Clethodim Resistance in *Lolium rigidum* (Annual Ryegrass) and its Management in Broadleaf Crops

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ABSTRACT

Lolium rigidum is one of the most troublesome herbicide resistant weeds in Australia, which has so far evolved resistance to eleven major herbicide groups. Clethodim, an inhibitor of acetyl–coenzyme A carboxylase (ACCase), is a selective post-emergent herbicide used to control annual and perennial grasses in a wide variety of broadleaf crops and has been used by many farmers to manage *L. rigidum* in continuous cropping rotations. However, repeated use of this herbicide during the last two decades has resulted in the appearance of *L. rigidum* populations that are highly resistant to clethodim. Studies on the resistance mechanisms, genetics, and fitness of resistant alleles were undertaken to better understand the evolution of clethodim resistance in *L. rigidum.* Field studies were also undertaken to investigate the performance of alternative herbicides for the management of clethodim-resistant *L. rigidum* in faba bean and canola. Dose–response experiments were conducted on twelve populations of *L. rigidum* collected from different locations in Australia. All the populations were confirmed resistant to clethodim with resistance levels ranging from 3-34-fold as compared to the susceptible control. These resistant populations have also evolved cross-resistance to butroxydim. Sequencing of the target-site *ACCase* gene identified five known ACCase mutations (Leu-1781, Asn-2041, Gly-2078, Arg-2088, and Ala-2096) in these populations. In the highly clethodim-resistant populations, the level of clethodim resistance was influenced by the occurrence of frost close to herbicide application. A significant reduction in the level of clethodim efficacy was observed in resistant populations when plants were exposed to frost for three nights before or after clethodim application. However, there was no effect of frost on the response of the susceptible population suggesting that the mechanism present within the resistant populations interacts with frost to further reduce clethodim efficacy. The inheritance of clethodim resistance was investigated by cross pollinating the susceptible and five resistant populations. The results of the inheritance study showed different

patterns of inheritance of clethodim resistance in *L. rigidum*; which included a single gene, partially dominant, nuclear encoded trait, two different patterns of two-gene inheritance and an example of maternal inheritance of the resistance trait. The fitness of three resistant alleles (Leu-1781, Asn-2041, and Gly-2078) was also studied by determining the change in the frequency of resistant alleles in two generations of *L. rigidum* in the absence of clethodim use. The results of this experiment showed that there was no significant change in the frequency of Leu- 1781 and Asn-2041 alleles in *L. rigidum* populations from one generation to other but the frequency of Gly-2078 allele increased significantly (7 to 16%; $P \leq 0.05$). Studies were also undertaken to identify alternative herbicides for the control of clethodim resistant *L. rigidum* with a range of pre-emergent herbicides in broadleaf crops. In both faba bean and canola crops, pre-emergent herbicides alone were insufficient to effectively manage clethodim-resistant *L. rigidum*. The application of effective soil residual herbicides followed by the post-emergent tank-mixture of clethodim and butroxydim provided acceptable control of some clethodim resistant *L. rigidum* populations.

DECLARATION

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ACRONYMS

ACCase: Acetyl coenzyme A carboxylase

AGRF: Australian Genome Research Facility

AHAS   Acetohydroxyacid synthase

ALS   Acetolactate synthase

ANOVA   Analysis of variance

APP   Aryloxyphenoxypropionate

BC   Back cross

BCCP   Biotin carboxyl carrier protein

C   Celsius

CHD   Cyclohexanedione

CLF   Clearfield^{TM}

CT   Carboxyl transferase

EPSPS   5-enolpyruvylshikimate-3-phosphate

F_1 First filial generation

FAS   Frost after spray

fb   Followed by

FBS   Frost before spray

GR_{50} Lethal dosage (herbicide dose causing 50% growth reduction of plants)

GSR   Growing season rainfall

KPa   Kilopascals

LD$_{50}$ Lethal dosage (dose required to control 50% of individuals in the population)

LSD   Least significant difference

N   Nitrogen

NF   No frost

NSW   New South Wales

P   Phosphorus

PCR   Polymerase chain reaction

POST   Post-emergence

PPI   Preplant incorporated

PPZ   Phenylpyraxoline

PSPE   Pre-sowing pre-emergence

R   Resistant

S   Susceptible

SA   South Australia

TT   Triazine tolerant

VIC   Victoria

WA   Western Australia

WAP   Weeks after planting

WAS   Weeks after sowing

CHAPTER 1

REVIEW OF LITERATURE

1.1   Introduction

Weeds are a continuing problem in global crop production (Oerke, 2006), because of their capacity to persist despite efforts to control them (Harper, 1977). The potential crop yield losses due to weeds are higher compared to any other pest species (Oerke, 2006). During the past 5 decades, herbicides have become the dominant means for weed control in most agricultural areas of the world. The introduction of selective herbicides since the 1940s has greatly facilitated farmers' work by suppressing the need for manual weeding and making weed management easier, because weeds could be selectively controlled in all major field crops. However, dependence on herbicides for weed control has resulted in shifts in the weed flora and in some situations intensive herbicide usage has led to development of herbicide resistant weed populations (Heap, 2015; Powles and Yu, 2010). Worldwide, 247 weed species have evolved herbicide resistance in many agro-ecosystems (Heap, 2015). In 1957, the first case of herbicide (2,4-D) resistance was reported in Hawaii (Hilton, 1957). However, the first confirmed report of herbicide resistance is commonly attributed to Ryan (1970) who confirmed triazine resistance in *Senecio vulgaris* L. (common groundsel) in 1968 in USA. Since then many weeds have evolved resistance to triazines due to their intensive usage, mainly in maize cropping systems (Gronwald, 1994b) Since then, the number of weed biotypes with resistance to various herbicides has been on the rise. Many novel herbicides were introduced by the end of the 1970s and 1980s, and the period since 1980s witnessed a large increase in herbicide resistant weeds across many herbicide groups (Heap, 2015). Recent estimates indicate that 461 biotypes belonging to 247 species (144 dicots and 103 monocots) have evolved resistance to various herbicides (Heap, 2015).

Acetohydroxyacid synthase (AHAS/ALS) inhibiting herbicides have turned out to be particularly prone to resistance development in weeds. At present, 157 weed species are resistant to ALS inhibiting herbicides (Heap, 2015). ALS/AHAS inhibiting herbicides are selective and have low mammalian toxicity, which has ensured their widespread adoption in many crops all over the world and has significantly contributed to the prevalence of herbicide resistance to this group (Powles and Yu, 2010; Tranel and Wright, 2002). Similarly, intensive usage of acetyl-coenzyme A carboxylase inhibiting herbicides (ACCase) for grass weed control has resulted in widespread evolution of resistance to these herbicides (Délye, 2005). So far, 47 grass weed species have evolved resistance to ACCase herbicides (Heap, 2015). Glyphosate is another important herbicide, which is used widely due to its non-selective weed control without any residual effects. This herbicide is extensively used in fallows, for spot application or directed spray in crops or for selective weed control in genetically-modified glyphosate tolerant crops (Powles et al., 1998). Glyphosate has been widely used since 1974, yet glyphosate resistance evolution has been slow compared to several other herbicides (Powles et al., 1998). However, this trend has changed with many recent reports of glyphosate resistance in weed populations (Preston et al., 2009).

The speed of herbicide resistance evolution depends upon several factors including agro-ecosystem and herbicide factors, as well as plant genetic factors, such as the number and initial frequency of resistance genes, the level of dominance of the resistance allele and gene flow (Darmency 1994; Diggle and Neve, 2001). Amongst the operational factors, herbicide selection intensity has an important role in herbicide resistance progression, and tends to be influenced by herbicide rate and time of application (i.e. stage of weed). Apart from operational factors, some environmental factors, such as temperature and relative humidity at application, can have some influence on the activity of many post emergence herbicides (Prasad et al., 1967; Ritter and Coble, 1981; Wills, 1984). The temperature

before and after herbicide application can have a subtle effect on the efficacy of herbicides (Wilcox et al., 1988). Low relative humidity prior to, during and after herbicide treatment may cause the cuticle to be dehydrated thus possibly reducing absorption of water soluble herbicides (Anderson et al., 1993).

Lolium rigidum (annual ryegrass) is one of the most troublesome herbicide resistant weeds in Australia. It is an obligate cross pollinated species that has evolved resistance to most herbicide modes of action. It is a widespread weed and some of its biological characteristics increase its ability to evolve resistance to herbicides ((Preston et al., 1999). Due to over reliance on herbicides for the control of *L. rigidum* since the 1980s, resistant populations now exhibit multiple and cross resistance across several modes of action herbicides (Boutsalis et al., 2012; Broster and Pratley, 2006; Powles et al., 1998; Preston and Powles, 2002b). This species has evolved resistance to eleven different modes of action of herbicides (Heap, 2015). Clethodim is a selective post emergence herbicide used to control annual and perennial grasses in a wide variety of broad leaf crops (Burke et al., 2004; Burke and Wilcut, 2003a; Vidrine et al., 1995). For many years, clethodim at recommended field rates provided effective control of many *L. rigidum* populations with resistance to other ACCase inhibiting herbicides and allowed farmers to continuously crop their fields. However, repeated use of this herbicide has resulted in the development of clethodim resistance in *L. rigidum* populations, which is making weed control even more challenging. The levels of resistance to clethodim vary among *L. rigidum* populations, with farmers increasing its dose to gain control of some resistant populations (Boutsalis et al., 2012).

Understanding the mechanisms and inheritance of resistance is necessary for the formulation of efficient strategies to slow down the rate of evolution of resistance or to manage resistant weeds. As the inheritance and mechanisms of clethodim resistance have

not yet been fully studied, this project will focus on these areas of research. Field studies will help to identify herbicide options for the control of clethodim resistant *L. rigidum* in broadleaf crops.

1.2 Biology and Ecology of *L. rigidum*

1.2.1 Botanical Description and Taxonomy

The genus *Lolium* belongs to family Poaceae and grows in temperate areas with its origin from Europe, temperate Asia and Africa (Terrell, 1968). This genus includes eight species worldwide. This genus includes the widely distributed *L. rigidum* Gaud. (annual ryegrass or Wimmera grass), *L. multiflorum* Lam. (Italian ryegrass), and *L. perenne* L. (perennial ryegrass) (Terrell 1968). *Lolium* includes five more species, *L. temulentum* L., *L. remotum* Schrank., *L. canariense* Steud., *L. persicum* Boiss. and *L. subulatum* Vis. The ploidy level of all these species is diploid with 14 chromosomes (2n = 14). *L. multiflorum*, L. *perenne* and *L. rigidum* are allogamous (self-incompatible and cross pollinated); *L. temulentum*, *L. remotum* and perhaps *L. rigidum* var. *rottbollioides (L. loliaceum)* are autogamous (self-compatible and self-pollinated) (Spoor and McCraw, 1984). The autogamous species are less widely distributed compared to the allogamous species (Terrell, 1968). Allogamous plants are self-incompatible and naturally outcross within or between species in the same genus (Terrell, 1968). The self-incompatible *Lolium* species are generally more troublesome in agriculture than the self-compatible species (Charmet et al., 1996).

L. rigidum is an erect or sprawling annual grass grows up to a height of 15 cm and sometimes it may also grow up to 90 cm in height (Willis, 1970). Leaves of *L. rigidum* are bright green in colour, hairless and are shiny on the back of the blade. During the early stages of growth the base of seedlings is reddish purple in colour. The emerging leaves of *L. rigidum* are folded. It has wide ligules and long auricles. It has spikelets with 3-5

florets, as broad as, or little broader than, the rachis. These are deeply embedded in the rachis which is slightly zigzagged and roughly square in section (Beadle et al., 1982).

1.2.2 Ecology of *L. rigidum*

1.2.2.1 Seed Dormancy

Seeds of *L. rigidum* are normally dormant at the time of maturity for 8-9 weeks after seed set (Gramshaw, 1972). Release from dormancy occurs following an after-ripening period and is fastest under warm, moist conditions. However, the seeds have a period of dormancy that prevents germination in summer rain periods (Steadman, 2004). Seedling emergence is therefore prominent from late autumn to early spring. However, if imbibed seeds are kept in constant darkness they may steadily lose dormancy within a few days (Steadman, 2004). Bolger et al. (1999) suggested that cooler conditions may enhance dormancy release. Different levels of seed dormancy within a population can lead to staggered seedling emergence throughout the growing season, which makes its control more challenging and often results in some weeds escaping control and replenishing the soil seed bank (Bolger et al., 1999).

1.2.2.2 Germination and Emergence

Once seeds have lost dormancy through dark stratification, light and alternating temperatures are required to increase germination (Steadman, 2004). Germination in *L. rigidum* is highly responsive to temperature. Gramshaw (1972) reported a positive relationship between germination and temperature; the rate of germination increased with temperature up to 8-26° C but decreased slightly if the temperature increased up to 35° C. The optimum temperature for germination of *L. rigidum* seeds differs under light and dark conditions. For buried seeds the optimum temperature is 11° C, whereas for surface seeds exposed to light the optimum temperature required for germination is 27° C (Chauhan et

al., 2006a; Cook et al., 2005). Generally, 70-80 % of after-ripened *L. rigidum* typically germinates following opening rains in autumn and germination may continue for a period of six months (McGowan, 1970).

Seeds buried in the soil up to 2 cm depth, germinate faster than seeds that are on the surface of the soil. Chauhan et al. (2006) reported a decrease in seedling emergence of *L. rigidum* with increase in burial depth and emergence ceased at 10 cm depth. After 2 years of burial at 10-15 cm, ryegrass seeds can lose their viability (Cheam and Lee, 2005). Narwal et al. (2008) reported that the seeds of *L. rigidum* remain viable for a period of sixteen months. However, tillage systems can have some influence on the persistence of *L. rigidum* seed bank. Seed decay in *L. rigidum* was found to be greater for seeds on the soil surface as compared to shallow buried seeds, suggesting that no-till may contribute to a quicker depletion of the *L. rigidum* seed bank in the soil (Chauhan et al., 2006b).

1.2.2.3 Seed Production and Dispersal

L. rigidum can produce a large number of seeds even in extremely competitive surroundings. McGowan (1967) observed that *L. rigidum* in crops averaged 1043 seeds per plant. Rerkasem et al. (1980) reported that under irrigated conditions in a wheat crop, *L. rigidum* can produce up to 45,000 seeds per m^{-2}. Also, Davidson (1990) in his study concluded that in an ungrazed pasture *L. rigidum* can set nearly 26,000 seeds m^{-2}. The seeds are held tightly to the rachis and not adapted for dispersal by wind (Walsh and Powles, 2007), but once removed from the plant seeds are very light and can be easily dispersed by wind, water and animals in fodder and grain and also sometimes by machinery and vehicles. Equipment, such as harvesters, can disperse *L. rigidum* seeds as far as 18 meters (Blanco-Moreno et al., 2004).

1.3 Historical Background in Australia

1.3.1 Introduction and Spread

L. rigidum is a widespread weed of Australia (Jones et al., 2005; Kloot, 1983), which was introduced in different parts of the Australia as a pasture plant (Terrell, 1968) and has been used in reclaiming saline areas (Carris, 1962) and in soil conservation (Quilty, 1972). It is well adapted to most soil types in the southern Australia. *L. rigidum* has been a valuable and broadly established pasture grass across southern Australia since the nineteenth century. With the decline of the Australian wool industry since the 1970s, the area was moved from sheep pasture to grain production. From the time when a shift occurred towards arable crops, this well regarded pasture plant became Australia's most important weed of crops (Gill, 1996). *Lolium* spp. commonly occur as weeds of crops on most continents (Charmet et al., 1996). These species are important weeds of cereal crops but also occur in other crops, vineyards, irrigation channels, roadsides, fence lines, and orchards. Even though *Lolium* spp. has developed into a weed problem for crop growers, it is still considered a desirable pasture species in Australia, as well as in other countries (Botha et al., 2008; McCartney et al., 2008; Pereira et al., 2008). It is a problem weed of cropping on soil types found in southern Australia. High genetic variability in this diploid and out-crossing species is likely to contribute to its widespread distribution (Charmet et al., 1996). Apart from successful adaptation to unstable habitats, high genetic variability in this species has also enabled it to quickly be selected for resistance to nearly all selective herbicides registered for its control.

L. rigidum occurs throughout the Australian grain belt and ranks as the most widespread and important weed (Jones et al., 2005). Figure 1 shows distribution of *Lolium* in Australia. The first incidence of herbicide resistance in *L. rigidum* was reported in Australia in 1982 (Heap and Knight 1982). At present, herbicide resistant populations of

L. rigidum have been reported almost throughout the world i.e. parts of Australia, America, Europe, Asia and Africa (Heap, 2015).
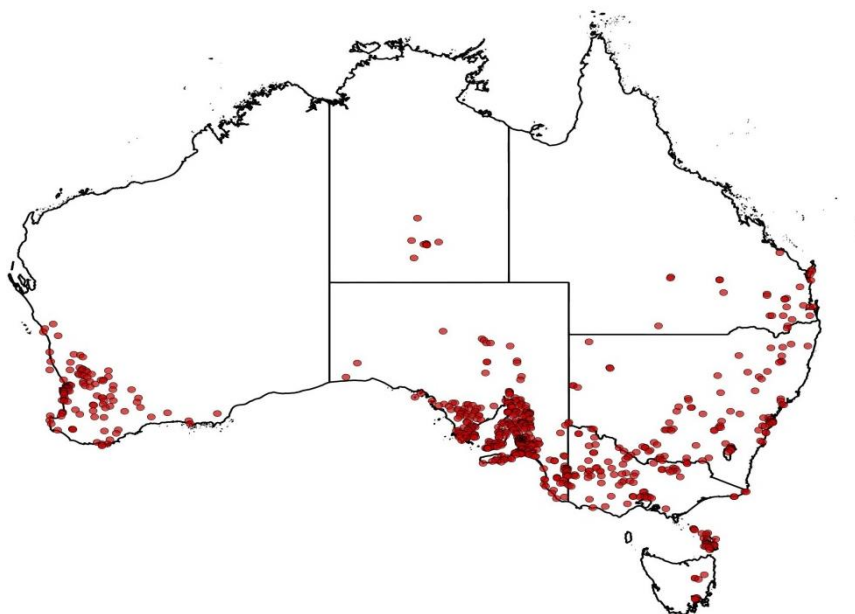
Figure 1: Distribution of *L. rigidum* in Australia (Anonymous, 2015)

1.3.2   Herbicide Resistance

Herbicide resistance is the inherited capacity of a plant to survive and reproduce following selection with a dose of herbicide normally lethal to the wild type (Heap, 1997; Powles et al., 1997). Resistance to a particular herbicide can be a natural property of the plant or a secondarily acquired mechanism (Heap et al., 2001). In 1957, the first case of herbicide resistance was reported against 2,4-D in Hawaii (Hilton, 1957). Almost a decade later, the first confirmed report of triazine resistance in *S. vulgaris* (common groundsel) was reported in 1968 in USA (Ryan, 1970). Since then, resistance to various herbicides has been reported in 144 dicot and 103 monocots weeds species (Heap, 2015). Over the last 20 years, ALS, ACCase and triazine resistant weeds have accounted for a large portion of the resistant species (Heap, 2015).
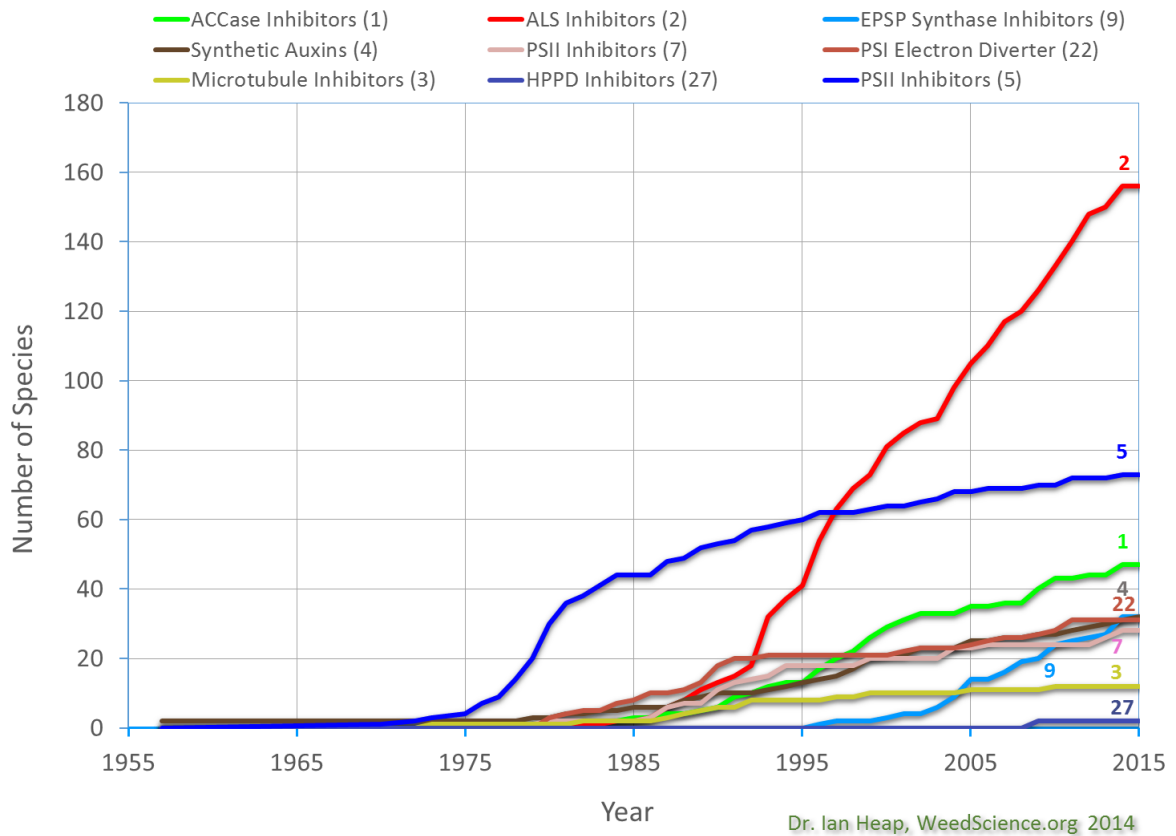
Figure 2: Resistance to different groups of herbicides

1.3.2.1 Evolution of herbicide resistance

Initial frequencies of herbicide resistance genes, allelic interactions of resistance genes (dominance), number of different resistance genes, mating system (inheritance pattern) and relative fitness of resistant individuals are the major genetic factors that govern the evolution of herbicide resistance (Jasieniuk et al., 1994). The initial frequency of resistance alleles has a greater influence on the evolutionary process when herbicides impose weak selection, as opposed to very strong selection (Preston and Powles, 2002a). The movement of resistance alleles by pollen flow has been reported to influence the evolution of resistance (Busi et al., 2011). Repeated use of one herbicide or herbicides with the same mode of action increases the risk of resistance to that herbicide (Owen and Zelaya, 2005). The frequency at which a selective agent is applied, either during a single season or over consecutive seasons also influences the rate of evolution, as does the specificity of the herbicides mode of action. Herbicides with a single target-site tend to be

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more prone to resistance evolution. Herbicide resistance can also occur through natural selection or may be induced by genetic engineering (Yuan et al., 2007).

1.3.2.2 Selection Pressure

Selection pressure may be defined as survival of the fittest and it is one of the main factors contributing to resistance. Efficiency and frequency of herbicide use are the major factors contributing to selection pressure for herbicide resistance (Maxwell and Mortimer, 1994). A few plants may survive an application of herbicide that is lethal to other members of the same species. If a few of these resistant individuals occur in a natural weed population, continual use of the herbicide would leave only the resistant biotypes (Jasieniuk, 1996). These resistant weeds grow and produce seed that germinate the following season and produce a new generation of resistant weeds. If the same herbicide were used year after year, the resistant weeds continue to thrive and reproduce, ultimately outnumbering the susceptible individuals. Continually using the same mode of action of herbicide will increase the selection pressure and the evolution of herbicide resistant weeds. Herbicides with long residual activity increase selection pressure over those without residual activity (Jasieniuk et al., 1996). This is because their greater persistence controls a greater percentage of the population.

1.3.2.3 Inheritance of resistance

The pattern of inheritance of resistance dictates how resistance from one generation will be passed to future generations. Two modes of inheritance have been reported i.e. nuclear and cytoplasmic inheritance (reviewed by Rao, 2000). Nuclear inheritance of herbicide resistance is conferred by alleles that are present on the nuclear genome. Until now, with the notable exception of the triazines, resistance to all herbicide classes is determined by nuclear inherited genes. However, in most species, triazine resistance has been

determined by cytoplasmic inheritance (Jasieniuk et al., 1994) where resistance alleles are encoded by the chloroplast genome. In nuclear inheritance, resistant alleles are transmitted through pollen and ovules, whereas transmission of the cytoplasmic resistance genes occurs through the ovules of the maternal parent, although a low level of transmission by pollen has been observed in a few species (Darmency and Gasquez, 1981). The sole exception to cytoplasmic inheritance of triazine resistance is found in *Abutilon theophrasti* (velvetleaf), where resistance is controlled by a single, partially dominant, nuclear gene (Anderson et al., 1993). Mostly resistance is controlled by a single major gene that can either be partially or completely dominant (Jasieniuk et al., 1996). However, multiple gene inheritance contributing to resistance has also been reported in glyphosate resistant *L. rigidum* populations from USA (Simarmata et al., 2005). In most weed species, when the resistance alleles occur on the nuclear genome, herbicide resistance is controlled by an allele(s) is at least partially dominant. An allele that is partially dominant will spread far faster within a population compared to the recessive allele, because heterozygotes will express at least part of the phenotype of the homozygote under selection pressure (Darmency and Gasquez, 1990).

1.3.2.4   Gene flow

Gene flow is the transfer of genes from one individual or population to another. Gene flow between and within plant populations occurs through two primary agencies: pollen dispersal and seed movement (Jasieniuk et al., 1996; Rieger et al., 1999). Gene flow together with the use of herbicides can play an important role in the spread of herbicides resistant populations. Resistance genes most likely arise in an area through mutation and are spread among individuals within that area by gene flow (Jasieniuk et al., 1996). Gene flow, through the movement of pollen or seed from resistant weed populations, may provide a source of resistance alleles to adjacent or nearby susceptible fields (Jasieniuk

and Maxwell, 1994). Seed movement between fields in agro-ecosystems due to agricultural implements can be extensive and dramatic in effect (Gardner, 1989) but, within fields, gene flow through natural seed movement may be relatively small (Shigematsu et al., 1989).

1.3.2.5 Fitness

Relative fitness of a genotype describes the ability of a one genotype to survive and produce offspring compared to other genotypes in the population as a result of natural selection (Maxwell and Mortimer, 1994).   When considering herbicide resistance, fitness may vary in the presence and absence of herbicides. Clearly, the fitness of an individual in the presence of herbicide considerably increases resistance alleles (Lande, 1983). According to Warwick and Black (1994), the relative fitness of individual plant also depends upon various biological as well as environmental conditions, such as genotype and population variation, intra and inter biotype competition and among environmental conditions temperature, light and management practices may also effect fitness. Fitness must be measured over the whole life cycle of a plant to encompass the effects of selection on mortality and seed production of survivors. Measurements of fitness are unlikely to be helpful for understanding the rate of resistance evolution or management of resistance unless they are conducted under field conditions with the crop with and without application of herbicide (Sanbagavalli et al., 2000).

1.3.3 Mechanisms of Resistance

Understanding the mechanisms of resistance is important in developing sound management strategies to reduce soil seed bank and intensity of resistant populations. Herbicide resistance in weeds can be due to a number of mechanisms: (1) modified target

site, (2) enhanced detoxification (metabolism), (3) reduced absorption/translocation, (4) sequestration or compartmentation and (5) gene amplification (Gronwald, 1994a; Heap, 2014). Mechanisms of resistance can be categorized into two groups: target site resistance and non-target site resistance (Preston et al., 2009; Yuan et al., 2007).

1.3.3.1 Target-site herbicide resistance

Target-site resistance is the result of a modification of the herbicide binding site, usually an enzyme, which precludes herbicides from effectively binding. This is the most common resistance mechanism and often the level of resistance is high. Sometimes, target-site based resistance can also occur which is due to over-production of herbicide-binding proteins.

1.3.3.1.1 Resistance to PSII - inhibiting herbicides

Photosynthetic electron transport is inhibited by a number of different herbicide classes, including the triazines, ureas and nitriles that block electron transport on the reducing side of photosystem II (Gronwald, 1994a). This blockage of electron flow leads to production of excess singlet oxygen, which results in destruction of lipids and chlorophyll (Powles and Yu, 2010). In most cases, triazine resistance is conferred by the amino acid substitution Ser-264-Gly (Gronwald, 1994a). However, this substitution (Ser-264-Gly) confers negligible resistance to phenyl urea herbicides (Devine and Shukla, 2000), whereas a slight variant Ser-264-Thr confers resistance to both triazines and urea herbicides (Masabni and Zandstra, 1999). In Australia, in many instances the intensive selection pressure from diuron and atrazine has resulted in selection for resistant *L. rigidum* to these herbicides and other photosystem II herbicides (Burnet et al., 1993a; Burnet et al., 1993b; Powles and Howat, 1990).

