



Herbicide resistance in *Conyza bonariensis* (L.) Cronquist (flaxleaf fleabane) populations from northeast Victoria and its management in mixed farming systems

By

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Table of Contents

Abstract.....	7
Declaration.....	9
Intended Publications from this thesis.....	10
Acknowledgements.....	11
Abbreviations.....	13
Chapter 1: Literature Review.....	15
1.1. Introduction.....	15
1.2. <i>Conyza bonariensis</i> (L.) Cronquist.....	16
1.2.1. Physical description and taxonomy.....	16
1.2.2. Distribution.....	17
1.2.3. Habitat.....	18
1.2.4. Weed Biology.....	19
1.2.5. Importance and Impacts.....	24
1.3. Herbicide Resistance.....	26
1.3.1. Resistance to EPSPS Inhibitors.....	29
1.3.2. Herbicide Resistant <i>Conyza bonariensis</i> (L.) Cronquist.....	35
1.4. Management of <i>Conyza bonariensis</i> (L.) Cronquist.....	41
1.4.1. Controlling <i>Conyza</i> species with herbicides.....	41
1.4.2. Non-chemical management tools for <i>Conyza bonariensis</i> (L.) Cronquist.....	45
1.4.3. Control of <i>Conyza Bonariensis</i> (L.) Cronquist on Channel Banks.....	48

1.5. Research Questions	48
References:	50
Chapter 2: Multiple-resistance to EPSPS and ALS inhibitors in northeast Victorian populations of <i>Conyza bonariensis</i> (L.) Cronquist	
Abstract.....	67
2.1. Introduction	68
2.2. Materials and Methods.....	71
2.2.1. Physical Survey	71
2.2.2. Screening for resistance to EPSPS inhibitors	71
2.2.3. Dose Response to EPSPS inhibitors.....	72
2.2.4. Screening for resistance to ALS inhibitors, synthetic auxins and PSI inhibitors	73
2.2.5. Dose response to ALS inhibitors, synthetic auxins and PSI inhibitors	74
2.2.6. Data Analysis	75
2.3. Results and Discussion	77
2.3.1. Resistance to EPSPS inhibitors.....	77
2.3.2. Resistance to ALS inhibitors	80
2.3.3. Synthetic auxin resistance.....	84
2.3.4. PS1 inhibitor resistance.....	86
2.4. Conclusions.....	86
References	88
Chapter 3: Exploring mechanisms of glyphosate resistance in <i>Conyza bonariensis</i> (L.) Cronquist populations from northeast Victoria	
	95

Abstract.....	95
3.1. Introduction	95
3.2. Materials and Methods.....	98
3.2.1. Seed source	98
3.2.2. Glyphosate Absorption and Translocation	98
3.2.3. Shikimate assays.....	100
3.2.4. Target Site Mutation	101
3.3. Results and Discussion.....	104
3.3.1. Absorption and Translocation of glyphosate	104
3.3.2. Shikimate Assays	107
3.3.3. Target Site Mutations in EPSPS.....	109
3.4. Conclusion	114
References:	115
Chapter 4: Control of <i>Conyza bonariensis</i> (L.) Cronquist in mixed farming systems.....	122
Abstract.....	122
4.1. Introduction	123
4.2. Materials and Methods.....	127
4.2.1. Management on irrigation channel banks	127
4.2.2. Management in grazed fallows	129
4.2.3. Data analysis	132
4.3. Results and discussion	133
4.3.1. Management on irrigation channel banks	133

4.3.2. Management in grazed fallows	138
4.4. Conclusions	144
References:	145
Chapter 5: Conclusions and Recommendations	149
REFERENCES:	153

Abstract

Conyza bonariensis (L.) Cronquist is a global weed and considered one of the most problematic species in modern agriculture. As a species it has developed resistance to herbicides of nine different active ingredients globally including 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) inhibitors, photosystem I (PSI) electron diverters, photosystem II (PSII) inhibitors and acetolactate synthase (ALS) inhibitors. Examination of 88 *C. bonariensis* populations collected across northeast Victoria identified that 40% of populations (or plants in specific populations) were resistant to 1080 g a.e. ha⁻¹ glyphosate. Multiple resistance was found to glyphosate and the ALS inhibitors chlorsulfuron, metsulfuron-methyl and sulfometuron-methyl in five of the nine populations fully characterised. This is the first reported case of multiple resistance to EPSPS- and ALS-inhibiting herbicides in *C. bonariensis*.

