of laboratory treatments

All subsequent work was conducted in pots with seedlings transplanted from seed germinated on 0.6% agar for 6 d in a growth cabinet with 16 h photoperiod at 20°C and a dark period at 15°C. Seedlings at the one leaf stage were planted in 17.5 cm pots of sterilised potting soil and grown outside during the normal winter growing season. Herbicides were applied to seedlings in pots at the correct growth stage for each herbicide using a laboratory cabinet sprayer in 113 L ha^{-1} of water carrier with adjuvants as required. Diclofop-methyl, sethoxdim, chlorsulfuron and simazine were the herbicides used.

The S_1 seedlings were treated at the 2-3 leaf stage with a range of application rates of diclofop-methyl and the S_2 selection was taken from survivors of two application rates of diclofop-methyl, 540 g a.i. ha^{-1} (low rate = L) and 2160 g a.i. ha^{-1} (high rate = H). The survivors from each of these rates were crossed to produce the S_2L and S_2H accessions respectively of the S_2 generation. About 20 survivors were transplanted into 30 cm pots at four plants per pot and allowed to grow to maturity. Pollen was excluded from pots at anthesis by 2 m tall translucent plastic sleeves, seed was harvested when dry and bulked for subsequent use. Selections from both of the S_2 accessions were made in the same way from survivors of the two application rates of diclofop-methyl to form accessions in the S_3 generations, S_3L and S_3H. Table 3.1 lists the herbicide rates and the resulting selections.

Table 3.1. Selections by diclofop-methyl and resulting accessions of *L. rigidum* populations.

Accession treated	Generation treated	Herbicide rate g a.i. ha^{-1}	Accession resulting
Susceptible (VLR2)	0	540	S_1
S_1	1	540 & 2160	S_2L & S_2H
S_2L	2	540	S_3L
S_2H	2	2160	S_3H

3.2.3 Statistical analysis

All herbicide dose response data were analysed and lethal dose concentrations with fiducial limits calculated using the probit analysis package "Polo PC" (LeOra

Software, 1119 Shattuck Ave., Berkeley, CA). Herbicide dose response data over a range of rates were used and the probit functions of slope, intercept and parallelism were used to compare the response to dose, the dose threshold and the similarity of response of the selected accessions by the likelihood ratio test of equality distributed as a Chi square (χ^2) value at the 0.05 level of probability. Dose response data was taken from two separate experiments with three replicates unless stated otherwise.

3.3 Results

3.3.1 The effect of selection with diclofop-methyl on resistance to diclofop-methyl

Selection from the susceptible population VLR2 with 540 g a.i. ha^{-1} of diclofop-methyl resulted in 1.4% survivors. These survivors were intercrossed and produced the S_1 accession. The S_1 accession showed a greater than 7 fold increased level of resistance when compared to the susceptible population as determined by comparison of the LD_{50} values (Table 3.2). Further selection of the S_1 generation with 540 g a.i. ha^{-1} resulted in a substantial increase in the calculated LD_{50} for the S_2L generation of about 800 times and a further increase of about 1.25 times between the S_2L and the S_3L generations (Table 3.2). The response to increasing doses of diclofop-methyl was significantly different for the susceptible, S_1, S_2 and S_3 generations. The S_2L and S_3L accessions responded similarly to increasing rates of herbicide as did the S_2H and S_3H accessions, the test for parallel transformed dose responses indicates that a dose by biotype interaction occurred in the first two generations of selection. The dose response curves are shown in Figure 3.1. Selection of survivors of herbicide applied at 540 g a.i. ha^{-1} over three generations resulted in about 90% of the S_3L generation being unaffected by 2.9 kg ha^{-1} of diclofop-methyl.

Selection of the S_1 and subsequent generations by 2160 g a.i. ha^{-1} of diclofop-methyl resulted in an increase in the LD_{50} values for the S_2H and S_3H accessions of

511 and 1.7 times respectively (Table 3.2 and Figure 3.2) when compared to the previous generation in each case. The responses of the S_2H and S_3H generations to increasing doses of diclofop-methyl were significantly different to each other and from the S_1 generation. The selection of survivors from 2160 g a.i. ha^{-1} in the S_1 and S_2 generations resulted in about 80% of the S_3H generation being unaffected by 2.9 kg ha^{-1} of diclofop-methyl.

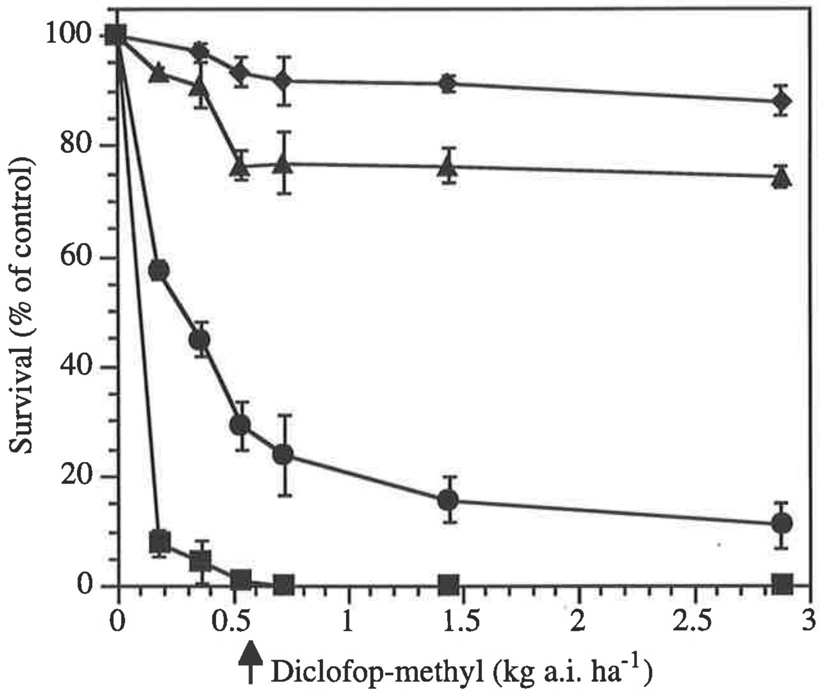
Table 3.2. The amount of diclofop-methyl calculated to cause 50% mortality (LD_{50}) in accessions of *L. rigidum* selected by successive applications of diclofop-methyl.

Accession	LD_{50} (kg a.i. ha^{-1})	Lower & Upper dose limits (kg a.i. ha^{-1})	Slope & intercept p=0.05*	Sig. parallel p=0.05*
VLR2	0.033	0.002-0.078	a	a
S_1	0.234	0.16-0.31	b	b
S_2L	184.748	25.9-4.6 x 10^4	c	c
S_3L	233.035	62.6-4.1 x 10^3	d	c
S_2H	119.664	22.3-2.2 x 10^3	c	c
S_3H	201.734	74.1-2.9 x 10^4	d	c

*Accessions with the same letter have similar log-probit regressions for the indicated function. Test for sig. of slopes and intercepts, L accessions χ^2=248.9; H accessions χ^2 =17.3, d.f. =156. Test for parallelism F_2 & F_3; χ^2=2.9, d.f. =3.

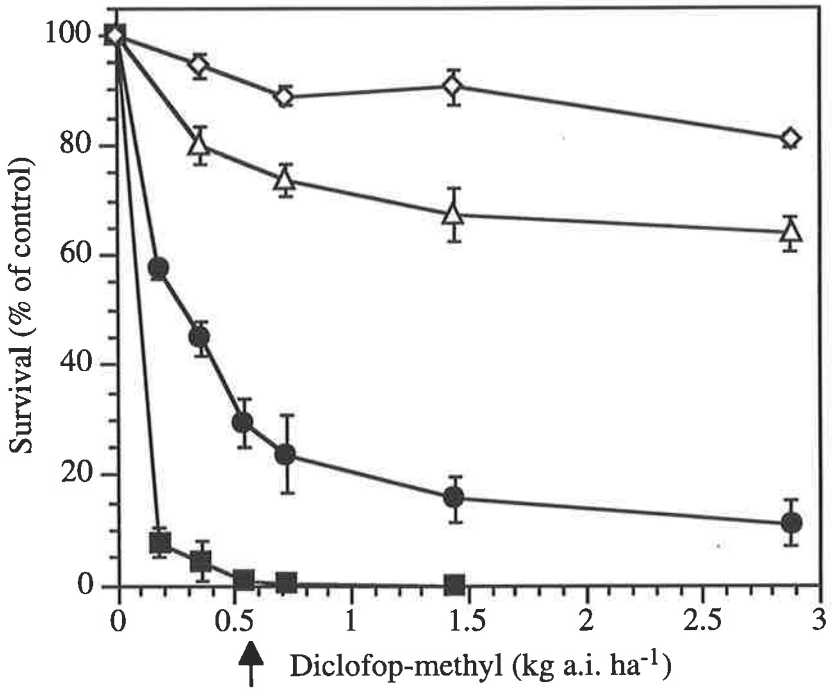
Regardless of the selection regime, all the selected accessions were significantly more resistant after each of three applications of the herbicide diclofop-methyl than the parent accession from which the selection was made. However, as shown by the comparison for slope and intercept of the probit transformation in Table 3.2, selection at a higher rate (S_2H and S_3H) of diclofop-methyl did not result in significantly different levels of resistance compared to selection at the lower, commercially used, rate (S_2L and S_3L). All the S_2 and S_3 accessions displayed parallel probit lines indicating no accession by dose interaction with increasing doses of diclofop-methyl. As the dose response experiments were carried out under consistent conditions to minimise environmental differences, parallel probit lines indicate the relative homogeneity of mechanisms conferring resistance in the later accessions.

Figure 3.1. Response to increasing rates of diclofop-methyl of VLR2 and the diclofop-methyl selected accessions S_1, S_2L and S_3L.



Legend: Susceptible VLR2, ■; S_1, ●; S_2L, ▲; and S_3L, ◆. Data are the means of two experiments with three replicates, bars represent S.E. of the mean. The arrow indicates the recommended field rate of diclofop for control of *L. rigidum*.

Figure 3.2. Response to increasing rates of diclofop-methyl of VLR2 and diclofop-methyl selected accessions S_1, S_2H and S_3H.



Legend: Response of susceptible VLR2, ■; S_1, ●; S_2H, △; and S_3H, ◇, to increasing doses of diclofop-methyl. Data are the means of two experiments with three replicates, bars represent S.E. of the mean. The arrow indicates the recommended field rate of diclofop for control of *L. rigidum*.

3.3.2 The effect of selection with diclofop-methyl on resistance to sethoxydim.

To assess whether selection with diclofop-methyl from the initially susceptible population VLR2 resulted in resistance to other herbicides other than diclofop-methyl, the accessions were treated with other herbicides commonly used for ryegrass control. Sethoxydim is a cyclohexanedione herbicide which inhibits ACCase. This herbicide has the same target enzyme as diclofop-methyl but is of different herbicide chemistry. Sethoxydim is not selective in cereals and is used to control ryegrass and other grass weeds in broadleaf crops.

Selection with diclofop-methyl resulted in increased resistance to sethoxydim in all of the accessions (Figures 3.3 and 3.4). The S_1 selection showed no significant increase in resistance to sethoxydim compared to the susceptible parent. Sethoxydim is a very effective herbicide on *L. rigidum* and causes high mortality at low application rates on susceptible populations, as a consequence the distribution of the herbicide doses was inappropriate for an accurate assessment of the fiducal limits of the LD_{50} of the susceptible VLR2. The LD_{50} values of the S_2L accession increased only 1.1 fold when compared to the S_1 generation (Table 3.3). No significant increase in the level of resistance was seen in the S_{2L} and S_{3L} accessions, but a significant difference was noted for the S_2H and S_3H accessions. The LD_{50} of both the S_3L and S_3H accessions increased about two times when compared to the previous accession (Table 3.3).

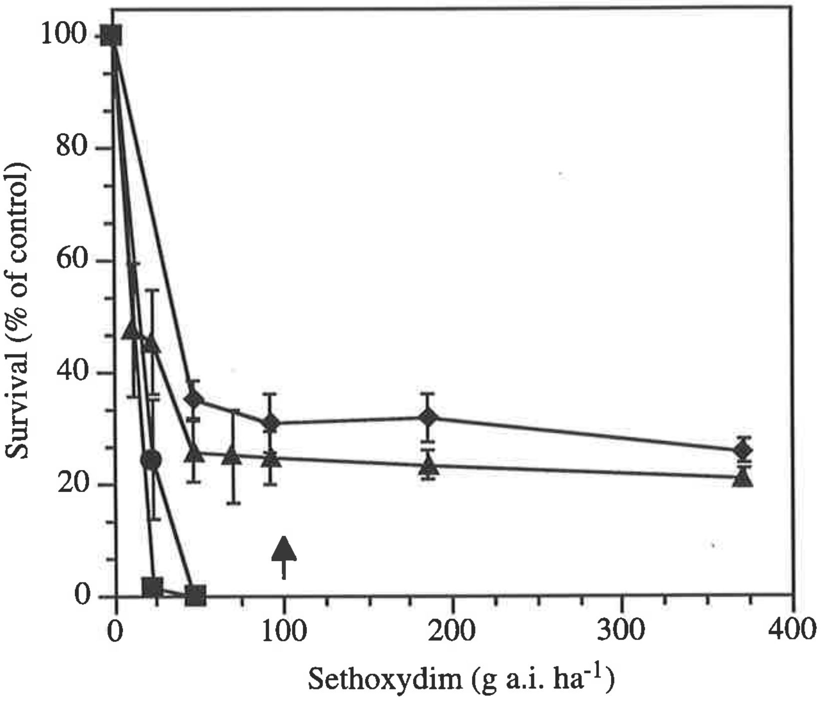
Table 3.3. The amount of sethoxydim calculated to cause 50% mortality in accessions of *L. rigidum* selected by successive applications of diclofop-methyl. Data is the mean of 2 experiments with three replicates.

Accession	LD_{50} (g a. i. ha^{-1})	Fiducal limits p=0.05 (g a .i. ha^{-1})	Sig. slope, intercept p=0.05*	Sig. parallel p=0.05*
VLR2	≅12.0			
S_1	21.8	3.2-45.5	a	a
S_2L	24.2	3.6-50.8	a	b
S_3L	45.8	22.9-77.3	a	b
S_2H	34.6	5.5-31.3	a	c
S_3H	54.3	33.5-72.9	b	c

*S_2 and S_3 accessions followed by the same letter have similar log-probit regressions for the indicated function. Test for slopes and intercepts for the S_2L and S_3L χ^2=13.6, d.f.=6; parallelism, χ^2=0.13, d.f.=71; S_2H and S_3H χ^2=293.9, d.f.=6; parallelism, χ^2=0.13, d.f.=3.

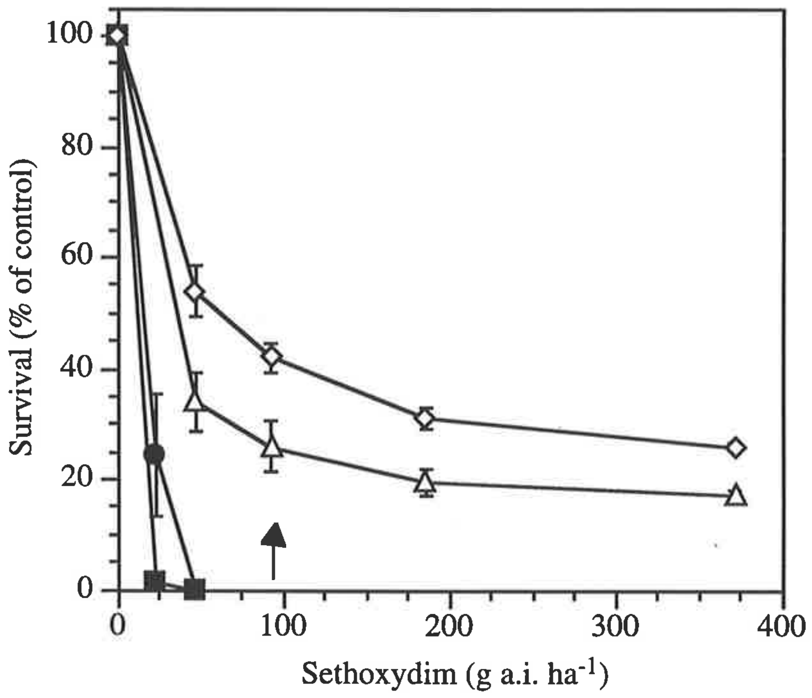
The S_2L and S_3L accessions displayed parallel probit lines as did S_2H and S_3H accessions, indicating there was no interaction within accessions by herbicide dose. However, the dose response of L and H accessions to sethoxydim were not significantly different from each other (Table 3.3), indicating that selection with different rates of diclofop-methyl resulted in accessions that displayed important increases in the LD50 responses to sethoxydim although the differences were not significant. The L and H accessions may contain resistance conferring mechanisms of different efficiency when challenged with sethoxydim or may contain different proportions of a similar mechanism. The lower survival rate of all accessions when exposed to sethoxydim

Figure 3.3. Response to increasing doses of sethoxydim of VLR2 and diclofop-methyl selected accessions S, S_2L and S_3L.



Legend: Susceptible VLR2, ■; S_1, ●; S_2L, ▲; and S_3L, ◆. Data are the means of two experiments with three replicates, bars represent S.E. of the mean. The arrow indicates the field rate of sethoxydim for *L. rigidum* control.

Figure 3.4. Response to increasing doses of sethoxydim of VLR2 and diclofop-methyl selected accessions S_1, S_2H and S_3H.



Legend: Susceptible VLR2, ■; S_1, ●; S_2H, △; and S_3H, ◇. Data are the means of two experiments with three replicates, bars represent S.E. of the mean. The arrow indicates the field rate of sethoxydim for *L. rigidum* control.

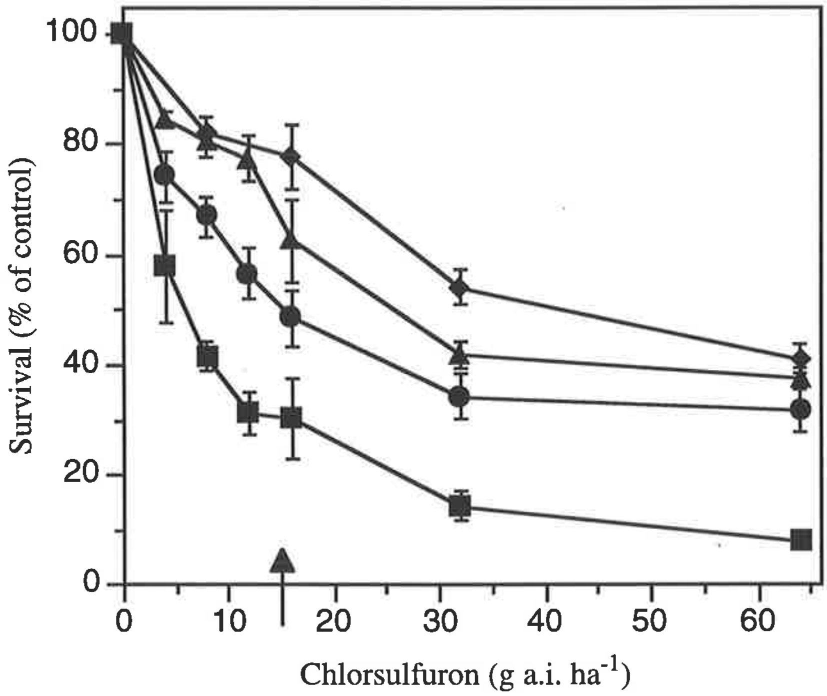
compared to the survival when exposed to diclofop-methyl indicates differences in efficacy of the resistance mechanism even though both herbicides inhibit ACCase. Both the diclofop-methyl selected accessions were resistant to sethoxydim, however the S_3H accession displayed a significant increase in resistance to sethoxydim compared to the S_2H.

The recommended application rate of sethoxydim for control of *L. rigidum* in broadleaf and legume crops is 93 g a.i. ha^{-1}. At that application rate 35% of the S_3L accession and 40% of the S_3H accession were resistant to sethoxydim. Selection on a susceptible population by diclofop-methyl substantially increased the resistance to sethoxydim of the final generation and indicates that cross resistance to sethoxydim can occur with repeated use of diclofop-methyl.