1.3.3.1.2 Resistance to Acetohydroxyacid synthase (AHAS/ALS) inhibiting herbicides

Acetohydroxyacid synthase also known as acetolactate synthase is the first enzyme in the biosynthetic pathway for the production of the branched-chain amino acids. These are the sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine, pyrimidinyloxybenzoates and sulfonyl-aminocarbonyl-triazolinone chemistries (Singh and Shaner, 1995). These herbicides stop synthesis of the branched-chain amino acids (valine, leucine and isoleucine) in susceptible plants (Kishore and Shah, 1988) and are often referred to as group B or group 2 herbicides. The site within the protein sequence where these changeovers occur in resistant weeds are Pro 197, Ala 205, Asp 376, Trp 574, Ser 653 and Ala 122 (Yu et al., 2008). So far, 157 weed species have evolved resistance to this group of herbicides, the most number of resistant weed species to any mode of action group (Heap, 2015).

1.3.3.1.3 Resistance to ACCase inhibiting herbicides

The ACCase-inhibiting herbicides inhibit the acetyl-coenzyme A carboxylase (ACCase) enzyme in monocotyledonous weeds. ACCase is the first dedicated enzyme in the biosynthetic pathway for lipid synthesis (Devine, 1997). There are two types of ACCase: prokaryotic and eukaryotic. Plants have both cytosolic and plastidic ACCase. In grasses the plastidic ACCase is homomeric and is target site for three herbicides classes (Tal and Rubin, 2004) arylophenoxypropanate (AHD) and cyclohexanedione (CHD) and pinoxaden (DEN). They are also known as group A or group 1 herbicides. Frequent use of ACCase herbicides for grass weed control has resulted in widespread evolution of resistance to ACCase herbicides (Heap, 2015). The target site resistance to ACCase herbicides is due to an amino acid substitution in the CT domain of the *ACCase* gene, which is the major cause of resistance to ACCase-inhibiting herbicides (Beckie and Tardif, 2012; Délye, 2005; Yu et al., 2007c). Seven target-site mutations (amino acid substitution) at positions 1781, 1999, 2027, 2041, 2078, 2088 and 2096 in the *ACCase* gene have been documented

to date in populations of several grass weed species (Beckie and Tardif, 2012; Kaundun, 2014). The mutation Ile-1781-Leu is associated with resistance to APP, some CHD (not to clethodim) and pinoxaden herbicides. The Trp-2027-Cys or Ile-2041-Asn mutations confer resistance to APP herbicides and pinoxaden. The Asp-2078-Gly mutation and Cys-2088-Arg mutation provides high-level resistance to all three classes of herbicides: APP, CHD and pinoxaden. The Gly-2096-Ala mutation confers resistance mainly to APP herbicides. The Trp-1999-Cys mutation confers resistance only to the APP herbicide fenoxaprop (reviewed by, Beckie and Tardif, 2012). Although selection of an altered ACCase through amino-acid substitution appears to be the most likely explanation for resistance, all mechanisms are possible. Resistance to ACCase herbicides has now been reported in 26 countries (Heap, 2015).

1.3.3.1.4 Resistance to EPSPS inhibiting herbicides

Glyphosate is by far the world's most widely used and important herbicide. It is non-selective systemic herbicide with no soil residual activity that is used for broad spectrum pre-seeding weed control. This herbicide is also widely used in fallows, or spot application or directed spray in crops or for broad application in genetically modified glyphosate tolerant crops (Powles et al., 1998). Glyphosate is an inhibitor of the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is responsible for the synthesis of the aromatic amino acids. Glyphosate has been widely used since 1974, yet glyphosate resistance evolution has been slow compared to several other herbicides (Powles, 2008).   Since first identified (Powles et al., 1998), glyphosate resistance has evolved in 29 weed species worldwide (Heap, 2015), and is now fast becoming a very significant problem globally (Powles, 2008). Resistance to glyphosate can be due to an alteration in the herbicide target site and also due to amplification of target-site gene: the genome of glyphosate resistant plants contained many more copies of the *EPSPS* gene

than that of glyphosate susceptible plants (Gaines et al., 2010). Glyphosate resistance can also be due to altered translocation of the herbicide in plants. In most cases, the resistance mechanism that endows glyphosate resistance in the majority of resistant weeds is altered translocation (Powles, 2008; Preston et al., 2009). Substitutions of Ser, Thr and Ala at Pro 106 have been found in *EPSPS* that results in glyphosate resistance in weeds (Sammons and Gaines, 2014; Yu et al., 2007a). Funke et al. (2009) found that a double mutation, Thr to Ile at position 97 and Pro to Ser at 101 in the *EPSPS* gene caused a glyphosate resistance in *Escherichia coli*. Similary, in recent studies, Yu et al. (2015) also reported double mutation (TIPS) in the *EPSPS* gene causing high level of glyphosate resistance in *Eleusine indica*.

1.3.3.1.5 *Resistance to inhibitors of photosynthesis in photosystem I*

The bipyridium herbicide, paraquat, the major photosystyem I inhibiting herbicide, is widely used for total vegetation control. In cropping systems, it is applied before planting annual crops, or during the dormant stage of perennial crops, or as a spray directed away from growing crops. It is active only when applied to foliage; it is not extensively translocated in plants and has no soil activity due to strong adsorption to soil colloids. So far, 31 weed species have evolved resistance to paraquat (Heap, 2015), including *L. rigidum* (Burnet et al., 1994). Paraquat disrupts the electron transport in photosystem I of photosynthesis in plants and subsequently increases the concentration of oxygen radicals, which destroy plant cell membrane and results in the death of plants (Preston, 1994). So far, two paraquat resistance mechanisms have been reported in plants. In most cases, resistance is due to reduced translocation of paraquat due to sequestration in plant tissues (Hawkes, 2014; Preston, 1994). In some other cases, paraquat resistance is due to detoxification of reactive oxygen (Fuerst and Vaughn, 1990; Preston, 1994).

1.3.3.2 *Non-target site resistance*

Non-target site resistance has been described due to rapid metabolism of a herbicide to non-toxic products. Non-target site resistance can be endowed by mechanisms such as enhanced metabolism, reduced rates of herbicide translocation, sequestration etc. Such mechanisms reduce the amount of herbicides reaching the target site (Powles and Yu, 2010). Weed species can be resistant to glyphosate by one or more of these mechanisms. Ferreira and Reddy (2000) showed that epicuticular waxes can reduce glyphosate absorption in some broadleaf species such as coca [*Erythroxylum coca* var. *coca* (Lam.)]. Previous research with glyphosate-resistant *L. rigidum*, exhibiting 10-fold glyphosate resistance (Pratley et al., 1999), found that neither uptake, translocation, nor metabolism was responsible for resistance (Feng et al., 1999). Subsequent studies showed no evidence for gene amplification or co-segregation of a specific *EPSPS* gene variant with glyphosate resistance, suggesting that the resistance mechanism may be non-target based (Baerson et al., 2002). Simarmata et al. (2005) provided evidence that difference in the sensitivity of EPSPS to glyphosate was a major contributor to glyphosate resistance in *L. rigidum* from California. Their research suggests that resistance to glyphosate is not due to reduced glyphosate uptake, glyphosate detoxification, or over expression of EPSPS, but due to reduced translocation (Feng et al., 2004).

1.3.3.3 *Herbicide detoxification –based resistance mechanisms*

Most plants have ability to metabolize some herbicides; some resistant plants have a greater ability to detoxify herbicides than susceptible plants. Various enzymes have been identified that are responsible for herbicide metabolism such as glutathione transferases, aryl acylamidases and the cytochrome P450. P450s are one of the largest superfamilies of enzymes and are found in almost all organisms, with plants having the highest number of P450 genes (Powles and Yu, 2010). The majority of examples of resistance due to herbicide metabolism are catalysed by cytochrome P450 monooxygenases. This is the

most common group of enzymes involved in the breakdown of different modes of action of herbicides in *L. rigidum* (Burnet et al., 1993a; Burnet et al., 1993b).

1.3.4 Clethodim Resistance in *L. rigidum*

The ACCase-inhibiting CHD herbicide clethodim is used post-emergent to control annual and perennial graminaceous weeds in broadleaf crops, as it is lethal to wheat and barley. This herbicide does not control broadleaf weeds or sedges (Devine and Shimabukuro, 1994). The selectivity of ACCase inhibiting herbicides is based on their effects at the herbicide target site - the plastidic ACCase that catalyzes the first committed step in *de novo* fatty acid biosynthesis (Burton et al., 1989; Gronwald et al., 1992). Unlike dicot plants in which the plastidic ACCase is a multisubunit protein complex, the *ACCase* gene in Gramineae is a single multifunctional enzyme encoded by one large nuclear gene and containing three distinct functional domains: a biotin-carboxylase (BC), a biotin-carboxyl carrier protein (BCCP) and a carboxyl-transferase (CT) (Gornicki et al., 1994; Konishi et al., 1996). This difference allows for the selective control of grasses, using classes of herbicides which specifically target the CT domain of the plastidic ACCase, inhibiting fatty acid biosynthesis and ultimately causing plant death (Sasaki et al., 1995; Herbert et al., 1996).

Most of the graminaceous weed species are susceptible to ACCase inhibiting herbicides, and the development of resistance to ACCase inhibiting herbicides in many cases is due to mutations in the CT domain of the *ACCase* gene leading to single amino acid substitutions in the plastidic ACCase. These changes reduce herbicide binding or interaction with ACCase, endowing resistance in many grass weed populations investigated (Marles et al., 1993; Shukla et al., 1997; Tal et al., 2000; Tal and Rubin, 2004). Metabolism-based resistance to ACCase inhibitors has been identified in a comparatively small number of weeds species (Bravin et al., 2001; Hidayat and Preston, 1997; Vila-Aiub et al., 2005) but

the level of resistance resulting from enhanced metabolism is usually relatively low compared with that of altered target site resistance.

In Australia, due to resistance to other post-emergent herbicides in *L. rigidum*, clethodim has become the most important grass herbicide for broadleaf crops. However, clethodim-resistant *L. rigidum* populations have appeared over the last two decades and are now present on many farms across the southern Australian wheat belt. Clethodim is the lowest resistance risk ACCase inhibiting herbicide, with only two of eleven target-site mutations in weed populations that confer resistance to this herbicide (reviewed by Beckie and Tardif (2012). Aspartate-2078-glycine and cystine-2088-arginine mutations in the plastidic ACCase enzyme have been identified as the main mutations that confer clethodim resistance at field rates of herbicide application (Kaundun, 2014; Yu et al., 2007b). Delye et al. (2008) reported that Leucine-1781, Glycine 2078 and Alanine-2096 mutations in *ACCase* gene may also confer resistance to clethodim in the field if the conditions are not optimal for herbicide efficacy, or at reduced clethodim use rates.

1.3.5 Management of Herbicide Resistance

The adoption of no-till in Australian cropping systems has increased reliance on herbicides for the control of various weed species, which may have increased the rate of evolution of herbicide resistant populations. The combination of various factors like high genetic diversity and cross pollination in *L. rigidum* populations has allowed this species to evolve resistance to eleven different modes of action of herbicides (Powles et al., 1996). Increasingly, multiple resistance across many herbicide chemistries is severely reducing herbicide options available for the control of *L. rigidum* in crop-production systems (Walsh and Powles, 2007). The sustainability of the existing and any future herbicide resources will be much improved with the development of effective alternative weed control technologies.

Effective management of herbicide resistance in weeds depends on reducing selection pressure for the evolution of resistance (Gressel and Segel, 1990; Maxwell, 1992), which of necessity involves reducing the frequency and amount of herbicide applied and increasing reliance on integrated practices. Crop rotations or competitive cover crops, where economically feasible, could be employed. The weed spectra associated with different crops differ due to differences in the competitiveness and life cycles of different crops. Rotations of crop may also permit the use of different herbicides.

The use of herbicide rotations are often advocated for resistance prevention and management. However, herbicide rotations and mixtures should comprise compounds from different classes with dissimilar modes of action that control the spectrum of weeds. They should have the same persistence in the environment, and be degraded in different ways. The idea behind the use of herbicide mixtures or rotation is to lessen the occurrence of resistant individuals in a weed population to a very low frequency equalling their initial frequencies of herbicide resistance for each compound used in mixtures or rotations before they were ever used.

1.4   Summary and Knowledge Gaps

During the last half century, herbicides have become the dominant method of weed control in most agricultural areas worldwide and the intensive use of herbicides has resulted in the evolution of resistant weeds to many herbicides with different modes of action across the world. *L. rigidum* is one of the most troublesome herbicide resistant weeds in Australia (Jones et al., 2005; Pannell et al., 2004). It is an obligate cross pollinated species, which has so far evolved resistance to herbicides with eleven different modes of action (Heap, 2015). Clethodim, an ACCase inhibitor, is a selective post-emergence herbicide used to control annual and perennial grasses in a wide variety of broadleaf crops (Burke et al., 2004; Burke and Wilcut, 2003b). Clethodim has the ability

to control *L. rigidum* populations with resistance to other ACCase-inhibiting herbicides and has been used to provide effective control of herbicide-resistant *L. rigidum* and allowed many farmers to continuously grow crops in their fields (Boutsalis et al., 2012). However, repeated use of this herbicide has resulted in the evolution of clethodim resistance in *L. rigidum* populations (Boutsalis et al., 2012; Yu et al., 2007b), making weed control even more challenging. The levels of resistance to clethodim vary among *L. rigidum* populations, with farmers increasing rates of use to gain control of some resistant populations. Understanding the mechanisms, inheritance of resistance, and the fitness of resistant alleles is necessary for the formulation of strategies to slow down the rate of evolution of clethodim resistance and to manage resistant weeds. Therefore, the work presented in this thesis is designed to address the following objectives:

i.   Biochemical and genetic characterization of clethodim-resistant *L. rigidum* populations.

ii.   To study the effect of environmental factors such as frost on the efficacy of clethodim on clethodim-resistant *L. rigidum* populations.

iii. To identify the inheritance of resistance in clethodim-resistant populations.

iv.   To determine the relative fitness of clethodim-resistant alleles.

v.   To investigate alternative herbicide options for the control of clethodim-resistant *L. rigidum* in faba bean and canola crops.

1.5   Outline of the Thesis

The starting point of this study was to investigate clethodim resistance in *L. rigidum* populations collected from different parts of Australia, where clethodim had provided inadequate control of *L. rigidum* at the recommended field rate. For this purpose, pot experiments were conducted at the University of Adelaide. The next chapter (Chapter 2) details the level of clethodim and butroxydim resistance in *L. rigidum* populations which

is followed by investigations of the mechanisms endowing clethodim resistance. The effect of frost on the efficacy of clethodim in clethodim-resistant *L. rigidum* populations is covered in Chapter 3. Chapter 4 presents the mode of inheritance of clethodim resistance in *L. rigidum* populations. The change in the frequency of clethodim mutant alleles in two generations of *L. rigidum* in the absence of clethodim use, to determine relative fitness is presented in Chapter 5. The results of field experiments conducted to investigate the alternative herbicide options for the control of clethodim-resistant *L. rigidum* in faba bean and canola is discussed in Chapters 6, 7 and 8. The thesis concludes in Chapter 9 where the main findings are summarized, and the conclusions from the research are presented, followed by recommendations for future research.

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CHAPTER 2

TARGET ENZYME-BASED RESISTANCE TO CLETHODIM IN *LOLIUM RIGIDUM* POPULATIONS IN AUSTRALIA

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Overall percentage (%)	85%
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Target Enzyme-Based Resistance to Clethodim in *Lolium rigidum* Populations in Australia

Rupinder Kaur Saini, Jenna Malone, Christopher Preston, and Gurjeet Gill*

Clethodim resistance was identified in 12 rigid ryegrass populations from winter cropping regions in four different states of Australia. Clethodim had failed to provide effective control of these populations in the field and resistance was suspected. Dose–response experiments confirmed resistance to clethodim and butroxydim in all populations. During 2012, the LD_{50} of resistant populations ranged from 10.2 to 89.3 g ha^{-1}, making them 3 to 34–fold more resistant to clethodim than the susceptible population. Similarly, GR_{50} of resistant population varied from 8 to 37.1 g ha^{-1}, which is 3 to 13.9–fold higher than the susceptible population. In 2013, clethodim-resistant populations were 7.8 to 35.3–fold more resistant to clethodim than the susceptible population. The higher resistance factor in 2013, especially in moderately resistant populations, could have been associated with lower ambient temperatures during the winter of 2013. These resistant populations had also evolved cross-resistance to butroxydim. The resistant populations required 1.3 to 6.6–fold higher butroxydim dose to achieve 50% mortality and 3 to 27–fold more butroxydim for 50% biomass reduction compared to the standard susceptible population. Sequencing of the target-site ACCase gene identified five known ACCase substitutions (isoleucine-1781-leucine, isoleucine-2041-asparagine, aspartate-2078-glycine, and cysteine-2088-arginine, and glycine-2096-alanine) in these populations. In nine populations, multiple ACCase mutations were present in different individuals. Furthermore, two alleles with different mutations were present in a single plant of rigid ryegrass in two populations.

Nomenclature: Clethodim; rigid ryegrass; *Lolium rigidum* Gaudin.

Key words: ACCase gene, butroxydim, clethodim, resistance mechanism, target-site mutation.

Rigid ryegrass is the most important grass weed of winter grain crops in Australia (Jones et al. 2005). It is an obligate outcrossing species with high genetic diversity (Charmet et al. 1996). As a result of its adaptation to climate, extensive planting as a pasture species, and prolific seed production (Rerkasem et al. 1980), it is present in high numbers across the southern Australian grain belt (Neve and Powles 2005). Over the past few decades, herbicides have become the most common method for the control of rigid ryegrass in crops. Because of over reliance on herbicides, this grass species has evolved resistance to at least nine different modes of action of herbicides (Heap 2015). Rigid ryegrass has many features that contribute to the evolution of resistance, including a short soil seed-bank life, a propensity to occur in large densities, high genetic diversity within populations, and self-incompatibility (Gill 1996; Gramshaw 1972). The most important classes of herbicides used in the control of rigid ryegrass are the aryloxyphenoxypropionate (APP/fops), cyclohexane-

diones (CHD/dims) (Délye 2005), and phenylpyraxoline (PPZ/dens) (Hofer et al. 2006; Muehlebach et al. 2009), which act specifically on grass weeds by inhibiting the acetyl-coenzyme A carboxylase (ACCase; EC 6.4.1.2) enzyme (Harwood 1988).

ACCase catalyzes the first committed step in de novo fatty acid biosynthesis in plants, which is essential for the production of cell membranes and secondary plant metabolites (Gronwald et al. 1992). In grasses, ACCase-inhibiting herbicides inhibit the homomeric plastidic ACCase; however, in most dicotyledonous species, their heteromeric chloroplastic ACCase is insensitive to ACCase-inhibiting herbicides. This allows these herbicides to be used for grass control in dicotyledonous crops (Devine 1997; Powles and Yu 2010). ACCase-inhibiting herbicides have been used extensively worldwide to control grass weed species (Devine and Shimabukuro 1994) and this frequent and widespread use has resulted in the evolution of resistance in many grass weed species including blackgrass (*Alopecurus myosuroides* Huds.), rigid ryegrass and wild oat (*Avena fatua* L.) (Heap 2015). Resistance to ACCase herbicides can occur because of insensitivity of target enzyme, sequestration of the herbicide away from the target site, increased rate of herbicide detoxification, or decreased rate of herbicide activation (Devine 1997),

and also because of an over-production of ACCase activity (Bradley et al. 2009). Target-site resistance results from a single amino acid change in the ACCase gene, which is the major cause of resistance to ACCase-inhibiting herbicides. Seven target-site mutations (amino acid substitution) at positions 1781, 1999, 2027, 2041, 2078, 2088, and 2096 in the ACCase gene have been documented to date in populations of several grass weed species (Beckie and Tardif 2012; Cruz-Hipolito et al. 2012). The mutation Ile-1781-Leu is associated with resistance to APP, some CHD (not to clethodim) and pinoxaden herbicides. The Trp-2027-Cys or Ile-2041-Asn mutations confer resistance to APP herbicides and pinoxaden. The Asp-2078-Gly mutation and Cys-2088-Arg mutation provides high-level resistance to all three classes of herbicides: APP, CHD, and pinoxaden. The Gly-2096-Ala mutation confers resistance mainly to APP herbicides. The Trp-1999-Cys mutation confers resistance only to the APP herbicide fenoxaprop (Beckie and Tardif 2012).

Clethodim, a CHD herbicide, is a selective POST herbicide typically used to control annual and perennial grasses infesting dicot crops (Burke et al. 2004). Depending on the mutations, clethodim has the ability to control rigid ryegrass populations with resistance to other ACCase-inhibiting herbicides and has been used extensively in Australia for the control of rigid ryegrass (Boutsalis et al. 2012). This has resulted in the evolution of clethodim resistance in rigid ryegrass populations in Australia (Yu et al. 2007), with as many as 60% of the fields across southeastern Australia having clethodim resistance (Boutsalis et al. 2012). Butroxydim became available several years later than clethodim and is sometimes used for the control of ACCase resistant rigid ryegrass (Yu et al. 2007). In an effort to achieve acceptable control of such populations, farmers have increased the clethodim dose over time, because many of the populations could be controlled with higher rates of clethodim. The objective of this study was to quantify the level of resistance to clethodim and a related herbicide butroxydim in 12 rigid ryegrass populations and to determine whether resistance in these populations was associated with mutations in the ACCase enzyme.

Materials and Methods

Plant Material. Twelve rigid ryegrass populations used in this study (Table 1) were collected from different farms in South Australia, Western

Table 1. Location of collection sites for *Lolium rigidum* populations investigated for clethodim resistance.

Population	Population code	Geographical location
SLR4	S1	Bordertown, SA (36.3°S, 140.7°E)
VLR1	S2	Serviceton, VIC (36.36°S, 140.98°E)
A1207.1	R1	Wubin, WA (30.1°S, 116.63°E)
A61	R2	Kellerberrin, WA (31.62°S, 117.77°E)
A52	R3	Ultima, VIC (35.48°S, 143.25°E)
A58	R4	Kellerberrin, WA (31.62°S, 117.65°E)
A1209.1	R5	Geraldton,WA (28.77°S, 114.60°E)
A48	R6	Cummins, SA (34.25°S, 135.73°E)
A1240.2	R7	Brocklesby, NSW (35.8°S, 146.41°E)
A91	R8	Moora, WA (30.63°S, 116.01°E)
A1264.1	R9	Lockhart, NSW (35.21°S, 146.71°E)
A370.1	R10	Booleroo Centre, SA (32.88°S, 138.35°E)
A56	R11	Bruce Rock, WA (31.881°S, 118.148°E)
A615	R12	Yendon, VIC (37.63°S, 143.96°E)
L739	R13	Geraldton, WA (28.77°S, 114.6°E)

Australia, Victoria, and New South Wales, where clethodim at $36-120$ g a.i. ha^{-1} had provided inadequate control of rigid ryegrass in the field and resistance was suspected. Seed from a large number of plants that had survived clethodim application in the field was collected during 2010 and 2011. Two known herbicide-susceptible ryegrass populations, SLR4 and VLR1 (S), hereinafter referred as S1 and S2 (McAlister et al. 1995; Wakelin and Preston 2006), and one resistant population R13 (Boutsalis et al. 2012), were used as standards.

Seed Germination and Plant Growth. Seeds of resistant and susceptible populations were germinated on 0.6% (w/v) agar and incubated in a germination cabinet with 12 h light and 12 h dark periods with 30 μmol m^{-2} s^{-1} at 20 C/15 C temperatures (Lorraine-Colwill et al. 2001). After 7 days, seedlings at the one-leaf stage were transferred to 9.5 cm by 8.5 cm by 9.5 cm punnet pots (Masrac Plastics, South Australia, Australia) containing cocoa peat potting mix (Boutsalis et al. 2012) with a density of nine seedlings per pot. There were three replicates for each herbicide dose and pots were arranged in a randomized complete block design. Two dose–response experiments were conducted outdoors under natural conditions during May 2012 and June 2013. Plants were watered and fertilized as needed. The mean monthly temperature data for both the growing seasons is presented in Table 2 (source: Australian Bureau of Meteorology).

Dose−Response Experiments. At the two to three–leaf stage, rigid ryegrass seedlings were treated with clethodim (Select®, 240 g L^{-1} clethodim,

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Table 2. Average maximum and minimum monthly temperature (C) at Waite Campus, University of Adelaide, Urrbrae, SA, Australia, during the growing seasons of 2012 and 2013.

| | Temperature | | | |
| | Year 2012 | | Year 2013 | |
Month	Maximum	Minimum	Maximum	Minimum
		C		
April	24.2	12.6	23.8	13.3
May	18.2	9.3	20.6	12.3
June	15.3	7.9	16.4	8.6
July	15.3	7.8	16.2	9.1
August	15.9	7.7	17.2	9.1

Sumitomo) or butroxydim (Factor®, 250 g kg^{-1} butroxydim, Crop Care). Clethodim was applied at 0, 7.5, 15, 30, 60, and 120 g ha^{-1} for S plants, and 0, 30, 60, 120, 240, and 480 g ha^{-1} for R plants. Butroxydim was applied to both resistant and susceptible populations at 0, 5.6, 11.3, 22.5, 45, and 90 g ha^{-1}. The recommended dose of clethodim in Australia is 60 g ha^{-1} and for butroxydim 45 g ha^{-1}. As recommended by the herbicide labels, ethyl, and methyl esters of vegetable oil (Hasten, Victorian Chemicals) at 1% (v/v) were added to clethodim and 1% (v/v) paraffin oil (Supercharge, Crop Care) was added to butroxydim. The herbicides were applied by using a laboratory moving boom sprayer equipped with twin nozzles (Tee-Jet 1100 Flat-Fan Spraying Systems, Wheaton, IL). The output volume of the sprayer was 103 L ha^{-1} at a pressure of 250 kPa and a speed of 1 m s^{-1}. Control plants were not treated with herbicide. Plants were returned and maintained outdoors after the treatment. Three weeks after spraying, the surviving plants were counted. Plants were recorded as alive if they had tillered and produced new leaves since herbicide application, and plants showing severe chlorosis, stunting, and mortality were considered as susceptible (Powles et al. 1998). The surviving plants were harvested and oven dried at 70 C for 2 d. The dry weight data were expressed as a percentage of the respective unsprayed control. Data (survival and dry weight) was analyzed by using a log-logistic equation

(Graphpad Prism v.6.0; GraphPad Software, San Diego, CA) and the dose of herbicide required to kill 50% of the plants (LD$_{50}$) and causing 50% growth reduction of plants (GR$_{50}$) with respect to the untreated control were calculated for each population, and R/S ratio was computed as LD$_{50}$ (R)/LD$_{50}$ (S). The model fitted was

$$y = 100 \Big/ \left[1 + 10^{(\log \mathrm{IC}_{50} - x) \times b} \right],$$

where y is the plant survival (%) or biomass reduction (%), x is the log-dose of the herbicide used, IC$_{50}$ is the dose of herbicide required to produce 50% reduction in plant survival or biomass, and b is the slope of the curve.

Sequencing of ACCase Gene. Fresh leaf material ($\sim$1 cm^2) was harvested from young leaves of at least 12 surviving plants from each resistant population, snap frozen in liquid nitrogen and stored at -20 C until its use. DNA was extracted with the use of the DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions. Primers were designed to amplify regions in the carboxyl tranferase (CT) domain known to be involved in sensitivity to ACCase herbicides. Two sets of primers covering all seven known mutations sites (1781, 1999, 2027, 2041, 2078, 2088, and 2096) were designed against the blackgrass (accession number AJ310767) plastidic ACCase gene sequence (Table 3) and used to amplify a 1.5-kb fragment covering nearly the entire CT domain without any intron. The range of amino acids covered by the fragment was equivalent to codons 1658–2157 in blackgrass. A nested PCR approach was employed with oligo set Acclr9 and Acclr6 (Zhang and Powles 2006) followed by oligo set AccCT 2F and AccCT 2R (Malone et al. 2014). MyFi^{TM} DNA polymerase kit (Bioline, Australia Pty Ltd., Alexandria, NSW, 1435) was used to run PCR reactions of 25 µl containing 80 to 100–ng DNA template, 1x MyFi reaction buffer, 0.8 µM of each specific primer and two units of MyFi DNA Polymerase (high fidelity *Taq*). Amplification was carried out in an automated DNA thermal cycler

Table 3. Primers sequences used for amplification and sequencing of the CT domain of the ACCase gene in *Lolium rigidum* from genomic DNA.