Nine populations collected as part of a resistance survey conducted across northeast Victoria showed varying levels of glyphosate resistance; glyphosate susceptible (GS) biotypes DL4, IR7 and IR11; low level glyphosate-resistant (Gr) biotypes DL3, DL13 and IR14 with Resistance Indices (RI's) between 2.3 and 2.8; and high level glyphosate resistance (GR) biotypes DL19, IR5 and IR10 with RI's over 6. Results of laboratory evaluation for herbicide translocation demonstrated that this was not involved in the resistance present in these populations. GR, Gr and GS populations showed differential accumulation of shikimate suggesting insensitive EPSPS may be involved in the resistance found in these *C. bonariensis* populations. Sequencing both genomic DNA and plasmid DNA identified Pro106-Thr and Pro106-Ser mutations, these mutations have previously been found to confer glyphosate resistance. As these mutations occurred in all three population groups, therefore (an)other mechanism(s) must be contributing to the resistance. Future investigation focused on expression of EPSPS and ABC transporter genes may provide greater insight into the mechanisms conferring resistance in these *C. bonariensis* populations.

C. bonariensis is a successful ruderal invader common on irrigation channel banks in Victoria and New South Wales, Australia. Options approved for herbicide control on channel banks are limited and field experimentation conducted in New South Wales over two years demonstrated that there are no effective herbicide control options for managing the weed in these sites. The lack of effective herbicide options highlights the need for further research into both new herbicides and non-chemical control options.

Little is known about the use of defoliation as a strategic management tool of *C. bonariensis* and information available shows variable results. Field experiments were therefore conducted in Dookie, Victoria and Goolgowi, New South Wales to investigate using defoliation in conjunction with herbicide applications. Greatest control over the two experiments was provided by the sequential applications of paraquat + diquat applied 5-10 days after defoliation; and MCPA + dicamba applied 8-9 days prior to defoliation. These experiments demonstrated effective control could be achieved by the use of defoliation with herbicide application in a double-knock system. In a region where widespread resistance to EPSPS and ALS inhibitors has been demonstrated, additional strategies for management of *C. bonariensis* are critical.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Intended Publications from this thesis

Multiple resistance to EPSPS and ALS inhibitors in hairy fleabane; Charlotte Aves, John Broster, Leslie A. Weston, Gurjeet Gill and Christopher Preston: Proposed journal – Weed Technology

Exploring mechanisms of glyphosate resistance in *Conyza bonariensis* (L.) Cronquist populations from northeast Victoria; Charlotte Aves, Jenna Malone, Mahima Krishnan, Christopher Preston, Gurjeet Gill and Leslie A. Weston: Proposed journal – Pesticide Biochemistry and Physiology

Control of *Conyza bonariensis* (L.) Cronquist in mixed farming systems; Charlotte Aves, Christopher Preston, Gurjeet Gill and Leslie A. Weston: Proposed journal – Crop and Pasture Science

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Abbreviations

ACCase	acetyl-coenzyme a carboxylase
a.e.	acid equivalent
a.i.	active ingredient
AGRF	Australian Genome Research Facility
ALS	acetolactate synthase
APMA	aminomethylphosphonic acid
ANOVA	analysis of variance
C	Celsius
ED ₅₀	median effective dose
EPSPS	enzyme 5-enolpyruvylshikimate-3-phosphate synthase
GR	glyphosate-resistant
GS	glyphosate susceptible
HAT	hours after treatment
IWM	integrated weed management
LB	luria-bertani broth
LSD	least significant difference
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate

PSI	photosystem I
PSII	photosystem II
RI	resistance index relative to sensitive biotype
S3P	shikimate-3-phosphate

Chapter 1: Literature Review

1.1. Introduction.

Weeds are among the most important biotic restraints in crop production and *Conyza bonariensis* (L.) Cronquist is one of the most problematic weed species globally in broadacre crop production (Bajwa et al. 2016). In Australia, *Conyza* spp. are ranked as the third most important weed in fallows infesting 2,793,252 hectares (ha) and costing farmers \$43.2 million in lost revenue. *C. bonariensis* is also a significant weed in grain crops, where it was ranked seventh in terms of infested area covering 597,531 ha and costing \$1.3 million in lost revenue (Llewellyn et al., 2016).

C. bonariensis is a successful ruderal invader, which occurs commonly in fallows, along roadsides, railway lines, irrigation channels, as well as in orchards, vineyards, forests and arable lands (Wu, 2007). Irrigation channels are important for seed dispersal (Charles, 1991) and therefore effective weed management in these areas is critical to managing the weed across districts. There is no current research on effective control options for *C. bonariensis* on channel banks. Management using herbicides in fallows has been widely investigated (Shrestha et al., 2008a; Wu et al., 2008; Widderick et al., 2012; Lamego et al., 2013; Sansom et al., 2013) and herbicide control options have been investigated in wheat, sorghum (Wu et al., 2010), lucerne pastures (Wu and Koetz, 2012) and soybean (Bajwa, 2016). There has been research conducted into integrated weed control options including cultivation (Shrestha et al., 2008a; McLean et al., 2012), cover crops (Paula et al., 2011) and crop competition (Widderick et al., 2012). There has been limited research conducted on management of *C. bonariensis* through use of a combination of herbicides and defoliation, and research results have shown conflicting results (Shrestha et al., 2008a; de Vargas Pereira et al., 2016).

C. bonariensis has developed herbicide resistance to nine active ingredients (glyphosate, paraquat, diquat, atrazine, simazine, chlorsulfuron, pyriithiobac-sodium Na, sulfometuron-methyl and imazapyr) in 13 countries (Matzrafi et al., 2015; Heap, 2017). Weed surveys across Australia have

found glyphosate resistance in South Australia, New South Wales and Queensland (Walker et al., 2011; Cook, 2013; Preston, 2014). So far none of the weed surveys have focused on northeast Victoria which is a diverse agricultural region.

1.2. *Conyza bonariensis* (L.) Cronquist

1.2.1. Physical description and taxonomy

C. bonariensis is an annual or biennial member of the Asteraceae family (Wu, 2007). Asteraceae is the largest family of flowering plants in the world, including over 1,600 genera and 23,000 individual species (Gao et al., 2010). The *Conyza* genus belongs to the Astereae, subtribe Conyzinae and is believed to have derived from the genus *Erigeron*. The *Conyza* genus consists of 50-80 species. (Thebaud and Abbott, 1995). The following eight *Conyza* species are naturalised in Australia: *Conyza aegyptiaca* (L.) Aiton, *C. bilbaoana* J. Remy, *C. bonariensis*, *C. canadensis* (L.) Cronquist var. *canadensis*, *C. leucantha* (D. Don) Ludlow & P.H. Raven, *C. parva* (syn. *C. canadensis* var. *pussila* (Nutt.) Cronquist), *C. primulifolia* (Lam.) Cuatrec. & Lourteig (syn. *C. chilensis* Spreng) and *C. sumatrensis* (syn. *C. albida* Willd. ex Spreng.). Among these species, *C. bonariensis* and *C. sumatrensis* are the most common species, with *C. bonariensis* being the most widespread in Australia (Wu and Zhu, 2014).