3.3.3 The effect of selection with diclofop-methyl on resistance to chlorsulfuron

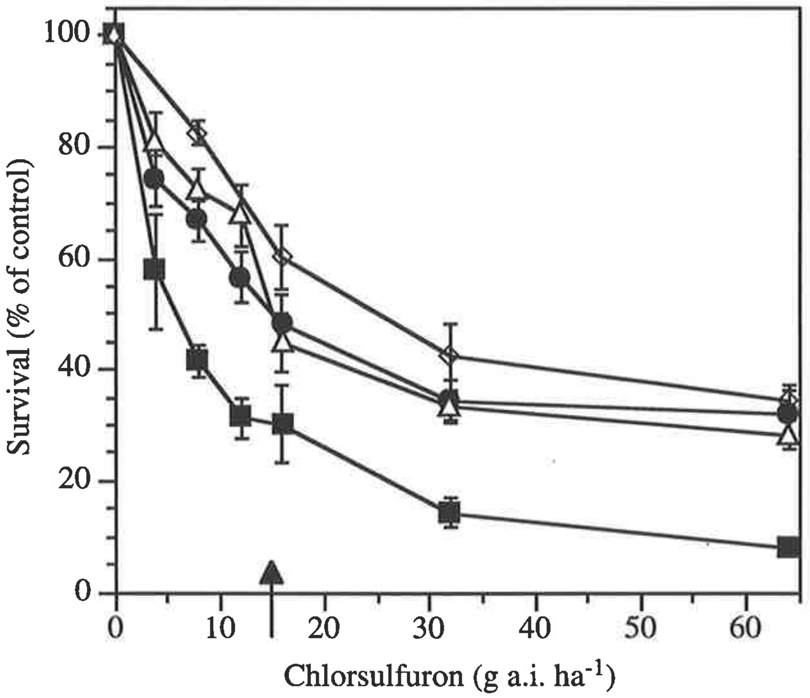
Selection from a susceptible population with the ACCase inhibitor diclofop-methyl not only resulted in resistance to othe ACCase inhibiting herbicides such as sethoxydim but also in cross-resistance to the ALS inhibitor chlorsulfuron. The accessions selected at both the low or the high rate of diclofop-methyl displayed resistance to chlorsulfuron as shown in Figures 3.5 and 3.6. Consistent and significant increases in the level of resistance to chlorsulfuron are evident as shown in Table 3.4. The S_1 generation was three times as resistant to chlorsulfuron as the susceptible parent, whereas the S_3L and S_3H generations were 6.2 and 4.8 fold as resistant as the susceptible parent respectively. The response to increasing doses of chlorsulfuron for both the high and low rate selected accessions were significantly different.

Figure 3.5. Response of VLR2, and diclofop-methyl selected accessions S_1, S_2L and S_3L to increasing rates of chlorsulfuron.



Legend: Susceptible VLR2, ■; S_1, ●; S_2L, ▲; and S_3L, ◆. Bars represent S.E. of the means of two experiments replicated three times. Arrow indicates the recommended rate for control of *L. rigidum* in the field.

Figure 3.6. Response to increasing rates of chlorsulfuron of VLR2 and diclofop-methyl selected accessions S$_1$, S$_2$H, and S$_3$H.



Legend: Susceptible VLR2, ■; S$_1$, ●; S$_2$H, Δ; S$_3$H, ◇. Bars represent the S.E. of the means of two experiments replicated three times. Arrow indicates the recommended rate for control of *L. rigidum* in the field.

Table 3.4. The calculated LD_{50} and confidence intervals, following application of chlorsulfuron to the diclofop-methyl selected accessions.

Accession	LD_{50} (g a. i. ha^{-1})	Fiducal limits p=0.05 (g a. i. ha^{-1})	Sig. slope, intercept p=0.05*	Sig. parallel p=0.05*
VLR2	6.0	4.7-7.3	a	a
S_1	18.2	14.9-22.5	b	b
S_2L	30.2	24.9-38.2	c	c
S_3L	37.2	32.8-42.1	d	c
S_2H	19.0	15.7-23.5	e	c
S_3H	28.6	23.1-36.8	f	c

* Rows followed by the same letter have similar log-probit regressions for the indicated function. Test for significance; L accessions χ^2=323.73, d.f.=6; H accessions χ^2=44.7, d.f.=6 ; parallelism χ^2=6.21, d.f.=3.

The S_3L diclofop-methyl selected accession displayed a higher level of resistance to chlorsulfuron than the accession selected by the higher rate of diclofop-methyl. The transformed probit lines are parallel for both the S_2 and S_3 accessions indicating that although the level of resistance is different, there is no accession-interaction with dose and therefore the mechanisms conferring survival in the accessions may be similar.

3.3.4 The effect of selection with diclofop-methyl on resistance to simazine.

Due to shortage of seed only the S_2L and S_2H accessions were tested with simazine. Figure 3.7 shows the response of these accessions and the susceptible parent

VLR2 to increasing doses of the PSII inhibitor simazine. Both accessions are significantly different from each other and from VLR2 (Table 3.5). The LD_{50} for S_2L shows a 2.9 fold increase and S_2H a 1.6 fold increase when compared to the susceptible, VLR2.

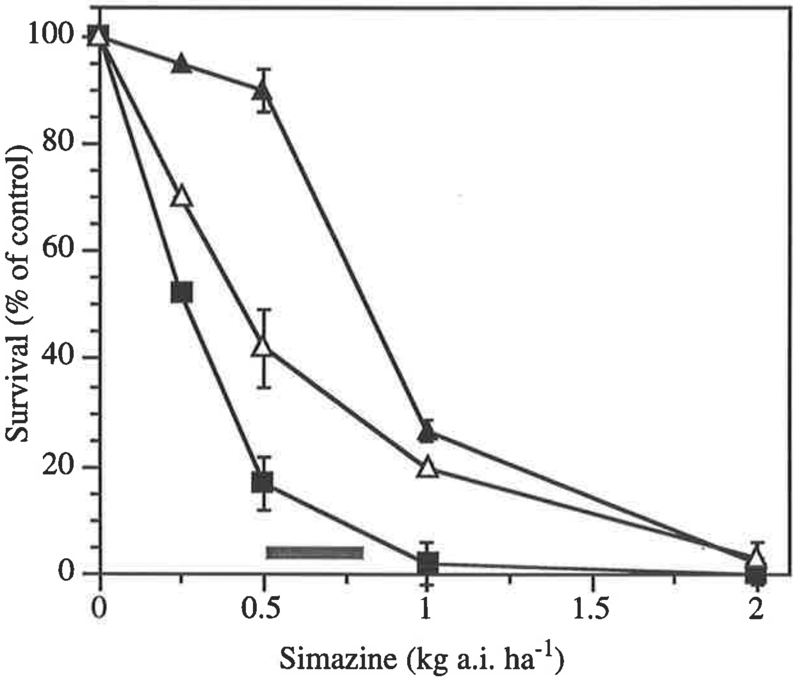
Table 3.5. Amount of simazine calculated to cause 50% mortality in accessions of *L. rigidum* selected by successive applications of diclofop-methyl.

Accession	LD_{50} (g a. i. ha^{-1})	Fiducal limits p=0.05 (g a. i. ha^{-1})	Sig. slope, intercept p=0.05*	Sig. parallel p=0.05*
VLR2	389.6	306.1-458.3	a	a
S_2L	1134.9	839.2-1575	b	b
S_2H	622.5	501.0-742.6	c	c

*Accessions followed by the same letter have similar log-probit regressions for the indicated function. Test for significance; all accessions χ^2=3.73, d.f. =6; parallelism χ^2=4.21, d.f. =2.

Compared with the susceptible parent, both accessions displayed significant cross-resistance to simazine as a result of selection by diclofop-methyl. Also, the S_2L accession displayed a higher level of resistance to simazine than the S_2H accession.

Figure 3.7. Response to increasing doses of simazine of VLR2 and the diclofop-methyl selected accessions S_2L and S_2H .



Legend: Susceptible VLR2, ■; and S_2L, ▲; and S_2H, △. Bars indicate S.E. of the mean, data are from two experiments of three replicates each. The hatched area indicates the recommended range of application rates of simazine for *L. rigidum* control.

3.4 Discussion

In the experiments reported here the onset of resistance in an initially susceptible population of *L. rigidum* when exposed to annual applications of the herbicide diclofop-methyl was shown to be extremely rapid. The dose response curves indicate that almost complete survival was observed after only three generations of selection. The development of herbicide resistance and cross resistance in a population is of great importance especially when trying to predict the onset and management of resistance.

Adaptation and evolution in plant communities is considered to be due to the selection of phenotypes conferred by many genes with small additive effects on the fitness of individuals (Lande, 1983). This is in accord with the classical Darwinian mode of evolution proceeding in a series of small steps. Certainly this would be the case for populations undergoing gradual change by natural selection, where the selection pressure is not high. However, at extreme selection pressure, genes with a major influence will be favoured (McNair, 1991; Roush and McKenzie, 1987). Under extreme selection pressure, an adaptive or genetic response depends on the likelihood of major genes containing genetic variation capable of conferring a selective response being present in the population. With annual ryegrass populations, this genetic potential was shown to be present in many of the populations tested (Chapter 2).

Many plant species, particularly weedy species, demonstrate a range of phenotypic responses to herbicides (Thai *et al.*, 1985; Price *et al.*, 1983). It has been suggested that selection within the phenotypic range is most likely to select for a predominantly polygenic response (Roush and McKenzie, 1987; Lande, 1983), while selection at the extreme of the range of tolerance may select for a major gene(s) response (McNair, 1991). The data in this chapter indicates that higher levels of resistance to diclofop-methyl were conferred by selection with the lower rate of diclofop-methyl while the opposite is the case with sethoxydim. The response to diclofop-methyl may be due to other mechanisms being co-selected at the lower rate of herbicide. However, selection at the high rate of herbicide application selected less effectively for resistance to chlorsulfuron and simazine (Tables 3.4 and 3.5) when compared to the lower rate of selection.

3.4.1 Estimation of frequency of resistance conferring allele(s)

The studies from the susceptible populations collected from a variety of sites (Chapter 2) indicate that the individual plants that survived the initial herbicide application were mainly heterozygous for the resistance conferring allele(s). Work on *Lolium* species, including *L. rigidum* by Cooper, (1954) supports this contention. "... in an obligate outcrossing species most individuals are the progeny of a cross between heterozygotes and are themselves heterozygous" (Cooper, 1954). In a species such as *L. rigidum,* an allele or alleles conferring resistance to a herbicide would occur in the heterozygous state. Using the Hardy-Weinberg principle a homozygote frequency frequency of 2.5 per 100,000 could be expected in a population with a 1% resistant heterozygotes present, assuming that the resistance alleles were present as heterozygotes and that there was no fitness costs for any resistant allele in the absence of a herbicide (see Chapter 5).

The percentage mortality of the susceptible component of the accessions selected at the field rate of diclofop-methyl, Figure 3.1, can be used to estimate the frequency of the susceptible allele in this population if resistance is dominant, as established in Chapter 2, and assuming the initial heterozygosity of resistance conferring alleles. Figure 3.1 show a distinct plateau at about the field rate of diclofop-methyl, 0.54 kg ha^{-1} for the S_2L and S_3L generations, this plateau is also observed if the log-dose*mortality data is plotted (not shown). This indicates that the susceptible component of the accessions have all been eliminated at this herbicide application rate and the survivors will be the component of the generations that are heterozygous or homozygous for resistance. The frequency of the allele(s) in the susceptible population VLR2, was calculated using the initial level of survivors observed, 1.4% (Section 3.2). The observed frequencies in each subsequent generation were calculated independently for each generation from the proportion killed by the herbicide using the Hardy-Weinberg principle and are shown in Table 3.5.

Table 3.5. The frequencies of diclofop resistant allele(s) calculated from Figure 3.1.

Generation	Survival %	Frequency of resistant allele(s)
0 (VLR2)	1.4	0.007
S_1	28	0.15
S_2L	77	0.53
S_3L	93	0.73

The estimate of the proportion of resistant survivors present as heterozygotes and homozygotes was made assuming resistance was conferred as a single gene. Using the Hardy-Weinberg principle the frequencies of a single dominant resistant allele with selection against the recessive susceptible allele were calculated using the frequency of 0.007 which was observed in the initial generation. Selection occurred before mating, and mating was considered to be at random. The observed frequencies of the R allele (considering resistance to observed as a discrete character) for the S_1, S_2L and S_3L generations (Table 3.5) were compared to the calculated allele frequencies and the comparison is shown in Table 3.6.

Table 3.6. Observed frequency of the resistant allele of the S_1, S_2L and S_3L accessions from Table 3 compared to the expected resistant allele frequency calculated as for a single gene in a population under selection.

Generation	Observed frequency of resistant allele(s)	Calculated frequency of resistant allele
0 (VLR2)	0.007	0.007
S_1	0.15	0.5
S_2L	0.53	0.67
S_3L	0.73	0.75*

* χ^2 test for conformity of genotypes; S_3L conformed to the calculated frequencies of genotypes, $\chi^2 = 0.30$, df=1.

Only the S_3L generation conformed to the expected frequencies of resistant alleles conferring survival at the field rate of diclofop-methyl (Table 3.6) considering the resistance phenotype as a single character. The S_1 and S_2L generations did not conform to the expected allele frequencies and showed a surplus of susceptible alleles, consistent with incomplete dominance of the resistant allele(s) in the early generations of selection or survival of susceptibles from the first herbicide application. This was overcome in later generations as the surplus of susceptible plants were eliminated. Incomplete dominance in the early generations of selection for resistance was noted in several of the susceptible populations undergoing selection reported in Chapter 2.

The onset of diclofop resistance in a population selected by three generations of exposure to diclofop-methyl had a similar final outcome as the enrichment of a single dominant allele in a population undergoing exclusive selection. This conclusion explains the rapid development of resistance to diclofop-methyl reported in histories of resistant biotypes.

3.4.2 The onset of cross resistance

The onset of cross resistance to sethoxydim and non-target site cross resistance to chlorsulfuron increased with each generation, Tables 3.3 and 3.4. The increase in LD_{50} of chlorsulfuron in each generation of the accession selected at the field rate of diclofop-methyl was related to the observed increase in resistance to diclofop-methyl. Similarly the increase in LD_{50} of sethoxydim in each generation of the accession selected at the field rate of diclofop-methyl was also related to the increase in resistance to diclofop-methyl. Although there was insufficient data to fully evaluate the response of these accessions to increasing doses of simazine it is obvious that resistance to simazine also evolved with resistance to diclofop-methyl.

3.5 Conclusion

In these experiments, approximately 760,000 field grown individuals of a herbicide susceptible *L. rigidum* biotype were treated with the commercial rate of diclofop-methyl. The survivors of the initial herbicide application occurred at about the frequency observed for other *L. rigidum* populations (Chapter 2). Two resistant accessions were generated, one by treatment with two more applications of the recommended field application rate of diclofop-methyl (540 g a.i. ha^{-1}), the other by two more applications of four times the field rate (2160 g a.i. ha^{-1}). The result was selection for substantial herbicide resistance. Each selection by diclofop-methyl increased the level of resistance in each of three generations of selection. The LD_{50}'s of the selected accessions indicate that control in the field would be poor after the first selection. The susceptible *L. rigidum* population VLR2 used here displayed the capacity to rapidly evolve resistance to diclofop-methyl as did the susceptible populations described in Chapter 2. It is likely that many *L. rigidum* populations have the capacity to evolve resistance and this capacity explains the widespread occurrence and rapid onset of herbicide resistance in Australia.

The repeated application of diclofop-methyl also selected for resistance to sethoxydim in the diclofop-methyl accessions. Selection with high or low rates of diclofop-methyl resulted in resistance to sethoxydim, however sethoxydim control remained superior to that achieved by diclofop-methyl in all accessions. Sethoxydim and diclofop-methyl have the same mode of action, inhibition of ACCase, however, selection by diclofop-methyl conferfed a higher level of resistance to diclofop-methyl than to sethoxydim. The difference in the level of resistance is not unexpected as sethoxydim is a more effective herbicide than diclofop-methyl on susceptible *L. rigidum* plants (Tables 3.2 and 3.3) and selection only by diclofop-methyl is more likely to select for mechanisms or target site alterations which give the highest protection against diclofop-methyl.

It is characteristic of cross resistance so prevalent in *L. rigidum* that some level of resistance is encountered to other herbicides. At the recommended rate for field use, sethoxydim controlled 55-65% of the S_3 accessions and diclofop-methyl controlled only 5-10%. As the susceptible population VLR2 and each generation of the resistant accessions were exposed only to diclofop-methyl, this is a well defined example of target site cross resistance to herbicides of dissimilar chemistry but similar mode of action. The tendency for resistance to sethoxydim to be more effective may be able to be exploited in the management of resistance, especially in the early generations. In contrast, resistance to chlorsulfuron will develop at a high rate due the more rapid evolution of resistance.

The propensity for *L. rigidum* to evolve cross resistance to herbicides with different modes of action is illustrated by the dose responses to the herbicides chlorsulfuron and simazine. The level of cross resistance to chlorsulfuron was more pronounced with the S_3L accession than the S_3H accession. Selection at the higher rate of diclofop-methyl selected a lower level of cross resistance to chlorsulfuron, although it was evident at the field rate under both selection regimes. The LD_{90}'s, a more reasonable measure of efficacy for field application were calculated to be 229.5 g a. i. ha^{-1} and 174.07 g a. i. ha^{-1} for S_3L and S_3H respectively, which are 14 and 11 times the recommended field rate of chlorsulfuron for the control of *L. rigidum* and substantially more than the crop tolerance level for wheat.

The level of cross resistance to simazine was also higher in the L accessions than the H accessions. The LD_{50} ratio (LD_{50} resistant /LD_{50} susceptible) for the low rate selection is 2.9 and 1.6 for the high rate selection. The calculated rate of simazine required for achieving 90% control in the field at is 2.6 and 2.3 times the rate required to cause 90% mortality of VLR2 for the low and high rate selection regimes respectively.

The expression of cross resistance to herbicides with other sites of action in the plant was influenced by the rate of the selective agent whereas the level of resistance to the ACCase inhibitors was not. The level of cross resistance to the ACCase inhibitor sethoxydim was higher in the high rate selected accession than in the low rate selected accession. However, the accessions selected with the low rate of diclofop-methyl displayed higher levels of cross resistance to herbicides with other target sites than did the accessions selected at the high rate. The high rate regime may have limited genetic diversity among the survivors by imposing a higher selection pressure. Roush and McKenzie (1987) suggest that laboratory selection which allows some survivors usually results in polygenic resistance. Even though the rate discrimination occurred subsequent to the first (S1) generation, this could explain the presence of higher levels of cross resistance in the low rate selection regime.

Resistance to diclofop-methyl, cross resistance to another ACCase inhibitor and to herbicides with other sites of action evolved very rapidly by the exclusive use of diclofop-methyl on a susceptible *L. rigidum* population. This contrasts to other weed species (except *Alopecurus myosuroides*) where cross resistance to herbicides with other modes of action has not occurred. The capacity of *L. rigidum* to evolve target site and non-target site resistance to more than one herbicide group has important consequences for the management and control of ryegrass populations in areas used for crop production. The potential for extensive cross resistance in *L. rigidum* suggests that with regular use of diclofop-methyl, control with other herbicides can be expected to decline also. The prospect of cross resistance casts doubt on the advisability of relying solely on alternate herbicides either from within the same chemical group or from other chemical groups following selection with diclofop-methyl.

Chapter four

Mechanisms of resistance in a herbicide cross-resistant ryegrass population developed from a susceptible population

4.1 Introduction.

The development of herbicide resistant accessions of *L. rigidum* from a susceptible biotype following exposure to three annual applications of diclofop-methyl was described in Chapter 3. The accessions developed resistance to the ACCase inhibiting herbicides diclofop-methyl and sethoxydim. Both these herbicides inhibit the same enzyme although they are from different chemical groups. In addition, non-target site cross resistance was observed to the ALS inhibitor chlorsulfuron and the PSII inhibitor simazine. These herbicides have target sites different to diclofop-methyl the herbicide used for selection of the resistant accessions, therefore, cross resistance has been demonstrated to herbicides with target sites other than the herbicide which was used in the selection process.

The development of cross resistance is an important aspect of the development of herbicide resistance in *L. rigidum* populations. Management problems are compounded by the appearance of cross resistance in a weed population that has initially become resistant to a commonly used herbicide. Although there are many herbicides which are active on *L. rigidum*, they represent only a limited number of chemical groups and few are selective in cereal crops (Section 1.3.5). Any reduction in the range of herbicides available for the control of *L. rigidum* due to the onset of herbicide resistance and cross resistance will reduce weed control options. In particular, the development of reliable herbicide application strategies based on the use of alternative herbicides can rapidly fail if *L. rigidum* populations display cross resistance to the alternative herbicides. Identifying the mechanisms conferring cross resistance in a population has shown the scope of the pre-existing genetic diversity capable of conferring resistance and the ability of the selection processes to enrich the genes within populations.

Cross resistance has been shown to develop rapidly in the laboratory selected populations (Chapter 3) and has also been described in resistant *L. rigidum* populations collected from the field (Gill, 1995; Powles and Matthews, 1991; Heap and Knight, 1986). The experience of many herbicide users has been that resistance and cross resistance have reduced the efficacy of ACCase inhibitors, the ALS inhibitors and other selective herbicides in many resistant populations. Several studies from field collected populations have identified mechanisms conferring cross resistance. Christopher *et al.* (1991) have reported on a mechanism of ALS cross resistance in a biotype of *L. rigidum* and Burnet *et al.* (1991) described mechanisms of cross resistance in two biotypes resistant to the PSII inhibitors.

Reports of cross resistance and non-target site cross resistance to herbicides in other resistant weed species are rare. Cross resistance and non-target site cross resistance have been reported in herbicide resistant *Alopecurus myosuroides,* Hall *et al.* (1994) and in *Phalaris paradoxa*, by Yaacoby *et al.,* (1986) both in populations collected from the field. In a controlled selection of several populations of *Alopecurus myosuroides* by four applications of the ACCase inhibitor fenoxaprop-*p*-ethyl, Chauvel *et al.* (1992) was only able to detect limited target site cross resistance to one other herbicide group.