Primer	Sequence 5′ 3′	Use
Acclr9	ATGGTAGCCTGGATCTTGGACATG	Amplification (run 1)
Acclr6	GGAAGTGTCATGCAATTCAGCAA	Amplification (run 1)
AccCT 2F	CCACTCCTGAATTTCCCAGTGG	Amplification (run 2)
AccCT 2R	CGCGATTTGAGTGTACAAAGGCTG	Amplification (run 2)
AccCT MidF	CCTGAGAATACATGTGATCCTCGTG	Sequencing
AccCT MidR	CCATTTCCCTTGGCTGTCATCAATGCC	Sequencing

Table 4. The dose of clethodim and butroxydim required for 50% mortality (LD_{50}) of resistant and susceptible *Lolium rigidum* populations with confidence intervals in parentheses. R/S is the ratio of LD_{50} of resistant and susceptible populations.[a]

Population	LD_{50} (g ai ha^{-1})								Resistance value[b]
	Experiment 1 (May 2012)	R/S	Experiment 2 (June 2013)	R/S	Experiment 1 (May 2012)	R/S	Experiment 2 (June 2013)	R/S	
	Clethodim				Butroxydim				
S1	2.7 (2.6, 2.8)	–	–	–	2.3 (2.2, 2.3)	–	–	–	–
S2	–	–	2.7 (2.6, 2.8)	–	–	–	5.5 (4.5, 6.7)	–	–
R3	10.2 (4.5, 22.9)	3.8	27.9 (22.3, 35.0)	10.5	3.9 (2.8, 5.4)	1.7	15.9 (14.2, 17.8)	2.9	7.2 (M)
R7	26.5 (22.1, 31.7)	9.9	20.2 (13.2, 32.3)	7.8	–	–	22.9 (19.4, 26.9)	4.2	8.9 (M)
R9	16.5 (10.2, 26.5)	6.2	39.2 (36.4, 42.2)	14.7	3.2 (2.1, 4.9)	1.4	20.1 (15.2, 26.5)	3.7	10.5 (M)
R10	31.3 (24.0, 40.7)	11.8	34.5 (29.4, 40.4)	13.0	–	–	30.5 (23.4, 39.6)	5.5	12.4 (H)
R6	18.2 (7.2, 45.5)	6.8	53.2 (44.3, 64.0)	20.0	6.2 (5.2, 7.3)	2.7	29.5 (18.2, 48.1)	5.4	13.4 (H)
R1	27.0 (23.2, 31.4)	10.2	44.8 (38.3, 52.5)	16.8	5.7 (5.1, 6.5)	2.5	26.3 (16.0, 43.5)	4.8	13.5 (H)
R12	–	–	37.1 (32.8, 41.9)	13.9	–	–	32.6 (23.1, 46.1)	5.9	13.9 (H)
R4	33.5 (23.9, 47.0)	12.6	40.2 (36.3, 44.6)	15.1	6.3 (5.3, 7.5)	2.7	17.9 (14.6, 22.0)	3.3	13.9 (H)
R2	36.0 (30.0, 43.0)	13.5	38.5 (34.7, 42.7)	14.5	3.1 (1.7, 5.4)	1.3	29.0 (25.5, 33.0)	5.3	14.0 (H)
R11	34.0 (27.8, 41.5)	12.8	46.9 (19.7, 111.1)	17.6	3.7 (2.6, 5.1)	1.6	21.6 (15.8, 30.0)	3.9	15.2 (H)
R13	29.1 (18.5, 45.7)	10.9	71.2 (59.8, 84.7)	26.8	5.2 (4.4, 6.1)	2.2	36.5 (14.2, 94.1)	6.6	18.9 (H)
R5	59.8 (42.9, 83.2)	22.5	72.3 (60.6, 86.1)	27.2	5.2 (4.4, 6.1)	2.2	27.9 (20.9, 37.4)	5.1	24.9 (H)
R8	89.3 (68.8, 115.8)	33.6	95.3 (69.2, 131.5)	35.3	6.3 (5.3, 7.5)	2.7	22.5 (16.4, 30.8)	4.1	34.5 (H)

[a] Abbreviations: L, low resistance (2–6); M, moderate resistance (> 6–12); H, high resistance (> 12).

[b] Resistance value presented for clethodim is the average of R/S ratio of the two experiments.

(Eppendorf Mastercycler® Gradient, Germany) with PCR conditions as follows: 3 min denaturing at 94 C; 35 cycles of 30 s denaturation at 94 C, 30-s annealing at 56 C, and 2 min elongation at 68 C, and a final extension for 7 min at 68 C.

The PCR products were examined on 1.5% agarose gels stained with 1x SYBR® Safe DNA gel stain. Samples were electrophoresed in 1x TAE Buffer (40 mM Trizma base, 1 mM Na$_2$EDTA, pH to 8 with glacial acetic acid) at 90 volts and photographed under UV light (λ302 nm). DNA fragment sizes were estimated by comparing their mobility to bands of known sizes in a low-mass molecular weight marker (Invitrogen, Australia). PCR products were sequenced in reverse and forward directions by Australian Genome Research Facility (AGRF) Ltd., Australia, with the use of primers AccCT Mid F and AccCT Mid R (Table 3) to obtain sequence data covering the full CT domain fragment. The DNA sequence data were assembled, compared, and analyzed with the use of ContiExpress from the Vector-NTi Advance 11.5 programs (Invitrogen) and all sequences visually rechecked using the chromatogram files.

Results and Discussion

Dose–Response Experiments. Dose–response experiments confirmed resistance to both clethodim and butroxydim in all the rigid ryegrass populations tested (Table 4). In both experiments (2012 and 2013), the plants of S populations S1 and S2 were completely killed by clethodim at the recommended rate of 60 g ha^{-1} and even at lower rates, while the known resistant population R13 had little or no mortality at this rate. All the putative resistant populations showed some survival at the recommended field rate (data not shown).

The clethodim rate required for 50% mortality (LD_{50}) for the S population was 2.7 g ha^{-1} in both the experiments (Table 4). The LD_{50} for the known resistant population R13 was 29.1 g ha^{-1} in 2012 and 71.2 g ha^{-1} in 2013, giving resistance levels (R/S) 10.9- and 26.8-fold higher than the S population. The resistance level to the herbicides for the resistant populations has been categorized according to the R/S index of LD_{50} values and populations are classified as having high resistance (> 12), moderate resistance (> 6 to 12), and low resistance (2 to 6) (Ahmad-Hamdani et al. 2012). The 12 putative resistant populations showed varying levels of resistance to clethodim. In Experiment 1, the LD_{50} of resistant populations ranged from 10.2 to 89.3 g ha^{-1}, making them 3 to 34–fold more resistant to clethodim than the S population, S1. However, higher doses of clethodim were required to control the resistant populations in Experiment 2. During 2013, all the populations except R3 and R7 were found to exhibit a high level of resistance to clethodim. The clethodim LD_{50} values of resistant populations ranged from 20.2 to 95.3 g ha^{-1}, making them 10 to 36−fold more resistant than the S population, S2. In

Table 5. The dose of clethodim and butroxydim required for 50% biomass reduction (GR_{50}) of resistant and susceptible *Lolium rigidum* populations with confidence intervals in parentheses. R/S is the ratio of GR_{50} of resistant and susceptible population.[a]

Population	GR_{50} (g ai ha^{-1})								Resistance value[b]
	Experiment 1 (May 2012)	R/S	Experiment 2 (June 2013)	R/S	Experiment 1 (May 2012)	R/S	Experiment 2 (June 2013)	R/S	
	Clethodim				Butroxydim				
S1	2.7 (2.6, 2.7)	–	–	–	2.3 (2.3, 2.4)	–	–	–	–
S2	–	–	2.7 (2.6, 2.7)	–	–	–	2.3 (2.3, 2.4)	–	–
R3	8.1 (4.3, 19.0)	3.1	12.7 (4.0, 40.6)	4.8	3.4 (2.3, 4.8)	1.5	16.9 (13.0, 22.0)	7.3	4.0 (L)
R7	21.4 (15.7, 29.3)	8.1	14.2 (8.8, 22.9)	5.4	–	–	24.7 (20.3, 30.1)	10.7	6.8 (M)
R9	8.0 (3.2, 20.0)	3.0	29.8 (21.2, 42.0)	11.2	3.1 (1.7, 5.4)	1.3	17.3 (12.9, 23.3)	7.5	7.1 (M)
R10	19.7 (12.0, 32.1)	7.4	18.9 (10.2, 35.2)	7.1		–	39.5 (33.6, 46.5)	17.2	7.3 (M)
R6	18.9 (8.8, 40.5)	7.1	47.2 (35.2, 63.2)	17.7	4.7 (3.5, 6.1)	2.0	39.7 (19.8, 79.4)	17.3	12.4 (H)
R1	15.4 (11.8, 20.0)	5.8	35.0 (27.7, 44.2)	13.2	5.0 (3.6, 7.0)	2.2	16.1 (9.2, 28.2)	7.0	9.5 (M)
R12	–	–	31.9 (27.6, 36.7)	12.0	–	–	32.0 (20.4, 50.0)	13.9	12.0 (M)
R4	19.2 (12.0, 30.8)	7.2	39.5 (26.8, 58.3)	14.9	3.9 (3.5, 4.3)	1.7	19.4 (15.6, 24.2)	8.4	11.1 (M)
R2	26.0 (18.6, 36.3)	9.8	19.7 (11.2, 35.0)	7.4	2.9 (0.7, 11.3)	1.3	15.1 (10.3, 22.0)	6.6	8.6 (M)
R11	17.4 (13.4, 22.5)	6.5	15.4 (5.6, 42.3)	5.8	5.1 (2.9, 8.7)	2.2	28.4 (13.2, 61.2)	12.3	6.2 (M)
R13	16.5 (8.9, 30.5)	6.2	42.6 (30.5, 59.5)	16.0	4.1 (3.5, 4.9)	1.8	54.7 (16.6, 180.5)	23.8	11.1 (M)
R5	24.1 (12.7, 45.8)	9.1	39.5 (26.8, 58.3)	14.9	4.3 (3.0, 6.3)	1.9	62.0 (25.9, 148.3)	27.0	12.0 (M)
R8	37.1 (28.9, 47.6)	13.9	72.9 (51.9, 102.4)	27.4	4.5 (3.8, 5.2)	1.9	13.9 (8.8, 22.0)	6.0	20.7 (H)

[a] Abbreviations: L, low resistance (2–6); M, moderate resistance (> 6–12); H, high resistance (> 12).
[b] Resistance value presented for clethodim is the average of R/S ratio of the two experiments.

both experiments, R8 was rated as the most resistant population having an LD_{50} of 89.3 and 95.3 g ha^{-1}, which is considerably greater than the recommended field rate and had a resistance level 34 to 36 times higher than the S populations.

Resistant populations showed variable levels of biomass reduction and GR_{50} varied from 8 to 37.1 g ha^{-1}, which is 3 to 13.9–fold higher than that of the S population in Experiment 1 (Table 5). In Experiment 2, clethodim at the recommended field rate (60 g ha^{-1}) was found to reduce the growth of all resistant populations except R8 population which did not substantially reduce the shoot dry weight at this rate. The lowest GR_{50} of clethodim for the resistant populations was observed in R3 (4.8-fold greater than the S population), whereas the highest GR_{50} (27-fold) was recorded in population R8 (Table 5). Varying levels of clethodim resistance have previously been observed in populations of rigid ryegrass (Broster et al. 2011; Yu et al. 2007), Italian ryegrass [*Lolium perenne* ssp. *multiflorum* (Lam.) Husnot] (Martins et al. 2014), wild oat spp. from the Western Australian grain belt (Ahmad-Hamdani et al. 2012; Owen and Powles 2009) and in an Asia minor bluegrass (*Polypogon fugax* Nees ex Steud.) population from China (Tang et al. 2014). This variation in the resistance level in rigid ryegrass populations may be associated with different ACCase mutations and/or presence of different resistance mechanisms because of the high genetic variation within the populations of this species.

Cross-resistance to butroxydim varied among the populations. As expected, the S population was killed at the recommended butroxydim field rate of 45 g ha^{-1}, with a LD_{50} of 2.3 and 5.5 g ha^{-1} in 2012 and 2013, respectively (Table 4). In Experiment 1, the butroxydim LD_{50} values for the resistant populations ranged from were 3.1 to 6.3 g ha^{-1}, making them 1.3 to 2.7–fold more resistant than the S populations. A higher dose of butroxydim was required to control all the resistant populations in Experiment 2 than in Experiment 1. Resistant populations had butroxydim LD_{50} values 2.9 to 6.6–fold higher than the S population in Experiment 2 (Table 4). Similarly, in Experiment 1, butroxydim was found to reduce the growth of all resistant populations effectively. The GR_{50} values for butroxydim for the resistant populations were 1.3 to 2.4–fold greater than the S population (Table 5) in 2012. The GR_{50} of all resistant populations was considerably higher in 2013 than 2012. The GR_{50} in 2013 varied from 13.9 to 62 g ha^{-1}, which was 6 to 27–fold greater than for the S population (2.3 g ha^{-1}) (Table 5).

Previous research has shown that some clethodim-resistant populations of rigid ryegrass with different ACCase target-site mutations have cross resistance to the CHD herbicide butroxydim (Yu et al. 2007). The level of resistance to butroxydim

Table 6. Comparison of nucleotide sequence and derived amino acid sequence of highly conserved region of the ACCase enzyme from susceptible and resistant populations of *Lolium rigidum*. Twelve plants per population were sampled.

Amino acid number	1781	2041	2078	2088	2096	
Amino acid	Ile	Ile	Asp	Cys	Gly	
Consensus sequence	ATA	ATT	GAT	TGC	GGG	Non-mutated plants
S1	–	–	–	–	–	–
R1	TTA (3)	AAT (2)	–	CGC (1)	–	6
R2	–	–	GGT (7)	–	–	5
R3	TTA (6)	–	GGT (1)	–	–	5
R4	TTA (2)	–	GGT (5)	–	–	5
R5	–	–	GGT (7)	–	–	5
R6	TTA (4)	AAT (2)	GGT (1)	–	–	5
R7	–	AAT (4)	GGT (1)	CGC (2)	–	5
R8	–	AAT (1)	GGT (6)	–	–	5
R9	–	AAT (5)	–	–	–	7
R10	CTA (4)	AAT (2)	GGT (1)	CGC (1)	GCG (1)	3
R11	CTA (1)	–	GGT (4)	CGC (4)	–	3
R12	–	AAT (1)	–	–	–	11
R13	TTA (4)	–	GGT (1)	–	–	7

[a] Dashes indicate identical codon to the consensus sequence. Figures in parentheses are number of individuals in which specific mutation(s) were identified. Samples without identified mutations are suspected as having non–target-site resistance mechanism(s).

is not high and some of these populations can still be controlled by using higher rates of butroxydim, if applied under optimum conditions. The variation in resistance levels between experiments might be due to the difference in environmental conditions. When these CHD herbicides were applied during the warmer conditions of autumn (May) in 2012 (Table 2), resistant populations showed a much lower level of resistance (LD_{50} and GR_{50}) than in cooler conditions (June) in 2013. Some previous studies also suggest that ACCase-inhibiting herbicides work better in warmer conditions, which results in greater herbicide efficacy (Johnson et al. 2002; Kells et al. 1984).

Sequencing of ACCase Gene. Target-site mutations for clethodim resistance were identified in all resistant populations of rigid ryegrass. The nucleotide sequences of the resistant populations differed from that of the susceptible population as shown in Table 6. Sequencing results revealed five known ACCase mutations (isoleucine-1781-leucine, isoleucine-2041-asparagine, aspartate-2078-glycine, cysteine-2088-arginine, and glycine-2096-alanine) in these populations (Table 6). Among 12 clethodim-resistant populations tested, Asp-2078-Gly mutation was found in clethodim survivors of 10 populations and was the most common mutation. Ile-1781-Leu and Ile-2041-Asn mutations were detected in seven populations, whereas Cys-2088-Arg was identified in four populations and Gly-2096-Ala in only one

population (Table 6). In nine populations, multiple ACCase mutations were present within a single population (Table 6). Furthermore, in populations R4 and R10 individual plants contained two separate mutations 1781/2078 and 1781/2096, respectively. Populations with both single and multiple amino acid substitutions had a similar level of resistance to clethodim, which can be clearly seen in the LD_{50} values of resistant populations (Table 4). In all populations tested, out of 12 individuals some resistant individuals had no known ACCase mutations and are suspected of having non-target-site resistance mechanism(s). Previous research has shown that the presence of a single ACCase mutation provides low-level resistance to ACCase inhibiting herbicides and resistance-level increases if there are multiple ACCase mutations (Yu et al. 2007). Conversely, we found that multiple mutations did not provide an additive effect on the level of resistance, which could be caused by homo/heterozygous status of plants for a specific mutation. Rigid ryegrass is a widespread obligate outcrossing species with high genetic diversity (Preston et al. 1999), so multiple mutations within the population and different mutant alleles in the single resistant plant can be expected (Malone et al. 2014; Yu et al. 2007). Six substitutions endowing resistance to clethodim have been previously reported in rigid ryegrass (Malone et al. 2014; Yu et al. 2007), Italian ryegrass (Martins et al. 2014), blackgrass (Délye 2005), wild oats (Cruz-Hipolito et al. 2011; Liu et al. 2007;

Yu et al. 2013a) and in hood canarygrass (*Phalaris paradoxa* L.) (Hochberg et al. 2009). Amino acid modification at positions 2078 and 2088 are known to provide strong resistance to clethodim and all other substitutions provide weak or no resistance to this herbicide (Jang et al. 2013; Yu et al. 2007). Malone et al. (2014) observed that there was an increase in the frequency of individuals carrying multiple amino acid substitutions in resistant individuals of rigid ryegrass, which may be related to the increased frequency of clethodim resistance. Repeated use of clethodim over an extended period on rigid ryegrass populations that already had resistance to other ACCase-inhibiting herbicides might have selected for the accumulation of amino acid modifications within ACCase that contribute to clethodim resistance.

In this research, we have not studied any non–target-site based clethodim resistance mechanisms in these populations. As the level of resistance to CHD herbicides was variable among rigid ryegrass populations and there were many plants without any known mutations, it is feasible that non–target-site resistance mechanisms may also be present in these resistant populations. For example, in population R12, out of 12 resistant plants tested only one individual had a known target site mutation. It has been previously confirmed that more than one mechanism of herbicide resistance can co-exist within a population of rigid ryegrass (Christopher et al. 1992). Non–target-site mechanisms endowing resistance to ACCase-inhibiting herbicides, specifically enhanced herbicide detoxification, have been previously reported in rigid ryegrass populations in Australia (Holtum et al. 1991; Preston et al. 1996; Yu et al. 2013b). Thus, we expect that all possible herbicide-resistance–endowing mechanisms can be present in large populations of rigid ryegrass under herbicide selection pressure.

In conclusion, this study has identified high levels of clethodim resistance in some of the rigid ryegrass populations investigated. Five previously reported amino acid substitutions (1781, 2041, 2078, 2088, and 2096) in the CT domain sequences of the plastidic ACCase gene were identified in clethodim-resistant rigid ryegrass populations investigated. Evolution of clethodim resistance in rigid ryegrass has made its management considerably more difficult in the cropping systems of Australia. Australian growers will need to employ alternative management strategies for the effective control of this weed in future.

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CHAPTER 3

FROST REDUCES CLETHODIM EFFICACY IN CLETHODIM-RESISTANT RIGID RYEGRASS (*LOLIUM RIGIDUM*) POPULATIONS

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Overall percentage (%)	85%
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By signing the Statement of Authorship, each author certifies that:

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    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Frost Reduces Clethodim Efficacy in Clethodim-Resistant Rigid Ryegrass (*Lolium rigidum*) Populations

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Rigid ryegrass, an important annual weed species in cropping regions of southern Australia, has evolved resistance to eleven major groups of herbicides. Dose-response studies were conducted to determine response of three clethodim-resistant populations and one clethodim susceptible population of rigid ryegrass to three different frost treatments (-2 C). Clethodim-resistant and susceptible plants were exposed to frost in a frost chamber from 4 pm to 8 am for three nights before or after clethodim application and were compared with plants not exposed to frost. A reduction in the level of clethodim efficacy was observed in resistant populations when plants were exposed to frost for three nights before or after clethodim application. In the highly resistant populations, the survival percentage and LD_{50} were higher when plants were exposed to frost before clethodim application compared to frost after clethodim application. However, there was no influence of the frost treatment on clethodim efficacy of the susceptible population. Sequencing of the acetyl co-enzyme A carboxylase (ACCase) gene of the three resistant populations identified three known mutations at positions 1781, 2041 and 2078. However, most individuals in the highly resistant populations did not contain any known mutation in ACCase, suggesting the resistance mechanism was non-target site. The effect of frost on clethodim efficacy in resistant plants may be an outcome of the interaction between frost and the clethodim resistance mechanism(s) present.

Nomenclature: Clethodim, rigid ryegrass, *Lolium rigidum* Gaudin

Keywords: *ACCase* gene, frost effect, resistance factor, resistance mechanism, target site mutation

Rigid ryegrass is the most economically important grass weed of winter grain crops in Australia (Jones et al. 2005). It is an obligate outcrossing species with high genetic diversity (Charmet et al. 1996). As a result of its adaptation to climate, extensive planting as a pasture species and prolific seed production (Rerkasem et al. 1980), rigid ryegrass is distributed across the southern Australian grain belt (Neve and Powles 2005). Over the past few decades, herbicides have become the most common method for the control of rigid ryegrass in crops. Due to over reliance on herbicides, this grass species has evolved resistance to herbicides belongs to eleven different modes of action (Heap 2015). The most important classes of herbicides used for the control of rigid ryegrass are the aryloxyphenoxypropionate (APP/fops), cyclohexanediones (CHD/dims) (Délye 2005), and phenylpyraxoline (PPZ/dens) (Hofer et al. 2006; Muehlebach et al. 2009), which act specifically on grass weeds by inhibiting the acetyl-coenzyme A carboxylase enzyme (ACCase; EC 6.4.1.2) (Harwood 1988).

Clethodim is a selective POST herbicide typically used to control annual and perennial grasses infesting dicot crops (Burke et al. 2004; Burke and Wilcut 2003; Vidrine et al. 1995). Among ACCase inhibiting herbicides, clethodim has the lowest risk of resistance, with only two of eleven target-site mutations in weed populations conferring resistance to this herbicide (Beckie and Tardif 2012). Aspartate-2078-glycine and cystine-2088-arginine mutations in the plastidic ACCase enzyme have been identified as the main mutations that confer clethodim resistance at the field rates of herbicide application (Yu et

al. 2007). However, leucine-1781 and alanine-2096 mutations in *ACCase* gene may also confer resistance to clethodim in the field if environmental conditions are not optimal for herbicide efficacy, or when herbicide rates are reduced (Délye et al. 2008). Depending on the mutations present, clethodim has the ability to control rigid ryegrass populations that are resistant to other ACCase-inhibiting herbicides (Yu et al. 2007), and has been used extensively in Australia for the control of rigid ryegrass (Boutsalis et al. 2012). Persistent use of clethodim has resulted in the evolution of resistance in rigid ryegrass populations in Australia (Yu et al. 2007), with as many as 60% of fields across south eastern Australia having clethodim resistance (Boutsalis et al. 2012).

Environmental factors are known to have an impact on the performance of various herbicides (Kudsk and Kristensen 1992). For example, temperature before and after application can have a profound influence on the efficacy of herbicides (Johnson and Young 2002; Waltz et al. 2004; Wilcox et al. 1988). The effect of temperature on herbicide efficacy largely depends on the herbicide chemistry and weed species in question. The absorption and translocation of most graminicides is greater under high temperatures (Coupland 1987; Kells et al. 1984; Xie et al. 1996a). Previous research identified a reduction in herbicide translocation as one of the mechanisms of herbicide resistance in various weed species, including rigid ryegrass (Lorraine-Colwill et al. 2002), Italian ryegrass (*Lolium multiflorum* L.) (Michitte et al. 2007; Perez-Jones et al. 2007), horseweed (*Conyza candensis* L.) (Feng et al. 2004), and barnyard grass (*Echinochloa colona* L.) (Nguyen et al. 2015). Reports on the effect of temperature on herbicide resistance levels in weeds are limited. Purba et al. (1995) reported that the mechanism of resistance to paraquat was dependent on temperature in resistant biotypes of hare barley (*Hordeum leporinum* Link). A reduction in the level of glyphosate resistance in horseweed when grown at cold temperatures (<10 C) has been reported (Ge et al. 2011). In recent studies, Nguyen et al. (2015) reported reduced efficacy of glyphosate in glyphosate-

resistant populations of barnyard grass at high temperature (30 C). The underlying mechanism(s) of different efficacy of herbicides under varying temperatures is poorly understood and needs investigation for better management of weeds. At present there are no published studies on the effect of temperature on the phenotypic expression of clethodim resistance in rigid ryegrass.

In southern Australia clethodim is often applied in late June or early July (winter). Frosts can occur on or near the date of application of this herbicide and may influence the efficacy of clethodim on rigid ryegrass. Growers have reported that clethodim efficacy can be variable on rigid ryegrass populations during winter. In the literature, no information is available on the effect of freezing temperature on the efficacy of clethodim and only limited information is available on the efficacy of other herbicides at low temperatures. The objective of this study was to determine the impact of frost on the efficacy of clethodim on rigid ryegrass and in particular on clethodim-resistant populations.

Materials and Methods

Plant Material. The seeds of three clethodim-resistant rigid ryegrass populations used in this study were collected from different farms in Western Australia, Victoria, and South Australia (Table 1) where clethodim at 60-120 g a.i. ha^{-1} had provided inadequate control of rigid ryegrass in the field. These populations were initially tested for clethodim resistance at the 2 to 3- leaf stage with 84 g a.i. ha^{-1} of clethodim (Table 1). There were two highly resistant and one moderately resistant populations of rigid ryegrass, as well as a known susceptible (S) population,VLR1 (McAlister et al. 1995) used as a reference (control) in this study.

Seed Germination and Plant Growth. Seeds of resistant and susceptible populations were germinated on 0.6% (w/v) agar and incubated in a germination cabinet with 12 h

light and 12 h dark periods with 30 µmol m^{-2} s^{-1} at 20 C/15 C temperatures, respectively (Lorraine-Colwill et al. 2001). After 7 days, seedlings at the one leaf stage were transferred to 9.5 cm by 8.5 cm by 9.5 cm punnet pots (Masrac Plastics, South Australia, Australia) containing cocoa peat potting mix (Boutsalis et al. 2012) with a density of nine seedlings per pot. Plants were watered and fertilized as needed. Winters in Adelaide are characterized by cool (average minimum 2 to 10 C, average maximum 10 to 20 C), wet (~ 400 mm rainfall between May and August), and moderately humid (average RH of 71 to 80%) conditions.

Table 1. Collection sites for rigid ryegrass populations and their level of resistance to clethodim in a screening study in 2012.[a]

Population	Geographical location	Primary phenotype	% Survival
571-12	Mingenew, WA (29.11°S 111.26°E)	High resistant	90
615-12	Yendon, VIC (37.38°S 143.58°E)	High resistant	80
48-12	Cummins, SA (34.15°S 135.44°E)	Moderately resistant	20
VLR1	Serviceton, VIC (36.22°S 140.59°E)	Susceptible	0

[a]Rigid ryegrass populations were tested for clethodim resistance with 84 g ha^{-1} of clethodim applied at 2-3 leaf stage. The labelled rate of clethodim in Australia is 60 g ha^{-1}.

Frost Treatments. Frost treatments included: no frost (NF), where plants were not exposed to frost; frost before spray (FBS), where plants were exposed to frost from 4 pm to 8 am for three nights before clethodim application; and frost after spray (FAS), where plants were exposed to frost for three nights after clethodim application from 4 pm to 8 am.

For NF treatment, rigid ryegrass plants were not exposed to frost and were grown outside under the natural conditions of winter. For FBS treatment, frost was applied for three nights before clethodim application by placing the rigid ryegrass plants inside the frost chamber overnight and lowering the temperature minimum to -2 C for 2 hours. At -2 C temperature there was a deposition of white ice crystals on the leaves of rigid ryegrass. The frost conditions inside the chamber were set to mimic a natural frost event at Clare, South Australia in 2013. Air temperature within the frost chamber was monitored using thermocouples positioned throughout the chamber and there was complete darkness inside the frost chamber. Each morning at 8.30 am the plants were taken outside the frost chamber under natural conditions, and later at 3.30 pm returned to the frost chamber during the night. After three nights of frost exposure, rigid ryegrass plants were taken out of the frost chamber and two hours later sprayed with clethodim. After the spray, plants were kept outside under natural conditions for the duration of the experiment.

For FAS treatment, frost was applied three nights after clethodim application by placing the rigid ryegrass plants inside the frost chamber overnight and each morning at 8.30 am the plants were taken outside the frost chamber into natural conditions, and later at 3.30 pm returned to the frost chamber during the night. Three nights after frost treatment, plants were maintained outside under natural conditions for the duration of the experiment. For all the frost treatments, clethodim was applied at the same time.

The experiment was conducted in randomised complete block design with four biotypes (three resistant and one susceptible) by seven clethodim rates with four replication. At 2 to 3- leaf stage, rigid ryegrass plants were treated with clethodim (Select®, Sumitomo) at 0 to 36 g a.i. ha^{-1} for susceptible population, and 0 to 480 g a.i. ha^{-1} for resistant populations. The recommended dose of clethodim in Australia is 60 g a.i. ha^{-1}, which is lethal to susceptible rigid ryegrass populations. Vegetable oil (Hasten,

Victorian Chemicals) at 1% (v/v) was added to all clethodim treatments. Dose-response experiments were conducted for each frost treatment and were repeated in 2013 and 2014. The herbicide was applied by using a laboratory moving boom sprayer equipped with twin nozzles (Tee-jet 1100 flat fan Spraying Systems, Wheaton, IL). The output volume of the sprayer was 103 L ha^{-1} at a pressure of 250 kPa. The control plants were not treated with clethodim. Plants were returned and maintained outdoors after the treatment. Three weeks after spraying, the surviving plants were counted. Plants were recorded as alive if they had tillered and produced new leaves since clethodim application and plants showing severe chlorosis, stunting, and mortality were considered as susceptible (Powles et al. 1998). The surviving plants were harvested and oven dried at 70 C for 2 days. The dry weight data were expressed as a percentage of the respective untreated control. All data were pooled from the two experiments and analysed by using a log-logistic equation (Graphpad Prism v.6.0; GraphPad Software, San Diego, California). LD_{50} (dose required to control 50% of individuals in the population) and GR_{50} (dose causing 50% growth reduction of plants) estimates generated from the log-logistic analysis were used to calculate the resistance factor (RF). The RF was computed as LD_{50} (FBS or FAS)/LD_{50} (NF). The model fitted was

$$y = \frac{100}{1 + 10^{(\log IC_{50} - x) \times b}} \tag{1}$$

where, y is the plant survival (%) or biomass reduction (%), x is the log-dose of the herbicide used, IC_{50} is the dose of herbicide required to produce 50% reduction in plant survival or biomass, and b is the slope of the curve.