C. bonariensis is reported to be an allopolyploid with a chromosome number of $2n = 6x = 54$ (Thebaud and Abbott, 1995; Wu, 2007; Okada et al., 2015). The genus *Conyza* has been suggested to contain numerous spp. with varying ploidy levels and the genus is referred to as containing complex polyploids. Different samples of *C. bonariensis* have been reported as having a diploid, tetraploid, hexaploid, and pentaploid chromosomal complement (Soares et al., 2015).

C. bonariensis grows up to 1m tall and has erect, grey, hairy stems often branched at the base. Leaves are greyish-green and hairy; the basal leaves are linear, oblong or narrow-oblong and 40-90 mm long, 5-15mm wide, with toothed margins. Leaves become progressively smaller going

up the stem, and are oblong to linear. *C. bonariensis* produces numerous flowers on pyramidal panicles. 50-200 ray florets per head surround each urceolate (urn-shaped) bract, approximately 3-4mm long. Inflorescences are white and sometimes tinged purple or red (Wu, 2007).



Figure 1: *Conyza bonariensis*: A – early rosette B - early flowering (taken at University of Melbourne, Dookie Campus)

C. bonariensis is often confused with other members of the *Conyza* species, especially when plants are young given its similar physical appearance (Alpen et al., 2014; Wu and Zhu, 2014). *C. bonariensis* does however have significantly higher trichome and stomatal densities than *C. sumatrensis*. *C. bonariensis* trichome densities ranged from 67.2 to 221.9 trichomes mm⁻² on the adaxial surface and 74.0 to 168.1 trichomes mm⁻² on the abaxial surface. Stomatal densities averaged 314.3 ± 12.5 stomata mm⁻² on the adaxial surface and 237.4 ± 9.1 stomata mm⁻² on the abaxial surface (Wu and Zhu, 2014).

1.2.2. Distribution

C. bonariensis is generally believed to have originated in South America (Wu, 2007) and is now a global weed having naturalized in warm areas throughout the world. It occurs in more than 40

crops in 70 countries (Zambrano-Navea et al., 2013) and has been endemic in Australia for a long time with a number of specimens collected in Australia dated back to the 1840s (Wu, 2007).

C. bonariensis is present in every Australian state and territory (Figure 1). It is the most widespread weed in Australia's northern region (Cook, 2013) infesting both summer and winter crops, such as wheat, chickpeas, cotton and sorghum (Wu, 2007), and has recently become a problem weed throughout the cropping belt of south-eastern Australia (Wu and Zhu, 2014).