There have been a great number of reports from field observations which indicate the propensity for *L. rigidum* to develop resistance to many herbicides. In most reported cases of resistance the field history was not conclusive and it is difficult to accurately ascribe patterns of resistance to the herbicides previously used. Also, in field collected samples the possibility of untreated or introduced individuals contributing to the gene pool cannot be ruled out. This problem can be overcome by selection of cross resistance under controlled conditions as was described in Chapter 3. This chapter describes the identification of the mechanisms conferring resistance in those accessions to the ACCase inhibitors and the mechanisms conferring cross resistance to the ALS inhibitors and the PS II inhibitors.

4.2 Materials and Methods.

4.2.1 Plant material for enzyme assay and metabolism study

Resistant accessions of *L. rigidum* were developed by exposing a susceptible population of *L. rigidum* at the field rate (540 g a.i. ha^{-1}) of diclofop-methyl. The initial selection was done in the field, however, further selections were performed under controlled conditions (see section 3.2). For details of the selection procedure resulting in the development of herbicide resistant accessions S_3L and S_3H see Table 3.1. The response to herbicides of accessions S_3L and S_3H are shown in Sections 3.3.1-3.3.4.

Seeds were germinated on 0.6% agar in a growth cabinet, at 20° C and a light intensity of 50 µmol photons m^{-2} s^{-1} and an 8hr 15° C dark phase. At the one leaf stage, seedlings were transplanted to sterilised potting soil and grown in a growth room at 20° C, 330 µmol photons $m^{-2}s^{-1}$ and 8hr, 16° C dark phase. For the enzyme extraction, 66 plants were grown in trays (30 cm X 45 cm), while for metabolism studies, 6 plants were planted in polystyrene cups. Plants were used at the 2 - 3 leaf stage, the developmental phase at which herbicide is usually applied in the field. For herbicide application, the transplanted seedlings were grown outdoors in the winter, the normal growing season for *L. rigidum*. Hand watering was done as required. Herbicides were applied at the 2 - 3 leaf stage as previously described.

4.2.2 Enzyme extraction

All extraction and partial purification procedures were performed at 0-4°C. Plant material was ground in a chilled mortar with 10 ml of buffer A (100 mM Tris-HCl [pH 8.0], 1 mM PMSF, 20 mM DTT, 1 mM Na_2EDTA, 0.5% [w/v] insoluble PVP, 0.5% [w/v] PVP-40, 10% [v/v] glycerol, and 2 mM isoascorbic acid). The brei was centrifuged for 15 min at 27000 g. ACCase activity precipitated from the supernatant between 10% and 40% $(NH_4)_2SO_4$ saturation. The pellet was resuspended in 2.5 ml of buffer B (50 mM Tricine [pH 8.0], 2.5 mM $MgCl_2$, 1 mM DTT, and 50 mM KCl) and loaded onto a chromatography column. The column contained Sephadex G-25 (PD10 column, Pharmacia) and was previously equilibrated with buffer B. Partially purified ACCase was eluted from the column with 2.7 ml of buffer B, kept on ice and assayed immediately for ACCase activity.

4.2.3 ACCase assay

ACCase activity was assayed by following the incorporation of ^{14}C from $NaH^{14}CO_3$ into acid stable product at 30° C. Assays contained, in 200 µl, 50 mM Tricine (pH 8.0), 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 1 mM ATP, 10 mM $NaH^{14}CO_3$ (containing 7.4 KBq of ^{14}C), 0.3 mM acetyl-CoA, enzyme sample and herbicide as required. Reactions were initiated by the simultaneous addition of ATP, acetyl-CoA and $NaH^{14}CO_3$ after 3 min incubation of enzyme with herbicide. Reactions were terminated by the addition of 25 µl of glacial acetic acid. A fluted glass fibre filter (Whatman GF-A) was added to each reaction vial and the sample dried under an air stream. Scintillant, (2 ml) was added to each vial and the acid stable radioactivity determined in a Beckman LS5000TD detector. The output from the scintillation counter was corrected for background and acetyl-CoA independent CO_2 fixation.

Herbicides for ACCase assay

All herbicides were made up as 1 mM stock in 70% (v/v) acetone and diluted prior to use such that the highest acetone concentration in any assay was 0.3% (v/v). Diclofop was a gift of Hoechst Australia and sethoxydim from Schering Australia.

4.2.4 Herbicide metabolism methods

Application of [^{14}C](U-Phenyl) Diclofop-methyl

2.5mM [^{14}C] (U-phenyl) Diclofop-methyl (50 Bq μL^{-1}) in 1μl of a solution that was similar to the commercial formulation of the product was deposited on the leaf blade 0.5 cm above the leaf axil of ryegrass plants at the two leaf stage of plants grown in soil in a growth chamber. After exposure for 6, 12, 28, 51 and 77 hours, tissue surrounding the site of deposition including the leaf axil and the meristem was separated from the roots and leaves. Each experimental unit consisted of five plants. The tissue was washed in 100ml of 20% methanol containing 0.02% Triton X-100, the washes were retained and the tissue was blotted dry and frozen in liquid N_2.

Application of [^{14}C](U-Phenyl) Simazine

A 1μM solution of [^{14}C] (U-Phenyl) simazine (37 MBq mM^{-1}) was prepared with 33% Hoaglands nutrient solution* and introduced to 25ml vials in which 2-3 leaf seedlings of *L. rigidum* had been established with nutrient solution only. The vials were placed in a growth room with conditions as above and the nutrient solution level maintained with 33% Hoaglands nutrient solution, if required, until harvest.

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* Hoaglands nutrient solution contained, KH_2PO_4 0.5mM, K_2SO_4 0.4mM, $MgSO_4$ 1mM, $Ca(NO_3)_2$ 1.67mM, KNO_3 1.67mM, EDTA Na_2 64μM, $FeSO_4$ 72μM, $CaSO_4$ 800μM, Na_2MoO_4 0.25μM, $CuSO_4$ 0.16μM, $ZnSO_4$ 0.38μM, $MnCl_2$ 4.6μM, H_3BO_3 23μM.

Extraction procedure

Following pulverisation of frozen tissue in a cold mortar, the powder was suspended in 5ml of 80% methanol in the mortar. The brei was centrifuged at 9000 x g for 20 min at 4° C. The pellet was resuspended in 2ml of 80% methanol, re-extracted and re-centrifuged. The supernatants were pooled and reduced in volume under vacuum to give suitable specific radioactivity. Samples were filtered through a 0.22µm nylon filter before injection onto the HPLC column.

HPLC analysis

Radio labelled metabolites were separated using reverse phase HPLC with a Brownlee Labs ODS-5 Spherisorb-5 column (250 X 4.6 mm i.d.). Radioactivity was detected with a flow-through scintillation detector (Radiomatic A140, Canberra Instruments, Tampa, FL.). Solvents used were Solvent A; 10% (v/v) acetonitrile; 89% (v/v) H_20; 1% (v/v) acetic acid, Solvent B; 90% (v/v) acetonitrile; 9% (v/v) H_20; 1% (v/v) acetic acid. Elution conditions involved a 10 min linear gradient from 30 to 35% Solvent B, followed by a 12 min linear gradient from 35 to 50% Solvent B, followed by a 3 min linear gradient from 35 to 100% Solvent B. The column was eluted with 100% Solvent B for 10 minutes. The combined flow rate of Solvents A and B was 1.5 ml min^{-1} at all times.

4.2.5 Application of malathion

The organophophate insecticide malathion was applied as a foliar application to two leaf *L. rigidum* seedlings germinated and transplanted as described previously and grown in 17.5 cm pots containing recycled potting soil. Technical grade malathion was emulsified with a hydrocarbon solvent and nonionic surfactant. Malathion was a gift from Incitec Australia.

4.3 Results

4.31 Mechanisms of resistance to ACCase inhibitors

Seedlings of the third generation of the high and low rate selections were assayed for the effect of two ACCase inhibiting herbicides on *in vitro* enzyme activity. The concentration of the herbicides required for 50% inhibition (I_{50}) of extracted ACCase activity and the resistance ratio (R I_{50}/S I_{50}) when compared to the parent susceptible population are shown in Table 4.1. The S_3H population had the higher LD_{50} ratio to diclofop acid, the biologically active derivative of diclofop-methyl. The ACCase activity extracted from S_3L accession was less inhibited by diclofop acid, although the level of tolerance at the whole plant level is similar to the S_3H accession. ACCase activity from the S_3H accession was 2.5 fold more tolerant of diclofop acid *in vitro* than that from S_3L, Table 4.1.

ACCase activity in enzyme peparations extracted from both the S_3L and the S_3H accessions had similar tolerance to sethoxydim. The LD_{50} ratios were 2.4 and 2.3 respectively, (Table 4.1).

Table 4.1. Herbicide doses required for 50% *in vitro* inhibition of ACCase activity (I_{50}) of S_3 generations selected from the susceptible VLR2 (S).

Resistant accessions	Herbicide	I_{50} (µM) R	I_{50} (µM) S	R/S Ratio
S_3 L	diclofop acid	14.0	0.19	74
S_3 L	sethoxydim	17.0	7.5	2.4
S_3H	diclofop acid	35.0	0.18	194
S_3H	sethoxydim	10.5	4.5	2.3

The data is the mean of two experiments replicated three times.

Both accessions contained ACCase enzyme activity that exhibited resistance to both diclofop acid and sethoxydim. The S3L accession contained an ACCase enzyme which was substantially less inhibited by diclofop than the susceptible parent population and the S3H enzyme was less inhibited by diclofop *in vitro* than the S3L accession. Both accessions were inhibited similarly by sethoxydim *in vitro* (Table 4.1). The resistance exhibited by the enzyme *in vitro* is almost certainly an important mechanism of resistance to the herbicides diclofop-methyl and sethoxydim *in vivo* and is similar in magnitude to other resistant forms of the ACCase enzyme extracted from *L. rigidum* (Tardif *et al.*, 1993).

It cannot be assumed that both accessions are homogenous for individuals possessing the resistant form of ACCase enzyme. In fact, the dose response curves for S3L and S3H (Figures 3.1 & 3.2) indicate that the resistant accessions comprise two groups of plants, of which the most resistant sub-group of each accession are not killed at extreme doses of the herbicides used.

Separation of these sub-groups was attempted by germination of the S3L accession on agar containing 1mM diclofop-methyl. A sub-group containing about 20% of the S3L accession was obtained and transferred to potting soil and grown in the absence of herbicide for 4 weeks. ACCase activity was extracted from these plants and assayed in the presence of diclofop and sethoxydim (Table 4.2). Selection of the population in this way resulted in an increase in the LD_{50} ratio of ACCase *in vitro* to diclofop acid but not to sethoxydim.

Table 4.2. Amount of herbicide required for 50% inhibition of *in vitro* ACCase activity (I_{50}) of the susceptible biotype VLR2 (S) and the selected sub-group of resistant biotype S_3L.

Herbicide	I_{50} (μM) S_3L subgroup	I_{50} (μM) S	R/S Ratio I_{50} (μM)
diclofop acid	20.0	0.15	133.3
sethoxydim	6	2.5	2.4

The data is the mean of two experiments replicated three times.

The S_3L population comprised at least two resistant phenotypes, one containing an ACCase enzyme highly resistant to diclofop *in vitro* and another, in which the resistant ACCase was not as resistant. The S_3H accession contained an ACCase enzyme more resistant than the sub-group from S_3L (Tables 4.1 and 4.2). The whole plant response (Table 3.2) however, shows that the S_3L accession is as resistant to diclofop as S_3H. Therefore, other mechanisms that enhance resistance must be present in the S_3L accession.

4.3.2 Metabolism of diclofop-methyl

The third generation of the two selected resistant accessions and the parent population were treated with U-^{14}C diclofop-methyl to establish if differential de-esterification of diclofop-methyl to diclofop acid or metabolism of diclofop acid to inactive compounds was a resistance mechanism. Differences in the rate of disappearance of radiolabel as diclofop-methyl and the appearance as diclofop acid, the rate of disappearance of radiolabel as diclofop acid and the appearance of diclofop metabolites or conjugates may endow resistance when biotypes are compared.

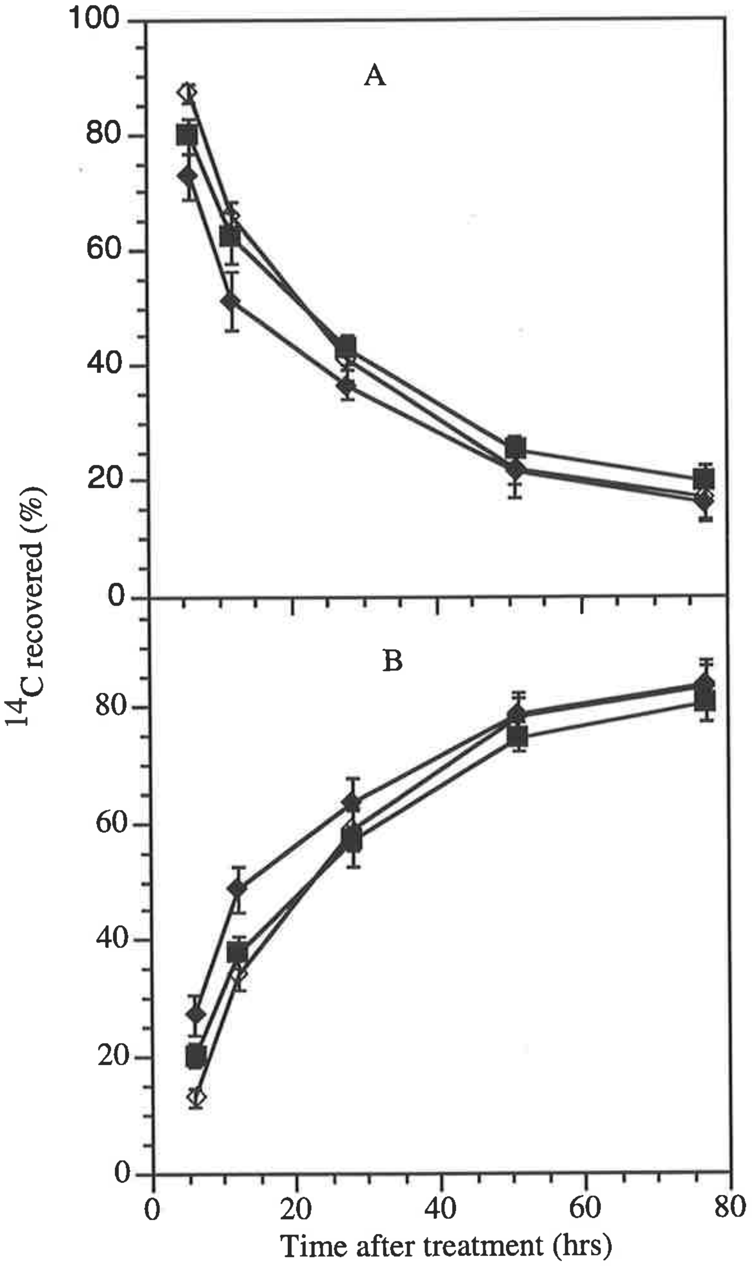
Figure 4.1A shows the disappearance of radiolabel as diclofop-methyl and diclofop acid for each biotype in one graph, as they were not different. Any differences between the accessions in demethylation was not detected in these experiments. Figure 4.1B shows the appearance of radiolabel as inactive herbicide metabolites in the cell and on the cell wall. There was a significant difference in the rate of metabolism of diclofop acid to inactive metabolites between the biotype VLR2 and S3L at six and twelve hours after application of the herbicide. Metabolism of diclofop was depressed in the S3H accession compared to the S3L.

4.3.3 Mechanism of resistance to chlorsulfuron

The S3L and S3H accessions displayed cross resistance to the herbicide chlorsulfuron, (Figures 3.5 and 3.6). Both accessions responded to increasing doses of chlorsulfuron indicating that resistance may be conferred by a dose dependent mechanism which is likely to be metabolism of the herbicide.

In a report by Christopher *et al.* (1991) a similar whole plant response to chlorsulfuron was present in a resistant biotype in which an increased rate of *in vitro* metabolism of the active herbicide molecule was considered to be the principle mechanism of resistance.

Figure 4.1. Metabolism of diclofop-methyl and recovery of radiolabel by VLR2, S3L and S3H.

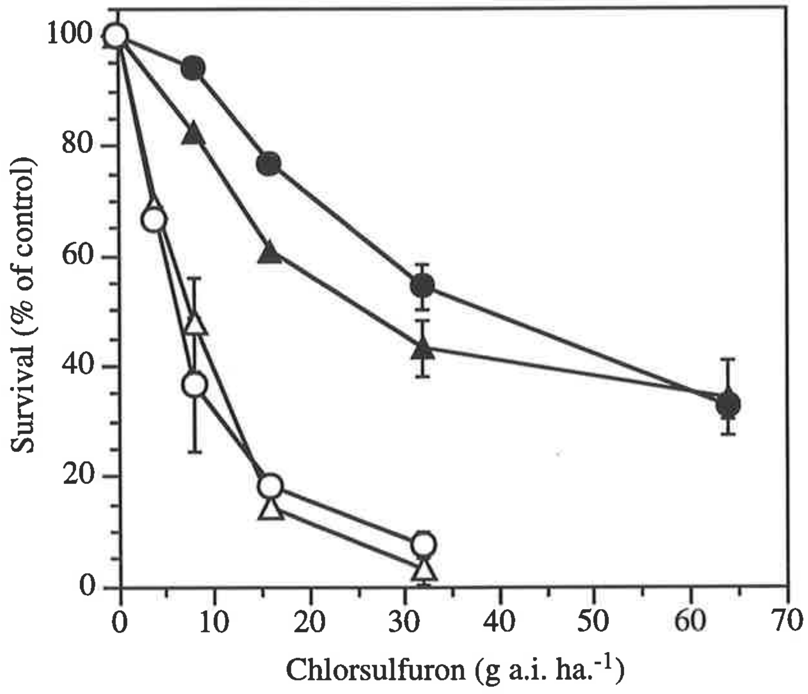


Legend: VLR2 ■; accession S3L ◆; accession S3H, ◇; Radiolabel recovered as (A), diclofop-methyl and diclofop acid; (B), total metabolites. Data is the mean of two experiments. There was a significant differences between biotypes VLR2 and S3L at 6 and 12hrs ($p<0.05$), d.f. = 10.

To investigate the basis of cross resistance to chlorsulfuron the S_3L accession was treated with the non-selective ALS inhibitor sulfometuron-methyl at doses ranging from 0 and 3 to 96 g. a.i. ha^{-1}. There were no survivors from the herbicide application 28 days after treatment (data not shown). Wheat and other grasses are unable to metabolise sulfometuron-methyl at a rate sufficient to protect the plant against herbicide action (Anderson and Swain, 1992). Also, Christopher *et al.* (1992) has demonstrated that a chlorsulfuron resistant biotype of *L. rigidum* which displayed an increased rate of metabolism of chlorsulfuron was easily controlled by sulfometuron-methyl. The F_3L accession contained an ALS enzyme susceptible to sulfometuron-methyl yet resistant to chlorsulfuron, therefore, metabolism of chlorsulfuron was most probably the mechanism of resistance. Both the S_3L and S_3H accessions appear to have the same mechanism of resistance to chlorsulfuron as there was no interactions between biotype and response to herbicide application rate (Table 3.4).

The organophosphate insecticide malathion has been shown to reduce the rate of cytochrome P450 metabolism of a sulfonylurea herbicide in *Zea mays* (Kreuz and Fonne-Pfister, 1992; Pruss and Johnson, 1992). Christopher *et al.* (1994) demonstrated that metabolism of chlorsulfuron in *L. rigidum* is reduced in the presence of malathion and that malathion synergises chlorsulfuron action *in vivo* in both a susceptible and resistant biotype. In addition, Christopher *et al.* (1994) demonstrated that malathion synergises chlorsulfuron activity in biotypes resistant to chlorsulfuron due to chlorsulfuron metabolism, but has no effect on biotypes containing an resistant form of the ALS enzyme. Figure 4.2 shows the effect of malathion in conjunction with chlorsulfuron on survival in the S_3L and S_3H accessions. Table 4.4 shows the LD_{50}'s of the accessions are similar when chlorsulfuron is applied in the presence and different when applied in the absence of a foliar application of 1000 g a.i. ha^{-1} malathion.

Figure 4.2. Response of diclofop-methyl selected accessions S3L and S3H in the presence or absence of malathion to increasing doses of chlorsulfuron.



Legend: Diclofop-methyl selected accessions S3L, (○, ●) and S3H, (△, ▲) in the presence (○, △) or absence (●, ▲) of 1000g a.i. ha^{-1} of malathion. Bars represent the S.E. of the mean of two experiments with three replicates each.

Table 4.4. The amount of chlorsulfuron calculated to cause 50% mortality in accessions of *L. rigidum* selected by successive applications of diclofop-methyl in the presence or absence of malathion.