One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (P = 0.05) was performed by using Graphpad Prism to assess differences between frost treatments within each population, separately.

Identifying Target-Site Mutations. Fresh leaf material (~1 cm^2) was harvested from young leaves of at least twelve surviving plants from each resistant population, snap frozen in liquid nitrogen and stored at -20 C. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions. Two sets of previously described primers (Malone et al. 2014; Saini et al. 2015a) were used, which amplified nearly the entire CT domain of plastidic *ACCase* gene covering all the seven known mutations sites (1781, 1999, 2027, 2041, 2078, 2088 and 2096). MyFi^{TM} DNA polymerase kit (Bioline, Australia Pty Ltd, Alexandria, NSW, 1435) was used to run PCR reactions of 25 μL containing 80-100 ng DNA template, 1x MyFi reaction buffer, 0.8 μM of each specific primer and 2 units of MyFi DNA Polymerase (high fidelity Taq). Amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany) with PCR conditions as follows: 3 min denaturing at 94 C; 35 cycles of 30 s denaturation at 94 C, 30 s annealing at 56 C and 2 min elongation at 68 C, and a final extension for 7 min at 68 C.

The PCR products were examined on 1.5% agarose gels stained with 1 x SYBR® Safe DNA gel stain. Samples were electrophoresed in 1 x TAE Buffer (40 mM Trizma base, 1 mM Na$_2$EDTA, pH to 8 with glacial acetic acid) at 90 volts and photographed under UV light (λ302 nm). DNA fragment sizes were estimated by comparing their mobility to bands of known sizes in a low mass molecular weight marker (Invitrogen, Australia). DNA sequences were assembled, compared and analysed using ContigExpress from the Vector-NTi Advance 11.5 programs (Invitrogen) and all sequences visually rechecked using the chromatogram files.

Results and Discussion

Frost Response Experiments. The results of the dose-response experiments conducted under natural conditions showed the S population completely controlled with 60 g ha^{-1}

clethodim (Figure 1D). Response of the resistant populations to clethodim was different. Population 48-12 had 10% survival; population 571-12 had 30% survival; whereas population 651-12 had more than 50% survival (Figure 1C, 1A, 1B). Addition of frost treatments did not markedly affect survival of the S population, nor the moderately clethodim-resistant population 48-12, as LD_{50} and GR_{50} were similar ($P \geq 0.05$) under both with and without frost treatments (Table 2). However, in the highly resistant populations 571-12 and 651-12, the survival percentage and LD_{50} were higher ($P < 0.05$) when plants were exposed to frost for 4 pm to 8 am for 3 nights before clethodim application (Table 2).

Among the rigid ryegrass populations, the highly resistant population 571-12 showed the greatest effect of frost on rigid ryegrass control. In this population, a high level of plant survival (82%) was noticed even at the labelled rate of clethodim (60 g ha^{-1}) when frost exposure occurred 3 nights before (FBS) clethodim treatment compared with the 30 and 50% survival, when plants were not exposed to frost, or exposed to frost for 3 nights after clethodim application, respectively (Figure 1A). Plants that were exposed to frost before or after clethodim application had significant decreased ($P < 0.05$) clethodim efficacy compared to the plants without frost exposure. The LD_{50} of clethodim for 571-12 population was 47.2 g ha^{-1} when plants were not exposed to frost. However, when plants were exposed to frost before clethodim application, the LD_{50} value was 130.2 g ha^{-1}. This population showed 2.8-fold more resistant to clethodim compared to NF treatment (Table 2). When this population was exposed to frost after clethodim application there was a smaller but significant increase in LD_{50} to 58.7 g ha^{-1}, making the plants 1.2-fold more resistant to clethodim than plants not exposed to frost.

Similarly, the plants of this population that were not exposed to frost displayed lower biomass ($P < 0.05$), with GR_{50} of 20.4 g ha^{-1} compared to plants that were exposed

to frost before or after clethodim application with GR_{50}, 35.0 and 23.5 g ha^{-1}, respectively (Table 2).

The population 615-12 also showed a reduced efficacy of clethodim in plants exposed to frost. Plants of this population without frost exposure and the plants exposed to frost after clethodim application showed 50% survival at the recommended field rate of clethodim (Figure 1B). However, the plants exposed to frost before clethodim application had 80% survival at the same rate of clethodim. Individuals of this population when exposed to frost before clethodim application had a higher ($P < 0.05$) LD_{50} (88.9 g ha^{-1}), 1.6-fold more resistant to clethodim than plants without frost exposure (57.2 g ha^{-1}) or those exposed to frost after clethodim application(LD_{50} 58.9 g ha^{-1}) (Table 2). In population 651-12, frost treatments increased GR_{50} ($P < 0.05$) compared to the no-frost control (Table 2).

These results clearly show that frost can have a significant effect on clethodim efficacy in some clethodim resistant rigid ryegrass populations. Additionally, clethodim efficacy was more affected by the occurrence of frost before clethodim application compared with after application. It is possible that the reduced clethodim efficacy in case of frost before clethodim application may be related to reduced absorption and translocation of clethodim as a result of frost stress in rigid ryegrass plants. As in the previous studies, it has been reported that sethoxydim (an herbicide with the same mode of action to clethodim) translocation in bermudagrass (*Cynodon dactylon* L.) was affected by low temperature (Wills 1984). Likewise, lower translocation of several foliar-applied grass herbicides at low temperature have been reported in quackgrass (*Agropyron repens*) (Harker and Dekker 1988). At lower temperatures, phloem translocation is reduced due to freezing of leaf tissues accompanied by damage of phloem cells (Weatherley and Watson 1969). It has been found previously that the cooling of whole plant or plant shoots to sub-

optimal temperatures reduces translocation of photosynthates (Hofstra and Nelson 1969; Whittle 1964). Some previous studies also suggest that temperature is one of the most important environmental factors affecting performance of ACCase inhibiting herbicides (Johnson et al. 2002; Kells et al. 1984; Kudsk and Kristensen 1992).

In general, uptake and translocation of foliage applied herbicides increases with increasing temperature (Godar et al. 2015; Kudsk and Kristensen 1992). This is due to effects on the rate of diffusion through the plant cuticle, increased rates of transpiration and subsequent apoplastic movement, and an increase in the general metabolic activity of the plant providing suitable energy sources for the active loading of assimilates in the translocation stream (Caseley 1987). Variation in herbicide translocation with temperature has also been observed with other herbicides. It has been reported that warm temperatures around the time of spray application enhanced the activity of sethoxydim leading to a greater reduction in shoot growth of couch grass (*Elymus repens* L.) (Coupland 1987). Likewise, Kowalczyk et al. (1983) reported that wild oat (*Avena fatua* L.) plants grown at low temperatures were less susceptible to difenzoquat than plants grown at high temperatures. Ivany (1981) found that glyphosate application after severe frost caused foliage necrosis and resulted in poor control of quackgrass (*Agropyron repens* L.). Similarly, Xie et al. (1996b) showed that translocation of imazamethabenz-methyl was increased in wild oat by high temperatures, whereas low temperature (5/10 C) decreased translocation of this herbicide. Kumaratilake and Preston (2005) also demonstrated that low temperature (5/10 C) reduces glufosinate activity and translocation in wild radish. In recent studies, Godar et al. (2015) found more sensitivity of Palmer amaranth (*Amaranthus palmeri*) to mesotrione at low temperature (25/15 C, day/night) and greater absorption, translocation, and metabolism of mesotrione at high temperature (40/30 C) compared to low temperature.

Table 2. The dose of clethodim required for 50% mortality (LD_{50}) and for 50% biomass reduction (GR_{50}) of resistant and susceptible rigid ryegrass populations under different frost treatments with confidence intervals (CI) in parentheses. RF is the ratio of LD_{50} and GR_{50} of frost before spray or frost after spray to no frost treatments.

Population	LD_{50} (g a.i. ha^{-1})	RF	GR_{50} (g a.i. ha^{-1})	RF
571-12 (R)				
NF	47.2 (37.9, 58.8)	-	20.4 (11.4, 36.6)	-
FBS	130.2 (105.8, 160.3)*	2.8	35.0 (27.0, 45.3)*	1.7
FAS	58.7 (49.9, 69.1)*	1.2	23.5 (18.5, 29.9)*	1.2
615-12 (R)				
NF	57.2 (49.8, 66.1)	-	26.8 (20.0, 36.2)	-
FBS	88.9 (76.3, 103.5)*	1.6	59.5 (48.5, 72.9)*	2.2
FAS	58.9 (53.8, 64.5)	1.0	40.0 (35.9, 44.4)*	1.5
48-12 (R)				
NF	8.7 (6.9, 11.1)	-	5.6 (4.2, 7.4)	-
FBS	9.6 (6.6, 13.8)	1.1	8.4 (7.6, 9.4)	1.5
FAS	9.5 (8.0, 11.5)	1.1	7.7 (5.2, 11.5)	1.4
VLR1 (S)				
NF	5.5 (4.6, 6.4)	-	4.1 (3.3, 5.1)	-
FBS	7.1 (6.0, 8.4)	1.3	4.8 (4.0, 5.7)	1.2
FAS	6.0 (8.0, 11.5)	1.1	4.4 (3.7, 5.2)	1.1

*Indicates values are significantly different (P < 0.05) from no frost treatment within each population according to Dunnett's multiple comparisons test.

Abbreviations: NF: No Frost; FBS: Frost Before Spray; FAS: Frost After Spray
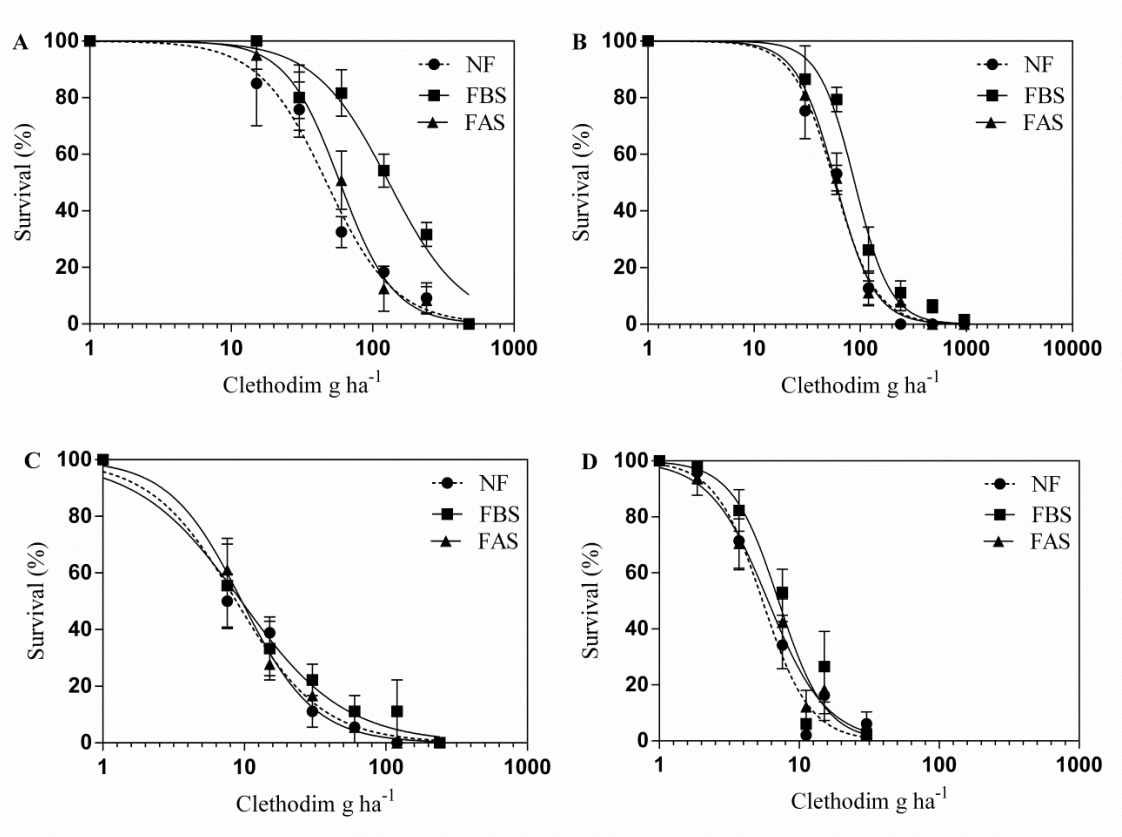
Figure 1. Survival of plants from several populations of rigid ryegrass, 1A: 571-12, 1B: 651-12, 1C: 48-12, and 1D: VLR1 treated with various rates of clethodim under different frost treatments. (●) plants were not exposed to frost, NF; (■) plants were exposed to frost for three nights before clethodim application, FBS; and (▲) plants were exposed to frost for three nights after clethodim application, FAS. Values are means of ± SE of eight replicates. The recommended dose of clethodim in Australia is 60 g ha^{-1}.

Furthermore in the previous studies, Purba et al. (1995) showed reduced translocation of paraquat as a mechanism of resistance in resistant biotypes of hare barley is associated with low temperature and this mechanism of resistance breaks down at higher temperatures due to increased translocation of herbicide. In our study, there was no significant effect of lowering of temperature due to frost on clethodim efficacy in the S population, however, a decrease in clethodim efficacy was observed in the two highly

resistant populations. Clearly there was an interaction between the effect of frost and the clethodim resistance mechanism(s) present in rigid ryegrass populations.

Identifying Target-Site Mutations. A target site mutation within *ACCase* gene was identified in all three resistant populations of rigid ryegrass. The nucleotide sequences of individuals within the resistant populations differed from that of the susceptible population, and three known ACCase mutations were identified in resistant populations (Table 3, Figure 2). The highly resistant population 571-12 had some individuals with a mutation at nucleotide position 2078 (8%); 651-12 population had individuals with a mutation at 2041 (8%), and 48-12 individuals contained three separate mutations at position 1781 (33%), 2041 (17%) and 2078 (8%). No other mutations were found. However, there were many individuals in the highly resistant population did not contain any of the known mutations within ACCase. As we have not amplified the entire *ACCase* gene, we cannot exclude the existence of mutations in other positions of the enzyme. In all clethodim-resistant populations of rigid ryegrass studied here, the presence of non-target site resistance mechanism appears to account for some or most of the resistance to clethodim. The resistant population with the most individuals carrying a target-site mutation (48-12) was the only population not to show a reduction in clethodim efficacy with frost treatments. The more resistant populations (571-12 and 651-12) contained few individuals with known target site mutations in ACCase and showed reduced clethodim efficacy with frost. It appears that the mechanism present within these two populations interacts with frost to further reduce clethodim efficacy. Previous research in rigid ryegrass has established a role of phytochrome P450 mediated herbicide degradation in herbicide resistance (Christopher et al. 1994; Preston and Powles 1998; Preston et al. 1996). Such enzymatic processes are likely to be highly sensitive to temperature and may have played an important role in clethodim response to frost observed in this study.

Table 3. Different target-site mutations identified in carboxyl transferase (CT) domain of ACCase enzyme in different populations of rigid ryegrass.

Population	Amino acid substitution	No. of individuals having mutation	Frequency of individuals without a mutation (%)
571-12 (R)	2078Gly	1	92
615-12 (R)	2041Asp	1	92
48-12 (R)	1781Leu/2041Asp/2078Gly	4/2/1	42

Amino acid modifications at position 1781, 2041 and 2078 associated with clethodim resistance have been previously reported in rigid ryegrass (Malone et al. 2014; Saini et al. 2015c; Yu et al. 2007). Amino acid modification at position 2078 is known to provide strong resistance to clethodim (Jang et al. 2013; Kaundun 2014; Saini et al. 2015a; Yu et al. 2007). The amino acid substitution at position 2041 has been reported to confer a low level of resistance to clethodim in wild oats (Cruz-Hipolito et al. 2011; Liu et al. 2007), Italian ryegrass (Martins et al. 2014) and Johnsongrass (*Sorghum halepense* L.) (Scarabel et al. 2014) but confers high level of resistance to clethodim in rigid ryegrass (Saini et al. 2015c). The Ile-1781-Leu mutation also confers moderate to high level of resistance to clethodim in rigid ryegrass (Saini et al. 2015b; 2015c; Yu et al. 2007).

Figure 2. Sequence alignment of partial *ACCase* gene from the resistant (571-12, 615-12 and 48-12) and susceptible (VLR1) rigid ryegrass populations and blackgrass (GenBank accession AJ310767). A: Amino acid substitution of Ile-1781-Leu (nucleotide change from ATA to TTA at position 5,344). B: Amino acid substitution of Ile-2041-Asn (nucleotide change from ATT to AAT at position 6,122) and C: Amino acid substitution of Asp-2078-Gly (nucleotide change from GAT to GGT at position 6,233).

In this research, we have not studied non-target site based clethodim resistance mechanisms in these populations. As the frequency of individuals without any of the known ACCase mutations is high in these populations, it is likely that resistance is due to some other mechanisms. It has also been previously confirmed that more than one mechanism of herbicide resistance can co-exist within a population of rigid ryegrass (Christopher et al. 1992). Non-target site mechanisms endowing resistance to ACCase-inhibiting herbicides have been previously reported in rigid ryegrass populations in

Australia (Holtum et al. 1991; Preston and Powles 1998; Preston et al. 1996; Yu et al. 2013). In our study, the biochemical basis of the temperature (frost) dependence of clethodim resistance remains unknown and warrants future research.

In conclusion, the efficacy of clethodim declined sharply in clethodim-resistant populations of rigid ryegrass especially if they were exposed to frost before clethodim application. The consequences of this study are significant, as despite clethodim resistance being widespread, growers still use clethodim to control rigid ryegrass by increasing the herbicide rate (Boutsalis et al. 2012). Clethodim is also typically applied to rigid ryegrass in South Australia during winters when frosts can occur. The combination of clethodim resistance and low temperatures likely explains the variable control of rigid ryegrass observed with this herbicide by growers.

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CHAPTER 4

INHERITANCE OF EVOLVED CLETHODIM RESISTANCE IN RIGID RYEGRASS (*LOLIUM RIGIDUM*) POPULATIONS FROM AUSTRALIA

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Inheritance of Evolved Clethodim Resistance in Rigid Ryegrass (*Lolium rigidum*) Populations from Australia

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Five clethodim-resistant populations of rigid ryegrass were analysed for the inheritance of clethodim resistance. Reciprocal crosses between resistant (R) and susceptible (S) biotypes were made and individuals of first filial generation (F_1) were tested for resistance to clethodim. Within crosses, dose responses of reciprocal F_1 families of all populations, except A61 were not significantly different from each other, indicating clethodim resistance is encoded on the nuclear genome. The level of dominance observed in the dose-response experiments ranged from partial to complete within the herbicide dose used. In population A61, within each cross, the response of F_1 from the maternal and paternal parent was significantly different, indicating that resistance is inherited through the female parent, with a minor proportion through pollen. F_1 individuals from four of the populations were crossed with susceptible individuals to create backcross (BC) progenies. All BC populations segregated in a different manner. Only one population, FP, fitted a single gene model (1:1). Two other populations, A91and E2, did not fit to the single gene model. A complete dose-response analysis on BC progeny of A91 population fitted a 1:3 gene inheritance model, indicated that two genes contributed to clethodim resistance, one dominant and second one recessive gene, whereas, in E2 population two dominant genes contributed to clethodim resistance and closely fitted 3:1 gene inheritance model. For the

A61 population the BC populations responded similarly to the female parents. Where the maternal parent was susceptible (F$_1$♀S) the BC progeny were susceptible and where the maternal parent was resistant (F$_1$♀R), the BC progeny were resistant. This confirmed maternal inheritance of clethodim resistance in this population. The results of this study indicate different patterns of clethodim resistance in rigid ryegrass exist and such variation in the pattern of inheritance indicates multiple resistance mechanisms may be present in rigid ryegrass populations.

Nomenclature: Clethodim; rigid ryegrass, *Lolium rigidum* Gaud. LOLRI.

Key words: Clethodim resistance, inheritance.

During the past 50 years, herbicides have become the dominant method of weed control in agriculture. The intensive use of herbicides for weed control has resulted in the evolution of herbicide resistance in many weed species. Rigid ryegrass is one of the most troublesome herbicide resistant weeds in the cropping regions of Australia (Jones et al. 2005; Pannell et al. 2004). The success of rigid ryegrass in Australian grain production is a result of its adaptability to diverse environments and its high fecundity (Gherekhloo et al. 2012; Rerkasem et al. 1980). It is an obligate cross-pollinated species (Charmet et al. 1996), which has so far evolved resistance to eleven different herbicide classes (Heap 2015).

Aryloxyphenoxypropionate (APP) and cyclohexanedione (CHD) herbicides are potent inhibitors of acetyl coenzyme A carboxylase (ACCase; EC 6.4.1.2) (Délye et al. 2005) and are commonly used for the control of various grass weeds. Resistance to ACCase-inhibiting herbicides in most cases has been attributed to insensitivity of target enzyme (Gherekhloo et al. 2012; Kuk et al. 1999; Leach et al. 1995; Martins et al. 2014).

However, metabolism-based resistance to ACCase inhibitors has been identified in a comparatively small number of weeds species (Bravin et al. 2001; Hidayat and Preston 1997; Preston et al. 1996). Clethodim, a CHD herbicide, is a selective post-emergent herbicide used to control annual and perennial grasses in a wide variety of broadleaf crops (Burke et al. 2004; Burke and Wilcut 2003; Vidrine et al. 1995). Due to resistance to other post-emergent herbicides in rigid ryegrass in Australia, clethodim has become the most important grass herbicide for canola and pulse crops but resistance to this herbicide is also increasing. While target site mutations that provide resistance to clethodim are known, they do not fully explain the level of resistance observed in rigid ryegrass.

Inheritance patterns of resistance to ACCase inhibitors have been determined in a few weed species. In most instances where resistance is encoded by a nuclear gene, it is expressed as either a dominant or partially dominant trait (Betts et al. 1992; Tardif et al. 1996). Nuclear inheritance of herbicide resistance is conferred by alleles that are present on the nuclear genome. Until now, with the notable exception of the triazines, resistance to all herbicide classes is determined by nuclear inherited genes. However, in most species, triazine resistance has been determined by cytoplasmic inheritance (Jasieniuk et al. 1994) where resistance alleles are present on the chloroplast genome. In nuclear inheritance, resistant alleles are transmitted through pollen and ovules, whereas transmission of the cytoplasmic resistance genes occurs only through the ovules of the maternal parent, although a very low level of transmission by pollen has been observed in a few species (Darmency and Gasquez 1981). The sole exception to cytoplasmic inheritance of triazine resistance is found in velvetleaf (*Abutilon theophrasti)*, where resistance is controlled by a single, partially dominant, nuclear gene (Anderson et al. 1993).

Understanding the mechanisms and inheritance of resistance is necessary for the formulation of efficient management strategies to slow down the rate of evolution of

resistance or to manage resistant weeds. As the inheritance of clethodim resistance has not yet been fully studied, the objective of this study was to determine the inheritance of resistance in rigid ryegrass populations, which are resistant to clethodim.

Materials and Methods

Plant material. Five rigid ryegrass populations used in this study (FP, E2, F4, A91, and A61) were obtained from different locations in Australia (Table 1), where clethodim 60-120 g a.i. ha^{-1} had provided inadequate control of rigid ryegrass in the field and resistance was suspected. Seeds from a large number of plants that had survived clethodim application in the field were collected. Dose response experiments were carried out on these populations (Saini et al. 2015a; Saini et al. 2015b), with SLR4 (Wakelin and Preston 2006a) as a standard susceptible (S) control. Plants which survived clethodim application at the recommended and higher rates (> 60 g ha^{-1}) in the dose response study were used as the parent material for the resistant (R) populations in this study.

Table 1. Location of collection sites for rigid ryegrass populations studied.

Population	Geographical location
FP	Roseworthy, SA (34.53^o, 138.74^o)
E2	Roseworthy, SA (34.53^o, 138.74^o)
F4	Roseworthy, SA (34.53^o, 138.74^o)
A91	Moora, WA (30.38^o 116°)
A61	Kellerberrin, WA (31.37°117.46°)

Generation of F$_1$ and Backcross Populations. Individuals from each resistant population and a known S population were crossed to produce the F$_1$ generations. One plant each of

the R and S populations were grown in a single pot (30 cm diameter) containing standard potting mix (Boutsalis et al. 2012). The distance between the two plants was 10 cm and replica pots (six of each population) were placed at 20 m intervals. The plants were maintained outside in normal growing conditions and watered once or twice a day, depending on the weather conditions. Prior to flowering, the pots were encased with a 1.2-m-high transparent plastic sleeve, supported by a wire mesh case which was open at the top. This plastic sleeve was used to reduce the chance of any external pollen from pollinating the two plants in the pot. Rigid ryegrass is an allogamous species (Terrell 1968), and a single plant sets almost no viable seed (Preston 2009). Seeds were harvested separately from the maternal R and maternal S parents within each cross and called F_1 progeny.

The amount of seed resulting from each individual cross varied widely. Although all plants used in crossing experiments were germinated at the same time and cultured under the same conditions, some individual crosses failed to produce viable seeds. Asynchronous flowering or incompatibilities of the individual plants are the likely causes of failure of these crosses. In the following year, to test the segregation of clethodim resistant traits, F_1 individuals were crossed with an individual from the S parent as described above to create back-cross (BC) families. Eight individual F_1 plants from each population were used, yielding five BC families with sufficient seed to test.

Dose Response on F_1 progeny and BC populations. Clethodim dose-response experiments were conducted on the S, R, F_1 (maternal S and R origins), and $F_1 \times S$ (BC) plants in the following year under normal winter growing season conditions. Seeds collected from F_1, $F_1 \times S$, S, and R plants were germinated on 0.6% (w/v) agar and incubated in a germination cabinet with 12 h light with 30 µmol m^{-2} s^{-1} and 12 h dark periods at 20 C/15 C temperatures (Lorraine-Colwill et al. 2001). After 7 days, seedlings at

the one leaf stage were transferred to 9.5 cm by 8.5 cm by 9.5 cm punnet pots (Masrac

Plastics, South Australia, Australia) containing cocoa peat potting mix (Boutsalis et al.

2012) with a density of nine seedlings per pot. Clethodim (Select®, Dow AgroSciences,

Australia Ltd., NSW) was applied at a range of rates from 0 to 480 g ha^{-1} (ten to eleven

different rates per population) to plants at the two to three-leaf stage. Ethyl and methyl

esters of vegetable oil (Hasten TM, Victorian Chemical Co., Vic., Australia) at 1% (v/v)

were added to clethodim as recommended in the herbicide label. There were three

replicates for each clethodim dose and pots were arranged in a randomized complete block

design. Clethodim was applied by using a laboratory moving boom sprayer equipped with

twin nozzles (Tee-jet 1100 flat fan Spraying Systems, Wheaton, IL). The output volume of

the sprayer was 103 L ha^{-1} at a pressure of 250 kPa and a speed of 1 m s^{-1}. Control plants

were not treated with clethodim. After treatment, the plants were maintained outdoors

under the normal growing conditions. Three weeks after spraying, surviving plants were

counted. Plants were recorded as alive if they had tillered and produced new leaves since

clethodim application and plants showing severe chlorosis, stunting and mortality were

considered as susceptible (Powles et al. 1998).

Statistical Analyses. The survival data from dose-response experiments were analysed by

using a log-logistic equation (Graphpad Prism v.6.0; GraphPad Software, San Diego,

California). This provides the dose-response curves to the graph and the LD$_{50}$ (dose which

controls 50% of the population). The model fitted was

$$y = \frac{100}{1 + 10^{(\log \text{IC}_{50} - x) \times b}}$$ (1)

where, y is the plant survival (%), x is the log-dose of the herbicide used, IC$_{50}$ is the dose

of herbicide required to produce 50% reduction in plant survival, and b is the slope of the

curve. The segregation of clethodim resistance within BC families was tested against

single-gene model with a dominant allele using G-test with Williams correction (Sokal

and Rohlf 1981). The response of single dominant allele was modelled by summing $0.50 \times$ survival of the F_1 hybrid and $0.50 \times$ survival of the susceptible population. The populations which did not show a good fit to the single gene model were then subjected to two-gene inheritance models. The first, for two dominant genes (3:1), was produced by summing $0.75 \times$ survival of the F_1 hybrid and $0.25 \times$ survival of the susceptible population. The second, for a dominant and a recessive gene (1:3), was produced by summing $0.25 \times$ survival of the F_1 hybrid and $0.75 \times$ survival of the susceptible population. These models were then compared with the actual response of the BC populations to determine whether the dose response fitted a single-gene or a two-gene effect at each of the eleven different rates of clethodim used.