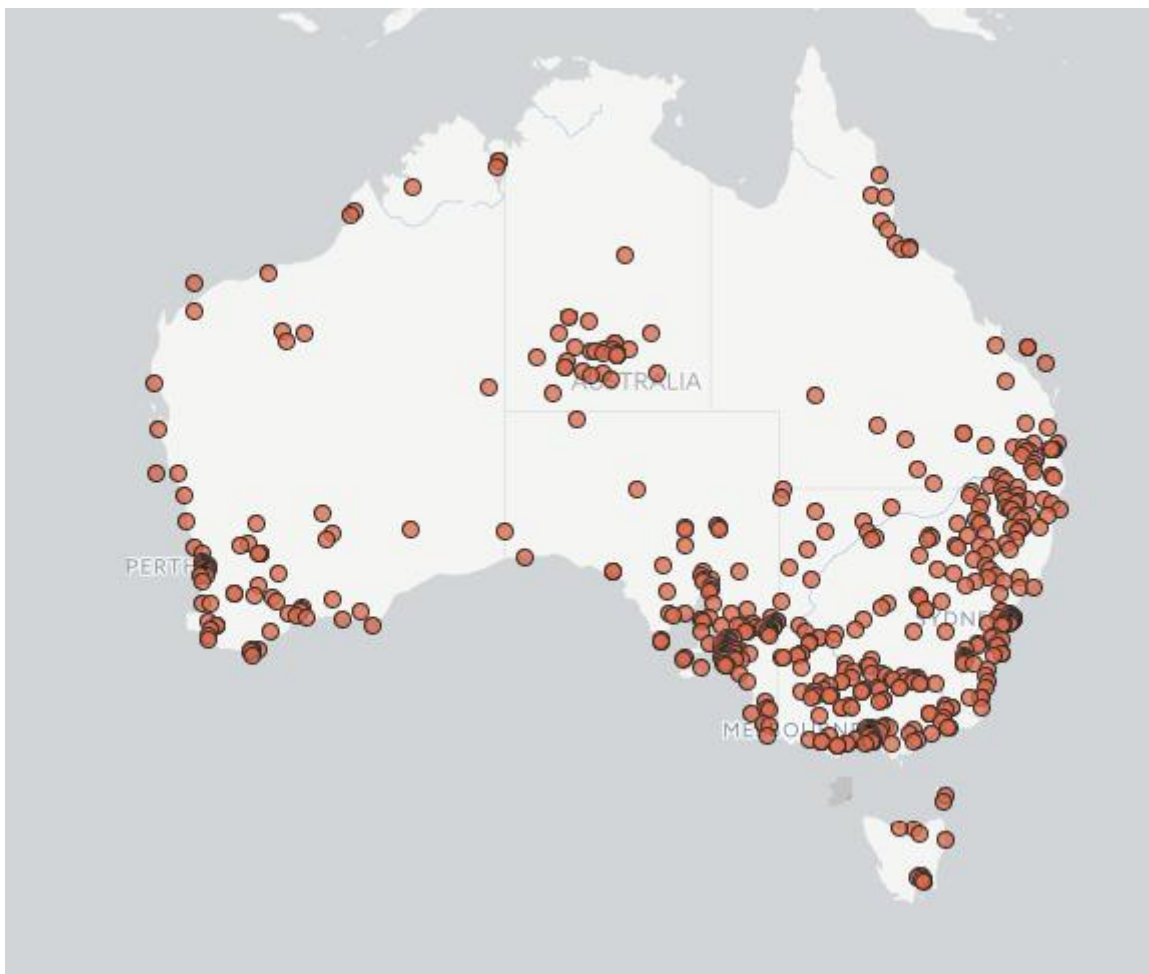


Figure 2: Distribution of *C. bonariensis* based on the 851 records in Australia's Virtual Herbarium (adapted from Australia's Virtual Herbarium, 2017)

1.2.3. Habitat

Conyza species are described by ecologists as successful ruderal invaders, which tolerate a wide range of climates, soils and habitats (Alpen et al., 2014). They have naturalized in warm areas

throughout the world, however, their broad geographical distribution suggests there is no specific climatic requirement for growth (Wu, 2007).

They infest arable land, orchards, vineyards, forests, roadside, fallow land, pastures, horticulture, abandoned fields, as well as industrial sites and irrigation channels (Wu, 2007). Although *C. bonariensis* is more common on lighter textured soils it can also be found on heavier soil types (Wu et al., 2007). *C. bonariensis* is a common component of pasture, particularly in areas that have been neglected or where ground cover is poor (Wu, 2007). High concentrations of nitrate associated with legume based pastures were a key trigger for increased biomass and reproductive ability of *C. bonariensis* (Prieur-Richard et al., 2000).

C. bonariensis has increased with the widespread adoption of conservation cropping as it is photoblastic, requiring light for germination (Wu et al., 2007; Walker et al., 2011). The abundance of *C. bonariensis* in no-till crops may be associated with an improved environment for germination and seed survival resulting from stubble retention, with soil moisture conditions favourable for longer periods (Wu et al., 2007). A species shift associated with glyphosate resistance and no-till farming has been found through physical survey of weeds in the same 50 paddocks across Queensland and New South Wales. *C. bonariensis* has risen from the fourteenth weed in the order of prevalence in 2001 to become most prevalent across Australia in 2010. Its prevalence is clearly seasonal and since 2010 it has declined in prevalence in some locations across southern Australia (Werth et al., 2012).