	LD_{50} [g a.i. ha^{-1}] (fiducial limits $p<0.05$, in brackets)	
Accession	chlorsulfuron	chlorsulfuron + 1000g a.i.ha^{-1} malathion
S_3L	42.99 (35.72-54.75)^{ax*}	6.02 (4.45-7.49)^{ay}
S_3H	32.97 (28.68-38.57)^{bz}	6.69 (5.34-8.02)^{ay}

* Treatments followed by the same letter (a-b) have parallel probit transformations. Accessions followed by the same letter (x-z) have similar transformed slopes and intercepts. Data is the mean of two experiments with 3 replicates.

The simultaneous application of malathion and chlorsulfuron reduced the LD_{50} of chlorsulfuron for both accessions to about 6 to 7 g a.i. ha^{-1} (Table 4.4). Both accessions responded similarly to the combination of chlorsulfuron and malathion (Table 4.4). In addition, the LD_{50}'s of the malathion plus chlorsulfuron treatments are similar to the LD_{50} of the parent susceptible population (6.0 g a.i. ha^{-1}), (Table 3.4). These observations, (Table 4.4) in conjunction with the susceptibility to sulfometuron-methyl and taken with the observations of Christopher *et al.* (1994) strongly suggest that the mechanism of resistance to chlorsulfuron in both S_3L and S_3H is increased metabolism of the herbicide.

4.3.4 Mechanism of resistance to simazine

The S_2L and S_2H accessions display resistance to the PS II inhibitor simazine, (Table 3.5 and Figure 3.7). Although the level of resistance was not high, indicated by the R/S LD_{50} ratio of 2.9 for S_2L and 1.6 for S_2H, the percentage of survivors at the usual field application rate (50% and 30% for S_2L and S_2H respectively) would be enough to cause serious problems in the field. Both accessions showed increasing mortality with increasing doses of herbicide (Figure 3.7), indicating that an altered site of action was not likely to be the mechanism of resistance.

Direct metabolism studies using U-^{14}C simazine to identify herbicide metabolites were performed and the results are presented in Table 4.5. The data shows that there was a four fold difference in the amount of non-herbicidal metabolites obtained from the diclofop-methyl selected accessions compared to the susceptible parent. The susceptible biotype had the lowest proportion and the S_2L accession had the highest proportion of simazine metabolites detected under this system. Total recovery from leaves was about 30% of the radiolabel added to the whole plants. The differences between the amounts of metabolites are consistent with the level of resistance to simazine shown in Figure 3.7.

Burnet (1992) has described metabolites of simazine from *L. rigidum* with low retention times from HPLC and concluded that a polar metabolite was likely to be di-de-ethylsimazine. The major metabolite referred to in Table 4.5 eluted at a similar time as the metabolite under similar HPLC gradient conditions used by Burnet (1992). This experiment shows that increased amounts of herbicide metabolites are present in resistant accessions which suggests that metabolism is likely be the basis of simazine resistance in the diclofop selected accessions.

Table 4.5. ^{14}C recovered as simazine and the only major metabolite from leaf tissue of susceptible VLR2 and diclofop selected accessions S_2L and S_2H following 26 hrs exposure to 2µM ^{14}C simazine in 33% Hoaglands hydroponic solution.

	% Radiolabel recovered	
Accession	^{14}C simazine	^{14}C simazine metabolites
VLR2	97.8	2.1
S_2L	90.2	9.8
S_2H	91.8	8.2

Values are the mean of one experiment with three replicates each of 15 plants.

4.4 Discussion

Chapter 3 described the responses of the selected populations to a range of herbicides which demonstrated that high levels of cross resistance had developed after only three applications of diclofop-methyl. The data presented in this chapter supported the whole plant response to the herbicides application by elucidating the mechanisms responsible for resistance. As shown in Table 3.2, the resistance of whole plants from both the S_3L and S_3H accessions to the ACCcase inhibitor diclofop-methyl was extremely high when compared to the susceptible parent population VLR2. From the data presented here it is evident that a resistant ACCase enzyme is a major mechanism of resistance in both accessions. Table 4.2 shows the I_{50} values of the S_3L and S_3H enzyme extracts were substantially greater than for enzyme extracts from the susceptible VLR2 population to diclofop acid. Thus both the S_3L and S_3H accessions have developed target site resistance to diclofop-methyl.

Even though the S3L accession had greater resistance to foliar applied diclofop-methyl (Table 3.2) than the S3H accession, the I_{50} for *in vitro* inhibition of ACCase was substantially less than for the S3H accession. Also, further selection on agar increased the I_{50} of the S3L accession to diclofop-methyl probably by elimination of the susceptible and the heterozygote components of the population. Other mechanisms conferring some resistance to diclofop in whole plants at moderate herbicide rates may not be expressed in bioassay due to the high herbicide concentration. Such mechanisms can be present and protected in a population when high levels of resistance are conferred by an insensitive target enzyme. The ability to acquire more than one mechanism of resistance to herbicides is a feature of the development of resistance in *L. rigidum.*

As well as displaying target site resistance to diclofop-methyl both accessions displayed target site cross resistance to the ACCase inhibitor sethoxydim. Sethoxydim was more effective on all accessions than diclofop both as a foliar application, Figures 3.3 and 3.4, and also on enzyme extracts *in vitro,* Table 4.2. Even though there was a substantial difference between the selection regimes, *in vitro* tolerance to sethoxydim was similar for all accessions including the highly resistant sub-group selected from the S3L accession. The change in the ACCase enzyme that conferred resistance to diclofop may not affect the sethoxydim binding domain on the ACCase enzyme to the same extent. Hence, the difference in the level of inhibition of the ACCase enzyme to sethoxydim and diclofop and the similarity of sethoxydim activity on the S3L and S3H accessions.

There was a difference in the rate of metabolism of diclofop to inactive compounds between the S3L and the parent population (Figure 4.1). So resistance to the ACCase inhibitor diclofop-methyl in the S3L accession appears to be due to both a tolerant form of the target enzyme and an increased level of metabolism. This supports the observation that the S_3L had a high level of resistance to foliar applications of diclofop-methyl but a lower level of resistance of the ACCase *in vitro* than S_3H.

All diclofop selected accessions displayed resistance to the ALS inhibitor chlorsulfuron. The data from section 4.3.3 and figure 4.2 shows indirectly that the resistance of whole plants to the herbicide (Figures 3.5 and 3.6) is almost certainly due to increased metabolism of chlorsulfuron. The probit analysis (Table 3.4) showed that accessions did not display an interaction with herbicide doses, thus, each accession probably has similar resistance conferring mechanisms and probably differ only in their relative frequency within each accession. Selection by an ACCase inhibitor herbicide only, which resulted in selection of an insensitive ACCase target site and diclofop metabolism has also selected for resistance to the ALS inhibitors endowed by enhanced herbicide metabolism. Thus, both the S_3L and S_3H accessions display non-target site cross resistance.

The diclofop selected accessions also showed cross resistance to simazine (Figure 3.7). Simazine is from a different herbicide group than either the ACCase or the ALS inhibitors and therefore has a target site unrelated to the target site of the herbicide used in the selection process. Table 4.5 shows directly that metabolism of the active simazine molecule is a mechanism of resistance in both diclofop-methyl selected accessions. Therefore it is safe to conclude that accessions S_2L and S_2H display non-target site cross resistance to the PS II inhibitor simazine.

All the herbicides used in this study are metabolised by the crop species in which they are used selectively. Diclofop-methyl, simazine and chlorsulfuron are also metabolised in *L. rigidum* plants, albeit at rates which are unable to protect susceptible populations from lethal damage (Burnet *et al.*, 1992; Christopher *et al.*, 1991; Matthews *et al.*, 1990). The evidence reported here shows that non-target site cross resistance conferred by increased capacity to metabolise these herbicides has been selected solely by the use of diclofop-methyl.

As shown in Figure 4.1 there is an increased level of herbicide metabolism in S_3L which contributes to resistance to diclofop-methyl. Increased metabolism of diclofop-methyl was not detected in S_3H. For the other non-target site mechanisms the S_3L displays significantly higher levels of resistance than S_3H (Tables 3.4 and 3.5) and the characterisation of the resistance mechanisms supports this observation (Tables 4.4 and 4.5). The low rate selection regime used co-selected the enhanced metabolism activity from the original population at a higher level of efficacy than the high rate selection regime. Selection with the higher rate of diclofop-methyl appears to have selected for a greater proportion within the population of the tolerant form of ACCase, but not for effective mechanisms of enhanced metabolism. Reduction of the genetic variation within the selected population due to increased selection pressure in the S_1 generation to form the S_2H accession is most likely the determining event. Thus the selection intensity has an influence on the presence or absence of non-target site cross resistance in herbicide resistant *L. rigidum.*

Non-target site cross resistance is not commonly observed in most of the species which have biotypes with evolved resistance to herbicides. This may be because the diversity of mechanisms are not present in the species or population under selection, or the necessary potential mechanisms are not co-selected with the principle mechanism of resistance. However, from the data shown in Chapter 3 and here, both extreme target site cross resistance and substantial non-target site cross resistance was induced by the application of the cereal selective graminicide diclofop-methyl.

Chapter five

Comparative fitness of herbicide resistant ryegrass biotypes

5.1 Introduction

The number of sites and area affected with herbicide resistant ryegrass has increased rapidly since herbicide resistance in *L. rigidum* was first reported by Heap and Knight (1982). Following the use of herbicides from the aryloxyphenoxypropionate, cyclohexanedione and sulfonylurea groups of herbicides, many populations of ryegrass have become resistant or partially resistant to herbicides from these chemical groups (Powles and Matthews, 1991). Many of the resistant biotypes, especially following exposure to the aryloxyphenoxypropionate group of herbicides, have been shown to be cross resistant to other selective herbicides registered for the control of ryegrass. This has severely limited the prospects of further herbicide use for weed control in crops. Evaluating the fitness of herbicide resistant *L. rigidum* is an essential first step in understanding the resistance pheneomenon. If the fitness characteristics of a new resistant weed population are understood it is possible to predict both the onset of resistance on a wider scale and the persistence of the resistant phenotype under competitive conditions and also to identify any physiological differences attributable to the resistance genotype. From the weed control practioners viewpoint, fitness studies enable careful management techniques to help prevent or control infestations to be instituted.

The fitness of an organism is a combination of many attributes contributing to survival and reproduction. In the case of annual plant species the ability of one genotype to leave more progeny compared to another will lead to an evolutionary advantage (Harper, 1977). For annual ruderal species with a limited seed-bank life, annual seed output is an important determinant of the species success. In a mediterranean climate, characterised by decreasing soil moisture during anthesis, the ability to accumulate sufficient biomass during the growing season greatly influences seed production and is therefore, important to the success of a species. Thus, relative seed production and relative biomass are likely to be good indicators of the fitness of unique subsets or biotypes selected from a population. For a species with high plasticity, such as *L. rigidum*, relative tiller number is also an indicator of potential success.

Many investigations into the fitness of herbicide resistant weeds have been carried out. These have been mainly on weeds resistant to the triazine herbicides and have established that most triazine resistant weed biotypes produce less dry matter and have lower reproductive capacity compared to herbicide susceptible populations (McCloskey and Holt, 1990; Holt, 1990; Warwick, 1990; Holt and Radosevich, 1983). This has been established for triazine resistant weeds where resistance is conferred by a single gene mutation on the D1 protein resulting in impaired electron flow between Qa and Qb in the photosynthetic apparatus of PS II, (Ort *et al.,* 1983; Holt *et al.,* 1981), see Section 1.5.

There are a few reports where target-site based resistance to triazine herbicides did not reduce the reproductive capacity and dry matter productivity (Holt and Thill, 1994; Schonfeld *et al.,* 1987). Fitness differences between the resistant and susceptible biotypes being compared could be masked (or enhanced) by environmental and genetic factors not related to resistance. In some studies on triazine resistance the variation between susceptible populations of diverse geographical origin was greater than the difference between the resistant and susceptible types conferred by the reduced Qb function (Warwick and Marriage, 1982, 1982b). However, most of the studies show a reduction in fitness associated with the mutation at the Qb binding site and this has been conclusively shown by comparison of isonuclear lines of many triazine resistant and susceptible weed species (McCloskey and Holt, 1991; Jacobs *et al.,* 1988).

Resistance to triazine herbicides not due to the mutation at the Qb binding site is less common and of the three weed species reported, only *Abutilon theophrasti,* has appeared in an environment and in a species where the PS II mutation might have been expected to occur. The PS II mutation confers a greater level of resistance to atrazine than the glutathione transferase mediated metabolism in *Abutilon theophrasti* but a comparison of fitness penalty due to either mechanism of resistance is difficult due to the comparative biotypes being taken from different geographic areas (Gronwald, 1994; Gronwald *et al.,* 1989). The other two weeds to develop non-target site resistance to the PS II inhibitors due to herbicide metabolism are *L. rigidum* and *A. myosuroides* reported by Burnet *et al.* (1993) and Kemp *et al.* (1990) respectively.

Most of the reports of fitness studies on populations with resistance to triazine herbicides conclude that there is a pleiotropic cost of target site resistance to triazine herbicides. However, there is also variation between populations from different management systems due to adaptation to seasonal differences or other ecological factors which may confound comparative whole plant studies. Variations in fitness between resistant populations with the same mutation may be due to different management activities which result in varied pressures for adaptation (Haldane, 1960). In other resistant weed species where the differences between resistant and susceptible may not be not so large the difficulties associated with the choice of biotypes for comparison may obscure any small differences in relative fitness.

In spite of these difficulties there is considerable interest in the relative fitness of a resistant biotype when compared to a susceptible counterpart. Fitness is important to the evolutionary phenomena exhibited by the onset of herbicide resistance. The persistence of novel genes is dependent upon relative fitness in the generations following the occurrence of the mutations conferring resistance and also on adaptation over subsequent generations. Evaluation of resistance in a competitive situation may also help identify any physiological differences between resistant and susceptible biotypes. Identification of a fitness penalty in established resistant populations or in populations developing resistance could contribute to management of herbicide resistance.

Because of the extensive literature on triazine resistant weeds there has been a tendency to assume that biotypes displaying resistance to herbicides are likely to be less fit than comparable susceptible populations and therefore management of resistant weeds is not difficult. Limited research has been performed on weeds resistant to other herbicides. Weiderholt and Stoltenberg (1996) showed that a biotype of *Setaria faberi* resistant to the ACCase inhibitors fluazifop-*p*-butyl and sethoxydim was not less fit than a susceptible biotype, either in competition with a corn (*Zea mays*) crop or in an intraspecific comparison. Alcocer-Ruthling *et al.* (1992) reported that an ALS resistant *Lactuca serriola* biotype was equally competitive as a susceptible biotype but accumulated biomass a greater rate in non-competitive conditions.

It is important to determine the relative competitiveness of the herbicide resistant *L. rigidum* biotypes from a practical viewpoint. Such information might aid the management of resistant weed populations and identify any exploitable differences between resistant and susceptible biotypes. If resistance was unequivocally associated with reduced fitness across a range of biotypes then a competitive crop or pasture phase without herbicide selection pressure could serve to decrease the frequency of the resistant biotype as postulated by Gressel and Segel (1978).

The relative fitness of resistant individuals in an unsprayed population of *L. rigidum* prior to initial herbicide selection would be important to the success of novel resistant allele(s). If the resistant component of an unsprayed population carried a fitness penalty that could be expected to lead to a reduction of the frequency of resistant individuals in a susceptible population. Similarly, when a cycle of selection by herbicide application is commenced, the fitness of the F_1 crosses between the resistant survivors and susceptible plants will have an important bearing on the persistence of the F_1 generation and will influence the rate of onset of resistance.

Although herbicide resistance has been well characterised in several *L. rigidum* biotypes and many of the resistance conferring mechanisms have been identified, little is known about the competitive ability of different herbicide resistant biotypes of *L. rigidum*. This series of studies was designed to compare several herbicide resistant *L. rigidum* biotypes with different herbicide resistance mechanisms to susceptible biotypes and identify if resistance conferred by a particular mechanism resulted in reduced fitness. The fitness of first generation crosses between a resistant and a susceptible biotype was assessed as was the fitness of the resistant component of an unsprayed susceptible *L. rigidum* population.

5.2 Materials and methods

5.2.1 Experimental design

Experiments one, two and three were conducted in the field and experiment four in the glasshouse.

5.2.2 Experiment one

Experiment 1 was a replacement series in the style of de Wit, (1960) with the multiple resistant biotype (SLR31) compared with a susceptible biotype (VLR2), at two densities with four replicates and various proportions as shown in Table 5.1. The herbicide resistance spectrum of the biotypes is listed in Table 5.2.

Table 5.1. Planting proportions of resistant (SLR31) and susceptible (VLR2) biotypes established in Experiment 1.

Planting densities (plants m^{-2})			
400		900	
Resistant	Susceptible	Resistant	Susceptible
1.0	0	1.0	0
0.9	0.1	0.9	0.1
0.75	0.25	0.7	0.3
0.5	0.5	0.5	0.5
0.25	0.75	0.3	0.7
0.1	0.9	0.1	0.9
0	1.0	0	1.0

The biotypes were also compared under non-competitive conditions. The replacement series format has some disadvantages for assessing competitiveness in plants that occur at a range of densities in the field, however the ability to identify an advantage at a low frequency is of importance in understanding the development of resistance in the field.

5.2.3 Experiment two

Experiment 2 was a partial diallel series with 4 biotypes in selected comparisons at 5 densities and two proportions with three replicates. The biotypes VLR1, SLR31, WLR1 and SLR3 were established in pure stands at densities of 900, 600, 300, 100 and

50 plants per square metre (1: 0 proportion). The following pair comparisons (1:1 ratio) were made SLR3 - SLR31, SLR3 - VLR1, SLR31 - WLR1 and WLR1 - VLR1, the comparison of SLR31 - VLR2 having been made in experiment 1. The design allowed all the resistant biotypes to be compared to the susceptible biotype and two resistant biotypes were compared to one common resistant biotype. This experimental design allowed the biotypes to be compared to each other over a range of densities both alone and in an equal mixture but with the mixed planting at the same density as each biotype when planted alone. The design, as executed here, was sensitive to responses to density but with limited mixture proportions than the replacement series.

5.2.4 Experiment 3

F_1 crosses between the resistant biotype (SR31) and the susceptible biotype (VLR1) were made in the previous season. Several pots containing a susceptible and a resistant plant were grown and prior to anthesis 2m high plastic barriers were placed around the pots to exclude foreign pollen. Seeds were harvested at maturity from both the susceptible and resistant parent and bulked and stored as above.

5.2.5 Herbicide resistance spectrum of the biotypes

The herbicide resistance spectrum and known resistance mechanisms of the biotypes are summarised in Table 5.2. Tardif and Powles (1994) describe the resistance mechanisms of biotype SLR31, which has the most complex array of resistance conferring mechanisms. This biotype has a 12% subset which has a resistant form of the ACCase enzyme and the biotype as a whole displays increased metabolism of diclofop-methyl. It also has non-target site cross resistance to the ALS inhibitors, Christopher *et al.*, (1992) and uncharacterised resistance to the dinitrolaniline herbicides

(McAlister *et al.*, 1994). SR31 also has the ability to recover from herbicide induced membrane depolarisation (Häusler *et al.*, 1991).

Table 5.2. Resistance spectrum and known resistance mechanisms of the biotypes.

Biotype	Resistance spectrum	Resistance mechanisms	Refs. *
SLR31	APP's and CHD's.	Insensitive ACCase subset; elevated metabolism	1
	ALS inhibitors	Enhanced herbicide metabolism.	2
	Dinitroanilines.	Unknown.	3
SLR3	APP's and CHD's.	Insensitive ACCase	4
WLR1	ALS inhibitors.	Insensitive ALS	2
VLR1	Susceptible		
VLR2	Susceptible		

* 1, Tardif and Powles, 1994; 2, Christopher *et al.,* 1992; 3, McAlister *et al.,* 1994; 4, Tardif *et al.,* 1993.

5.2.6 Seed preparation and production

Experiment 1

To minimise any differences in seed protein, macro and micronutrient content and environmental influences during maturity, the seed used for both experiments was collected from plants grown at a common site in the year prior to establishment. The seed of both resistant and susceptible biotypes was multiplied during the season prior to the experiment at a site free of *L. rigidum*. The resistant biotype was treated with 540 g a.i. ha^{-1} of the commercial formulation of diclofop methyl at the 2-4 leaf stage in the field to maintain the herbicide selection pressure. Plantings were separated with a 50m border of triticale (*Triticale* x *triticosecale*) to prevent pollen exchange during anthesis.

Experiment 2

Seeds of each biotype were germinated on 0.06% agar and seedlings transplanted into 15.0 cm pots containing recycled potting soil. The plants were treated with the appropriate herbicides at the 2-4 leaf stage. SLR31 seedlings were survivors from 540 g a.i. ha^{-1} of the commercial diclofop methyl and SLR3 seedlings from 93 g a.i. ha^{-1} sethoxydim as the commercial formulation and WLR1 seedlings were survivors of 15 g a.i. ha^{-1} of chlorsulfuron. Following herbicide application fifty plants of each biotype were grown in groups of five in the field and screened from pollen contamination at anthesis with 2m high plastic barriers. The seed was harvested by hand, cleaned and bulked.