Results and Discussion

Parent and F1 Dose-Response Curves. Dose-response experiments were performed on the parental susceptible (SLR4), parental resistant (R), F_1 maternal resistant (♀R), and F_1 maternal susceptible (♀S) populations to determine the dominance of the resistance alleles and whether the gene was nuclear encoded. These experiments showed that when the populations were treated with clethodim, the S parent was completely controlled with the recommended rate (60 g ha^{-1}) of clethodim and also at much lower rates, whereas higher rates of clethodim were required to control the parental resistant and both F_1 progenies (except for population A61). In all the populations except A61, the response of F_1 from the maternal parent was almost similar to that of F_1 from the paternal parent (Figure 1, Table 2). These results show that the resistance to clethodim in some rigid ryegrass populations is nuclear encoded and was transferred by pollen during the cross pollination. These results are consistent with the majority of the herbicide-resistant species studied, where resistance genes are mostly located on the nuclear genome (Chandi et al. 2012; Okada and Jasieniuk 2014; Preston and Mallory-Smith 2001; Wakelin and Preston 2006b).

In contrast, in both F_1 families derived from the A61 population, the response of the F_1 from the maternal parent and the paternal parent were different (Figure 1E and 1F, Table 2). The F_1 hybrid produced with the clethodim-susceptible biotype as the female parent (♀S) showed a response similar to the maternal parent, whereas when the clethodim-resistant biotype was the female parent (♀R), the F_1 hybrid had a response more similar to the resistant parent. This clearly indicates that a major component of resistance in this population is inherited through the female parent, with a minor proportion through pollen. The source of maternal inheritance is normally chloroplasts or mitochondria and the source of resistance in pollen is normally the nucleus (Darmency and Gasquez 1981). Maternal inheritance of resistance has been widely reported for triazine herbicides (Darmency and Gasquez 1981; Jasieniuk et al. 1996; Machado and Bandeen 1982; Scott and Putwain 1981). Apart from triazine herbicides, in the recent studies, maternal inheritance has also been reported in flupropanate herbicide in serrated tussock (*Nassella trichotoma*) (Ramasamy et al. 2010). However, this is the first example of maternal inheritance for resistance to ACCase-inhibiting herbicides.

From the dose response, the LD_{50} values calculated for the F_1 families (calculated by dividing the LD_{50} of F_1 progeny by the LD_{50} of the S parent) of FP, E2, and F4 populations were 1.2 to 4.8 fold higher than the S parent (Table 2). The LD_{50} values of most of the F_1 families were lower than the R parent (Figure 2A and 2B, Table 2). The lower resistance of the F_1 families when compared with the clethodim-resistant populations indicates that clethodim resistance in rigid ryegrass is conferred by an allele(s) that is not fully dominant over susceptibility within the dose range used. Therefore, in these populations of rigid ryegrass clethodim resistance mechanism is inherited in a semi-dominant manner at the rates used.

In A91 population, both F_1 families had LD_{50} values similar to the R parent (Figure 1C and 1D, Table 2), and in this population, resistance is best described as fully or completely dominant. In cases where the F_1 dose-response curve is similar to the S curve, a recessive gene would control the R plant; and if it were similar to the R curve, it would indicate presence of a dominant gene controlling resistance to clethodim (Murray et al. 1995). Dominance of the allele conferring clethodim resistance in rigid ryegrass is consistent with the inheritance of herbicide resistance observed in many other herbicide resistant weeds (Jasieniuk et al. 1995; Jugulam et al. 2005; Wakelin and Preston 2006b).

Incomplete dominance of herbicide resistance is common in weed species, but the degree of dominance demonstrated by the resistance allele varies significantly (Betts et al. 1992; Lorraine-Colwill et al. 2002; Murray et al. 1995; Tardif et al. 1996). It has been described previously that resistance to inhibitors of ACCase can be inherited in a variety of ways. In populations of Italian ryegrass (*Lolium multiflorum* Lam.) and wild oat (*Avena fatua* L.) having ACCase inhibitor resistance, a single nuclear, semi-dominant to dominant gene was responsible (Betts et al. 1992; Maneechote et al. 1994; Murray et al. 1995; Seefeldt et al. 1998). According to Jasieniuk et al. (1996) a dominant trait spreads more rapidly in populations, as both homozygous dominant and heterozygous individuals will carry the resistance trait. In addition, because rigid ryegrass is highly allogamous, the spread of resistance conferred by a dominant or semi-dominant gene will spread rapidly through the population. The pattern of inheritance of clethodim resistance in these four populations (FP, E2, F4 and A91) is consistent with the majority of other herbicide resistant weeds species studied, where resistance gene were mostly located on the nuclear genome, and inherited in a dominant or semi-dominant manner (Chandi et al. 2012; Ng et al. 2004; Okada and Jasieniuk 2014; Volenberg and Stoltenberg 2002). Likewise, Wakelin and Preston (2006b) in the previous study, also reported that glyphosate resistance in rigid ryegrass populations is partial and completely dominant and is under nuclear control.

Table 2. LD_{50} values for F_1 dose-response experiment. R/S is the ratio of LD_{50} of resistant parent or F1 progeny (♀S and ♀R) to susceptible parent.[a]

Population	LD_{50} (g a.i. ha^{-1})	95% C.I.		R/S
		Lower	Upper	
FP × SLR4				
2-F_1 (♀S)	6.8	5.6	8.3	3.6
2-F_1 (♀R)	9.2	6.9	12.3	4.8
FP (R)	18.4	14.0	24.1	9.7
SLR4 (S)	< 1.9	-	-	-
E2 × SLR4				
2-F_1 (♀S)	3.7	3.3	4.2	1.9
2-F_1 (♀R)	2.3	1.9	2.9	1.2
5-F_1 (♀S)	5.3	3.9	7.2	2.8
5-F_1 (♀R)	5.2	4.6	5.9	2.7
E2 (R)	23.0	20.7	25.6	12.1
SLR4 (S)	< 1.9	-	-	-
F4 × SLR4				
2-F_1 (♀S)	4.7	3.4	6.4	2.5
2-F_1 (♀R)	5.6	4.0	7.9	2.9
3-F_1 (♀S)	5.1	3.5	7.4	2.7
3-F_1 (♀R)	3.3	2.2	4.9	1.7
F4 (R)	36.2	32.7	40.2	19.1
SLR4 (S)	< 1.9	-	-	-
91 × SLR4				
2-F_1 (♀S)	5.9	5.0	7.0	3.1
2-F_1 (♀R)	5.3	4.9	5.8	2.8
4-F_1 (♀S)	6.2	5.1	7.4	3.3
4-F_1 (♀R)	6.9	6.0	8.1	3.6

Table 2 (continued). LD_{50} values for F_1 dose-response experiment. R/S is the ratio of LD_{50} of resistant parent or F1 progeny (♀S and ♀R) to susceptible parent.[a]

Population	LD_{50} (g a.i. ha^{-1})	95% C.I.		R/S
		Lower	Upper	
91 (R)	7.6	5.6	10.2	4.0
SLR4 (S)	< 1.9	-	-	-
61 × SLR4				
1-F_1 (♀S)	3.0	2.4	3.8	1.6
1-F_1 (♀R)	11.6	9.6	14.1	6.1
4-F_1 (♀S)	2.3	1.9	2.8	1.2
4-F_1 (♀R)	25.4	21.0	30.7	13.4
61 (R)	29.4	25.7	33.7	15.5
SLR4 (S)	< 1.9	-	-	-

[a] Abbreviations: LD_{50}, the dose lethal to at least 50% of the populations, F_1, first filial generation; S, susceptible; R, resistant; ♀S, seed from maternal S plant; ♀R, seed from maternal R plant. Numbers represent the family number of population.

For susceptible population, SLR4, data could not be fitted to the log logistic equation, but all individuals were killed by 1.9 g a.i. ha^{-1} clethodim.
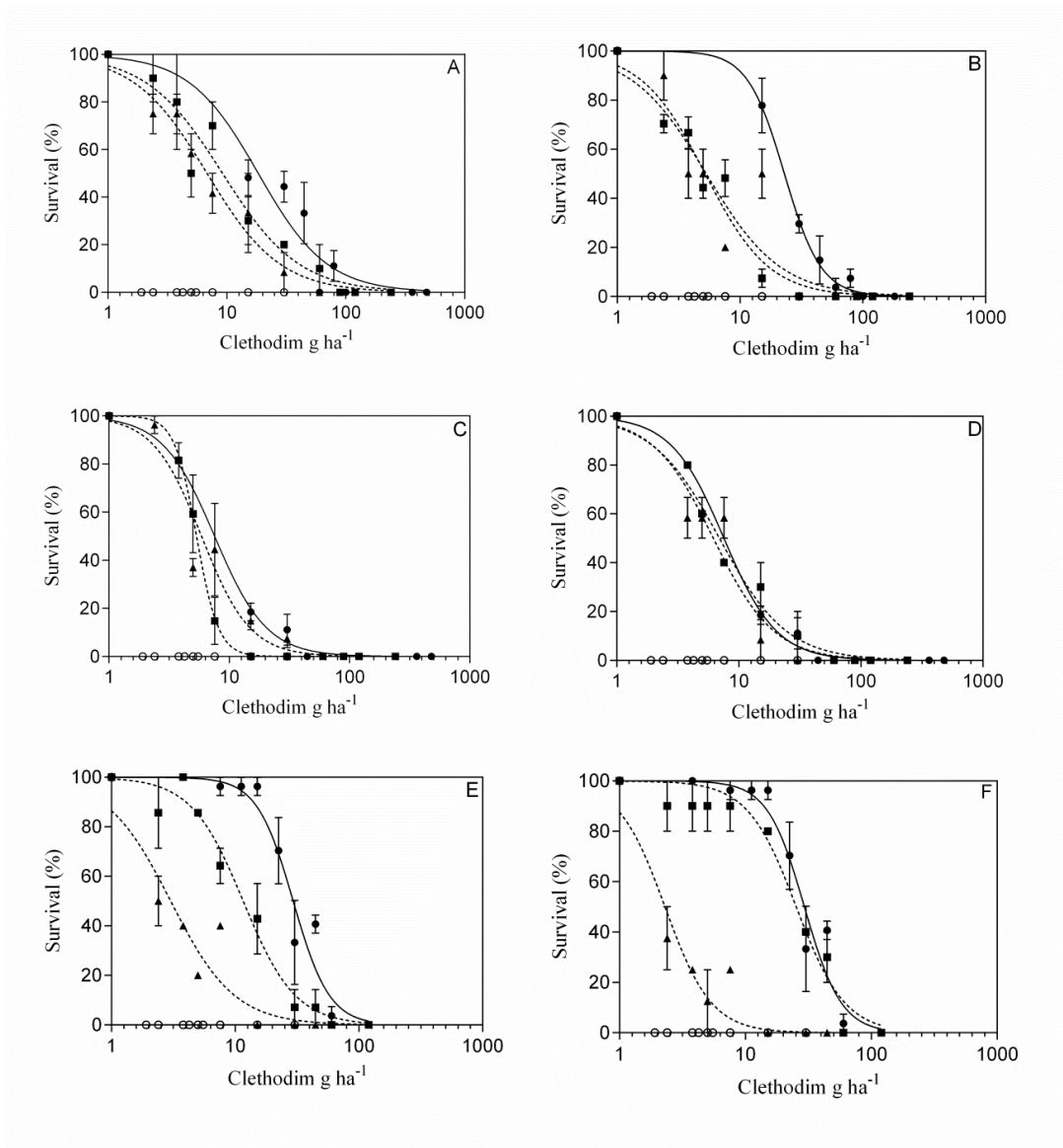
Figure 1. Dose-response curves for first filial generation (F_1) individuals of four different clethodim-resistant populations of rigid ryegrass. Populations were (A) FP, (B) E2, (C) 2-A91, (D) 4-A91, (E) 1-A61, and (F) 4-A61. Graph C and D are different families of same population, A91 and graph E and F are different families of A61 population. R parent, —●—; S parent, —○—; F1 (from R parent), – –■– –; F1 (from S parent), – –▲– –. Each data point is a mean of three replicates and bars represent SEM. The susceptible population, SLR4, had complete mortality at the lowest rate used (1.9 g a.i. ha^{-1}) and consequently data did not fit the log logistic equation, therefore we were unable to generate curves of those data.

Segregation of the Resistance Trait in $F_1 \times$ S BC population. Backcrosses were made with F_1 of four of the populations (FP, E2, A91, and A61) to ascertain whether the genetic control of clethodim resistance resides in a single gene or multiple genes. The BC plants were treated with 11 different clethodim rates ranging from 0 to 4 times the recommended field rate. The number of survivors of the treated S, F_1 and BC plants was used to calculate the expected mortality at each rate. The single gene model assumed that 50% of the BC progeny behaved like F_1 population and 50% behaved like S parent. On the basis of the single gene hypothesis of inheritance of clethodim resistance, the dose response curve of the BC progeny of FP population was similar to the theoretical curve expected and fitted a 1:1 resistant : susceptible (R:S) segregation pattern for clethodim (Figure 2). Additionally, the BC data was also tested for goodness of fit to one gene model at each rate of clethodim used. The results revealed that segregation at the two out of four lower rates of clethodim was not significantly different to the expected 1:1 ratio for single gene inheritance. This ratio is consistent with the segregation ratio for a single nuclear gene (Table 3).

However, the dose response curve of the BC progeny of E2 and A91 population significantly deviated from the theoretical curve expected for a single gene model (Figure 3). A similar response was observed when BC data of these populations was tested for goodness of fit to a 1:1 segregation ratio at each rate of clethodim (Table 3). For the A91 population at all the rates and for the E2 population at two out of four rates, there was a poor fit to the model (Table 3), indicating that resistance in these two populations is not due a single gene. As a result, a series of two-gene models of resistance were tested. For the E2 population, the dose response curve of BC progeny was similar to the expected curve and fitted a 3:1 segregation pattern for clethodim, indicating that clethodim resistance was likely controlled by two additive genes, both dominant (Figure 3B). However, for the A91 population the segregation pattern fitted more closely a 1:3 segregation pattern, indicating two genes are involved in clethodim resistance in this

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population as well, except one of the genes is recessive at the rates of clethodim used (Figure 3A).

Herbicide resistance due to two or more genes has been previously reported in a glyphosate resistant rigid ryegrass population from California (Simarmata et al. 2005). Similarly, in a diclofop-methyl resistant population of tame oat (*Avena sativa*) resistance was controlled by two genes with recessive action (Warkentin et al. 1988), whilst in three tolerant maize inbred lines, the response to diclofop-methyl was highly heritable and controlled by a minimum of three genes (Geadelmann and Andersen 1977). This indicates that even within the same species, the number of genes controlling resistance can be different across resistant populations.
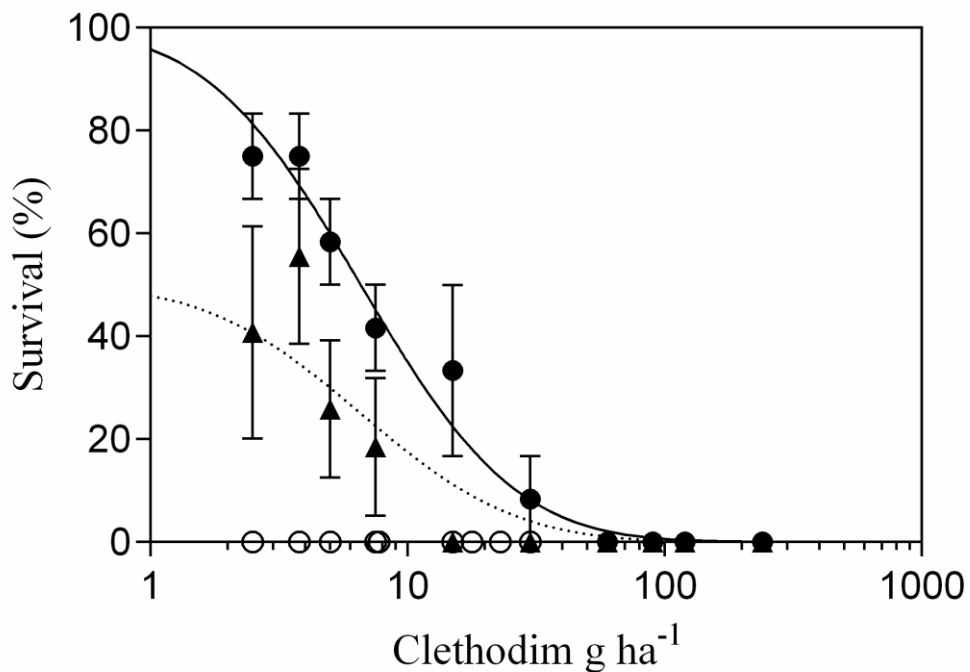

Figure 2. Dose-response of susceptible (○), F$_1$ (●), and backcross (▲) progeny of FP population of rigid ryegrass to clethodim. The dotted line is the predicted response for resistance to be the result of a single allele dominant at low doses. Data points are means ± 95% confidence intervals for three replicates.

Table 3. Number of backcross individuals designated as dead or alive to different clethodim treatment and G-test values for goodness of fit to the expected ratio for three different inheritance models.[a]

| Backcross family	Clethodim treatment (g ha^{-1})	Observed plant numbers		Model					
				1:1 R:S		3:1 R:S		1:3 R:S	
		Dead	Alive	G-statistic	P	G-statistic	P	G-statistic	P
2-F$_1$FP × SLR4	2.4	7	11	0.87	0.35	1.63	0.20	10.19	0.00
	3.8	12	15	0.33	0.57	4.72	0.03	11.19	0.00
	5.0	20	7	6.41	0.01	28.06	0.00	0.01	0.91
	7.5	23	5	11.34	0.00	37.31	0.00	0.63	0.43
2-F$_1$E2 × SLR4	2.4	10	8	0.22	0.64	7.39	0.01	3.12	0.08
	3.8	20	7	6.41	0.01	28.06	0.00	0.01	0.91
	5.0	15	12	0.33	0.57	11.19	0.00	4.72	0.03
	7.5	25	2	22.75	0.00	55.18	0.00	5.57	0.02
4-F$_1$91 × SLR4	2.4	15	3	8.50	0.00	26.36	0.00	0.71	0.40
	3.8	23	4	18.25	0.00	48.53	0.00	3.23	0.07
	5.0	23	4	14.51	0.00	42.63	0.00	1.64	0.20

[a]Abbreviation: F$_1$, first filial generation
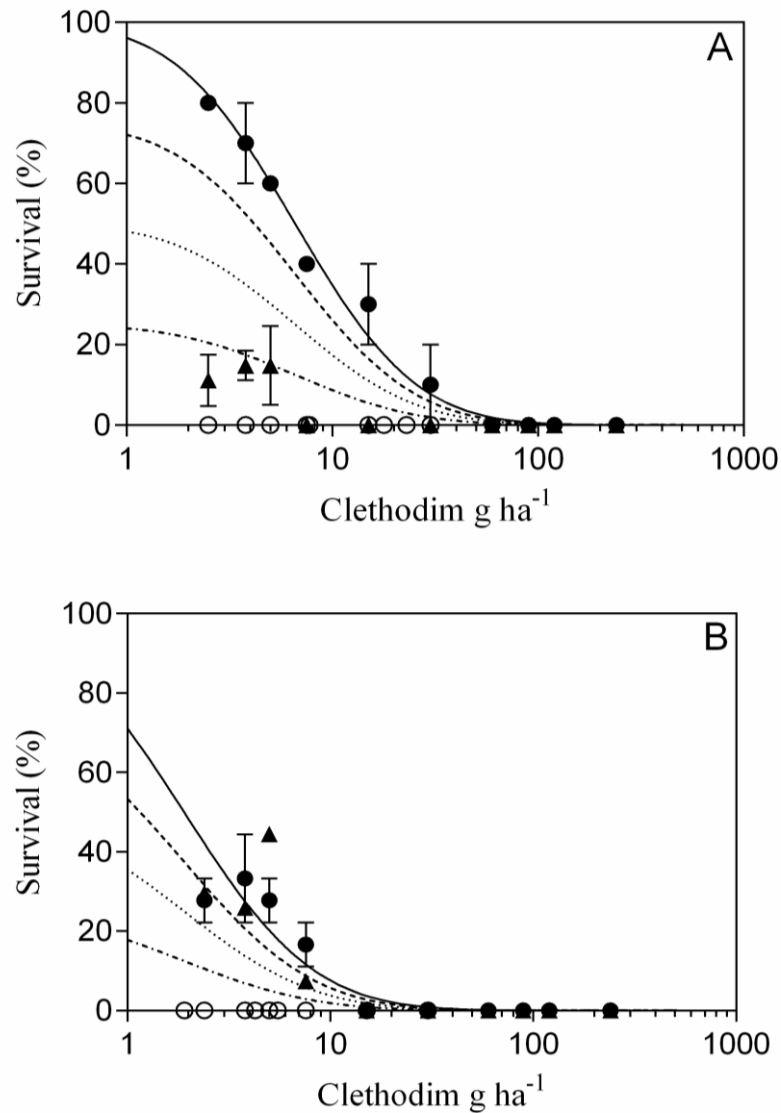
Figure 3. Dose-response of susceptible (○), F$_1$ (●), and backcross (▲) progeny of (A) A91 and (B) E2 populations of rigid ryegrass to clethodim. The dotted line is the predicted response for resistance to be the result of a single allele dominant at low doses. The dashed line is the predicted response for resistance to be the result of alleles at two genes both dominant at low doses. The dash and dotted line is the predicted response for two genes, one dominant and one recessive at low doses. Data points are means ± 95% confidence intervals for three replicates.

For the A61 population, the response of the BC progeny of this population was similar to the female parents. In BC population where the maternal parent was a

susceptible biotype (F$_1$♀S), produced susceptible progeny but when the maternal parent was a resistant biotype (F$_1$♀R), the BC progeny were resistant (Figure 4). These BC results confirmed the resistance trait was largely controlled by cytoplasmic DNA.
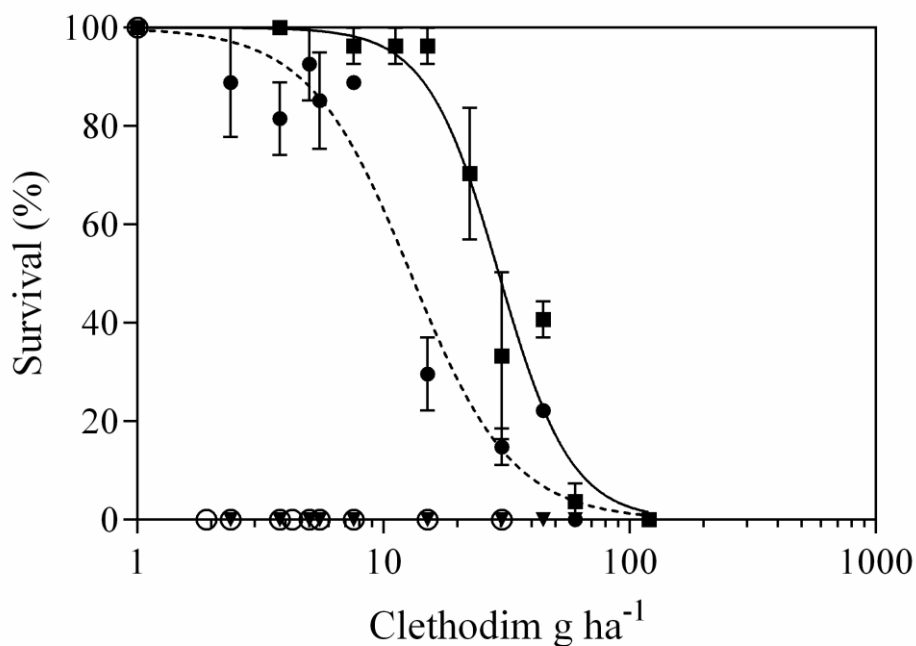

Figure.4. Dose-response of S parent (○), R parent (■), and backcross progeny F$_1$♀R × S (●), F$_1$♀S × S (▼) of A61 population of rigid ryegrass to clethodim.

The predominance of major gene inheritance can be attributed to the fact that herbicides are highly target site specific, interfering with a single enzyme in major metabolic pathways. Mutations with the gene encoding the target enzyme may decrease sensitivity to the herbicide and result in resistance (Jasieniuk et al. 1996). Secondly, repeated application of these highly effective herbicides imposes strong selection pressure on susceptible populations and if resistant weeds are present, even at very low frequencies,

rapid selection for resistance can occur. In contrast, polygenic inheritance would require recombination among individuals for many generations to bring together a sufficient number of alleles, each with a small additive effect, to produce a highly resistant population of weeds (Chauvel and Gasquez 1994).

In conclusion, this work demonstrates several different patterns of inheritance of clethodim resistance in rigid ryegrass. Variation in the pattern of inheritance indicates that different resistance mechanisms may be present in different rigid ryegrass populations. The patterns of resistance included a single gene, partially dominant, nuclear encoded trait, two different patterns of two-gene inheritance and an example of maternal inheritance of the resistance trait. It has been previously confirmed that more than one mechanism of herbicide resistance can co-exist within populations of rigid ryegrass (Christopher et al. 1992) and so the inheritance patterns for resistance may vary within and between populations. It can be important to determine the inheritance mechanism of resistant weed population to aid management. The management options for resistance that is inherited as a recessive allele can be different to those where inheritance is inherited as a dominant allele (Huang et al. 1999). Therefore, the knowledge of inheritance pattern of clethodim resistance in rigid ryegrass may help in the development of possible management strategies, judicious use of herbicides as well as preventing the spread of resistance.

Acknowledgements

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CHAPTER 5

PERSISTENCE OF RESISTANCE ALLELES–1781, 2041 AND 2078 IN CLETHODIM-RESISTANT RIGID RYEGRASS POPULATIONS IN THE ABSENCE OF HERBICIDE SELECTION

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Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Rupinder Kaur Saini
Contribution to the Paper	Planned the study, conducted all experiments, analysed and interpreted data, and wrote the manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised development of work, reviewed the studies, helped in data interpretation.
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CHAPTER 6

ALTERNATIVE HERBICIDES FOR THE MANAGEMENT OF CLETHODIM RESISTANT RIGID RYEGRASS (*LOLIUM RIGIDUM*) IN FABA BEAN (*VICIA FABA* L.) IN SOUTHERN AUSTRALIA

Rupinder Kaur Saini, Samuel Kleemann, Christopher Preston and Gurjeet Gill

The University of Adelaide, Waite Campus, South Australia

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Overall percentage (%)	85%
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Alternative Herbicides for the Management of Clethodim-Resistant Rigid Ryegrass (*Lolium rigidum*) in Faba Bean (*Vicia faba* L.) in Southern Australia

Rupinder Kaur Saini, Samuel G. L. Kleemann, Christopher Preston, and Gurjeet S. Gill*

Two field experiments were conducted during 2012 and 2013 at Roseworthy, South Australia to identify effective herbicide options for the management of clethodim-resistant rigid ryegrass in faba bean. Dose–response experiments confirmed resistance in both field populations (B3, 2012 and E2, 2013) to clethodim and butroxydim. Sequencing of the target site of acetyl coenzyme A carboxylase gene in both populations identified an aspartate-2078-glycine mutation. Although resistance of B3 and E2 populations to clethodim was similar (16.5- and 21.4-fold more resistant than the susceptible control SLR4), the B3 population was much more resistant to butroxydim (7.13-fold) than E2 (2.24-fold). Addition of butroxydim to clethodim reduced rigid ryegrass plant density 60 to 80 % and seed production 71 to 88 % compared with the standard grower practice of simazine PPI plus clethodim POST. Clethodim + butroxydim combination had the highest grain yield of faba bean (980 to 2,400 kg ha^{-1}). Although propyzamide and pyroxasulfone plus triallate PPI provided the next highest levels of rigid ryegrass control ($<$ 60 %), these treatments were more variable and unable to reduce seed production (6,354 to 13,570 seeds m^{-2}) to levels acceptable for continuous cropping systems.
Nomenclature: Clethodim; rigid ryegrass, *Lolium rigidum* Gaudin; faba bean, *Vicia faba* L.
Key words: Herbicide strategies, PPI herbicides, target site mutation, weed control.

En 2012 y 2013, se realizaron dos experimentos de campo en Roseworthy, en el sur de Australia, para identificar opciones de herbicidas efectivos para el manejo de *Lolium rigidum* resistente a clethodim en campos de haba. Experimentos de respuesta a dosis confirmaron la presencia de resistencia a clethodim y butroxydim en ambas poblaciones de campo (B3, 2012 y E2, 2013). La secuenciación del sitio activo del gen de acetyl coenzyme A carboxylase identificó la mutación aspartate-2078-glycine en ambas poblaciones. Aunque la resistencia a clethodim de B3 y E2 fue similar (16.5 y 21.4 veces más resistentes que el control susceptible SLR4), la población B3 fue mucho más resistente a butroxydim (7.13 veces) que E2 (2.24 veces). La adición de butroxydim a clethodim redujo la densidad de *L. rigidum* 60 a 80% y la producción de semilla 71 a 88%, al compararse con la práctica estándar de los productores de aplicar simazine PPI más clethodim POST. La combinación de clethodim + butroxydim tuvo el mayor rendimiento de grano de haba (980 a 2,400 kg ha^{-1}). Aunque propyzamide y pyroxasulfone más triallate PPI brindaron los segundos niveles de control de *L. rigidum* más altos ($<$ 60%), estos tratamientos fueron más variables e incapaces de reducir la producción de semillas (6,354 a 13,570 semillas m^{-2}) a niveles aceptables para sistemas de cultivo continuo.