1.2.4. Weed Biology

Knowledge of weed biology is essential for development of both economically and environmentally acceptable weed management systems. Weed biology relates to plant attributes such as morphology, seed dormancy and germination, physiology of growth, competitive ability, and reproductive biology. Concepts of population biology can be used to predict infestations and evaluate sustainable management strategies (Bhowmik, 1997).

1.2.4.1 Seed biology

A single *Conyza bonariensis* inflorescence consists of multiple small, white capitula (seed heads), predominately branching off the main stem. Each seed head is hemispherical with a diameter of 8–12 mm. Individual seeds are singly enclosed in small, hard achenes, each of which is equipped with a tuft of bristles (pappus). The number of seeds (achene/pappus units) has been estimated at 290–400 seeds per head (Wu, 2007; Borger et al., 2012). The initial viability of the seeds in Australia is estimated to be 80% (Wu et al., 2007), seeds are non-dormant and rapidly germinate when conditions are suitable (Zambrano-Navea et al., 2013).

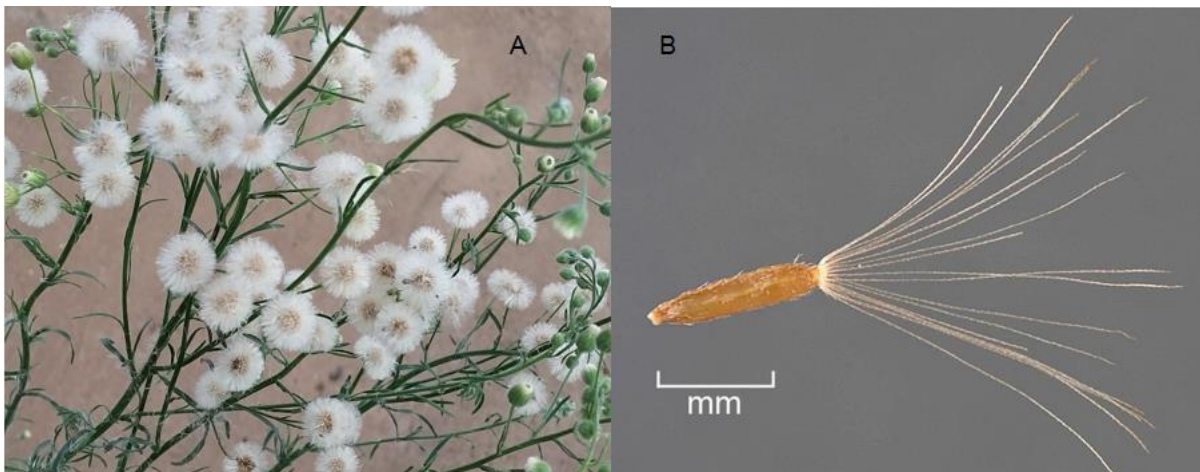


Figure 3: *Conyza bonariensis* seeds. A - Seed heads (taken at University of Melbourne, Dookie) B - individual seed (Source: Walters and Southwick, n.d.)

C. bonariensis is self-compatible and not reported to be pollinated by insect activity, suggesting either autogamy or wind-pollination is important for seed production (Wu, 2007; Zelaya et al., 2007). *C. bonariensis* has a selfing rate of 0.88 (Okada et al., 2015). *C. bonariensis* is also able to flower all year round although flowering is promoted by longer day length and high light intensity (Wu, 2007). It is a prolific seed producer with estimated seed production varying from 375,561 seeds per plant (Kempen and Graf, 1981), 119,100 seeds per plant (Wu et al., 2007) to 5,965 seeds per plant (Borger et al., 2010).

1.2.4.2. Seed Production, Dispersal and Colonisation Ability

Dispersal is an important driver of invasions, metapopulation dynamics, spatial pattern formation, and species movement (Skarpaas et al., 2006). *C. bonariensis* is wind dispersed and has high rates of potential dispersal due to its pappus and low settling velocities of $0.2911\text{m}\cdot\text{sec}^{-1}$ (Andersen, 1992). Estimating the dispersal ability of *C. bonariensis* can be difficult; laboratory experiments have shown there is great variability between plants and among inflorescences and seeds within plants (Andersen, 1992). Borger et al. (2012) also found different plants had different base release thresholds and were affected differently by wind speed, orientation and plant age.