Experiment 3

Seedlings of biotypes SR31, VLR1 and the F_1 crosses were planted as described below. The seedlings were established at a 1:1 ratio, with the F_1 compared to both the resistant and susceptible parent. The planting density was 900 m^{-2}.

5.2.7 Field conditions and planting details

The experiments were conducted in separate years on prepared and solarised field sites at the Waite Campus, Adelaide, South Australia (latitude 34° 58' S., longitude 138°.0 65' E.). The soil is an Urrbrae red-brown earth, a calcic rhodoxeralf soil, with a pH of 7.4 in the 0-100mm surface layer. The sites were evenly fertilised at a rate of 200 kg ha^{-1} of agricultural (N-P-K, 20-10-0) fertiliser incorporated by hand raking prior to planting. In both experiments the plants were established within wire grids with individuals of one biotype identified at planting with a 2.5cm diameter plastic ring. Four borders or boundary rows of the same adjacent biotype separated or surrounded the sub-plots.

Seedlings of similar size and age for all biotypes were used. Seeds were germinated in plastic trays on 0.06% w/v water agar in a growth room, with a 16hr day at 20°C and 15°C night. At the one leaf stage the seedlings were acclimatised outside for two days prior to planting and individual seedlings were hand planted. The few seedlings that died within 7 days of planting were replaced by similar age plants of the same biotype. The experiments relied on natural rainfall. Until canopy closure, limited hand weeding was performed to eliminate other species. Weeding was done with minimal disturbance to the soil surface.

The field experiments were conducted during the winter and spring, the normal growing season for *L. rigidum*. The growing season rainfall was 450mm and 567mm during Experiments 1 and 2 respectively. The mean annual rainfall for the same growing season period at the Waite Campus measured over 65 years was 452mm.

All experiments were harvested by hand when plants were mature. Individual plants were identified, the above ground parts were cut and placed in a paper bag with an identifying label and air dried for 48 hrs. The packaged material was stored under cover at room temperature and above ground parts of individual plants were weighed, counted and seed output per plant evaluated.

5.2.8 Statistical analysis

Experiment 1 was a randomised complete block design with four replicates of each proportion. Experiment 2 was a split plot design with biotypes randomised within densities and replicated three times. For both experiments the dry weight, seed number and tiller number from each variety and density was measured. For Experiment 1, 50 plants from each treatment were analysed. The number of seeds per gram dry weight was counted from a random sample of plants and a harvest index calculated. For experiment 2, 10 plants from each biotype per treatment were measured for dry weight, seed number and tiller number. An analysis of variance was performed on transformed data ($\log_n(X + 1)$) as the dry weight and seed number data was not normally distributed.

The data from the no competition plots of Experiment 1 was normally distributed and t-tests were performed on the dry weight and seed number data. Experiment 3 was harvested similarly to Experiment 1 and the dry weight and seed number per plant compared with anova.

5.2.9 Experiment 4

An investigation was done to establish if the resistant component of a susceptible population such as were reported in Chapter 2 displayed any difference in fitness from the whole population. A susceptible population (population number 2, Chapter 2) was chosen because of the high frequency of survivors.

Materials and methods

Seeds of the susceptible population number 2 were germinated in plastic trays on 0.06% w/v water agar in a growth room, with a 16hr day at 20°C and 15°C night. At the one leaf stage the seedlings were fixed in plastic foam plugs on a floating perforated tray. The seedlings were grown on 33% Hoaglands nutrient solution and the growth rate of individual plants measured from 21 days to 28 days after transfer to the nutrient solution. After 28 days the plants were treated with 187 g a.i. diclofop methyl ha^{-1}, half the recommended field application rate as a foliar application. The survivors were counted 21 days after treatment and the survivors recorded in the growth rate class achieved before treatment.

5.3 Results

5.3.1 Experiment 1

There was no significant difference, t test, ($p < 0.05$) between the dry weight of each biotype in the non competitive plots, t = 0.255, (data not shown). There was no difference in plant biomass expressed as plant dry weight or seed production at maturity between the herbicide resistant (SLR31) and susceptible (VLR2) biotypes used in this experiment. The results of the replacement series experiment for the density treatments of 400 and 900 plants m^{-2} are shown graphically in Figure 5.1. The tables of means is presented in Tables 5.4 and 5.6 and anova summary in Tables 5.3 and 5.5.

Table 5.3. Anova table dry weight per plant, density 400 m^{-2} (experiment 1).

Source	d. f.	MS.	F pr.
Replicate	3	0.622	
Biotype	1	1.335	0.399 ns
Proportion	5	1.277	0.22 ns
Biotype*prop.	5	1.185	0.255 ns
Error	30	0.851	

The F values indicate no significant difference between the biotypes and no biotype*proportion interaction at P< 0.05.

Table 5.4. Table of transformed means of plant dry weight, untransformed data (g) in parenthesis from experiment 1, planting density 400 m^{-2}.

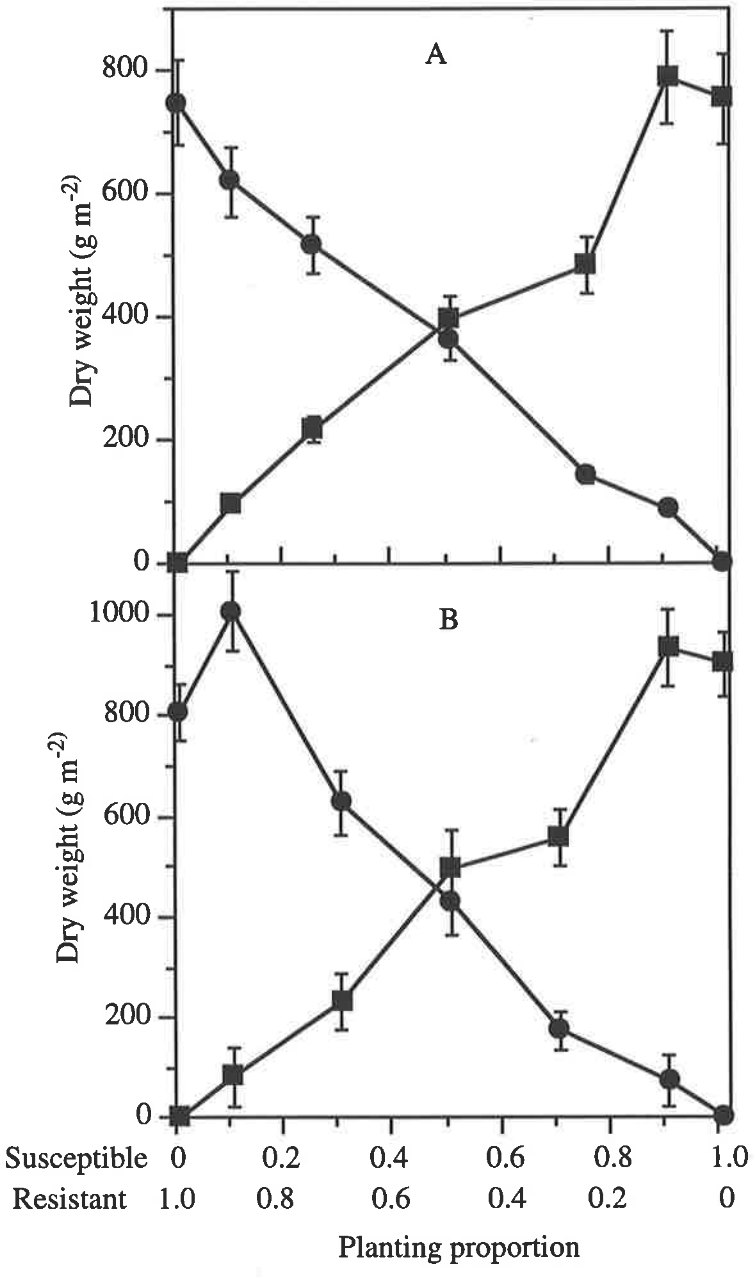
	Mean plant dry wt. (g)					
	Planting proportions					
Biotype	1.0	0.9	0.75	0.5	0.25	0.1
Resistant	0.962	0.929	0.939	0.940	0.791	1.034
(SLR31)	(1.868)	(1.722)	(1.863)	(1.817)	(1.406)	(2.194)
Suscept.	0.969	0.904	0.897	0.986	1.048	1.092
(VLR2)	(1.867)	(1.634)	(1.611)	(1.839)	(2.155)	(2.353)

Table 5.5. Anova table for plant dry weight, density 900 m^{-2} (experiment 1).

Source	DF	MS.	F pr.
Replicate	3	3.565	
Biotype	1	0.652	0.086 ns
Proportion	5	1.970	<0.001
Biotype*prop.	5	0.339	0.350 ns
Error	30	0.291	

The F values indicate no significant difference between the biotypes and no biotype*proportion interaction at $P< 0.05$. There were no biotype*proportion interaction on the relative biomass of each biotype.

Figure 5.1. Dry weights of plants from planting densities of 400 and 900 m^{-2} (Experiment 1).



Legend: Dry weight of susceptible VLR2, ■; and resistant SLR31, ●; at various planting proportions. Graph A, 400 plants m^{-2}, graph B, 900 plants m^{-2}. Data is the mean of 4 replicates, error bars show S.E. of the mean.

Table 5.6. Table of transformed means of plant dry weight, untransformed data (g) in parenthesis, from experiment 1, planting density 900 m^{-2}.

	Mean plant dry wt.(g)					
	Planting proportion					
Biotype	1.0	0.9	0.7	0.5	0.3	0.1
Resistant	0.573	0.7390	0.619	0.572	0.460	0.512
(SLR31)	(0.912)	(1.26)	(1.007)	(0.911)	(0.652)	(0.807)
Susceptible	0.629	0.692	0.587	0.625	0.550	0.589
(VLR2)	(1.026)	(1.159)	(0.896)	(1.054)	(0.900)	(0.946)

A harvest index was established for each biotype at each proportion. The seed number to whole plant dry weight ratio was established using a random selection from each biotype and density treatment. The harvest index and correlation are presented in Table 5.7.

Table 5.7. Correlation and coefficient of determination of harvest index for all densities, experiment 1.

	Correlation r		Harvest index seeds $^{-g}$ (dry wt)	
Density (m^{-2})	SLR31	VLS2	SLR31	VLS2
No competition	0.431	0.797	95.68	106.55
400	0.874	0.850	98.78	95.72
900	0.951	0.896	103.45	99.230

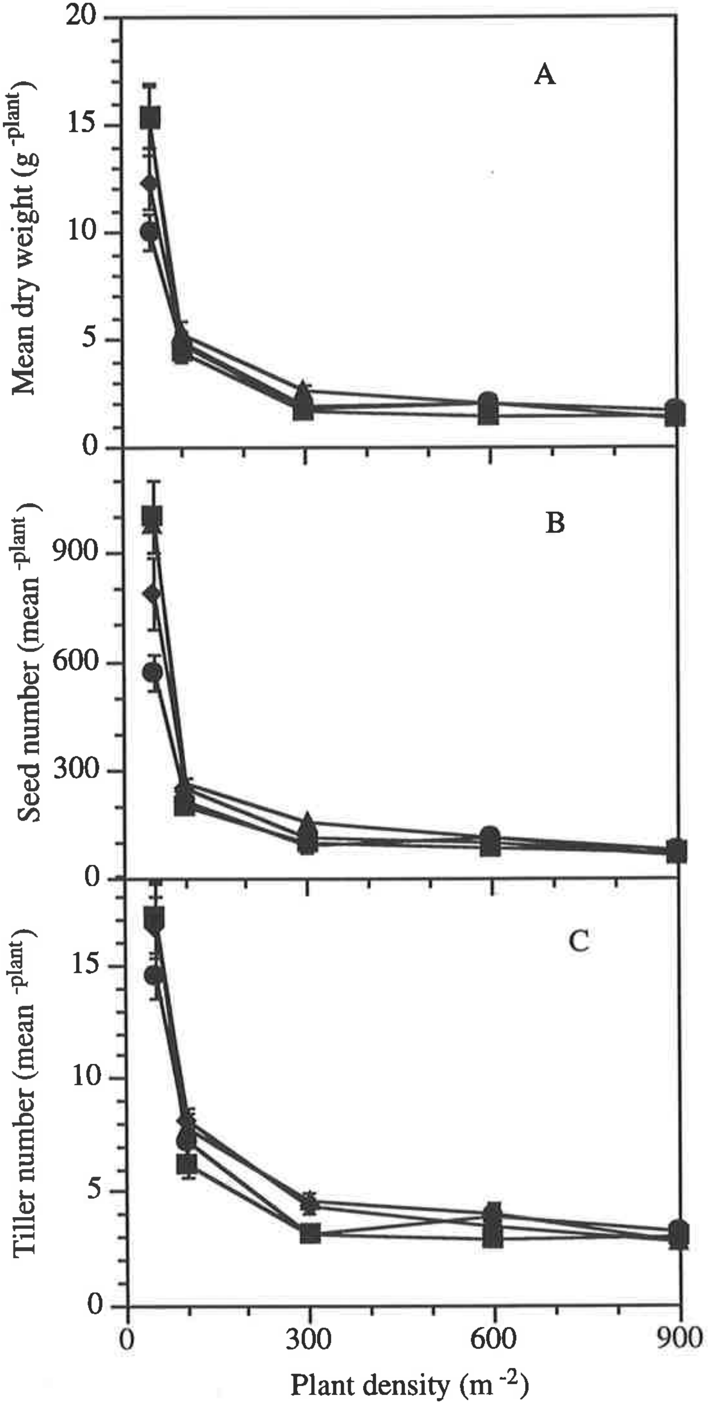
From the results presented in Figure 5.1 and Tables 5.3 to 5.7, it is evident that the capacity for accumulation of biomass and the reproductive capacity of the resistant population SLR31 was not different from the susceptible biotype VLR2. As SLR31 has multiple resistance, it is concluded that the presence of mechanisms that confer multiple resistance in this biotype are not associated with inferior fitness compared to the susceptible biotype used in this experiment.

The experiment was conducted at three densities of planting, the densities were within the range observed for mature plants in field infestations of *L. rigidum*. There was no consistent effect of density on the relative seed output of the two biotypes as seen by the harvest index in Table 5.7.

5.3.2 Experiment 2.

Dry weight, seed number and tiller number per plant were determined for each density when the biotypes were grown alone, the results are shown in Figure 5.2. There was no significant differences between biotypes for dry weight per plant when grown in alone or in paired plantings at 1:1 ratio. The results of the paired plantings are shown in Figure 5.2.

Figure 5.2. Comparison of various indicators of plant growth between the biotypes VLR1, WLR1, SLR3 and SLR31 over a range of densities (Experiment 2).



Legend: VLR1 ■; WLR1▲; SLR3 ◆; and SLR31●; grown alone. Graph A; mean dry weight, graph B; mean seed number and graph C; tiller number. Bars represent SE of the mean, data is the mean of three replicates.

The anova summary is shown in Table 5.8 and the table of means of the dry weights of the biotypes for the range of densities is presented in Table 5.9.

Table 5.8. Anova table for plant dry weight data, (Experiment 2).

Source	DF	MS.	F pr.*
Replicate	2	0.6928	
Replicate*whole plot			
Density	4	190.1084	<0.001**
Rep*w. plot*split plot			
Biotype	11	0.6101	0.062^{ns}
Density*biotype	44	0.4967	0.056^{ns}
Error	110	0.3384	

*The F values indicate no significant difference between the biotypes and no biotype*density interaction ($P< 0.05$). The coefficient of variation was 24.5%.

Table 5.9. Transformed means of the dry weights plant^{-1} across all densities, (Experiment 2).

Biotype *alone*	Dry weight	Biotype *paired planting*	Dry weight
VLR1	1.350	(SLR3-	1.333
SLR31	1.361	SLR31)	1.292
WLR1	1.468	(SLR3-	1.384
SLR3	1.392	VLR1)	1.422
		(SLR31-	1.367
		WLR1)	1.480
		(WLR1-	1.440
		VLR1)	1.279

As indicated in the analysis of variance table for mean seed number (Table 5.10) there were differences in seed production between biotypes. The differences in seed output of the biotypes tested were not consistent over all densities. The means of the biotype*density interactions are shown in Table 5.11. When planted alone at a density of 50 m^{-2} the susceptible biotype VLR1 had a greater mean seed number per plant by 8.4% than the resistant biotypes SLR31. In all other comparisons there was either no difference in relative seed output, or the resistant biotypes produced more seed than the susceptible. The differences in seed number shown in Tables 5.9 and 5.11, are due to minor differences in the harvest index for biotypes at some densities. It is likely that the inconsistent results were anomalies caused by losses at harvest.

Table 5.10. Anova table for seed number per plant, (Experiment 2).

Source	DF	MS	F pr.
Replicate	2	1.1521	
Replicate*whole plot			
Density	4	360.4946	<0.001**
Rep*w. plot*split plot			
Biotype	11	1.7112	0.024*
Density*biotype	44	1.6746	0.001**
Error	110	0.8037	

The F values indicate a significant difference between the biotypes* at ($P< 0.05$) and biotype*density interaction ($P<0.01$). CV = 18.18%.

Table 5.11. Means of biotype * density for seed number per plant, (Experiment 2).

Biotype	Density (plants m^{-2})				
alone	50	100	300	600	900
VLR1	6.77a	5.202b	4.362bcd	4.101b	3.938cd
SLR31	6.201d	5.286ab	4.058d	4.477ab	4.249abc
WLR1	6.702abc	5.436ab	4.895a	4.62a	3.953cd
SLR3	6.513abcd	5.476ab	4.651abc	4.408ab	3.956cd
paired planting					
(SLR3	6.224bcd	5.59ab	4.788abc	3.85c	3.998cd
SLR31)	6.171d	5.545ab	4.471abcd	3.9c	4.097abcd
(SLR3	6.299bcd	5.667a	4.29cd	4.122ab	4.478ab
VLR1)	6.393abcd	5.183b	4.389ab	4.479ab	4.072abc
(SLR31	6.241cd	5.414ab	4.464abcd	4.234ab	4.048abc
WLR1)	6.389abcd	5.551ab	4.256cd	4.18ab	4.543a
(WLR1	6.734ab	5.306ab	4.39bcd	4.268ab	4.244abcd
VLR1)	6.584abcd	5.276ab	4.418bcd	3.533c	3.783abcd

The LSD for biotypes*density interaction is 0.4584 ($P<0.05$). Transformed means followed by the same letter in the same density treatment are not different.

Table 5.12. Anova table for tiller number per plant, (Experiment 2).

Source	DF	MS.	F pr.
Replicate	2	0.6886	
Replicate*whole plot			
Density	4	142.9711	<0.001**
Rep*w. plot*split plot			
Biotype	11	0.5114	0.083
Density*biotype	44	0.4723	0.031*
Error	110	0.3009	

*The F value indicates a significant biotype*density interaction ($P<0.05$).

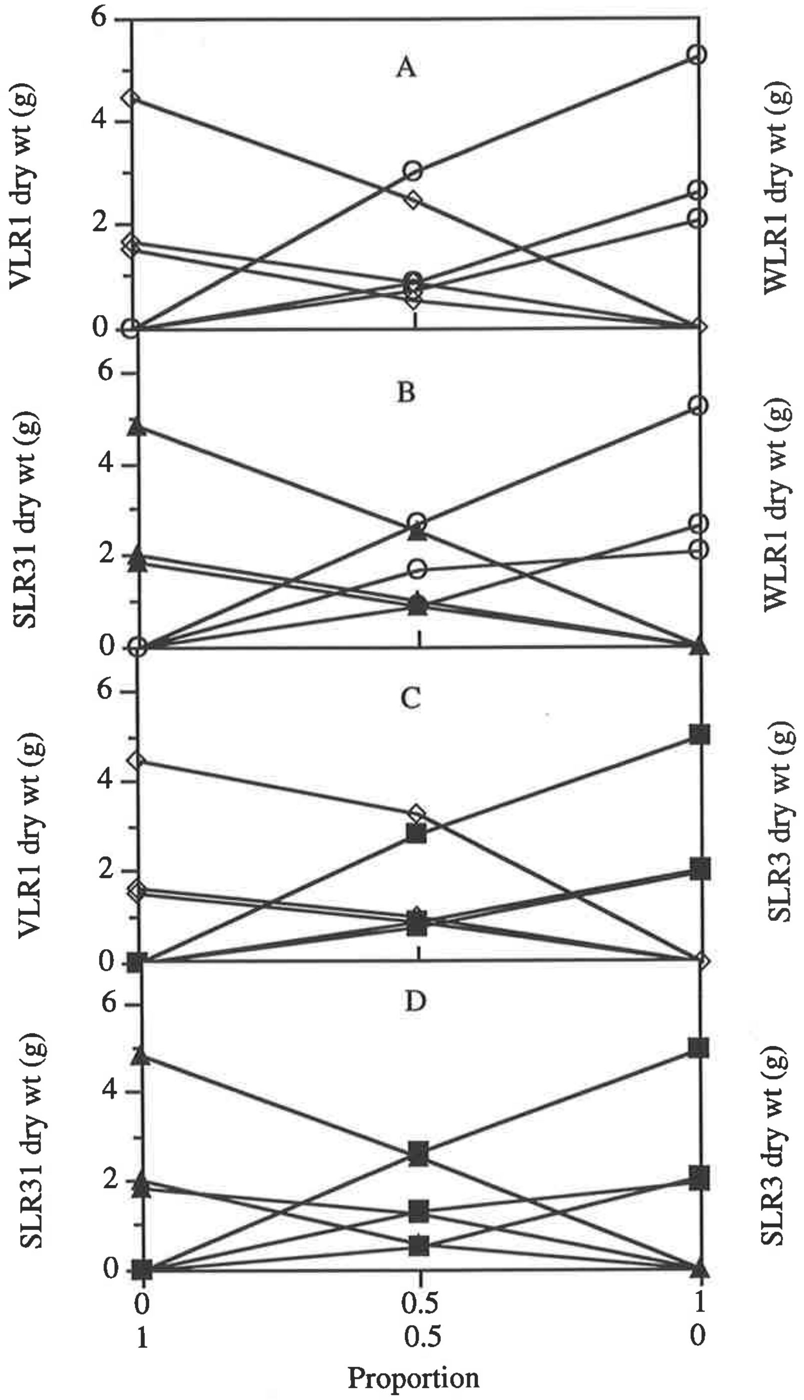
Table 5.13. Means of biotype*density for transformed tiller number per plant, (Experiment 2).