Rigid ryegrass is one of the most troublesome herbicide-resistant weeds in Australia (Jones et al. 2005), and it has evolved resistance to at least 11 major herbicide mode-of-action groups (Heap 2015). As a result of its suitability to the climate, its extensive use as a pasture species, and its prolific seed production (Rerkasem et al. 1980), rigid ryegrass is present in high numbers across the southern Australian grain belt (Pannell et al. 2004). It is highly competitive against pulse crops and can significantly reduce grain yield (Hashem et al. 2011;

Lemerle et al. 1995). Rigid ryegrass can produce more than 1,000 seeds per plant even in extremely competitive surroundings (McGowan 1967), which enables the plants surviving weed control to readily replenish the seed bank and reinfest subsequent crops. In early to late 1980s rigid ryegrass was effectively controlled in crops with grass-selective herbicides. However, overreliance on the acetyl coenzyme A carboxylase (ACCase)-inhibiting and acetolactate synthase-inhibiting herbicides has led to the evolution of widespread resistance to these important herbicide groups (Boutsalis et al. 2012).

As many populations of rigid ryegrass have evolved resistance to the grass-selective herbicides, control options for rigid ryegrass in broadleaf crops have become far more limited. Clethodim, an

ACCase-inhibiting cyclohexanedione herbicide, has been widely used to provide selective control of grass weeds in many broadleaf crops (Burke et al. 2004; Burke and Wilcut 2003; Vidrine et al. 1995). Clethodim is deemed to be the lowest-resistance-risk herbicide, with only 2 of 11 target-site mutations found in weed populations endowing resistance to this herbicide (Beckie and Tardif 2012). Aspartate-2078-glycine and cystine-2088-arginine mutations in the plastidic ACCase enzyme have been identified as the main mutations endowing clethodim resistance at field rates (Yu et al. 2007). Délye et al. (2008) reported that leucine-1781, glycine-2078, and alanine-2096 mutations in the ACCase gene may also confer resistance to clethodim in the field if conditions are not optimal for herbicide efficacy, or with reduced application rates. Repeated use of clethodim over the last 2 decades has resulted in the evolution of clethodim resistance in rigid ryegrass populations (Yu et al. 2007), with as many as 60% of fields across southeastern Australia having some level of clethodim resistance (Boutsalis et al. 2012). In the past, clethodim resistance was managed by increasing its dose; however, this is no longer a viable option for growers as several populations of rigid ryegrass are now resistant to clethodim doses greater than the recommended field rate (120 g ai ha^{-1}).

For growers with clethodim-resistant rigid ryegrass, there are few herbicide options available for its selective control in broadleaf crops. Consequently, the use of PPI herbicides has increased to reduce reliance on grass-selective herbicides like clethodim. Traditionally trifluralin PPI was used to control rigid ryegrass in southern Australia. However, many rigid ryegrass populations across southern Australia have now evolved resistance to trifluralin (Boutsalis et al. 2012). Given the increasing prevalence of clethodim-resistant rigid ryegrass in southern Australia, there is an urgent need to identify alternative herbicides for its control. Therefore, the objective of this study was to identify effective herbicide options for the control of clethodim-resistant rigid ryegrass in faba bean, which is an important pulse crop in South Australia.

Materials and Methods

Dose–Response Experiments. Dose–response experiments were conducted to determine the resistance status of two field populations (B3 and E2) to

clethodim and butroxydim during the growing seasons of 2012 and 2013. Seedlings at one-leaf growth stage were randomly collected from two different experimental sites at Roseworthy located in the Lower North region of South Australia (34.51°S, 138.68°E at 68 m above sea level) and transplanted into 9.5 cm by 8.5 cm by 9.5 cm pots containing cocoa peat potting mix (Boutsalis et al. 2012). There were nine seedlings per pot and the pots were arranged in a randomized complete block design with three replications. The plants were maintained outdoors under natural conditions and watered and fertilized as needed. A highly characterized herbicide-susceptible (S) rigid ryegrass biotype (SLR4) served as the control population (Wakelin and Preston 2006). At the two- to three-leaf growth stage, rigid ryegrass seedlings were treated with clethodim and butroxydim. Clethodim was applied at 0, 7.5, 15, 30, 60, and 120 g ai ha^{-1} for S plants, and 0, 30, 60, 120, 240, and 480 g ha^{-1} for R plants. Butroxydim was applied to both resistant and susceptible populations at 0, 5.6, 11.3, 22.5, 45, and 90 g ai ha^{-1}. The recommended dose of clethodim in Australia is 120 g ha^{-1} and for butroxydim 45 g ha^{-1}. As per label recommendations, esterified canola oil mixed with nonionic surfactant Hasten^{TM} (Victorian Chemical Co. Pty. Ltd., Victoria, Australia) at 1% (v/v) was added to clethodim and 1% (v/v) paraffinic oil mixed with nonionic surfactant Supercharge® (Crop Care Australasia Pty. Ltd., Queensland, Australia) to butroxydim. The herbicides were applied by using a laboratory moving boom sprayer (Tee-Jet 110° flat fan, Spraying Systems, Wheaton, IL) equipped with a single nozzle (Hardi ISO F-110-01, Hardi, Adelaide). The output volume of the sprayer was 103 L ha^{-1} at a pressure of 250 kPa and a speed of 1 m s^{-1}. Control plants were not treated with herbicide. Plants were returned and maintained outdoors after herbicide treatment. Three weeks after spraying, surviving plants were counted and recorded as alive if they had strongly tillered since herbicide application and plants showing chlorosis, stunting, and mortality were considered as susceptible (Powles et al. 1998). The surviving plants were harvested and oven dried at 80 C for 2 d. The dry weight data were expressed as a percentage of the respective unsprayed control.

Sequencing of ACCase gene. Fresh leaf material ($\sim$ 1 cm^2) was harvested from young leaves of at least 12 resistant plants of both field populations

Table 1. Primer sequences used for amplification and sequencing of the carboxyl transferase (CT) domain of the *acetyl coenzyme A carboxylase* (*ACCase*) gene in *Lolium rigidum* from genomic DNA.

Primer	Sequence 5' 3'
Acclr9	ATGGTAGCCTGGATCTTGGACATG
Acclr6	GGAAGTGTCATGCAATTCAGCAA
AccCT 2F	CCACTCCTGAATTTCCCAGTGG
AccCT 2R	CGCGATTTGAGTGTACAAAGGCTG
AccCT MidF	CCTGAGAATACATGTGATCCTCGTG
AccCT MidR	CCATTTCCCTTGGCTGTCATCAATGCC

(B3 and E2), snap frozen in liquid nitrogen, and stored at −20 C until its use. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions. Primers were designed to amplify regions in the carboxyl tranferase (CT) domain known to be involved in sensitivity to ACCase herbicides. Two sets of primers covering all seven known mutation sites (1781, 1999, 2027, 2041, 2078, 2088, and 2096) were designed against the blackgrass (*Alopercurus myosuroides* Huds.) (accession number AJ310767) *ACCase* gene sequence (Table 1) and were used to amplify a 1.5-kb fragment covering nearly the entire CT domain without any intron. The range of amino acids covered by the fragment was equivalent to codons 1658–2157 in blackgrass. A nested polymerase chain reaction (PCR) approach was used with oligo set Acclr9 and Acclr6 (Zhang and Powles 2006) followed by oligo set AccCT 2F and AccCT 2R (Malone et al. 2014). MyFi DNA polymerase kit (Bioline, Australia Pty Ltd, Alexandria, NSW, 1435) was used to run PCR reactions of 25 μl that contained 80 to 100 ng of DNA template, 1× MyFi reaction buffer, 0.8 μM of each specific primer, and 2 units of MyFi DNA polymerase. Amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany) with PCR conditions as described by Malone et al. (2014).

The PCR products were examined on 1.5% agarose gels stained with 1× SYBR® Safe DNA gel stain. Samples were electrophoresed in 1× TAE buffer (40 mM Trizma base, 1 mM Na₂EDTA, pH to 8 with glacial acetic acid) at 90 V and photographed under UV light (λ302 nm). DNA fragment sizes were estimated by comparing their mobility with bands of known sizes in a low-mass molecular weight marker (Invitrogen, Australia). PCR products were sequenced by Australian Genome Research Facility Ltd., Australia using primers CT Mid F and CT Mid R (Malone et al. 2014) to obtain sequence data covering the full CT domain fragment. DNA sequence data were assembled, compared, and analyzed using ContigExpress from the Vector-NTi Advance 11.5 programs (Invitrogen) and all sequences visually rechecked using the chromatogram files.

Field Experiments. Two field experiments were conducted in different fields (B3 and E2) over the growing seasons of 2012 and 2013 at Roseworthy located in the Lower North region of South Australia (34.51°S, 138.68°E at 68 m above sea level). The soil at the field sites was a calcarosol (McKenzie et al. 2001) with organic matter content of 2 to 2.5% and a pH (water) of 7 to 7.5 in 0- to 20-cm layer. The long-term average annual rainfall at Roseworthy is 434 mm and average growing season rainfall (April to October) is 321 mm. Rainfall received at the site in 2012 and 2013 is shown in Table 2 (Australian Bureau of Meteorology 2014). Before the start of the experiment, the field sites were treated with glyphosate (900 g ai ha⁻¹) and oxyfluorfen (22 g ha⁻¹) for preplant weed control. Faba beans (cv. Nura) were sown at a depth of 5 cm at a seed rate of 150 kg ha⁻¹ (for a target of

Table 2. Summary of rainfall, cropping history, harvest, and herbicide application dates for the two study sites.

Year	Rainfall		Previous crop	Harvest date	Spray application date	
	Annual	GSR[a]			PPI	POST[b]
	mm					
2012	337	243	Lentil	November 27	May 21	July 20
2013	417	341	Barley	November 18	May 16	July 2

[a] Abbreviation: GSR, growing-season (April to October) rainfall.
[b] POST herbicide treatments were applied to rigid ryegrass at three- to four-leaf stage of growth.

Population	LD_{50} (g ha^{-1})	R/S	GR_{50} (g ha^{-1})	R/S
Experiment 1 (May 2012)				
Clethodim				
B3	68.2 (54.5, 85.3)	16.5	38.1 (30.4, 47.7)	9.7
SLR4	4.13 (2.5, 6.8)	-	3.9 (2.4, 6.4)	-
Butroxydim				
B3	26.9 (24.2, 29.9)	7.1	6.3 (3.6, 11.1)	1.6
SLR4	3.8 (3.0, 4.8)	-	4.0 (3.4, 4.7)	-
Experiment 2 (May 2013)				
Clethodim				
E2	58.1 (49.9, 67.7)	21.4	32.7 (26.4, 40.4)	12.3
SLR4	2.7 (2.7, 2.8)	-	2.7 (2.6, 2.7)	-
Butroxydim				
E2	7.7 (6.9, 8.7)	2.2	5.0 (4.3, 5.8)	1.6
SLR4	3.5 (2.6, 4.7)	-	3.0 (2.1, 4.5)	-

30 plants m^{-2}). The crop was sown on May 22, 2012 and June 5, 2013 using a no-till plot seeder fitted with knife-point openers (16 mm) and press wheels. Plots were 10-m long and contained six crop rows spaced 25 cm apart. Fertilizer rate was consistent with the local grower practice of 100 kg ha^{-1} of diammonium phosphate (18 kg N and 20 kg P ha^{-1}) banded below the seed at sowing. The experiments were established in a randomized complete block design with four replicates. Herbicides (PPI and POST) were applied using an all-terrain vehicle fitted with a spray boom delivering 100 L ha^{-1} water volume at a pressure of 200 kPa. Herbicide treatment of PPI simazine (1,350 g ha^{-1}) followed by POST clethodim (120 g ha^{-1}) is considered the standard grower practice for the district. The dose and timing of other herbicide treatments are presented in Table 4. As per label recommendations, clethodim was applied with spray adjuvant Hasten at 1% (v/v) (vegetable oil, Victorian Chemicals) and butroxydim with non-ionic surfactant Supercharge when rigid ryegrass had reached the three- to four-leaf growth stage.

Effect of Herbicides on Rigid Ryegrass Density and Seed Production and Grain Yield of Faba Bean.

Rigid ryegrass plant and spike density were assessed in a 0.25-m^2 quadrat placed at four random locations in each plot. Assessments on rigid ryegrass plant density were taken 12 weeks after sowing (WAS) before and after POST herbicide application. Spike density was assessed 16 to 18 WAS in October when all spikes had emerged. Seed production of rigid ryegrass was determined by

Table 4. Effect of herbicide treatments on rigid ryegrass plant and spike density, and seed production in faba beans grown at Roseworthy in 2012 and 2013.[a]

Treatment	Rate	Rigid ryegrass[b] Weed density 2012	Weed density 2013	Spike density 2012	Spike density 2013	Seed production 2012	Seed production 2013
	g ai ha^{-1}	——Plant m^{-2}——		——Spikes m^{-2}——		——Seed m^{-2}——	
Simazine PPI + clethodim POST	1,350, 120	181 d	112 bc	350 c	8 a	15,873 cd	73 a
Simazine PPI + clethodim + butroxydim POST	1,350, 120 + 45	73 ab	22 a	105 a	2 a	4,539 a	9 a
Dimethenamid PPI	720	300 e	185 bcd	748 d	281 bc	3,5169 e	15,074 b
Pyroxasulfone PPI	100	113 bc	208 bcd	271 bc	219 b	1,2498 bc	10,067 ab
Pyroxasulfone + triallate PPI	100 + 800	83 ab	199 bcd	149 ab	252 bc	6,354 a	13,171 b
Prosulfocarb + S-metolachlor PPI	2,000 + 300	173 d	325 d	393 c	449 c	18,704 d	20,775 b
Prosulfocarb + S-metolachlor + triallate PPI	2,000 + 300 + 800	147 cd	221 cd	335 c	287 bc	16,134 cd	15,032 b
Propyzamide PPI	500	64 a	101 b	172 ab	256 bc	7,742 ab	13,570 b

[a] Means within the same column followed by the same letters are not significantly different according to P = 0.05.
[b] Rigid ryegrass plant density m^{-2} data were square root transformed before mean comparisons. Data presented are the nontransformed mean values.
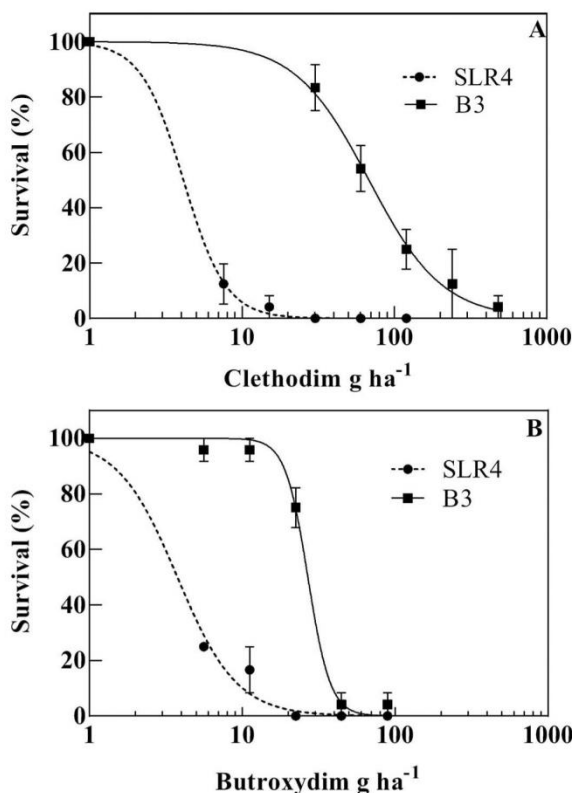
Figure 1. Dose–response experiments undertaken in May 2012 (1A and 1B) showing the response of field populations of ryegrass B3 (■) and E2 (▼) and a known susceptible population (●) to clethodim and butroxydim. Recommended rate of clethodim is 120 g ha^{-1} and butroxydim is 45 g ha^{-1}. Each data point is a mean of three replicates and bars represent standard error of the mean. Nonlinear regression analysis was performed using the equations (1A): $y = 100/1 + 10^{(1.833 - x) \times -1.795}$, (1B): $y = 100/1 + 10^{(1.430 - x) \times -5.902}$, where y is the plant survival (%) or biomass reduction (%) and x is the log dose of the herbicide used.

counting the number of spikes of 20 rigid ryegrass plants randomly sampled from each plot. The number of seeds produced per plant was determined by multiplying the number of spikes by the average number of seed in each spike. The number of seed produced per unit area was based on the weed density and shown as number of seeds per square meter. Faba bean grain yield was determined with a small plot harvester when the crop had reached physiological maturity and 12% grain moisture content. Harvest dates in each experiment are

shown in Table 2. A grain subsample of 500 g was used to determine the average grain weight (g [100 seeds]$^{-1}$).

Statistical Analyses. Weed control (plant and spike density, and rigid ryegrass seed production) and crop data were analyzed separately with ANOVA (Genstat 5 Committee 2003). A square-root variance-stabilizing transformation was used for rigid ryegrass plant density data before analysis. Original means are reported with mean separation of the transformed data. Means were separated using LSD at P = 0.05. Data (plant survival and dry weight) from dose–response experiments were analyzed using log-logistic equation (Graphpad Prism v.6.0; GraphPad Software, San Diego, CAia) to calculate the dose of herbicide required to produce 50% reduction in plant survival (LD$_{50}$) and dry weight (GR$_{50}$). The model fitted was

$$y = 100/\left[1 + 10^{(\log IC_{50} - x) \times b}\right] \qquad [1]$$

where y is the plant survival (%) or biomass reduction (%), x is the log dose of the herbicide used, IC$_{50}$ is the dose of herbicide required to produce 50% reduction in plant survival or biomass, and b is the slope of the curve.

Results and Discussion

Dose–Response Experiments. Dose–response experiments confirmed resistance in both field populations (B3 and E2) to clethodim, whereas the susceptible population (SLR4) was controlled with less than the recommended field rate (< 120 g ha^{-1}) of clethodim (Figures 1A and 2A). The LD$_{50}$ values for clethodim were 68.2 and 58.1 g ha^{-1} for R populations B3 and E2, respectively. In contrast, the S population showed 50% mortality at 4.13 and 2.71 g ha^{-1}, respectively (Table 3). On the basis of the LD$_{50}$ values, the R populations B3 and E2 were approximately 16 and 21 times more resistant to clethodim than the S population SLR4 (Table 3). Similar levels of resistance (20-fold) were reported for several populations of rigid ryegrass from the Western Australian wheat belt (Yu et al. 2007). According to Yu et al. (2007), the ACCase mutation present, the homo-/heterozygous status of a plant for a specific mutation, combinations of different resistance alleles, and herbicide rate used in the field contribute to the overall level of resistance
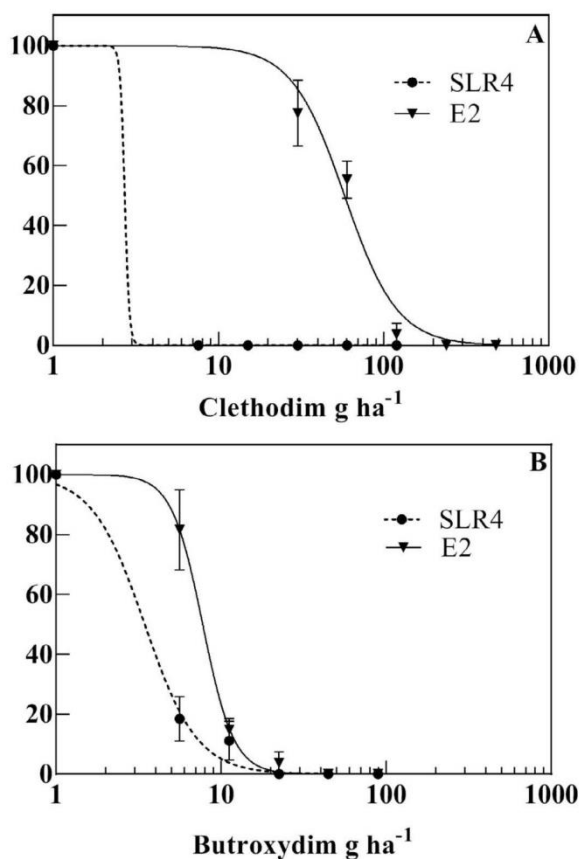
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Figure 2. Dose–response experiments undertaken in May 2013 (2A and 2B) showing the response of field populations of ryegrass B3 (■) and E2 (▼) and a known susceptible population (●) to clethodim and butroxydim. Recommended rate of clethodim is 120 g ha^{-1} and butroxydim is 45 g ha^{-1}. Each data point is a mean of three replicates and bars represent standard error of the mean. Nonlinear regression analysis was performed using the equations (2A): $y = 100/1 + 10^{(1.766 - x) \times -2.743}$, (2B): $y = 100/1 + 10^{(0.8882 - x) \times -4.629}$, where y is the plant survival (%) or biomass reduction (%) and x is the log dose of the herbicide used.

to clethodim. The clethodim rate causing 50% reduction of shoot growth (GR$_{50}$) for the S population was 3.93 and 3.97 g ha^{-1} as compared with 38.1 and 32.7 g ha^{-1} for R populations B3 and E2, respectively.

Dose responses of the R populations (B3 and E2) and the S (SLR4) to butroxydim (Figures 1B and 2B) were different. The susceptible population (SLR4) was easily controlled at the recommended butroxydim field rate of 45 g ha^{-1}, with LD$_{50}$ values

of 3.77 and 3.45 g ha^{-1}, whereas R populations B3 and E2 required 26.9 and 7.73 g ha^{-1} of butroxydim, respectively (Table 3). Population B3 was more resistant to butroxydim when compared with the E2 population in terms of plant survival (LD$_{50}$), but plants that survived herbicide treatment were stunted in growth as indicated by the modest GR$_{50}$ values (1.60) of both field populations. Cross-resistance to butroxydim in clethodim-resistant field populations is not surprising given that many of the ACCase mutations can endow resistance to other ACCase-inhibitor herbicides including clodinafop, diclofop, fluazifop, haloxyfop, sethoxydim, tralkoxydim, and pinoxaden (Yu et al. 2007).

Sequencing of *ACCase* gene. A target-site mutation for clethodim resistance was identified in both field populations of rigid ryegrass. The nucleotide sequences of the field populations differed from that of the susceptible population by a single nucleotide. Sequencing results revealed an aspartate-2078-glycine substitution in the CT domain of plastidic ACCase in both field populations of rigid ryegrass. Amino acid modification at position 2078 is known to provide strong resistance to clethodim (Délye et al. 2008) and has been already confirmed in rigid ryegrass (Yu et al. 2007), black-grass (Délye 2005), and wild oats (*Avena fatua* L.) (Cruz-Hipolito et al. 2011). The continuous use of clethodim over an extended period on rigid ryegrass populations that already had resistance to other ACCase-inhibiting herbicides might be selecting for the accumulation of amino acid modifications within the *ACCase* gene that contribute to clethodim resistance.

Effect of Herbicides on Rigid Ryegrass Density and Seed Production. Rigid ryegrass density was affected (P < 0.05) by herbicide treatments in both years of the study (Table 3). PPI simazine followed by POST clethodim plus butroxydim had the lowest density of rigid ryegrass in 2012 (73 plants m^{-2}) and 2013 (22 plants m^{-2}). Even though rigid ryegrass was confirmed resistant to both clethodim and butroxydim (Table 3), the mixture of the two reduced rigid ryegrass density relative to the standard grower practice of simazine PPI followed by clethodim POST by 60% in 2012 and 80% in 2013. As mentioned earlier, plants surviving butroxydim in the dose–response study were stunted (GR$_{50}$ = 1.6) and indicated modest levels of resistance in both of these populations. Conse-

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quently, application of clethodim–butroxydim mixture significantly reduced rigid ryegrass plant and spike density in the field (Table 4). Among PPI herbicide treatments, propyzamide was the most effective option against rigid ryegrass; however, additional weed control relative to the grower practice was much higher in 2012 (65%; $P < 0.05$) than in 2013 (10%). In 2012, there was 42 mm of rainfall in the week after herbicide application as compared with 23 mm of rainfall within this period in 2013. In previous research, Walker and Roberts (1975) showed that weed control with propyzamide was positively correlated with the amount of rainfall received in 7 d after application. Therefore, greater rainfall after herbicide application in 2012 may be responsible for better weed control with propyzamide in 2012 than in 2013. Kleemann and Gill (2012) reported much higher levels of rigid ryegrass control with propyzamide ($> 85\%$) in faba bean; however, the herbicide was applied early postsowing PRE rather than PPI as in this study. Failure of propyzamide to control rigid ryegrass in 2013 because of dry conditions reflected in high spike density (256 spikes m^{-2}) and seed set ($> 13,000$ seeds m^{-2}; Table 4). PPI applications of dimethenamid-P, prosulfocarb plus S-metolachlor, and pyroxasulfone alone or in combination with triallate provided modest ($\sim 54\%$) and in some instances no reduction in rigid ryegrass density relative to the standard grower practice in 2012 and 2013. Pyroxasulfone and prosulfocarb plus S-metolachlor in wheat were shown to provide 64 to 94% control of rigid ryegrass (Boutsalis et al. 2014; Kleemann et al. 2014). The combination of limited competitive ability of faba bean with weeds (Felton et al. 2004) and severe weed infestation at the experimental sites could have been responsible for the ineffectiveness of PPI herbicides used alone.

Rigid ryegrass spike number and seed production were significantly ($P < 0.05$) influenced by herbicide treatment in both years of the study (Table 4). Similar to the trend for weed control, PPI simazine followed by POST clethodim plus butroxydim had the lowest spike density (2 to 105 spikes m^{-2}). High rigid ryegrass plant density was recorded early in the growing season during 2013. However, greater rainfall received in late winter (July through August) in 2013 (111 mm) than in 2012 (59 mm) appeared to favor faba bean growth and its ability to suppress weed growth in the wetter season. Consequently, rigid ryegrass produced much greater spike density in simazine followed by POST clethodim plus butroxydim in 2012 than in 2013. This herbicide treatment also proved more effective in reducing seed production of rigid ryegrass in 2013 (two spikes m^{-2}; < 50 seeds m^{-2}) than in 2012 (105 spikes m^{-2}; 4,539 seeds m^{-2}). The lower level of resistance to butroxydim in the E2 population (2013) as compared with the B3 population (2012) may have contributed to this difference in rigid ryegrass density. Furthermore, faba beans are known for their high sensitivity to water stress (Mwanamwenge et al. 1999), which may have reduced their competitive ability against rigid ryegrass under drier conditions experienced in 2012. PPI herbicides resulted in a large buildup in the seed bank of rigid ryegrass, which could have serious effects on the productivity of subsequent crops in the rotation. These results suggest that PPI herbicides alone are inadequate for rigid ryegrass management in faba bean.

Effects of Herbicides on Grain Yield and Grain Weight of Faba Bean. In the absence of effective control, rigid ryegrass was extremely competitive against faba bean and caused large reductions ($P < 0.05$) in grain yield (Table 5). Reduction in grain yield due to rigid ryegrass competition has also been previously reported in other pulse crops (Hashem et al. 2011; Lemerle et al. 1995; McDonald 2003). In 2012, faba bean produced up to 50% less grain yield in treatments with low herbicide efficacy as compared with simazine PPI followed by clethodim plus butroxydim POST. However, the yield penalty in treatments with low weed control was $< 15\%$ in 2013. Greater sensitivity of faba bean to rigid ryegrass in 2012 could be the result of greater weed density and lower growing-season rainfall (Table 2), which could have exacerbated competition for soil water. In both years, the highest grain yields were recorded in simazine followed by clethodim plus butroxydim (980 to 2,400 kg ha^{-1}), which was most effective against rigid ryegrass. Faba bean grain yield in propyzamide (890 to 2,350 kg ha^{-1}) was similar to the simazine PPI followed by clethodim plus butroxydim POST treatment. Even though pyroxasulfone plus triallate PPI controlled rigid ryegrass 54% in 2012, faba bean yield at 550 kg ha^{-1} was lower than the standard grower practice simazine

Table 5. Effect of herbicide treatments on grain yield (kg ha^{-1}) and grain weight (g [100 seeds]$^{-1}$) of faba beans grown at Roseworthy in 2012 and 2013.[a]

| | | Grain yield | | Grain weight | |
		2012	2013	2012	2013
Treatment	Rate				
	g ai ha^{-1}	——kg ha^{-1}——		—g (100 seeds)$^{-1}$—	
Simazine PPI/clethodim POST	1,350 , 120	900 bc	2,110 a	576 bc	57.9 a
Simazine PPI/clethodim + butroxydim POST	1,350, 120 + 45	980 c	2,400 c	582 c	55.8 a
Dimethenamid PPI	720	490 a	2,170 ab	534 a	56.8 a
Pyroxasulfone PPI	100	680 ab	2,190 abc	552 abc	57.3 a
Pyroxasulfone + triallate PPI	100 + 800	550 a	2,190 abc	547 ab	55.1 a
Prosulfocarb + S-metolachlor PPI	2,000 + 300	860 bc	2,160 ab	559 abc	54.9 a
Prosulfocarb + S-metolachlor + triallate PPI	2,000 + 300 + 800	860 bc	2,060 a	572 bc	55.2 a
Propyzamide PPI	500	890 bc	2,350 bc	553 abc	57.6 a

[a] Means within the same column followed by the same letters are not significantly different according to LSD at the P = 0.05 level.

PPI plus clethodim and butroxydim POST at 900 kg ha^{-1}. Failure of faba bean to benefit from weed control as a result of treatment with pyroxasulfone could be associated with crop injury (data not shown). Similarly in previous research, Walsh et al. (2011) observed biomass reduction in faba bean with pyroxasulfone application.