Biotype	Tiller number per plant (transformed)				
	Density (plants m^{-2})				
alone	50	100	300	600	900
VLR1	2.993a	1.904b	1.36b	1.242ab	1.25ab
SLR31	2.985a	2.03ab	1.329b	1.516a	1.372ab
WLR1	2.794ab	2.092ab	1.668a	1.524a	1.228ab
SLR3	2.787ab	2.146ab	1.626a	1.38a	1.246ab
paired planting					
(SLR3	2.785ab	1.99ab	1.721a	1.094b	1.195ab
SLR31)	2.758ab	2.084ab	1.577ab	1.119b	1.241ab
(SLR3	2.724ab	2.152ab	1.409b	1.419a	1.384ab
VLR1)	2.682b	2.268a	1.442ab	1.537a	1.234ab
(SLR31	2.68b	2.102ab	1.439b	1.304ab	1.233ab
WLR1)	2.679b	1.974b	1.366b	1.428a	1.42a
(WLR1	2.631b	2.138ab	1.423b	1.223b	1.348ab
VLR1)	2.532b	2.082ab	1.457ab	1.289ab	1.129b

The LSD for biotypes*density interaction is 0.2804 ($P<0.05$). Transformed means followed by the same letter in the same density treatment are not different.

Representative results of Experiment 2 are shown in Figure 5.3. The dry weights of the biotypes planted in monoculture are plotted against each other with the dry weights of each biotype in the equal mixture scaled by 0.5 for the 1:1 planting. The lack of consistent deviation of the yields of the equal mixtures from a line of slope +1 or -1 shows the equal competitiveness of the tested biotypes when grown in 1:1 mixtures as indicated in Table 5.9. The 50 m^{-2} and 900 m^{-2} data for the dry weights of each biotype is not presented in Figure 5.3 due to difficulties with scale, (they were not different).

Figure 5.3. Dry weights of all biotypes and planting densities (Experiment 2).



Legend: All biotypes and diallel combinations for planting densities 100, 300, 600 per m^{-2} in descending order down each graph. A; WLR1, O, VLR1, ◇: B; SLR31, ▲, WLR1, O: C; SLR3, ■, VLR1, ◇: D; SLR3, ■, SLR31, ▲. Dry weights per plant from the 1:1 mixture are scaled by 0.5 for drawing.

Data for tiller number per plant was presented in Tables 5.13 and anova Table 5.12, and shows the resistant biotypes when grown alone at any densities tested did not show reduced tiller number compared to the susceptible.

5.3.3 Experiment 3

The dose response to diclofop-methyl of the maternal and paternal F_1 crosses are shown in Figure 5.4. The reciprocal F_1 crosses of the resistant SLR31 and the susceptible VLR1 were bulked and grown together at a density of 900 plants per square metre. This was the highest planting density used and was chosen to simulate conditions of high competitive pressure. The F_1 was compared to each of the parent biotypes to assess if it was inferior to the parents in growth or seed output per plant. The F_1 sample did not show any reduced capacity to accumulate biomass or produce seeds per plant when compared to either of its parents, (Tables 5.14 and 5.15). In fact, the F_1 was more vigorous for both those parameters than its parents (Table 5.14).

Table 5.14. Table of means of plant dry weight and seed number from experiment 3, planting density 900 m^{-2}.

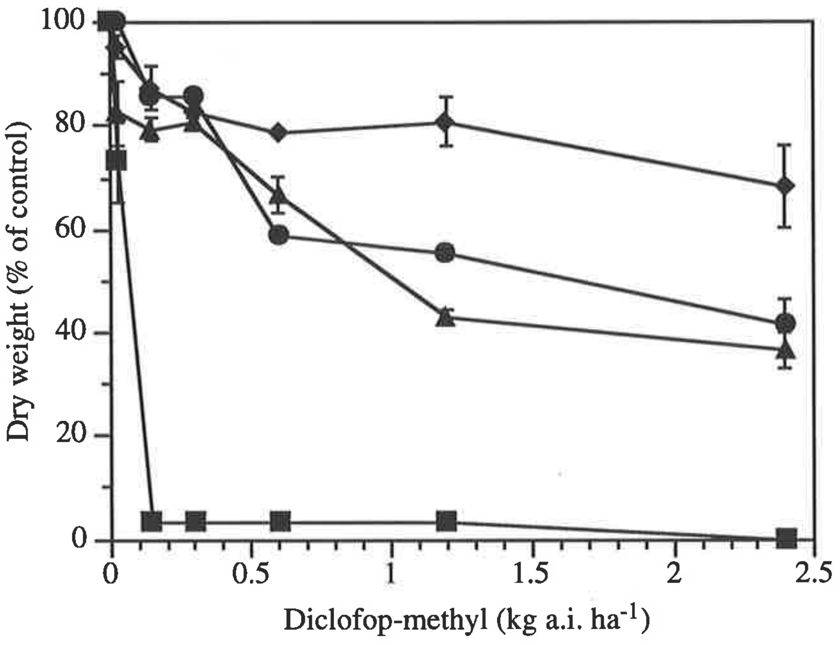
	Parameter assessed	
Biotype	Mean dry wt (g)	Mean seed number per plant
SLR31	1.02	103
VLR1	1.06	100
F_1	1.47	156

Table 5.15. Combined anova table showing the mean squares (MS) for dry weight and seed number per plant of resistant and susceptible parent plants and the F_1 generation, density 900 m^{-2} (Experiment 3).

Source	M S Dry wt. (Res. and F1)	MS Dry wt. (Sus. and F1)	MS Seed number (Res. and F1)	MS Seed number (Sus. and F1)
Replicate	3.25	0.07	60360	15145
Biotype	6.39^x	2.57^x	54781^x	76620^x
Error	0.44	0.47	6059	6624

^{x} indicates a significant difference $P< 0.05$, d.f. = 84, mean c.v. = 28.9%.

Figure 5.4 Response of susceptible, resistant and reciprocal crosses to diclofop methyl.

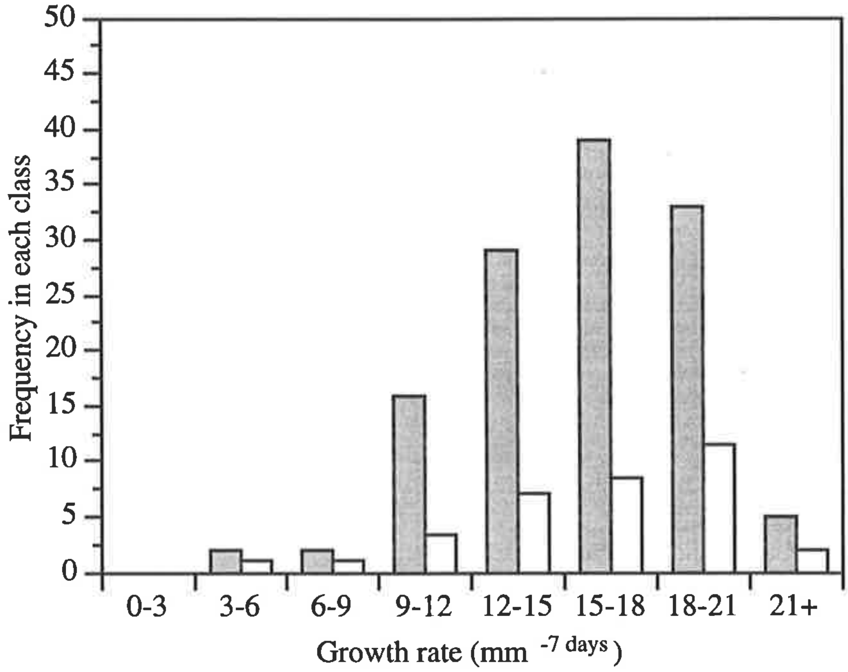


Legend: Susceptible VLR1, ■; resistant SR31, ◆; $F1_i$, ●; $F1_{TM}$, ▲. Data are the mean of three replicates, bars represent S.E. The field rate of diclofop-methyl is 0.6 kg ha^{-1}. VLR1 was sig. dif. from other biotypes, which were not dif., d.f.= 154.

5.3.4 Experiment 4

A susceptible population that had never been treated with herbicide was grown on 33% Hoaglands nutrient solution and the length of the leaves measured and recorded for each plant. After recording the growth over a period of seven days, the population was treated with 187 g a.i. ha^{-1} diclofop-methyl, which is half of the recommended field application rate. Survivors were identified and the growth rate of the survivors compared with the growth rate of the whole population and the results presented in Figure 5.14. The proportion surviving (19.9%) was influenced by the herbicide application rate.

Figure 5.4. Frequency distribution of survivors and dead *L. rigidum* plants from a susceptible field population when treated at 187 g a.i. of diclofop methyl ha^{-1}.



Legend: Susceptible population ▨; resistant survivors☐;following treatment with 187 g a.i. per ha diclofop-methyl. Data is from one experiment, n = 156.

There was no significant difference between the pre-treatment growth rate of the group that died or survived the herbicide application. The results are shown in Figure 5.4. The proportion of survivors were significantly correlated ($r=0.95$, $p< 0.01$, d.f.= 6) with the total of each growth rate class. This results suggests that survivors of an application of the herbicide diclofop-methyl to an untreated population were not drawn from a group that was either more or less fit than the population as a whole. The growth rate of the surviving group, the resistant plants, was not different from that of the whole population.

5.4 Discussion

The competition experiments reported in this chapter were carried out to investigate the hypothesis that resistant plants may be less fit than susceptible representatives of the same species. The pleiotropic cost to fitness of resistance to a herbicide has been shown in the cases of resistance to the triazine herbicides where resistance is due to the mutation in PS II. Thus, an important aspect of fitness studies especially in organisms where the selection pressure may be very intense is the adaptation of the gene conferring resistance. Did the mechanism or altered target site confer an observable fitness penalty to the organism? The mechanisms conferring resistance represented in the resistant biotypes used to compare fitness and biomass are not unique among the many resistant biotypes that have developed across southern Australia. Similar resistant biotypes occur across the southern Australian cropping zone and these resistant populations are drawn from areas with similar crop production systems and similar herbicide application histories.

5.4.1 Relative fitness of biotype SLR31

Relative fitness of a biotype with many resistance mechanisms

The biotype SLR31 has a diversity of resistance mechanisms (Table 5.2). The biotype SLR31 was compared to a susceptible biotype VLR2. Data from Experiment 1 show there is no difference in the dry weight and seed production of SLR31 and VLR2 when grown together at any proportion and at three densities. SLR31 was compared to two other resistant biotypes in Experiment 2 and there was no consistent differences between them either for dry weight, seed number or tiller number at any density. When grown with the other biotypes at a 1:1 proportion, each biotype contributed close to half of the total biomass, seed number and had about equal tiller numbers. SLR31 did not suffer from competition from either the susceptible or the resistant biotypes to which it was compared.

5.4.2 Relative fitness of biotype SLR3

Relative fitness of a biotype with a resistant ACCase enzyme

With the occurrence of herbicide resistance due to specific selection by one herbicide, biotypes with quite discrete mechanisms have been selected over time. Experiment 2 was conducted to evaluate the relative fitness of the biotypes SLR3 and WLR1 compared to SLR31 and the susceptible VLR1.

The resistant population SLR3 was selected with 3 years use of sethoxydim, an ACCase inhibitor that is not selective in cereal species (Tardif *et al.,* 1993). As a result of this restricted but intensive selection pressure this biotype has developed resistance to sethoxydim due to the increased frequency within the population of an insensitive ACCase target site (Table 5.2). SLR3 was selected by sethoxydim use in a continuous legume seed (*Trifolium subterranean* L.) production growing system where any relative fitness disadvantage as a result of the enrichment of the gene encoding for a modified

ACCase would be less likely to be subjected to selection for improved fitness as this species is considered to be uncompetitive against grass species (Donald, 1951). Despite this, there was no consistent fitness difference observed between the resistant biotype SLR3 and the susceptible biotype VLR1, only at a density of 300 plants m^{-2} was output reduced. SLR3 also showed no reduction in fitness when compared with other resistant biotypes.

5.4.3 Relative fitness of biotype WLR1

Relative fitness of a biotype with a resistant ALS enzyme

WLR1 was selected with 7 annual applications of chlorsulfuron, a cereal selective ALS inhibiting herbicide (Christopher *et al.,* 1992). As a result of this specific selection regime WLR1 has developed resistance to ALS inhibitors due to an insensitive ALS herbicide target site but it is not resistant to herbicides with other modes of action (Table 5.2). There was no consistent difference between the resistant biotype WLR1 and the susceptible biotype VLR1 to which it was compared, nor was it reduced in seed yield, tiller number or dry weight when compared to the other resistant biotypes in 1:1 mixtures.

This study demonstrates that *L. rigidum* biotypes with either an ACCase which is insensitive to ACCase inhibiting herbicides or an insensitive ALS, or a multiple resistant biotype with a diversity of mechanisms do not have a fitness penalty when compared to susceptible biotypes. None of the resistant biotypes were consistently reduced in seed yield, tiller number or dry weight when compared to a susceptible biotype or when compared to each other, either alone, or in 1:1 mixture over a range of densities. The presence of any of the resistance mechanisms which confer high levels of resistance to the particular herbicides did not impose a detectable fitness penalty on any of the recipient resistant populations in these series of experiments.

5.4.4 Competitive abilities of F_1 and unselected resistant plants

Competitive conditions are usually present when selection by herbicide application is occurring, so selection for fitness is likely to be proceeding during selection of herbicide resistance. As the resistant biotypes SLR31, SLR3 and WLR1 were collected from agricultural environments they may have any initial fitness differential masked by continuing selection for fitness. To establish if the early phase in the development of resistance causes any fitness differential may, more reliably, indicate the pleiotropic effects of genes conferring resistance. The results of Experiment 3 (Figure 5.4) show that the pre-treatment growth rate of the survivors of an application of diclofop methyl from an unsprayed susceptible population was not different to the growth rate of the whole population. Herbicide resistant individuals were not from a portion of the population displaying either high or low rates of growth, but were similar to the population as a whole. Any pleiotropic cost to early growth rate by the presence of allele(s) conferring herbicide resistance as assessed by plants surviving an initial herbicide application was not detected in this experiment.

As stated previously, *L. rigidum* is an allogamous species, the outcrossing nature of the species ensures that all plants are at the progeny of two different parents (see Section 1.1.2). Therefore the fitness of F_1 plants is of importance to the persistence of a novel genotype. The fitness of an F_1 is important in the case of herbicide resistance both where selection is occurring as shown in Chapter 2, but also under non-selective environments when herbicide applications may be either intermittent or reduced due to herbicide failure. The fitness of the F_1 crosses between biotype SLR31 and VLR2 was not inferior to either of the parents, as shown by the data in Table 5.14. The prospects for reducing the rate of onset of resistance by manipulation of the plant density are therefore low. Given the growth and fecundity of the F_1 crosses it is extremely unlikely that resistance will recede from a resistant population in favour of susceptible plants in the absence of herbicide applications due to reduced fitness of the resistant component. In this experiment heterosis was identified (Table 5.14), this may be due to the different biotypes involved and may be less likely to be identified in F_1 crosses from resistant and susceptible plants from the same population.

5.5 Conclusion

In an annual ruderal species a relative fitness difference can be expected to be important to the persistence of a species. Examination of the relative fitness of similar plants to identify pleiotropic effects as in the experiments reported in this chapter which utilised competition studies between resistant and susceptible populations ideally should involve isogenic lines with the resistance conferring genes being the only difference. This is difficult to obtain with allogamous plants. In this study the major biotypes were from field populations covering the range of resistance mechanisms and were grown at a common site for one generation to reduce differences such as nutritional influences and seasonal conditions. The resistant biotypes are all strongly resistant to the herbicides by which they were selected, and the results indicate they have not suffered any fitness penalty due to the presence of mechanisms conferring resistance to a range of herbicides.

The success of the genetic basis for herbicide resistance in a population depends in part, on the pleiotropic cost to fitness of the resistance genotype, although this may be balanced over time by the adaptation of resistant individuals during selection. As these biotypes are from field environments, competition from both crop species or inter specific competition can be assumed to have been present when selection in the field was occurring. However, the survivors of susceptible population (with no herbicide selection) in which herbicide resistance individuals were present at the frequency identified in Chapter 2, displayed no pleiotropic cost to fitness in the early stages of growth.

A gene that replaces another in a population due to an imposed management practice, if it has not gone to fixation, may decline in frequency in the absence of selection if there is a substantial fitness penalty. In the biotypes tested here no fitness penalty was observed and there is little likelihood that the resistant individuals will be replaced by susceptible plants following the cessation of selection by herbicide application. The implication for the management of herbicide resistant ryegrass populations is that in the absence of herbicide application the herbicide resistant biotypes reported here can be expected to persist indefinitely in a mixed population containing resistant and susceptible biotypes.

These biotypes, each with discrete biochemical mechanisms conferring resistance, have been shown not to be reduced in fitness when compared to susceptible biotypes. There have been many reports of populations of weeds that have developed resistance to herbicides and many of these populations have reduced biomass and reproductive output when compared to representative susceptibles. This study has shown that three distinct biotypes of herbicide resistant *L. rigidum* do not carry a fitness penalty and it is therefore unlikely that fitness penalties will be associated with resistance to these herbicides in *L. rigidum*.

Chapter six

The potential for removal of *Lolium rigidum* seed during crop harvesting operations and the effect of seed removal on the *L. rigidum* soil seedbank

6.1 Introduction

It is a characteristic of members of the species *Lolium rigidum* to display an erect habit during growth and anthesis, hence the appelation "rigidum" and the common name "rigid ryegrass" in some countries. The spike infloresences of *L. rigidum* tend to stay erect after anthesis until degeneration by weather or damage by physical contact breaks the stout rachis. This characteristic is referred to in botanical keys to differentiate *L. rigidum* and hybrids of *L. rigidum* from other types. For example, "rachis flexuose, slender to somewhat rigid to 1.8mm diameter" from (Kloot, cited in *Flora of South Australia*, 4th edition, 1986). Terrel (1968) from world-wide herbarium sources describes *L. rigidum* as follows, "Culms erect to widespreading or decumbent, or subprostrate". At maturity the seeds are firmly held in the glumes and "glumes tightly appressed against the rachis especially after anthesis" (Terrel, 1968). The characteristic of *L. rigidum* to retain seed in the culm is observed in field populations.

When growing as a weed of a planted crop the *L. rigidum* plants are usually as tall as the surrounding crop due the developmental plasticity of *L. rigidum*. The seed heads are often retained at a suitable height for machine harvesting, particularly when grown with crops which stand erect, such as cereal crops. As a consequence, considerable amounts of *L. rigidum* seed may be harvested with the grain and has been frequently observed as a contaminant of harvested grain or seeds. Under normal commercial practice in Australia much of the seed that is harvested is immediately returned to the field with the screenings from the primary cleaning sieves of the harvester. This seed represents a source of reinfestation for subsequent crops. Control of *L. rigidum* infestations by removal of seed from the field at harvest may represent a possible method of controlling population growth.

There is only a sparse literature related to the effects of the harvesting process on weed seed dispersal. Petzold (1956) reports that the potential for weed seeds to be spread by the the new "Combine Harvester" was recognised at the time of introduction to Germany in 1928-29. Petzold investigated the dispersal of seeds of three species of weeds by combine harvesters and found that a mean value of 14% of weed seeds were not collected in the grain or retained cleanings when trials were carried out. There were substantial differences in the proportion of the seed retained between the weed species. *Galium aparine* was most effectively retained with 86% of the seed in the grain and cleanings, and *Agrostis spica venti* least with 6.5% of the seed being similarly accounted for. The differences were attributed to the weight to surface area ratio differences of the seeds.

Ballare *et al.* (1987) modelled the influence of dispersal of *Datura ferox* within a field of *Glycine max* (soybeans) and Maxwell and Ghersa (1992) discussed the influence of seed dispersal of *Setaria viridis* within a crop of *Triticum aestivum* (spring wheat). Maxwell and Ghersa modelled the effect of dispersal and the resulting crop competition on reducing crop yield. They found that a weed which has seed totally dispersed by the harvesting process may reduce crop yield more than seed that is of limited dispersion irrespective of the competitiveness of the weed.

These reports of the role of machine harvesting in weed seed dispersal within a crop or field suggest a dramatic influence of harvesting on weed dispersal. Thurston, (1964) reported that a substantial proportion of wild oat seed (96%) was harvested, and 75% was restrained from reinfesting the field by moving with the grain in the harvester.

Wilson (1972) found the reverse, with 77% of wild oat seed shed prior to harvest and only 23% of the original population being retained in the grain. As wild oats shed seed at maturity the proportion of seed capable of retention during harvest may vary with the relative maturity of the crop and the wild oat plants. Other authors have discussed theoretical weed dispersal based upon biological factors between farms (Auld and Coote, 1980) or observations on weed contamination of crop seed as a means of dipersal (Horne, 1953).

Harvesting machines are designed to cut the crop and separate the grain from the rest of the crop. The unwanted portion, the crop residues, including weed seeds is spread back on the field. In this process *L. rigidum* infested fields are constantly contaminated by returning weed seeds to the soil surface during the harvesting operation. The proportion of *L. rigidum* seed captured during the harvesting operation is dependent in part, upon the cutting height of the crop and the type of cutting platform used in the harvesting process. In Australia, older style harvesting equipment, often called "Headers," cut only the heads of crops and tend to dislodge ryegrass seed from the glumes or break the rachis. Modern style harvesters with an open cutting platform cut a few centimetres of stalk below the grain bearing spike causing minimal disturbance to the spike of the crop and the spikes of the weeds such as *L. rigidum*.

Normally *L. rigidum* tends to retain seed in the head, especially when crops are harvested soon after the *L. rigidum* has matured. If harvesting is postponed the risk of seed shedding or breaking of the rachis increases. The erect habit of *L. rigidum* is observed to a lesser extent when it occurs as a component of crops or mature pastures which tend to lodge. However, the upstanding growth habit and seed retention of *L. rigidum* until about crop maturity increases the likelihood of harvesting and retaining ryegrass seed within the harvester to reduce the recontamination of the field in tall, early maturing crops.

The retention of *L. rigidum* seed with the crop grain during harvesting is also dependent upon the sieving capacity of the harvesting equipment. Modern harvesters have greater crop cleaning capacity when compared to older style harvesting machines. These machines are able to discharge much of the lighter seed material such as *L. rigidum* seed when sieving seeds of high density such as wheat, barley or other large or high density grains. When lighter grain or seeds of similar density to *L. rigidum* are harvested the machine settings usually allow *L. rigidum* seed to move with the crop seed.

When viable *L. rigidum* seeds are harvested, threshed and then returned to the soil the weed infestation can continue unabated. The potential to capture weed seed at harvest time and reduce reinfestation has the prospect to decrease the size of the *L. rigidum* seed bank in the soil. *L. rigidum* is an annual species, so if the proportion of seeds that are caught in the harvesting process is sufficient then the emergence of *L. rigidum* from the soil in subsequent years will be progressively reduced. To investigate the potential of retaining *L. rigidum* seed during the harvesting process and the rate of decline of *L. rigidum* seedbank a series of harvesting trials and a field experiment were conducted.

6.2 Materials and methods

6.2.1 Preliminary field trials

Two preliminary trials (trials 1 and 2) were conducted in agricultural fields to assess the proportion of *L. rigidum* seed cut by the harvester which then had the potential to be retained on the harvesting equipment. These experiments were conducted using a small plot harvester adapted to contain all the material usually discharged from the sieves of the harvester. The harvester was an open front reel type harvester with a cutting width of 1.4 m. *L. rigidum* seeds were separated by hand from the retained cleanings and expressed as a proportion of the total above ground seed population at the time of harvest.

6.2.2 Trials with commercial equipment

Following the trials with a small plot harvester, a seed collection device suitable for use with commercial harvesters was developed and became available for testing on several different crops at different locations. The equipment comprised an adjustable sieve extension to the top sieve of a commercial harvester with an elevating auger to transfer weed seeds into a holding bin mounted on the harvester that could be emptied away from the harvested field.

An evaluation trial was undertaken to identify the amount of ryegrass seed that was captured during commercial harvesting. Seven assessments were done within barley, wheat, oat and pea crops. The harvesters were from four different owners but each were fitted with a similar seed catching device, a "Ryetec" catcher is commercially available from "Harvestaire Corporation" 18 Mumford Place, Perth, Western Australia 6021. The trial consisted of sampling the preharvest population of *L. rigidum* seeds, evaluation of the number of seeds caught in the seed catching device, the number of seeds passed through the machine and returned to the soil, the number collected with the grain and the number of seeds not harvested. Data was collected from seven sites with 3 replicate blocks at each sites, seed number was evaluated on a square metre basis and the percentage effectiveness was determined. Arcsine transformed data, where appropriate, was evaluated by anova.

6.2.3 Seed bank reduction experiment

Following the successful trials with the plot harvester, an experiment involving wheat, barley and pea crop species was established to identify the relative effect of catching *L. rigidum* seed between the crop species and to quantify the decline in the *L. rigidum* seedbank number following seed catching during harvest. A site with ryegrass seed present in the soil was planted with wheat, barley and pea crop species to evaluate seed catching over the course of a typical crop rotation. The field plots were established at Roseworthy Campus (34.26°S: 138.42° E), approximately 50 kms north-west of Adelaide. The plots were harvested with a small plot harvester that was fitted with 300mm length of 12.5 mm raised lip sieve extension attached and a collection tray underneath the sieve extension. Ryegrass seeds were sieved from the airstream and collected in the tray before being returned to the field. Four replicate plots measuring 10m by 2m of each crop species had ryegrass seed caught during harvest and the reduction in the ryegrass seed bank was assessed and compared to plots without seed catching. The ryegrass seed bank was evaluated each autumn by taking 4, 10cm deep 11.5 cm diameter soil cores from each plot and separating the ryegrass from the soil by sieving the soil samples in a water bath. The number of ryegrass seeds per soil core was established by counting. The data was transformed by square root (x+1) and analysed by anova.

6.3 Results

6.3.1 Preliminary trials 1 & 2

The proportion of ryegrass seed cut and passed through an "open front" style harvester was evaluated in two preliminary trials. Each trial was conducted by harvesting a wheat crop infested with *L. rigidum* and retaining all the crop residues which included ryegrass seed. The amount of *L. rigidum* seed was separated by hand and counted and the results presented in Table 6.1.

Table 6.1. Amount and proportion of seed harvested from *L. rigidum* infestations in wheat crops. Data are the mean of 3 replicates ± S.E. of the mean in parenthesis for each trial.

	Pre-harvest density (m^{-2})	Seed harvested (m^{-2})	Seed remaining m^{-2})	% of seed harvested
Trial 1	2509 (311)	1807 (62.4)	666 (54.3)	72.3 (2.56)
Trial 2	2262 (270)	1266 (220)	1442 (190)	55.4 (4.49)

The results in Table 6.1 indicate that a substantial proportion of the *L. rigidum* seed in the field was cut, harvested and retained during the grain harvesting process. For Trial 1, it was estimated that of the seed on the soil surface not passed through the harvester about 75-80% was present in short segments of rachis which had broken prior to harvest. In Trial 2, about 25% was present in short segments of rachis which had broken prior to harvest. About 98.6% (4.2) of the *L. rigidum* seeds on the site were accounted for in Trial 1 and slightly more than 100% in Trial 2. The crops were harvested at a height of about 140 mm above the ground. Both trials were conducted in wheat (*Triticum aestivum* L.) crops in which the grain yielded about 3 - 3.25 tonnes /ha.

6.3.2 Commercial Evaluation

The data from the evaluation of commercial harvesting operations involving ryegrass seed catching is presented in Table 6.2. There was substantial variability in the proportion of seeds harvested or the proportion of seeds retained depending on the crop type and the period of time between *L. rigidum* maturity and crop harvesting. For example, early harvesting in the barley crop (Barley 1) which was windrowed at maturity and therefore at the earliest opportunity for ryegrass removal, reduced the ryegrass seeds not harvested to zero.

Table 6.2. Means of sampling evaluations performed on seed catching trials with commercial harvesters. Data is the mean of 3 replicates with S.D. in parenthesis.

	L. rigidum seed number (m^{-2})			
Crop spp.	Pre-harvest	Seeds harvested and caught	Seeds harvested and not caught	Seeds not harvested
Barley (1)	1687 (288)	1368 (426)	319 (89)	0
Barley (2)	1236 (322)	989 (217)	357 (106)	178 (62)
Durum wheat	2218 (269)	693 (343)	836 (406)	1487 (249)
Bread wheat	1862 (397)	775 (438)	362 (152)	138 (113)
Oats	7996 (1100)	3250 (279)	2009 (112)	448 (104)
Peas (1)	3268 (681)	490(188)	931 (450)	1643 (409)
Peas (2)	2281 (1033)	123 (39)	772 (429)	116 (119)

The calculated components of the seed catching trial were subject to separate anova with percentages transformed where appropriate; site was included as a source of error to accommodate the different sites that were a feature of the trial. There were significant differences between crops for each of the calculated proportions, (Table 6.3).

Barley crops (*Hordeum vulgare* L.)were harvested first in these trials as is the usual commercial practice. The greater effectiveness of harvesting, catching and retaining seed in barley crops was due, in part, to the *L. rigidum* seedheads still standing and not escaping the harvesting process (Table 6.3). Barley is also threshed vigorously during the harvesting process which increased the proportion of seeds retained at harvest, (Table 6.2). Barley 1, was a feed style barley and is usually threshed more vigorously than Barley 2, a malting style.

Table 6.3. The proportion of ryegrass seed harvested and caught compared to the amount present before harvest and also compared to the amount harvested from Table 6.2.

	Factor calculated		
Crop identity	% of total seeds harvested^{X}	% caught of preharvest density	% caught of amount harvested
Barley 1	100 a*	81 a	81 a
Barley 2	100 a	80 a	73 a
Durum wheat	69 b	31 b	45 c
Bread wheat	61 b	42 b	68 b
Oats	66 b	41 b	62 bc
Peas 1	44 c	15 c	34 d
Peas 2	39 c	5 c	15 e

^{X} percentages were adjusted to 100% where sampling error indicated more had been harvested.

* Within column differences indicated by letters are sig. dif. at $p<0.015$.

The durum wheat (*Triticum durum* Desf.) is not threshed as vigorously as bread wheat (*Triticum aestivum* L.) and consequently fewer *L. rigidum* seeds were retained within the seedcatching device. More ryegrass seeds were left unharvested within the durum crop than the other cereal crops, due to the relative time of ripening of the durum wheat and *L. rigidum*. Oat (*Avena sativa* L.) grain is not usually threshed vigorously at harvest and Table 6.2 shows a high proportion of *L. rigidum* seed was harvested in the oat crop but only 62% retained in the catching device. The lowest percentage of seed harvested and caught was from the pea crops. The grain of pea (*Pisum sativum* L.) crops are threshed very lightly to minimize splitting and therefore the *L. rigidum* seed was not threshed from the rachis and was not separated from the crop residues by the seed catching device. Also, the percentage of *L. rigidum* seed harvested was lowest for the pea crops due to lodging or shedding of the *L. rigidum* plants prior to harvest.

6.3.3 Seed bank reduction experiment

To test the efficacy of seed catching and to monitor any reduction in the *L. rigidum* seedbank attributable to seedcatching a series of field plots were established. Wheat, barley and pea crops were sown in a site infested with *L. rigidum*. The *L. rigidum* seedbank was 4214 m^{-2} (SE 4.41) measured during the autumn prior to crop establishment. *L.rigidum* numbers were reduced prior to crop establishment by application of the non-selective herbicide glyphosate. However, the crops were not treated with herbicide during the growing season resulting in a mean ryegrass plant population measured at crop anthesis of 97 m^{-2} (SE 4.1). There was no significant difference in the number of *L. rigidum* plants present between the crop species. All the crops were harvested on the same day as soon as practical after they had matured. The efficacy of seedcatching in these experiments was assessed by measuring the proportion of *L. rigidum* seeds caught during the harvesting operation. The effect of seed catching was assessed by measuring the relative change in seed bank numbers after harvest. The number of seeds caught during the harvesting operation is presented in Table 6.4. A comparison and analysis of the numbers of *L. rigidum* seeds in the soil following seed catching is presented in Tables 6.5 and 6.6.

Table 6.4. Number of ryegrass seeds present at harvest and the number caught during the harvesting operation. Data is the mean of 4 replicates ± SE of the mean in parenthesis.

	Seeds m^{-2}	
Crop spp.	Seeds caught at harvest	Seeds present at harvest
Barley	1146 (12.8)	1437 (37.3)
Wheat	5304 (31.7)	6791 (43.0)
Peas	3766 (32.5)	5382 (58.4)

The proportion of ryegrass seed caught in this experiment was 86.0% in barley plots, 70.0% in peas plots and 78.1% in the wheat plots. The proportion of ryegrass seed caught during the harvest operation was similar for these three crop species which contrasts with the commercial trial (Table 6.3). In the commercial trial, poor capture of *L. rigidum* seed occurred in peas and durum wheat crops. All the crops in this experiment (Table 6.4) were harvested at the same time with noe delay for the crops that are usually harvested later and with similar equipment which might explain the better relative proportion of catching in the pea and wheat crops.

The numbers of *L. rigidum* seed in the soil were examined following harvest and the relative seedbank determined (Table 6.5). Catching *L. rigidum* seed at harvest resulted in a reduction in the seed bank of between 50 and 55%. There was a difference between crop species (Table 6.6) in the proportion of reduction in seedbank size. The difference between species was related to the lower seed levels in the barley crop compared to the other the other crop species (Table 6.4).

Table 6.5. Mean density of *L. rigidum* seed bank in treated and control plots following seed catching at harvest. Data is untransformed.

	L. rigidum seed density (m^{-2})		
Crop spp.	Seed caught at harvest*	Control plots	% reduction
Barley	710 $^{a\,x}$	1561 $^{b\,x}$	55
Wheat	2875 $^{a\,y}$	5716 $^{b\,y}$	50
Peas	2411 $^{a\,y}$	5243 $^{b\,y}$	54

* Means followed by different letters are different at 5% level (a-b) within species and 1% (x-y) between species.

Table 6.6. Partial anova table for ryegrass seed bank density following seed catching at harvest. Data is from 4 replicates.

Source	d.f.	C.V.%	M.S.	F pr.
Crop species	2	47.8	6751.6	5.729*
Catching seed	1	9.5	6635.7	13.643**
Error	48		474.8	

* Significant at the 5% level; ** significant at the 1% level.

6.4 Discussion

Observations from farmers fields suggested that a proportion of *L. rigidum* seed was harvested during normal harvesting operations. In commercial harvesting operations this seed is immediately returned to the field with the crop residues where it forms the basis of further weed infestations. The results of Trials 1 and 2 presented here support these observations and demonstrated that harvesting and retaining a substantial proportion of *L. rigidum* seed at harvest was possible and that removing the caught seed from cropped areas was a potential method of reducing *L. rigidum* populations.

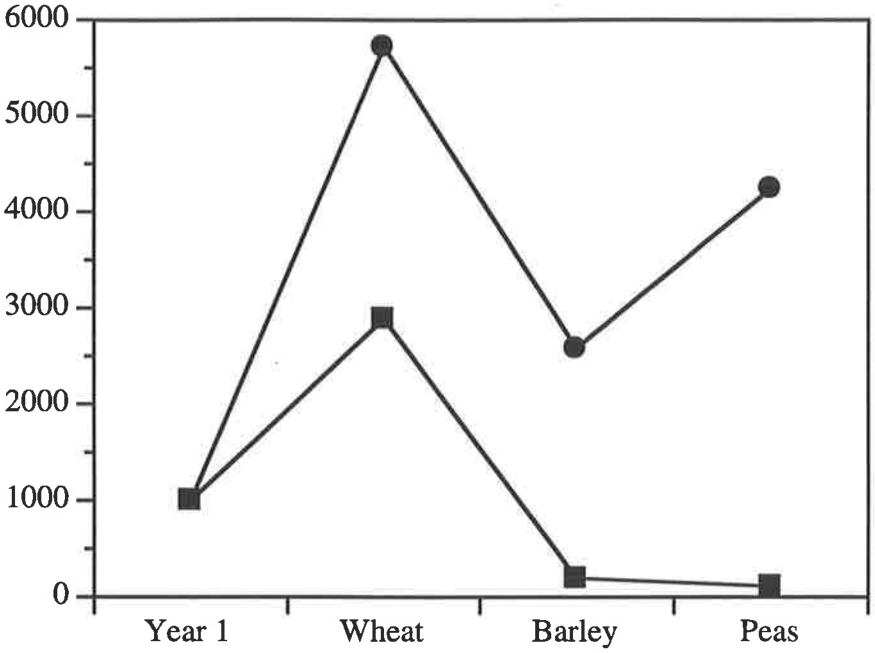
When this technology was used on commercial harvesting operations the results, shown in Table 6.3, were variable due to the diversity of field conditions and timing of harvest operations which affected the amount of ryegrass harvested or the amount threshed during harvest. However, the results were encouraging and showed the potential of the concept when applied to commercial agricultural fields. The results from a controlled field experiment (Table 6.4) indicate that a substantial proportion of ryegrass seed can be harvested and removed from the cropped area. Similar levels of performance could be achieved in commercial harvesting situations if sufficient priority is given to areas with *L. rigidum* infestations. The reduction in *L. rigidum* seed numbers in the soil, (Table 6.5) indicates that an average of 55% reduction in the *L. rigidum* seed infestation was achieved by one year of seed catching in barley crop, a 50% reduction in wheat cropped areas and 54% in areas under peas. This experiment was one years duration only, other weed reduction measures such as pre-seeding herbicides also reduced the ryegrass seed density, hence the discrepancy between the seed density indicated by Table 6.5 compared to the initial density of 4214 seeds per m^2.

L. rigidum is an annual species and because of high recruitment within a cohort in the first year following seed fall it can be considered a short-term annual. Previous work has documented the emergence of *L. rigidum* in the first year following seedfall at about 80% of the total emerging seed bank (Heap, 1989) and also 80% about the total seed bank population (Davidson, 1994). Therefore, the seed bank declines by germination at about 80% of the total in one year. If reinfestation from seedfall does not occur then the seedbank decline can be expected to exceed 80% per annum. The use of seedcatching at harvest has the potential to reduce the level of reinfestation from weeds surviving in the crop and therefore to maximize the rate of seedbank decline.

6.4.1 Projected seedbank decline in a crop rotation

A model of the changes in *L. rigidum* seed density in the seed bank due to seedcatching over a three crop rotation is shown in Figure 6.1. A constant seedling recruitment rate (*K*) of 0.023 as was identified in Section 6.33 was linked to the seed return rate (*B*) of *L. rigidum* grown with each of the crop species as reported in Table 6.4. The proportional reduction in each crop due to seed catching was as reported Table 6.4. The data was linked together to simulate a continuous crop rotation of wheat, barley and peas. Assuming a starting *L. rigidum* seed population of 1000 seeds m^{-2} the growth or decline are as shown for the simulated crop rotation with and without seedcatching.

Figure 6.1. Simulated changes in the *L. rigidum* seedbank (seeds m^{-2}) with and without seedcatching following a three crop sequence of wheat, barley and peas.



Legend: Simulated treatments, with seedcatching ■; without seedcatching ●.

Reducing the seed fall to the soil by catching ryegrass seed at harvest is a potential method for managing ryegrass populations as part of an IWM approach. The simulation shown in Figure 6.1 shows that an overall decline of approximately 90% with seed catching occurred compared to a fourfold increase without seed catching. The data is from the trial results (Table 6.4) and the efficacy of seed catching in peas may not be as high in commercial crops, Table 6.3. The effects of the crop species in influencing the fecundity of *L. rigidum* in parts of the rotation are also evident in Figure 6.1.

Maxwell and Gersha (1992) suggested that reducing dispersal of a weed of poor competitive ability but with high dispersal characteristics will reduce crop yield loss more than reducing dispersal of a weed with high competitive ability but low dispersal ability. Those predictions were generated by a weed dispersal and competition model. Seed catching at harvest has a potential role for reducing the quantity and limiting the dispersal of *L. rigidum* seed as shown in Figure 6.1. Seedcatching, therefore, has the potential to reduce crop yield loss as seed densities can easily be reduced to below crop loss thresholds, especially in cereal crops.

6.5 Conclusions

Catching seed at harvest is a technique very well suited to *L. rigidum* control because the erect habit and seed retention in the glumes and on the rachis makes machine harvesting possible. The technique is also well suited to *L. rigidum* control because the population density in the year following seedfall is sensitive to a reduction in seed bank replenishment rate due to the high proportion of seed which usually germinates in the first year. Therefore, seedcatching has great potential to reduce reinfestation of *L. rigidum* seeds in infested fields and may play an important part in the future management of this important weed.