Faba bean grain weight was not affected by the herbicide treatments in 2013 (54.9 to 57.9 g [100 seeds]$^{-1}$). However, in 2012, poor weed control with dimethenamid-P (53.4 g [100 seeds]$^{-1}$) significantly reduced faba bean grain weight as compared with simazine followed by clethodim plus butroxydim (58.2 g [100 seeds]$^{-1}$) (Table 5). Variation in crop response over the 2 yr is likely due to more competition between rigid ryegrass and the crop for water during the reproductive phase.

At present, there are no new grass-selective herbicides registered for use in broadleaf crops for the control of clethodim-resistant rigid ryegrass in Australia. The field studies reported here examined the efficacy of some currently registered herbicides for controlling clethodim-resistant rigid ryegrass in faba bean. From the herbicides examined, simazine PPI followed by clethodim plus butroxydim POST was the most effective treatment for control of clethodim-resistant rigid ryegrass. Of the herbicides applied PPI, propyzamide appeared to be the most effective; however, rigid ryegrass was still able to set a large amount of seed, which could have serious implications for weed management and productivity of subsequent crops in the rotation. Although the combination of simazine PPI with clethodim plus butroxydim POST was effective for the control of clethodim-resistant rigid ryegrass in faba bean,

resistance to both of these herbicides is steadily increasing in field populations of rigid ryegrass. There is an urgent need for the adoption of nonchemical weed control tactics to reduce the current heavy dependence on herbicides for weed control in Australian cropping systems. However, herbicides are likely to remain an important component of Australian cropping systems. Therefore, a serious effort is needed to identify effective alternatives to clethodim and butroxydim for POST use in broadleaf crops in southern Australia.

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CHAPTER 7

CONTROL OF CLETHODIM-RESISTANT *LOLIUM RIGIDUM* (RIGID RYEGRASS) IN TRIAZINE-TOLERANT CANOLA (*BRASSICA NAPUS* L.) IN SOUTHERN AUSTRALIA

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Overall percentage (%)	85%
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Signature	Date 14.12.2015

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Control of clethodim-resistant *Lolium rigidum* (rigid ryegrass) in triazine-tolerant canola (*Brassica napus* L.) in southern Australia

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ABSTRACT

Two field experiments were conducted during 2013 and 2014 at Roseworthy, South Australia, to identify effective herbicide options for the control of clethodim-resistant *Lolium rigidum* in triazine-tolerant canola. Dose response experiments in pots confirmed resistance in both field populations (F4-2013 and S2-2014) of *L. rigidum* to clethodim and butroxydim. At the recommended field rate of clethodim (120 g ha^{-1}), F4 and S2 populations had 45% and 67% survival, respectively. Therefore, the field populations F4 and S2 were approximately 16- and 26-fold more resistant to clethodim than the susceptible population, SLR4. Also, the S2 population had greater resistance to butroxydim (7-fold) than the F4 population (4.5-fold). Sequencing of the target site of acetyl co-enzyme A carboxylase gene identified two known mutations (aspartate-2078-glycine and cysteine-2088-arginine) in the F4 population and three known mutations (isoleucine-2041-asparagine, aspartate-2078-glycine and cysteine-2088-arginine) in the S2 population. Even though *L. rigidum* was confirmed resistant to clethodim and butroxydim, use of these (POST) herbicides in combination with preplant incorporated (PPI) herbicides improved weed control and canola yield relative to PPI herbicides only. Atrazine PPI followed by (fb) clethodim + butroxydim POST and atrazine PPI fb atrazine + clethodim POST reduced *L. rigidum* plant density (~52%) and seed production relative to the standard grower practice of atrazine PPI fb clethodim POST. These two herbicide combinations produced the highest seed yield of canola (1839–2196 kg ha^{-1}). Propyzamide alone (PPI) or as a split application (PPI fb POST) and in a mixture with clethodim improved *L. rigidum* control (32–63%) as compared to the standard grower practice but had limited impact on *L. rigidum* seed production. PPI herbicides alone were unable to effectively control clethodim-resistant *L. rigidum* in triazine-tolerant canola.

1. Introduction

Clethodim, a cyclohexanedione (CHD) herbicide, is widely used to control annual and perennial grasses in many broadleaf crops (Burke et al., 2004; Burke and Wilcut, 2003; Vidrine et al., 1995). In most broadleaf species, the selectivity of CHD herbicides results from an insensitive target enzyme acetyl coenzyme A carboxylase (ACCase; EC 6.4.1.2). ACCase catalyses the first committed step in de novo fatty acid biosynthesis in plants, which is essential for the production of cell membranes and secondary plant metabolites (Gronwald et al., 1992). In grasses, ACCase-inhibiting herbicides inhibit the homomeric plastidic ACCase. However, in most dicotyledonous species, their heteromeric chloroplastic ACCase is insensitive to ACCase-inhibiting herbicides (Devine, 1997; Powles and Yu, 2010).

Herbicides that inhibit ACCase include aryloxyphenoxypropionate (APP), CHD (Délye, 2005), and phenylpyraxoline (PPZ) chemistries (Hofer et al., 2006; Muehlebach et al., 2009). Due to the good crop tolerance and excellent efficacy of ACCase inhibitors, these herbicides have been extensively used to control grass weed species (Devine and Shimabukuro, 1994). Unfortunately, this frequent and widespread use of ACCase inhibitors has resulted in the evolution of resistance in 47 grass weed species worldwide (Heap, 2015). Weed species with high incidence of ACCase inhibitor resistance include *Alopecurus myosuroides* Huds. (Délye et al., 2005), *Avena fatua* L. (Cruz-Hipolito et al., 2011; Yu et al., 2013), *Lolium rigidum* Gaudin (Malone et al., 2014; Yu et al., 2007) and *Lolium perenne* ssp. *multiflorum* L. (Beckie and Tardif, 2012; Kaundun, 2014; Martins et al., 2014).

As Australia's most troublesome herbicide resistant weed

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L. rigidum has now developed resistance to eleven different groups of herbicides (Heap, 2015). Due to climatic adaptation and extensive use as a forage species in the past, *L. rigidum* is present in high densities across the southern Australian grain belt (Boutsalis et al., 2012; Pannell et al., 2004). *L. rigidum* is a prolific seed producer (Rerkasem et al., 1980), only a few plants need to survive weed control efforts to replenish the seed bank for re-infestation of subsequent crops. It is a highly competitive weed with broadleaf crops and can significantly reduce their grain yield (Hashem et al., 2011; Lemerle et al., 1995). In the past, *L. rigidum* was effectively controlled by clethodim in a wide variety of broadleaf crops.

Among ACCase inhibiting herbicides, clethodim has the lowest risk of development of resistance, with only two of eleven known target-site mutations in weed populations endowing resistance to this herbicide (Beckie and Tardif, 2012). Aspartate-2078-glycine and cystine-2088-arginine mutations in the plastidic ACCase enzyme have been identified as the main mutations that confer clethodim resistance at field rates of herbicide application (Yu et al., 2007). However, leucine-1781 and alanine-2096 mutations in ACCase gene may also confer low level of resistance to clethodim in the field if environmental conditions are not optimal for herbicide efficacy, or when herbicide rates are reduced (Delye et al., 2008). Depending on the mutations present in the population, clethodim has the ability to control *L. rigidum* populations resistant to other ACCase inhibiting herbicides (Yu et al., 2007), and has been extensively used in Australia for the control of *L. rigidum* in many broadleaf crops (Boutsalis et al., 2012). This over reliance on clethodim has resulted in the evolution of resistance in *L. rigidum* populations in Australia (Boutsalis et al., 2012; Yu et al., 2007). Initially, clethodim resistance was managed by the growers by simply increasing the herbicide rate from 60 g ha^{-1} to 120 g ha^{-1}. However, this is no longer possible as several clethodim-resistant *L. rigidum* populations can no longer be controlled with rates greater than the current recommended field rate (120 g ha^{-1}) (Boutsalis et al., 2012; Saini et al., 2015b, 2014). Furthermore, high rates of clethodim (>120 g ha^{-1}) use in canola can have various phytotoxic effects on the canola such as delayed flowering, distorted flower buds and grain yield suppression (Zerner, 2013). As a consequence, growers are reluctant to further increase the rates of clethodim in canola, and are finding it increasingly difficult to effectively control *L. rigidum* in this important crop.

Triazine-tolerant (TT) canola offers an opportunity to use a different herbicide mode of action for the control of *L. rigidum*. The productivity of TT canola can be affected by the fitness penalty associated with lower grain yield and oil content (Robertson et al., 2002), and the persistence of triazine herbicides in the soil can impact subsequent crops. However, TT canola is sown over a large area in the southern wheat-belt of Australia, mainly due to the weed control benefits it offers. Typically, growers use clethodim to control *L. rigidum* escapes from triazine herbicides when growing TT canola. However, with increasing resistance to clethodim it is not clear how effective planting TT canola alone will be for managing herbicide resistant *L. rigidum* populations. Therefore, the objective of this study was to identify effective herbicide options for the control of clethodim-resistant *L. rigidum* in TT canola.

2. Materials and methods

2.1. Dose response experiments

Dose response experiments were conducted at the University of Adelaide during the growing seasons of 2013 and 2014 to confirm the resistance to clethodim and butroxydim in the two populations (F4 and S2) present at the field sites. Based on previous field observations, both populations were suspected of being resistant to

clethodim. Seedlings of each *L. rigidum* field population were randomly collected from two different experimental sites at Roseworthy, South Australia (34.51°S, 138.68°E at 68 m above sea level) at the 1-leaf growth stage. Seedlings were transplanted into 9.5 cm by 8.5 cm by 9.5 cm plastic pots containing sterile cocoa peat potting medium (Boutsalis et al., 2012). Each pot had nine seedlings and the pots were maintained outdoors under natural conditions. The plants were watered and fertilized as needed. The experiments were conducted in a completely randomized block design with three replications. A standard susceptible (S) population, SLR4 (Wakelin and Preston, 2006) was included in each experiment for comparison.

At the 2 to 3-leaf growth stage, *L. rigidum* seedlings were treated with clethodim (Select®, Dow AgroSciences, Australia Ltd., NSW) and butroxydim (Factor* WG, CropCare Australasia, Queensland, Australia). Clethodim was applied at 0, 7.5, 15, 30, 60 and 120 g ha^{-1} for S plants, and 0, 30, 60, 120, 240 and 480 g ha^{-1} for the two field populations. Butroxydim was applied to both the suspected resistant and susceptible populations at 0, 5.6, 11.3, 22.5, 45 and 90 g ha^{-1}. The recommended rate for clethodim in Australia is 120 g ha^{-1} and for butroxydim 45 g ha^{-1}. Esterified canola oil plus non-ionic surfactant (Hasten TM, Victorian Chemical Co., Vic., Australia) at 1% (v/v) was added to clethodim and 1% (v/v) paraffinic oil (Supercharge®, CropCare Australasia, Qld., Australia) was added to butroxydim as recommended by the herbicide manufacturers. The herbicides were sprayed with a laboratory track boom sprayer equipped with a twin-nozzle (Hardi ISO F-110-01standard flat fan, Hardi, Adelaide) moving boom situated 40 cm above the pots and delivering 103 L ha^{-1} at 1 m s^{-1}and 250 kPa. Control plants were not treated with any herbicide. Plants were returned and maintained outdoors after herbicide treatment. Twenty eight days after herbicide treatment (DAT), the plants were scored as susceptible (dead) or resistant (alive). The surviving plants were harvested and oven dried at 80 °C for 2 days. The dry weight data were expressed as a percentage of the unsprayed control.

2.2. Sequencing of ACCase gene

To sequence the ACCase gene, shoot material (~1 cm^2) from at least 12 resistant plants of both field populations (F4 and S2) was harvested, snap frozen in liquid nitrogen and stored at −20 °C until its use. DNA was extracted from the shoot tissues using a DNeasy Plant Mini Kit (Qiagen, Australia) in accordance with the manufacturer's instructions. Standard PCR conditions and primers designed against the blackgrass (accession number AJ310767) ACCase gene sequence (Table 1) were used to amplify a 1.5 kb fragment covering nearly the entire CT domain, known to be involved in the sensitivity to ACCase-inhibiting herbicides. The range of amino acids covered by the fragment was equivalent to codons 1658–2157 in blackgrass. A nested PCR approach was employed with oligo set Acclr9 and Acclr6 (Zhang and Powles, 2006) followed by oligo set AccCT 2F and AccCT 2R (Malone et al., 2014). MyFi DNA polymerase kit (Bioline, Australia Pty Ltd,

Table 1
Primers used for the amplification and sequencing of the CT domain of the ACCase gene in *L. rigidum* from genomic DNA.

Primer	Sequence 5′ 3′
Acclr9	ATGGTAGCCTGGATCTTGGACATG
Acclr6	GGAAGTGTCATGCAATTCAGCAA
AccCT 2F	CCACTCCTGAATTTCCCAGTGG
AccCT 2R	CGCGATTTGAGTGTACAAAGGCTG
AccCT MidF	CCTGAGAATACATGTGATCCTCGTG
AccCT MidR	CCATTTCCCTTGGCTGTCATCAATGCC

Alexandria, NSW, 1435) was used to run PCR reactions of 25 μL contained 80–100 ng DNA template, 1x MyFi reaction buffer, 0.8 μM of each specific primer and 2 units of MyFi DNA Polymerase. Amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany) with PCR conditions as described by Malone et al. (2014).

PCR products were visualised on 1.5% agarose gels stained with 1 x SYBR® Safe DNA gel stain. Samples were electrophoresed in 1 x TAE Buffer (40 mM Trizma base, 1 mM Na$_2$EDTA, pH to 8 with glacial acetic acid) at 100 V and photographed under UV light (λ302 nm). DNA fragment sizes were estimated by comparing their mobility to bands of known sizes in a low mass molecular weight marker (Invitrogen, Australia). PCR products were sequenced (Australian Genome Research Facility, Australia) both in reverse and forward direction using primers CT Mid F and CT Mid R (Malone et al., 2014) to obtain sequence data covering the full CT domain fragment. Nucleotide sequences data were analysed using the Vector-NTi ContigExpress and AlignX software programmes (Invitrogen, USA) and all sequences were visually rechecked using the chromatogram files. The ACCase gene sequence from blackgrass was used as the reference sequence.

2.3. Field experiment

Two field experiments were conducted in different fields (F4 and S2) at Roseworthy, located in the Lower North region of South Australia, over the growing seasons of 2013 and 2014 to identify alternative herbicides for the control of clethodim resistant *L. rigidum* in TT canola. The soil at the field sites was a calcarosol (McKenzie et al., 2001) with organic matter content of 2–2.5% and a pH (water) of 7–7.5 in 0–20 cm layer. In 2013, 417 mm of annual and 341 mm of growing season (April–October) rainfall was recorded at the field site while in 2014 annual and growing season rainfall were 470 mm and 311 mm, respectively (Anonymous, 2015). A pre-seeding application of glyphosate at 900 g ha^{-1} and oxyfluorfen at 22 g ha^{-1} was made to control existing weeds present in the field.

TT canola (cv. Stingray) was sown at a depth of 2 cm at a seed rate of 5 kg ha^{-1} targeting 50 plants m^{-2} on May 17, 2013 and May 23, 2014 using a no-till plot seeder fitted with knife-point tines and press-wheels. For both years the previous crop was lentils. Plots were 10-m long and contained 6 crop rows spaced 25 cm apart. Fertilizer rate was consistent with the local grower practice of 80 kg ha^{-1} of diammonium phosphate (18 kg N and 20 kg P ha^{-1}) banded below the seed at sowing. The experiments were established in a randomized complete block design with four replicates. All herbicides [preplant incorporated (PPI) and post-emergent (POST)] were applied using an all-terrain vehicle fitted with a spray boom delivering 100 L ha^{-1} spray volume at a pressure of 200 kPa. PPI treatments were applied on May 15 and 22 in 2013 and 2014, respectively while the POST treatments were applied on July 2 and 20 in 2013 and 2014, respectively when the *L. rigidum* was at the 3–4 leaf growth stage. Application of PPI atrazine (1350 g ha^{-1}) followed by (fb) POST clethodim (120 g ha^{-1}) is common grower practice for the district and was used as comparator in this study. Herbicide rates used and timing (PPI or POST) of other herbicide treatments is presented in Table 3. Adjuvants were added to clethodim and butroxydim as previously described for the dose response experiments (Section 2.1).

L. rigidum plant and spike density were assessed throughout the growing season by using a 0.25 m^2 quadrat placed at four random locations in each plot. Assessments on *L. rigidum* plant density were taken 5 and 12 weeks after sowing (WAS) before and after POST herbicide application. Spike density was assessed 14 or 16 WAS when all the spikes had emerged. *L. rigidum* seed production was
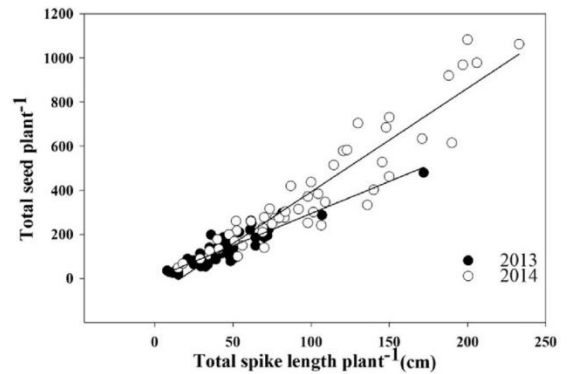

Fig. 1. The linear relationship between total spike length of *L. rigidum* per plant and total seeds produced per plant over two years. The line of best fit for the linear regression in 2013 (●) was $y = 2.9x$; $r^2 = 0.85$ and in 2014 (○), $y = 4.7x$; $r^2 = 0.87$.

assessed (16 or 18 WAS) in October prior to seed shatter. Seed production of *L. rigidum* was determined by measuring the spike length of 20 *L. rigidum* plants randomly from each plot and average spike length per plant was calculated. Separately, 50 plants from the experiment were randomly collected and total spike length per plant and numbers of seeds per plant were counted. The relationship between total spike length per plant and seed number per plant was determined using SigmaPlot version 12.5 (Systat Software Inc., Vic., Australia) with the equation y = bx, where y is the seed number per plant in x spike length per plant and b is the slope of the regression line obtained. Equations obtained (2013: y = 2.9x, r^2 = 0.85 and 2014: y = 4.7x, r^2 = 0.87) were used to estimate weed seed production m^{-2} (see Fig. 1). The number of seeds produced per unit area was based on the average spike length and spike density m^{-2} of *L. rigidum* and shown as number of seeds m^{-2}. Canola seeds were harvested with the use of a plot combine harvester on October 30 in 2013 and November 2 in 2014. Seed was then dried to uniform moisture and cleaned, and yield was determined. A seed subsample of 500 g was used to determine the 1000 seed weight.

2.4. Statistical analyses

Weed control data (plant and spike density, and *L. rigidum* seed production) and crop data (seed yield and seed weight) were subjected to the ANOVA with the use of GenStat version 15.3 (VSN International Ltd., UK). A square-root transformation was used for *L. rigidum* plant density data before analysis to normalise the distribution of residuals. Original means are reported but the means of the transformed data were compared by using LSD at P = 0.05. Relationships between spike density of *L. rigidum* and seed production, and spike density of *L. rigidum* and seed yield of canola were derived using SigmaPlot. Data (plant survival and dry weight) from dose response experiments analysed using log-logistic equation (Graphpad Prism v.6.0; GraphPad Software, San Diego, California) to calculate the dose of herbicide required to produce 50% reduction in plant survival (LD$_{50}$) and dry weight (GR$_{50}$). The model fitted was

$$y = \frac{100}{1 + 10^{(\log IC_{50} - x) \times b}} \qquad (1)$$

where, *y* is the plant survival (%) or biomass (%), *x* is the log-dose of the herbicide used, IC$_{50}$ is the dose of herbicide required to produce 50% reduction in plant survival or biomass, and *b* is the slope of the curve.

3. Results and discussion

3.1. Dose response experiments

Dose-response experiments confirmed resistance in both field populations (F4 and S2) to clethodim (Table 2). In both experiments (2013 and 2014), the plants of S population were completely killed by clethodim at the recommended rate (120 g ha^{-1}) while the F4 population had 45% and S2 population had 67% survival at this rate (Fig. 2a and c). The LD$_{50}$ and GR$_{50}$ values for both the populations are shown in Table 2. Based on the LD$_{50}$ values, the field populations F4 and S2 were approximately 16- and 26-fold more resistant to clethodim than the susceptible population SLR4. A similar level of resistance to clethodim has been reported in other populations of *L. rigidum* from Australia (Saini et al., 2015b, 2014; Yu et al., 2007) and *A. fatua* spp. from Western Australian (Ahmad-Hamdani et al., 2012) and in *L multiflorum* population from Oregon (Martins et al., 2014). The clethodim rate causing 50% reduction of shoot growth (GR$_{50}$) of population F4 and S2 was significantly higher than those of the susceptible population, resulting in R/S ratios of 9.1 and 16.7.

Both field populations exhibited cross-resistance to butroxydim (Fig. 2b and d). As expected, the susceptible population was killed at the recommended butroxydim field rate of 45 g ha^{-1}, with the LD$_{50}$ of 3.4 and 4.4 g ha^{-1} in 2013 and 2014, respectively (Table 2). However, the field populations were able to survive at the field rate having LD$_{50}$ of 15.4 g ha^{-1} for the F4 population and 30.9 g ha^{-1} for the S2 population. On the basis of R/S ratio, the S2 population was more resistant to butroxydim than the F4 population. Cross resistance to butroxydim in clethodim-resistant *L. rigidum* was not unexpected. Yu et al. (2007) previously reported that the *L. rigidum* plants homozygous for the mutant alleles at position 1781, 2078 and 2088 were resistant to clethodim and cross-resistant to several other ACCase inhibiting herbicides including butroxydim.

Table 2
Clethodim and butroxydim rate required for 50% mortality (LD$_{50}$) and for 50% biomass reduction (GR$_{50}$) of resistant and susceptible *L. rigidum* populations with confidence intervals (CI) in parentheses. R/S is the ratio of LD$_{50}$ and GR$_{50}$ of resistant and susceptible populations.

Population	LD$_{50}$ (g ha^{-1})	R/S	GR$_{50}$ (g ha^{-1})	R/S
2013				
Clethodim				
F4	43.2 (23.2, 80.3)	16.0	24.7 (12.3, 49.7)	9.1
SLR4	2.7 (2.6, 2.7)	—	2.7 (2.6, 2.7)	—
Butroxydim				
F4	15.4 (13.3, 17.8)	4.5	10.7 (7.0, 16.2)	3.6
SLR4	3.4 (2.6, 4.7)	—	3.0 (2.1, 4.5)	—
2014				
Clethodim				
S2	104.4 (79.1, 137.8)	26.1	63.5 (51.3, 78.5)	16.7
SLR4	4.0 (2.6, 6.4)	—	3.8 (2.9, 5.1)	—
Butroxydim				
S2	30.9 (25.4, 37.5)	7.0	24.8 (18.0, 34.1)	6.9
SLR4	4.4 (3.4, 5.7)	—	3.6 (2.6, 4.9)	—

3.2. Sequencing of ACCase gene

The CT domain of the plastidic ACCase enzyme was amplified by PCR and sequenced to identify any change in the nucleotide sequence. The presence of amino acid substitutions in seven previously characterized positions (1781, 1999, 2027, 2041, 2078, 2088 and 2096) in the CT domain of the ACCase gene of the grass species, known to be associated with resistance to ACCase-inhibiting herbicides (Kaundun, 2014; Malone et al., 2014), was analysed. Sequencing results revealed that the nucleotide sequences of the field populations differed from that of the susceptible population. Population F4 had two mutations at nucleotide positions 2078 (75%) and 2088 (17%), while population S2 had three mutations at position 2041 (8%), 2078 (67%) and 2088 (25%). No other mutations were found. Because the entire ACCase enzyme was not amplified,
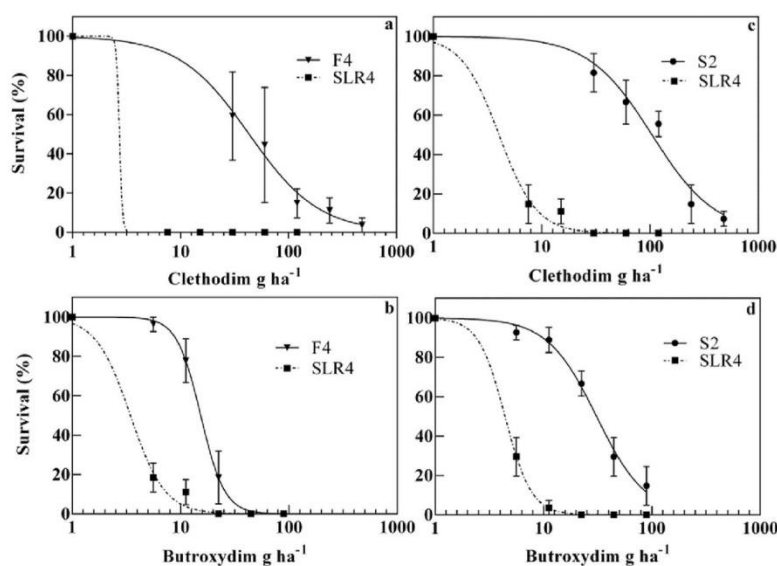

Fig. 2. Dose-response experiments conducted in 2013 (1A and 1B) and 2014 (2A and 2B) showing the response of field populations of *L. rigidum* F4 (▼) and S2 (●) and a known susceptible population SLR4 (■) to clethodim and butroxydim. Error bars represent SEM. Regression analysis was performed using the Equation (1A): $y = 100/1 + 10 \char`^ (1.635 - x) * -1.340$, (1B): $y = 100/1 + 10 \char`^ (1.186 - x) * -3.946$, (2A): $y = 100/1 + 10 \char`^ (2.019 - x) * -1.484$, (2B): $y = 100/1 + 10 \char`^ (1.490 - x) * -1.916$ where, y is the plant survival (%) or biomass reduction (%), x is the log-dose of the herbicide used.

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the existence of mutations in other positions of the enzyme cannot be excluded. Amino acid modification at position 2041, 2078 and 2088 have been reported to be associated with clethodim resistance in *L. rigidum* (Yu et al., 2007). The asp-2078-gly and cys-2088-arg substitution have been associated with resistance to clethodim (Beckie and Tardif, 2012; Kaundun, 2014). Plants homozygous for the mutant allele at position 2078 and 2088 were found to be resistant to clethodim and cross-resistant to many other ACCase-inhibiting herbicides, including butroxydim at field rates (Yu et al., 2007). The amino acid substitution at position 2041was reported to confer a low level of resistance to clethodim in *A. fatua* (Cruz-Hipolito et al., 2011; Liu et al., 2007), *L. multiflorum* (Martins et al., 2014) and *Sorghum halepense* L. (Scarabel et al., 2014) and confer high level of resistance to clethodim in *L. rigidum* (Saini et al., 2015b, 2014). The over reliance on clethodim for the control of *L. rigidum* populations that already have resistance to other ACCase-inhibiting herbicides may be selecting for the accumulation of amino acid modifications within the ACCase gene that contribute to clethodim resistance.

3.3. Effect of herbicides on L. rigidum density and seed production

In both years of field trials there were significant (P < 0.05) differences between herbicide treatments in *L. rigidum* density (Table 3). A significant reduction in *L. rigidum* density (P < 0.05 in 2013) relative to the standard grower practice was recorded with propyzamide (1000 g ha^{-1}) PPI applications or its split application (PPI fb POST) and as a mixture with clethodim. Atrazine PPI fb clethodim + butroxydim POST, and atrazine PPI fb atrazine + clethodim POST treatments also showed significant reduction in *L. rigidum* density compared to standard grower practice. Even though *L. rigidum* was confirmed resistant to clethodim and butroxydim (Fig. 2), their mixture in 2013 and 2014 provided 54 and 46% reduction in *L. rigidum* compared to the standard grower practice of atrazine PPI fb clethodim POST. Atrazine PPI fb atrazine + clethodim POST improved *L. rigidum* control by 31–52% compared to atrazine PPI fb clethodim treatment (P < 0.05 in 2013). Similarly, addition of atrazine to pethoxamid (POST) significantly (P < 0.05) increased *L. rigidum* control by 64–72% compared to pethoxamid applied alone. Increasing the propyzamide rate to 1000 g ha^{-1}, applied alone or as a split application (PPI fb POST), increased *L. rigidum* control by almost 35% compared to the lower rate of propyzamide (500 g ha^{-1}), but the differences were not-significant (P > 0.05). During both the growing seasons, the rainfall received was above the long-term average for the site and the wet winter conditions would have favoured the activity of propyzamide on *L. rigidum* (Walker and Roberts, 1975). Kleemann and Gill (2012)

also reported effective control of *L. rigidum* with propyzamide (>85%) in faba bean. PPI trifluralin fb carbetamide POST (81 plants m^{-2}, 2013; 876 plants m^{-2}, 2014) provided better weed control than the standard farmer practice in 2013 (P < 0.05) but was inferior than the standard practice in 2014 (P < 0.05). Dimethenamid-P and pethoxamid PPI were the least effective treatments in reducing *L. rigidum* density (P < 0.05 in 2013 when compared to standard grower practice). Lower efficacy with these treatments appears to be related to poor activity of these herbicides on *L. rigidum* in general.