Chapter seven

Summary and discussion

7.1 Weediness of *L. rigidum*

The management of *L. rigidum* is one of the major practical issues facing Australian agriculture. The geographic range of the weed is a contributor to the immensity of the problem. *L. rigidum* is present across most of the southern Australia cereal and legume cropping area, an east-west distance of about 3,000 km and a total area of about 14 m ha. The area of the infestation is only one aspect of the problem; there are other monocotyledenous weeds widely distributed without the impact of *L. rigidum* (Mayfield and Edwards, 1991). The weediness of *L. rigidum* is due to a combination of factors. The fecundity of *L. rigidum*, a combination of the plasticity of form and intrinsic harvest index is one facet of this (Rerkasem, 1980). Morphological plasticity contributes to the ability to produce many seeds given suitable space and resources and also limits the risk of death due to lack of resources. This is an important quality in an environment where spring and summer droughts are common, a characteristic which enables some seed to be produced even in extremely adverse conditions.

The emergence pattern following autumn or winter rains occur is also a contributor to the weediness of *L. rigidum*. A substantial proportion of an *L. rigidum* population germinates and emerges rapidly to be present in the crop or pasture. However a proportion of the population emerges later and is, therefore, present in crops throughout the growing season. A proportion of the population, about 20% of the population, also remains in the soil and germinates during the second and third year (Heap, 1988).

L. rigidum is well known for its adaptability to daylength and soil nutrient conditions (Forcella, 1984). The adaptability of populations to herbicides, as reported in Chapter 3 is another example of the adaptability of the species. The combination of the adaptability, extended emergence patterns and plasticity makes annual *L. rigidum* a persistent weed of crops.

7.2 Herbicide resistance

Coupled with inherent weediness of *L. rigidum* is the propensity to generate resistance and cross resistance to herbicides commonly used for its control. Following the advent of resistance in *L. rigidum* it became evident that cross resistance was a feature of many herbicide resistant *L. rigidum* populations. The onset of resistance and cross resistance, first described by Heap and Knight, (1986) had not been previously encountered in other herbicide resistant weed populations at that time. Non-target site cross resistance is still rare, having been reported only in *A. myosuroides* (Hall *et al.*, 1994; Kemp *et al.*, 1990) and to a limited extent in *P. paradoxa* (Yaacoby *et al.*, 1986). The onset of cross resistance posed many difficulties both practical and associated with the understanding of the phenomenon. The major practical problem was to design management strategies to contain the spread and reduce the density of herbicide cross resistant populations and to describe the rate of onset of cross resistance so farmers could be aware of the risks associated with the onset of cross resistance. Important to that, and to the general understanding of the phenomenon, was to elucidate the mechanisms conferring resistance.

The spectrum of herbicides to which resistance had developed in many of the populations encompassed most of the herbicides which were available for selective use. The practical considerations associated with cross resistance were a rapid decline in efficacy of major herbicide chemical groups (the aryloxyphenoxypropionates) and the very high risk of failure of alternative herbicides. The management of *L. rigidum* posed many difficulties because of the frequent failure of alternative herbicides due to cross resistance. With those prospects, the general outlook for long term control of *L. rigidum* with herbicides was pessimistic.

7.3 Initial frequency of resistance in unsprayed populations

There has been little previous work done on the frequency of resistant plants in natural weed populations or the frequency of resistance conferring genes in field populations. Price *et al.* (1983, 1985) and Thai *et al.* (1985) investigated the variability of herbicide performance and concluded that substantial interpopulation variability existed. This work was done chiefly with *A. fatua* yet little attention was paid to the findings due to substantial interpopulation variability in other morphological traits. Chauvel *et al.* (1992) found a low frequency of ACCase resistance occurred in four populations of *A. myosuroides* treated with fenoxaprop-*p*-ethyl, an ACCase inhibitor. However, the observed resistance was at a low frequency in the subsequent generations and cross resistance did not occur in the populations tested. The frequency of resistant *L. rigidum* plants within populations collected from the various sites as reported in Chapter 2 was high from some sites, and indicates the high probability of resistance occurring on most farm sites that are consistently treated with herbicides. The frequency of resistant individuals from farm populations is about 0.5-1%, and this represents a very high proportion within a population subjected to selection. This information substantiates the observations that the selection for resistance to diclofop-methyl can be extremely rapid and may occur in discrete populations of *L. rigidum* separated by distance such that gene flow between resistant populations would not be possible.

The observed frequency of the alleles bestowing resistance may be due to mutations of structural genes or to the presence of residual alleles within the species genotype conferring an adaptive advantage in certain conditions. The higher incidence of resistance conferring alleles in populations drawn from wetter sites as compared to those from drier sites tends to support the latter view. If this observation is substantiated, then this has serious implications for the prospect of the continued efficacy of weed control in arable areas. Successful crop production areas are those with an adequate rainfall and the natural response over time of the resident *L. rigidum* population may have been an increase in the frequency of genes which are also capable of conferring resistance.

Other explanations for the observed frequency of resistant plants from wet sites and farmland sites need to be canvassed and solutions found if the phenomenon of the rapid onset of resistance is not to be repeated for other weed species. Many of the populations discussed in Chapter 2, had been subjected to grazing by animals and other farm management practices which may have selected for robust phenotypes. However, the outcome of the assortative matings indicate that resistance can be inherited as a discrete factor. The observation that the majority of survivors of herbicide application from previously unsprayed populations were heterozygous is consistent with the allogamous mating behaviour of the species. Also, the observation that resistance to diclofop-methyl is largely inherited as a dominant character, even in the first generation of selection, tends to maximise the rate of onset of resistance in a population under selection (Jasieniuk *et al.*, 1996). Understanding the rate of evolution of herbicide resistance in the field where the survivors from an initial herbicide application behaved as heterozygotes with resistance as a dominant factor again casts doubt on the long term efficacy of herbicide application as a means of control of *L. rigidum*.

7.4 Fitness of herbicide resistant phenotypes

The potential for the persistence of an novel genotype in a population is dependent to a large degree on the fitness of the new phenotype (Maxwell and Mortimer, 1994). The persistence of a genetic trait in a population is influenced by many factors but in an annual weedy species such as *L. rigidum* a relative fitness difference would strongly influence biotype survival in the absence of herbicide application. The competition studies between resistant and susceptible populations reported in Chapter 5 were done to establish if any fitness differential existed between the resistant and susceptible biotypes. Management of resistant populations would be simplified if a substantial and exploitable difference between resistant and susceptible biotypes could be identified. Lessons from the extensive literature on triazine resistance indicated that a substantial pleiotropic cost to fitness of these weeds was associated with triazine resistance (Holt, 1990). This was not the case with resistance to the selective herbicides to which *L. rigidum* has developed resistance.

A range of biotypes were compared with both susceptible and resistance biotypes in such a way as to establish the relative productivity at a wide range of densities and at a range of frequencies. No differences were identified. A novel genotype may display a frequency dependent advantage which could explain the persistence of such a genotype at a low frequency within a population. The experiment conducted did not identify such an advantage. The conclusion from Chapter 5 is that the resistant biotypes were not less fit than the susceptible biotypes to which they were compared and persistence of the resistance phenotype would not be compromised by any associated genetic costs to fitness.

The biotypes tested also represented a range of resistance mechanisms which occur in *L. rigidum*. A fitness penalty may be associated with a particular resistance conferring mechanism such as the fitness penalty associated with target site resistance to the triazine herbicides (Holt, 1990); however, no fitness penalty was identified in the *L. rigidum* biotypes. As mentioned in Chapter 5, competitive conditions are usually present when selection by herbicide application is occurring, so selection for fitness is almost certainly proceeding during selection for herbicide resistance. As the biotypes were collected from agricultural environments they may be well adapted by continuing selection. In a population under selection, if the rare resistant biotypes were less fit than the remaining susceptible population in the early generations of selection then suppression by crop or intraspecific competition in the absence of herbicide application may selectively remove or reduce the frequency of the resistant individuals. The few resistant plants in an unselected population, those that will be the basis of a resistant population, were, however, no less fit than the population as a whole. The data in Section 5.5 shows that survivors of a herbicide application to a previously unselected population had a similar seedling growth rate as the whole population.

The fitness of the F_1 is an important consideration in the persistence of the resistant genotype; a less fit heterozygote could be expected to be suffer from competition and the resistant allele(s) could be lost if selective mortality occurred. The F_1 crosses from a susceptible and a resistant biotype showed no fitness penalty when compared to either of the parents, as shown in Section 5.4.

The data from Chapter 5 did not identify a fitness differential between the herbicide resistant *L. rigidum* biotypes tested. Even if the resistant genes have not become fixed within the population, herbicide resistant *L. rigidum* biotypes can be expected to persist in a mixed population.

7.5 Selection for herbicide resistance from a susceptible population

L. rigidum populations displayed a remarkable capacity to develop resistance to diclofop-methyl when treated with the field rate of the herbicide. The population described in Chapter 3 achieved a high level of resistance with three applications of diclofop-methyl. This population displayed an increase in the level of resistance to diclofop-methyl in each of three generations of selection. An increase in resistance to diclofop-methyl was also found when selections were made from survivors of a higher rate. It is obvious from the LD_{50}'s of each generation that the resistant component would not be controlled in the field. The level of visual control in the field after the first selection may be acceptable depending on the proportion of susceptible *L. rigidum* that emerged in the next season.

The repeated application of diclofop-methyl also selected for resistance to sethoxydim. The resistance to sethoxydim induced by the use diclofop-methyl is a clear cut case of target site resistance. The efficacy of sethoxydim remained superior to that of diclofop-methyl even though both sethoxydim and diclofop-methyl are ACCase inhibitors. These differences appear to be due the target site mutation which was less affected by sethoxydim than diclofop. The observation that sethoxydim activity is better correlated with homozygosity of the diclofop resistance conferring allele(s) in the resistant accessions is consistent with this. Sethoxydim is a more effective herbicide than diclofop-methyl on susceptible *L. rigidum* plants, however, its use is restricted to broadleaf crops only. In spite of the differences in efficacy between these herbicides the appearance of target site cross resistance has important ramifications for herbicide control of *L. rigidum*. Sethoxydim when used at sufficient rate on diclofop-methyl selected populations would be effective on these populations. However, in this case, its effectiveness would be limited to the first generation after selection with diclofop-methyl. Use of sethoxydim on established herbicide resistant biotypes would rapidly lead to the failure of this herbicide as selection operates on the survivors. In such a situation, the pre-conditions for failure of sethoxydim which were initiated by the use of diclofop-methyl are a well defined example of the problems of target site cross resistance

A unique feature of *L. rigidum* resistance in Australia has been the appearance of non-target site cross resistance. The capacity of *L. rigidum* to develop non-target site cross resistance is illustrated by the appearance of resistance to the herbicides chlorsulfuron and simazine, as shown in Chapter 3. Resistance to both these herbicides which have different sites of action from each other and from diclofop-methyl, was significant and at a sufficient level to cause serious problems in the field.

The level of cross resistance to chlorsulfuron was more pronounced with the accession selected at a lower rate of herbicide (S_3L) than with the higher rate (S_3H). Even though resistance to chlorsulfuron was evident at the field rate under both selection regimes, selection at the higher rate of diclofop-methyl reduced the relative level of cross resistance to chlorsulfuron. There may be an opportunity to use a herbicide from the aryloxyphenoxypropionate group at the maximum possible rate to restrict the genetic diversity of the survivors, and in this way reduce the level of cross resistance. This remains to be investigated on a wider scale.

Selection with diclofop-methyl also caused resistance to the PSII inhibitor simazine. The accession selected at the lower rate of diclofop (S_2L) generated almost twice the level of resistance to the PSII inhibitor simazine, than the high rate regime (S_2H). Again, the increased selection pressure of the high rate regime may have limited genetic diversity among the survivors by imposing a higher selection pressure. The rapid onset of both target site and non-target site cross resistance shown in Chapter

3 illustrates the capacity of *L. rigidum* to generate resistance to a range of herbicides when selected by a single herbicide.

The evidence presented in Chapter 4 showed that target site resistance conferred by an insensitive ACCase enzyme was an important mechanism of resistance to the ACCase inhibitor herbicides. The insensitive target site was selected by only three applications of diclofop-methyl. The diclofop selected accessions also displayed target site cross resistance to the ACCase inhibitor sethoxydim. However, *in vitro* resistance to sethoxydim was similar for all accessions and even for the highly resistant subgroup selected from the S_3L accession.

As well as target site resistance to the ACCase inhibitor diclofop-methyl, a difference in the rate of metabolism of diclofop-methyl to inactive compounds in the S_3L accession was identified which was not detected in the S_3H accession. The presence of resistance conferred by metabolism in S_3L in conjunction with an altered target site gave a higher level of resistance to foliar applications of diclofop-methyl than target site resistance alone as seen in accession (S_3H). Substantial levels of metabolic inactivation of diclofop-methyl by *L. rigidum* has been described by Holtum *et al.*, (1991).

Resistance to both chlorsulfuron and simazine was also due to increased metabolism (Section 4.3.3 and 4.3.4). Simazine and chlorsulfuron are from different herbicide groups and have unrelated target sites and unrelated modes of action, so the description of non-target site cross resistance to two herbicides in the diclofop-methyl selected populations is of considerable interest. The rapid selection of a mechanism or mechanisms able to metabolise three herbicides in the one population is also of considerable interest. The resultant increased capacity to metabolise herbicides following a brief period of selection suggests that the alleles may be closely linked or, perhaps the increased metabolic capacity is conferred by a family of genes coding for metabolic function but controlled at a single locus. The elucidation of the genetic basis of increased metabolic capacity demands further work.

Selection with diclofop-methyl increased the frequency of the allele or alleles conferring phenotypic resistance to diclofop-methyl. Resistance to diclofop-methyl may be caused by more than one allele but the resistance phenotype increased at a rate largely consistent with the rate that would be apparent with selection of a single dominant gene within a population. Selection with diclofop-methyl also caused resistance to other herbicides. Resistance to sethoxydim increased in proportion to the increase in the resistant allele(s) in the diclofop-methyl selected accessions and was slightly better related to the homozygosity of the diclofop-methyl resistance conferring allele for the onset of resistance. The increase in resistance to chlorsulfuron was also proportional with the increased frequency of the diclofop-methyl resistance conferring allele(s) in the population but was better described by the frequency of both heterozygous and homozygous alleles in the S_3L accession. The onset of cross-resistance to all herbicides was, therefore, proportional to the selection of the factor or factors in the population that conferred resistance to diclofop-methyl. Consistent use of diclofop-methyl for *L. rigidum* control will not only select for diclofop-methyl resistance and resistance to other ACCase inhibitors, but importantly, concurrent failure of other herbicides. This will occur rapidly if the dynamics of resistance reported in Chapter 3 are mirrored in the field.

Much of the data presented here and the conclusions drawn from it suggest that the use of herbicides, especially from the ACCase inhibitor group will have limited long term efficacy on populations of *L. rigidum*. The onset of non-target site cross resistance in ACCase treated population may also reduce the usefulness of important herbicides from other unrelated groups. The management of *L. rigidum* will certainly be more difficult in the future due to complex patterns of cross resistance.

7.6 Seed catching, its role in *L. rigidum* management

The options for control of *L. rigidum* in the absence of effective herbicides are limited to the influence of pre-sowing management activities and other crop and land management techniques that may have an effect on *L. rigidum* density or seed production. The targeting of specific management activities at a particular weed and the linking of these together to form an effective management system is called "Integrated Weed Management". The concept presented in Chapter 6 is one part of such an approach.

Seed catching during the crop harvesting operation can make an important contribution to integrated management of *L. rigidum* infestations. The results presented in Chapter 6 indicate that a substantial proportion of *L. rigidum* seed can be harvested and removed from the cropped area. The performance of such a technique will certainly vary with field conditions and operator skill, however the technique has considerable potential. Seed catching reduced the density of *L. rigidum* seeds in the soil as shown in Chapter 6, and over the course of a simulated three crop rotation demonstrated the potential for successfully reducing the *L. rigidum* seed bank.

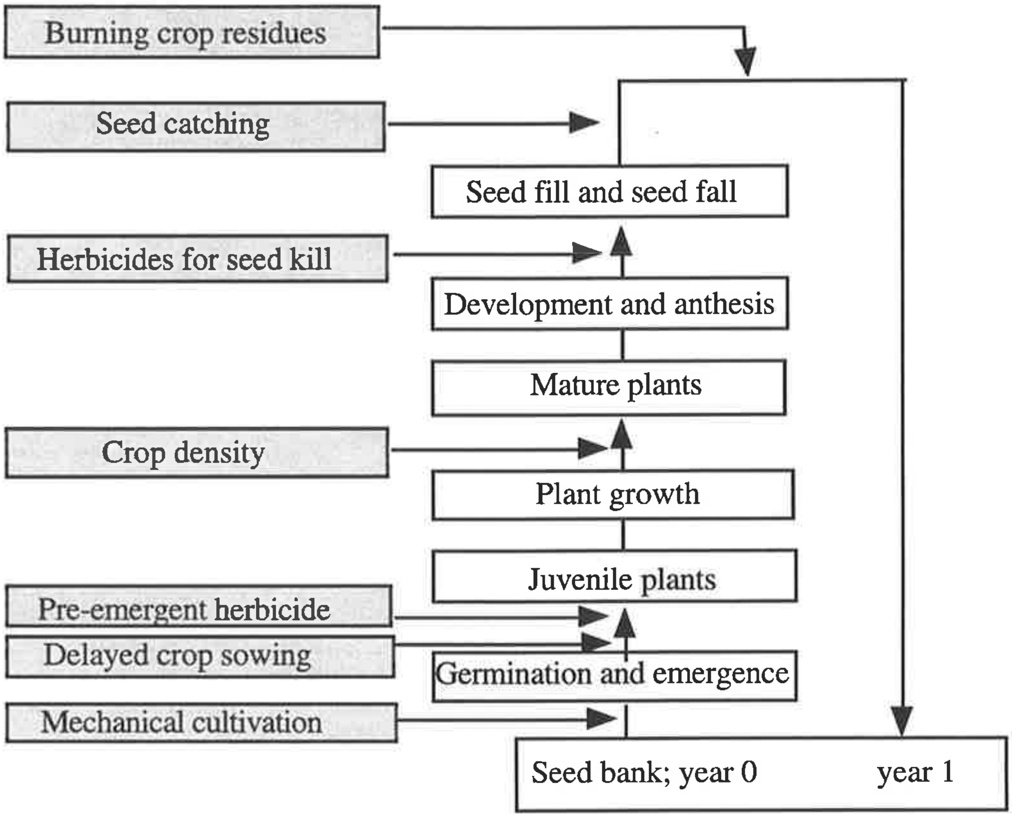
Seed catching has several aspects which make it an exciting prospect for control of *L. rigidum*. In addition to removing seeds from the field at the end of the growing season which will rapidly be reflected in a reduced seedling density in the next season, seed catching exerts no selection pressure for herbicide resistance. Seed catching may have the potential to delay the onset of resistance in populations of *L. rigidum* that are developing resistance by the reducing the reinfestation of the seed from resistant individuals. There may be the opportunity for catching seed at harvest to selectively remove a higher proportion of resistant individuals from within a crop. Resistant plants having survived an application of herbicide may be more robust plants than the later germinating susceptible plants and may be more effectively removed by seed catching than the remaining susceptible plants. The potential of seed catching to reduce the rate of onset of herbicide resistance requires further research.

Seed catching may, however, exert selection for other traits in *L. rigidum*, such as early seed fall or less robust stems or rachis, or even a tendency to reduced final plant height. It would seem, however, these adaptations may reduce the competitiveness and fecundity of *L. rigidum* and in such cases would only be acquired slowly. Again, careful observations would need to be undertaken to identify any genetic adaptations within *L. rigidum* or other weed species to seed catching.

7.7 Integrated Management for *L. rigidum* control

In addition to seedcatching there are other management techniques that have the potential to reduce to either the density or the fecundity of *L. rigidum* populations on a particular site. Some of these methods are, delayed crop establishment, pre-emergent herbicide, increasing crop density, the use of non-selective herbicides for reduction of seed viability at weed anthesis and burning of crop residues. A generalised scheme of weed growth and the steps in the plant population establishment processes where IWM methods could be effective are shown in Figure 1.

Figure 1. General scheme of the state and process links and IWM methods. The IWM methods in shaded boxes are arranged on the left hand side of the figure.



Many of these IWM methods require research to define the benefits and the interactions between them for long term weed control. Benefits need to be described for a variety of crop species, crop varieties and crop production environments.

The occurrence of herbicide resistance in *L. rigidum* populations to many of the selective herbicides commonly used for its control demands a different approach to weed management. There are limited prospects for continued widespread use of selective herbicides for control of *L. rigidum* weed infestations. The frequency of resistant plants in unsprayed populations, the capacity of survivors to confer high levels of resistance, and the high rate of onset of herbicide resistance following consistent exposure to herbicides all combine to limit the effectiveness of selective herbicides.

The inevitability of an increasing incidence of herbicide resistance when selective herbicides are the sole method of weed management presages an important role for "Integrated Weed Management" strategies for successful management of *L. rigidum*.

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