L. rigidum spike number and seed production were significantly (P < 0.05) influenced by the herbicide treatments in both years of the study (Tables 3 and 4). In general, *L. rigidum* spike density closely followed the trends observed for *L. rigidum* plant density (Table 3). In treatments where POST clethodim and/or butroxydim was used, there was a large reduction in spike density compared with the plant density. This result clearly indicates that these POST herbicides were still providing suppression of seed production even though these populations have evolved resistance. A significant reduction in spike density of *L. rigidum* in faba bean with POST application of clethodim and/or butroxydim has also been reported previously (Kleemann and Gill, 2012; Saini et al., 2015a). Dimethenamid-P and pethoxamid PPI were ineffective in reducing *L. rigidum* spike density relative to the standard grower practice (P < 0.05), as was the case for plant density. In both years, PPI atrazine fb clethodim POST or combination of clethodim + butroxydim produced significantly (P < 0.05) lower spike density (33–94 spikes m^{-2}) and seed production of *L. rigidum* (1286 to 6785 seeds m^{-2}) relative to dimethenamid-P and pethoxamid. Among the herbicide treatments, atrazine fb atrazine + clethodim POST and atrazine fb clethodim + butroxydim POST were the most effective treatments at reducing *L. rigidum* seed production by 29–55% compared to standard grower practice (PPI atrazine fb POST clethodim) and by >90% compared to pethoxamid. PPI herbicides alone were less effective (P < 0.05) in reducing spike density and seed production of *L. rigidum* than their combination with POST clethodim or clethodim + butroxydim. All herbicide treatments allowed *L. rigidum* to produce well in excess of 1000 seeds m^{-2}, which will undoubtedly cause production problems in the next crops in the rotation. These results indicate that PPI herbicides alone are inadequate for *L. rigidum* management in TT canola.

There was a strong linear relationship (r^2 = 0.97 in 2013; r^2 = 0.99 in 2014) between spike density of *L. rigidum* and its seed production (Fig. 3) during both years of the study. The slope of relationships indicates that in 2013 each *L. rigidum* spike produced 68 ± 14 seeds and 75 ± 4 seeds in 2014. Any *L. rigidum* plants that survived herbicide application were capable of massive seed

Table 3
Effect of herbicide treatments on *L. rigidum* plant and spike density in triazine-tolerant canola grown at Roseworthy in 2013 and 2014.[a]

Treatments[b]	Rate (g ai ha^{-1})	Weed density (plant m^{-2})		Spike density (spikes m^{-2})	
		2013	2014	2013	2014
Atrazine PPI fb clethodim POST (Standard grower practice)	1350 fb 120	171 c	522 ab	51 a	94 a
Atrazine PPI fb atrazine + clethodim POST	1350 fb 900 + 120	83 ab	361 a	33 a	54 a
Atrazine PPI fb clethodim + butroxydim POST	1350 fb 120 + 20	79 ab	282 a	60 ab	43 a
Dimethenamid PPI	540	269 d	864 bc	326 d	651 c
Propyzamide PPI	500	96 abc	354 a	231 cd	433 b
Propyzamide PPI fb clethodim POST	500 fb 120	67 a	324 a	79 ab	188 a
Propyzamide PPI	1000	63 a	264 a	150 abc	252 ab
Propyzamide PPI fb Propyzamide POST	500 fb 500	65 a	228 a	178 bc	165 a
Pethoxamid PPI	1880	381 e	869 bc	477e	705 c
Pethoxamid + triallate PPI	1880 + 1000	133 bc	767 bc	308 d	723 c
Pethoxamid PPI fb Atrazine POST	1880 fb 2250	106 abc	317 a	103 ab	81 a
Atrazine PPI fb carbetamide POST	1350 fb 1200	81 ab	876 c	67 ab	840 c

[a] Means within the same column followed by the same letters are not significantly different according to LSD at the P = 0.05 level.
[b] Abbreviations: fb, followed by; PPI, Preplant Incorporated; POST, post-emergence application of herbicides.

Table 4
Effect of herbicide treatments on seed yield of triazine-tolerant canola and *L. rigidum* seed production at Roseworthy in 2013 and 2014.[a]

Treatments[b]	Rate (g ai ha^{-1})	Canola seed yield (kg ha^{-1})		*L. rigidum* seeds (seeds m^{-2})	
		2013	2014	2013	2014
Atrazine PPI fb clethodim POST (Standard grower practice)	1350 fb 120	2145 f	1691 de	1821 a	6785 a
Atrazine PPI fb atrazine + clethodim POST	1350 fb 900 + 120	2149 f	1882 e	1286 a	2956 a
Atrazine PPI fb clethodim + butroxydim POST	1350 fb 120 + 20	2196 f	1839 e	2343 a	3274 a
Dimethenamid PPI	540	1622 b	1145 ab	21697 c	51473 c
Propyzamide PPI	500	1678 bc	1492 cd	15825 bc	32781 b
Propyzamide PPI fb clethodim POST	500 fb 120	2015 ef	1741 de	5588 a	13396 a
Propyzamide PPI	1000	1871 cde	1502 cd	7099 a	19148 ab
Propyzamide PPI fb Propyzamide PPI	500 fb 500	1774 bcd	1708 de	8638 ab	12401 a
Pethoxamid PPI	1880	1301 a	1255 bc	34817 d	51192 c
Pethoxamid + triallate PPI	1880 + 1000	1645 bc	1311 bc	22308 c	54532 c
Pethoxamid PPI fb Atrazine POST	1880 fb 2250	1986 def	1783 e	4997 a	6152 a
Atrazine PPI fb carbetamide POST	1350 fb 1200	2156 f	997 a	3040 a	63124 c

[a] Means within the same column followed by the same letters are not significantly different according to LSD at the P = 0.05 level.
[b] Abbreviations: fb, followed by; PPI, Preplant Incorporated; POST, post-emergence application of herbicide.

production and increasing the seed bank, which would have serious effects on the productivity of subsequent crops. Therefore, growers need to seriously consider the use of glyphosate or another non-selective herbicide after physiological maturity of canola to minimise seed production of the survivors of selective herbicides. This practice has been recently registered for use in canola production in Australia.

3.4. Effect of herbicides on seed yield of canola

There were significant differences among herbicide treatments in seed yield of canola (P < 0.05) in both years of the study (Table 4). In the absence of effective control, *L. rigidum* was extremely competitive and reduced seed yield by up to 41% in 2013 and 46% in 2014 relative to the best treatment of atrazine fb clethodim + butroxydim. Significant yield penalties due to *L. rigidum* interference have been previously reported in canola (Lemerle et al., 1995; Reeves and Lumb, 1974). In both years, seed yield was significantly (P < 0.05) greater in plots where atrazine was followed by POST herbicides (1691 to 2196 kg ha^{-1}) as compared to dimethenamid-P or pethoxamid alone (1145 to 1622 kg ha^{-1}). Seed yield in atrazine fb carbetamide POST treatment was similar to the standard grower practice and atrazine fb clethodim + butroxydim in 2013 (2156 kg ha^{-1}), but its seed yield was significantly lower

(P < 0.05) in 2014 (997 kg ha^{-1}). The reasons for poor efficacy of PPI atrazine fb carbetamide in 2014 compared to 2013 could be due to low growing season rainfall in 2014. Failure to control *L. rigidum* with PPI dimethenamid-P and pethoxamid resulted in significant (P < 0.05) yield reductions of up to 40% compared to atrazine fb clethodim + butroxydim. These results are consistent with the findings of Lemerle et al. (1995), who found that *L. rigidum* at a density of 300 plants m^{-2} reduced rapeseed yield by 32%.

An exponential relationship between *L. rigidum* spike density and the seed yield of canola was found and demonstrated severe interference of increasing *L. rigidum* spike density with canola seed yield (2013: r^2 = 0.73 and 2014: r^2 = 0.72) (Fig. 4). Canola seed weight was unaffected by the herbicide treatments in 2013 [2.8–2.9 g (1000 seed^{-1})] and 2014 [2.3–2.5 g (1000 seed^{-1})] (data not presented). The lower average seed weight in 2014 appears to be associated with a much higher *L. rigidum* density and lower spring rainfall in 2014 than in 2013.

4. Conclusions

The results from this study clearly demonstrated that no PPI herbicide treatment was able to prevent *L. rigidum* seed production. Even though clethodim resistance was confirmed in *L. rigidum* populations at both the field sites, use of clethodim in combination
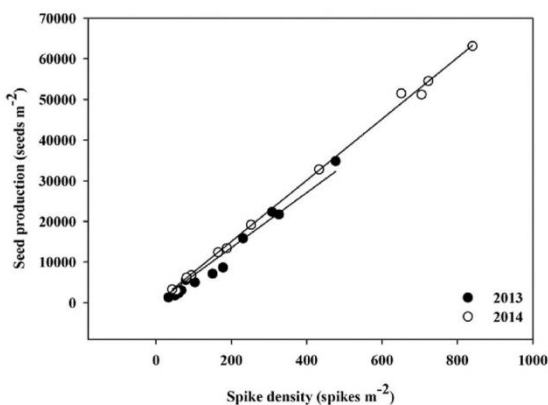
Fig. 3. The relationship between mean spike density of *L. rigidum* and seed production over two years. The line of best fit for the linear regression in 2013 was $y = 67.7x$; R^2 = 0.97 and in 2014, $y = 75.3x$; R^2 = 0.99.
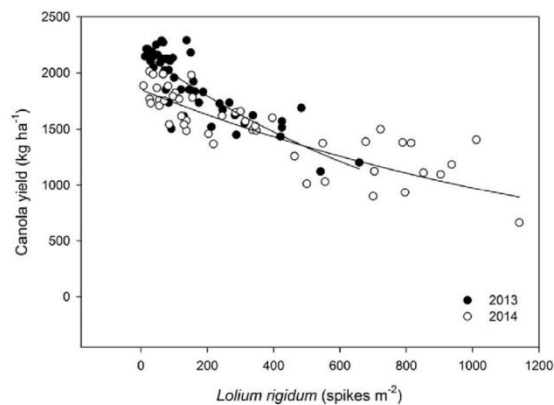
Fig. 4. Effect of spike density of *L. rigidum* on the seed yield of triazine-tolerant canola over two years. An exponential regression analysis was performed using the equation, in 2013, $y = 2183.9\exp^{-0.001x}$; r^2 = 0.73 and in 2014 $y = 1850.8\exp^{-0.0006x}$; r^2 = 0.72.

with atrazine provided the best weed control and seed yield of canola. Among the PPI herbicides, propyzamide appeared to be the best option, but *L. rigidum* was able to produce large amount of seed, which could reduce productivity of subsequent crops. None of the alternative treatments investigated were consistently more effective than the combination of atrazine and clethodim. The agronomic benefit of clethodim is likely to decrease over time as *L. rigidum* populations evolve higher levels of resistance. Currently, in Australia, there are no new selective grass herbicides registered for the control of clethodim-resistant *L. rigidum* in canola. Therefore, a serious effort is needed to identify effective alternatives to clethodim and butroxydim for post-emergence use in canola. Research is also needed to find out cultural and mechanical methods for the control of resistant populations of *L. rigidum* in southern Australia.

Acknowledgements

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CHAPTER 8

ALTERNATIVE HERBICIDES FOR THE CONTROL OF CLETHODIM-RESISTANT RIGID RYEGRASS (*LOLIUM RIGIDUM*) IN CLEARFIELD® CANOLA (*BRASSICA NAPUS* L.) IN SOUTHERN AUSTRALIA

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Statement of Authorship

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Name of Principal Author (Candidate)	Rupinder Kaur Saini
Contribution to the Paper	Planned the study, conducted all experiments, analysed and interpreted data, wrote the manuscript and acted as corresponding author.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 7.12.2015

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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CHAPTER 9

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

9.1 Discussion of Results

Weeds are a major threat to food security because of their capacity to persist and reduce crop yields (Harper 1977). The potential crop yield losses due to weeds are higher than other pest species (Oerke 2006). During the last half century, herbicides have become the most common tool used by the farmers for weed control in most agricultural areas of the world due to their efficacy, ease of use and low cost (Heap 1997). However, increased reliance on herbicides for weed control has resulted in an increase in herbicide resistant weed populations around the world (Heap 2015; Powles and Yu 2010). Currently herbicide resistance has appeared in more than 246 weed species and many thousands of crop fields worldwide (Heap 2015). Major factors that influence the evolution of herbicide resistance include intensity of selection by herbicides, the initial frequency of herbicide resistant individuals in the population, fitness of resistant plants, gene migration, and inheritance of resistance (Jasieniuk et al. 1996).

In Australia, *L. rigidum* is a major annual grass weed of cropping systems (Jones et al. 2005). This species was introduced into Australia in the 19th century and is now present at high densities across vast areas. *L. rigidum* reduces crop yields dramatically at high densities and is a major target of herbicides in this area. Herbicide resistance first appeared in *L. rigidum* in 1980 (Heap and Knight 1982) and is now widespread across the intensively cropped regions of southern Australia (Preston et al. 1999). So far *L. rigidum* has evolved resistance to at least eleven major herbicide groups (Heap 2015). The majority of cases of herbicide resistance in *L. rigidum* populations are to herbicides that inhibit acetyl-coenzyme A carboxylase (ACCase) or acetolactate synthase (ALS). As

many populations of *L. rigidum* have evolved resistance to the grass selective herbicides, control options for *L. rigidum* in broadleaf crops have become far more limited.

Clethodim, an ACCase inhibiting cyclohexanedione herbicide, is a selective post-emergent herbicide typically used to control annual and perennial grasses infesting dicot crops but exhibits little activity against dicots or non-graminaceous monocots (Burke et al. 2004). Clethodim has an ability to control populations with resistance to other ACCase-inhibiting herbicides (Bradley and Hagood 2001). As a result of widespread resistance to other post-emergent herbicides in *L. rigidum* in Australia, clethodim has become the most important grass herbicide for canola and pulse crops (Boutsalis et al. 2012). As has been the case for other herbicides, over-reliance on clethodim has resulted in the evolution of clethodim resistance in *L. rigidum* populations in Australia (Yu et al. 2007). In a recent survey, Boutsalis et al. (2012) have reported that 60% of the fields across south-eastern Australia have some level of clethodim resistance. In the past, clethodim resistance was managed by increasing its dose, however this is no longer a viable option for growers as several populations of *L. rigidum* are now resistant to clethodim doses greater than the highest recommended field rate. It is also well known that clethodim can cause damage to canola at rates higher than 120 g a.i. ha^{-1} but this is not the case in pulses. Butroxydim is an alternative herbicide that is also sometimes used for the control of ACCase resistant rigid ryegrass (Yu et al. 2007).

The recent sharp increase in clethodim resistance in the southern Australian grain belt was one of the reasons for this study. Understanding the mechanisms and inheritance of resistance is necessary for the formulation of strategies to slow down the rate of evolution of resistance and to manage resistant weeds. The mechanisms and inheritance of clethodim resistance and the alternative herbicides options for the control of clethodim resistant *L. rigidum* in broadleaf crops have not been studied previously.

In this project, studies were undertaken on twelve *L. rigidum* populations collected from different farms in South Australia, Western Australia, Victoria and New South Wales, where clethodim at 36-120 g a.i. ha^{-1} had provided inadequate weed control in the field and resistance was suspected. The dose-response experiments clearly demonstrated that all these populations of *L. rigidum* were resistant to clethodim and butroxydim (Chapter 2). The resistant populations exhibited various levels of resistance from low to high. The clethodim resistance level of resistant populations ranged between 3 to 35.3-fold as compared to the susceptible populations, SLR4 and VLR1. All the resistant populations had a low degree of resistance to butroxydim (Table 4, Chapter 2). Previously varying levels of clethodim resistance have been documented in the populations of *L. rigidum* (Broster et al. 2011; Yu et al. 2007), Italian ryegrass (*Lolium perenne ssp. multiflorum* L.) (Martins et al. 2014), wild oat (*Avena fatua* L.) (Ahmad-Hamdani et al. 2012; Owen and Powles 2009) and in an Asia minor bluegrass (*Polypogon fugax*) population from China (Tang et al. 2014). It has been also reported that some clethodim resistant populations of *L. rigidum* with different ACCase target site mutations have cross resistance to the CHD herbicide butroxydim (Yu et al. 2007).

Resistance to ACCase inhibiting herbicides can occur due to insensitivity of the target enzyme, sequestration of the herbicide away from the target site, increased rate of herbicide detoxification, or decreased rate of herbicide activation (Devine 1997), and also due to an overproduction of *ACCase* activity (Bradley et al. 2009). Target-site resistance from a single amino acid change in the *ACCase* gene is the major cause of resistance to ACCase-inhibiting herbicides. Seven target-site mutations (amino acid substitution) at positions 1781, 1999, 2027, 2041, 2078, 2088 and 2096 in the *ACCase* gene have been documented to date in populations of several grass weed species (Beckie and Tardif 2012; Cruz-Hipolito et al. 2012) providing resistance to ACCase inhibiting herbicides. *L. rigidum* populations with clethodim-resistance have evolved low to high levels of

resistance and target-site resistance mechanism was recognized in these populations (Chapter 2). Sequencing of the target-site *ACCase* gene identified five known ACCase substitutions (isoleucine-1781-leucine, isoleucine-2041-asparagine, aspartate-2078-glycine, and cysteine-2088-arginine, and glycine-2096-alanine) in clethodim-resistant *L. rigidum* populations (Table 6, Chapter 2). As we have not amplified the entire ACCase enzyme, we cannot exclude the existence of mutations in other positions of the enzyme. In all populations tested, some of the resistant individuals had none of the known ACCase mutations. This suggests presence of additional clethodim resistance mechanisms in *L. rigidum.* It has been previously confirmed that more than one mechanism of herbicide resistance can co-exist within a population of *L. rigidum* (Christopher et al. 1992). Previously, Yu et al. (2007) has reported that the presence of a single ACCase mutation provides low-level of resistance to ACCase inhibiting herbicides and resistance level increases if there are multiple ACCase mutations. Conversely, in our study we found that multiple mutations did not provide an additive effect on the level of resistance, which could be due to differences in the homo/ heterozygous status of plants for a specific mutation.

A significant reduction in the level of clethodim efficacy in some clethodim-resistant populations was observed (Table 2, Chapter 3), when plants were exposed to frost for three nights (4 pm to 8 am) before and after clethodim application. However, clethodim efficacy on clethodim-resistant populations was affected more by the occurrence of frost before than after clethodim application. There was no significant effect of frost conditions on clethodim efficacy in the susceptible population. The reduced clethodim efficacy in the case of frost before clethodim application could be related to reduced absorption and translocation of clethodim as a result of frost stress in *L. rigidum* plants. At lower temperatures, phloem translocation is reduced due to freezing of leaf tissues accompanied by damage of phloem cells (Weatherley and Watson 1969). In the previous studies, it has

been documented that temperature is one of the most important environmental factors affecting the performance of ACCase inhibiting herbicides (Johnson et al. 2002; Kells et al. 1984; Kudsk and Kristensen 1992). Further investigations showed three known target-site ACCase mutations in the survived individuals of clethodim-resistant populations (Table 3, Chapter 3). As explained earlier, most individuals in the highly resistant populations did not contain any of the known mutation in *ACCase* gene, suggesting a non-target site resistance mechanism. The reduction in clethodim activity in resistant plants by may be an outcome of the interaction between frost and the clethodim resistance mechanism(s) present. Effects of temperature on resistance levels have been previously reported in resistant biotypes of hare barley (*Hordeum leporinum* Link) where resistance to paraquat is greater at lower temperature (Purba et al. 1995). Also a reduction in the level of glyphosate resistance in horseweed was observed when plants were grown at cold temperatures (<10 C) (Ge et al. 2011).

The mode of inheritance of the resistance trait is one of the factors needed to predict and manage the evolution and spread of herbicide resistance (Jasieniuk et al. 1996; Maxwell et al. 1990). In *L. rigidum* several different patterns of inheritance of clethodim resistance were observed (Chapter 4). The patterns of resistance included a single gene, partially dominant, nuclear encoded trait, two different patterns of two-gene inheritance and maternal inheritance of the resistance trait. Variation in the pattern of inheritance indicates that different resistance mechanisms may be present in different *L. rigidum* populations. According to Jasieniuk and Maxwell (1994), the pattern of inheritance plays an important role in resistance evolution. The number of genes controlling resistance can impact on the speed of resistance evolution in weeds (Maxwell 1992; Roush et al. 1990). Jasieniuk et al. (1996) concluded that a dominant traits spread more rapidly in populations, as both homozygous dominant and heterozygous individuals will carry the resistance trait. In addition, because *L. rigidum* is highly allogamous, the spread of resistance endowed by a

dominant or semi-dominant trait will spread rapidly in the population. Inheritance of resistance in *L. rigidum* (Tardif et al. 1996) and Italian ryegrass (Betts et al. 1992) with an insensitive ACCase is conferred by a single nuclear, semi dominant allele. Target site-based resistance to ACCase inhibitors in wild oat is conferred by a single nuclear allele, with resistance being dominant at lower herbicide doses and susceptibility being dominant at increased rates (Murray et al. 1995; Seefeldt et al. 1998). Herbicide resistance due to two or more genes has been previously reported in a glyphosate resistant *L. rigidum* population from California (Simarmata et al. 2005). Similarly, in a diclofop-methyl resistant population of tame oat (*Avena sativa*) resistance was controlled by two genes with recessive action (Warkentin et al. 1988), whereas in three tolerant maize inbred lines, the response to diclofop-methyl was controlled by a minimum of three genes (Geadelmann and Andersen 1977). Until now, with the notable exception of the triazine herbicides resistance to all herbicide classes endowed by nuclear inherited genes. However, our study is the first example of maternal inheritance of resistance to ACCase-inhibiting herbicides.

The possession of resistance genes is commonly associated with an alteration of fitness of resistant individuals in relation to normal individuals in the absence of herbicide application (Darmency 1996; Holt and Thill 1994). In some weed species the presence of the resistance gene is occasionally associated with a reduction in fitness (Plowman et al. 1999). The best measure of fitness costs associated with resistance would be to determine the change in the frequency of the resistance allele for several generations (Vila-Aiub et al. 2009). There is a relationship between the relative fitness of an allele and its equilibrium frequency in populations. Additionally, alleles carrying larger fitness penalties will be less common within the population than those carrying smaller fitness penalties (Jasieniuk et al. 1996). Literature suggests that in the absence of herbicide selection pressure, herbicide resistance alleles are rare in weed populations, and this is a

consequence of fitness penalties the alleles carry (Jasieniuk et al. 1996; Preston and Powles 2002). Fitness costs in the absence of the relevant herbicide can help explain the low frequency of resistance alleles and may guide field management to control resistant weeds (Menchari et al. 2008; Vila-Aiub et al. 2009). In *L. rigidum* populations investigated, there was no significant change in the frequency of Leu- 1781and Asn-alleles over one generation but significant increase in the frequency of Gly-2078 alleles was identified in two populations in the absence of clethodim use (Chapter 5). The absence of fitness penalties associated with these resistant alleles suggests that there should be no decrease in the frequency of these resistant alleles over time in the absence of herbicide selection pressure (Menchari et al. 2008; Vila-Aiub et al. 2009).

Although herbicide resistance is a serious and escalating worldwide phenomenon, major research effort is aimed at confirming resistance and to determine the physiological and genetic basis of evolved practices, and controlling weed seed set have been reported as effective management tactics for the control of resistant weed populations (Beckie 2006; Christoffoleti et al. 2005; Gressel and Segel 1990; Preston et al. 2009). Clethodim is the last ACCase-inhibiting herbicide that provides effective control of herbicide resistant *L. rigidum*. Given the increasing prevalence of clethodim-resistant *L. rigidum* populations in southern Australia, there is an urgent need to identify alternative herbicide strategies to clethodim in dicot crops. The loss of clethodim to resistance will make *L. rigidum* management more difficult. In order to address this problem, field experiments were conducted in faba bean and canola (triazine-tolerant and Clearfield canola) crops at Roseworthy, South Australia from 2012 to 2014. Various pre-emergent and post-emergent herbicides were applied to determine the most effective treatment.

In faba beans, sole reliance on pre-emergent herbicides failed to adequately manage *L. rigidum* and large yield penalties occurred (Chapter 6, Table 4 & 5). Among the pre-

emergent herbicides, propyzamide appeared to be the most effective herbicide option in controlling *L. rigidum* (>60%); however *L. rigidum* was still able to set large amount of seed (>13,000 seeds m^{-2}), which could have serious implications for weed management and productivity of subsequent crops. The best control of clethodim-resistant *L. rigidum* was achieved with pre-emergent simazine followed by (fb) the mixture of clethodim + butroxydim post-emergent. Addition of butroxydim to clethodim significantly reduced *L. rigidum* plant density (60-80%) and seed production (71-88%) as compared to simazine fb clethodim alone. Importantly, higher grain yield of faba bean was achieved where pre-emergent herbicides were followed with post-emergent herbicides. The combination of poor competitive ability of faba bean with weeds (Felton et al., 2004) and severe weed infestation at the experimental sites could have been responsible for the ineffectiveness of pre-emergent herbicides used alone.

Likewise, the pre-emergent herbicides alone were inadequate to effectively control clethodim-resistant *L. rigidum* in both triazine-tolerant (TT) and Clearfield$^{®}$ (CLF) canola (Chapter 7 & 8). However, pre-emergent herbicides performed better in the CLF canola than in the open-pollinated TT-canola due to the increased competition provided by the CLF hybrid. Similar to the faba bean study, in both TT-and CLF-canola propyzamide was the best of the stand-alone pre-emergent herbicide options examined. Addition of clethodim to propyzamide tended to stunt *L. rigidum* plants and reduce their competitiveness, but had limited impact on *L. rigidum* seed production. The use of post-emergent herbicides in combination with pre-emergent herbicides improved weed control and canola yield relative to pre-emergent herbicides alone. In TT-canola, pre-emergent atrazine fb post-emergent clethodim + butroxydim was the best treatment in reducing *L. rigidum* plant density (~52%) and seed production (2300 to 3200 seed m^{-2}). This herbicide combination also produced the highest seed yield of canola (1839 to 2196 kg ha^{-1}). Similarly, in CLF-canola, pre-emergent trifluralin + triallate fb post-emergent imazamox +

imazapyr + clethodim + butroxydim provided superior control of *L. rigidum* compared to all other herbicide combinations tested. The differences in weed control between treatments in CLF-canola had very little effect on grain yield.

In both faba bean and canola crops, the use of soil residual herbicides in conjunction with clethodim and butroxydim provided the most effective control of clethodim-resistant *L. rigidum* despite resistance to clethodim and butroxydim being present; however no herbicide treatment was able to completely prevent seed production. Currently there are no effective herbicides to control clethodim-resistant *L. rigidum* in broadleaf crops. Therefore, the most effective strategy is to start with an effective pre-emergent herbicide and then use clethodim or its mixture with butroxydim to control and retard the growth of *L. rigidum* plants present in the crop. Use of a hybrid canola variety provides greater crop competition and may improve the efficacy of the pre-emergent herbicides and reduce seed set.

9.2   Conclusions

In conclusion, this research identified low to high level of clethodim resistance and low to moderate level of butroxydim resistance in *L. rigidum* populations from the southern Australian grains belt. From the resistance mechanism studies, the target-site mutations within the *ACCase* gene at position 1781, 2041, 2078, 2088, and 2096 were detected in clethodim-resistant populations. A reduction in the level of clethodim efficacy was observed in clethodim-resistant populations when plants were exposed to frost for three nights before the clethodim application. The reduced clethodim efficacy in case of frost before clethodim application may be related to reduced absorption and translocation of clethodim as a result of frost stress in *L. rigidum* plants. This study determined several different patterns of inheritance of clethodim resistance in *L. rigidum* populations. The patterns of resistance include a single gene, partially dominant, nuclear encoded trait, two

different patterns of two-gene inheritance and an example of maternal inheritance of the resistance trait. Furthermore, this study reports first case of maternal inheritance in ACCase-inhibiting herbicides. In the absence of clethodim use, no fitness cost is associated with Leu-1781, Asn-2041 and Gly-2078 alleles. In faba bean and in canola crops, pre-emergent herbicides alone are inadequate to effectively manage clethodim-resistant *L. rigidum.* The combination of effective soil residual herbicides with tank-mixtures of clethodim and butroxydim as post-emergent can still provide acceptable control of some clethodim resistant *L. rigidum* populations. Identification of a new post-emergence herbicide option could be highly beneficial for the management of clethodim resistant *L. rigidum* populations in broadleaf crops.

9.3   Recommendations for Future Research

In our study, only one mechanism i.e. target-site mechanism has been identified in clethodim resistant *L. rigidum* populations. However, many of the resistant individuals did not contain any known target-site mutation and are suspected of having non-target-site mechanisms. Therefore, non-target-site mechanisms need further investigation. Additionally, in clethodim-resistant populations of *L. rigidum* a significant reduction in the efficacy of clethodim was observed when plants were exposed to frost for three nights before or after clethodim application. However, in susceptible population there was no effect of frost on the efficacy of clethodim. As we did not investigate the biochemical basis of the temperature (frost) dependence of clethodim resistance, further research is required to identify the mechanism. In this study, none of the alternative pre-emergence herbicides investigated were able to completely prevent seed production of *L. rigidum.* At present, in Australia, there are no new selective grass herbicides registered for the control of clethodim-resistant *L. rigidum* in broadleaf crops. Therefore, a serious effort is needed

to identify effective alternatives to clethodim and butroxydim for post-emergence use in faba bean and canola crops.

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