Seed dispersal is influenced by wind, with more seed typically released at higher wind velocities. This however, is influenced by the age of the seed head with average seed dispersal ranging from 49.4% for 0 day old heads to 92.8% for 10 old day heads. Seeds are more likely to be released in updrafts or horizontal winds than downdrafts (Borger et al., 2012). Turbulence has also been found to increase seed release over laminar flows (Skarpaas et al., 2006).

Seed release is significantly greater where humidity is low, however, temperature typically was not shown to have a significant impact (Borger et al., 2012). Other influences on *C. bonariensis* dispersal include seed release height, local topography and vegetation structure (Andersen, 1992). Vehicle movement may also influence dispersal along roadsides and railway lines (Weaver, 2001; Borger et al., 2010).

Field studies in Spain have shown that the majority of *C. bonariensis* seed lands close to the parent plant, but a percentage of seeds are still airborne at 100 m (Bastida et al., 2007). In Merredin, Western Australia, colonisation occurred up to 1842 m from the parent population with up to 5% of seed landing more than 100 m away (Borger et al., 2010).

It is difficult to distinguish between *C. canadensis* and *C. bonariensis* as both have the characteristic pappus that aids their dissemination (Shrestha et al., 2008). Aerial collection of multiple

C. canadensis seeds at heights ranging from 41 to 140 m above ground level suggests that seeds are entering the Planetary Boundary Layer (PBL) of the atmosphere, where long-range transport of aerial biota frequently occurs. With wind speeds in the PBL frequently exceeding 20 m s^{-1} , seed dispersal can easily exceed 500 km in a single dispersal event (Shields et al., 2006).

C. bonariensis seeds are also successfully dispersed by water movement through surface runoff, irrigation channels and waterways (Wu, 2007). Glyphosate-resistant fleabane infests hundreds of km of glyphosate-treated irrigation channel banks in California's central valley (Powles, 2008). Channel banks are a source of weed infestation (Charles, 1991) and 15 *C. canadensis* seeds were found per 254 kL of water in a survey of weed seeds in irrigation water in the Yakima Valley and Columbia River, Washington. The germination percentage of these seeds was 80% (Kelley and Bruns, 1975).

Dispersal distance does not indicate the full extent of the ability of *C. bonariensis* to establish or colonise an area. Colonisation rates within the farming landscape will influence invasion rates of this species into new areas and the spread of herbicide-resistant genes through existing populations. The largest proportion of rosettes was found within 20 m of the parent plant. Sites with poor summer weed control are favourable for colonisation as well as roadsides, fence lines and standing stubble (Borger et al., 2010).

Studies into the ability of *C. bonariensis* to invade Mediterranean self-seeding meadows showed that species richness and the presence or absence of legumes played a role in establishment. *C. bonariensis* biomass decreased with increased species richness, associated with competitive processes. However, this effect was limited as above ground biomass and Leaf Area Index (LAI) at peak biomass showed no relationship to species richness. Biomass and net fecundity increased with an increase in legume species and this is attributed to additional nitrate concentrations found under legumes. Biomass of *C. bonariensis* decreased when the community under study contained grasses,

light interception at ground level was lower in species mixes containing grasses and competition for water was greater (Prieur-Richard et al. 2000). In the subhumid subtropics of Queensland, the build-up of soil fertility following improvement of pastures by introduction of legumes led to enhanced invasion by *C. bonariensis* (Tohill and Berry, 1981).

1.2.4.3. Germination, emergence and growth

The range of temperatures that promote germination are critical when estimating the time of emergence under field conditions (Karlsson and Milberg, 2007). The base, optimum and maximum temperatures for germination of *C. bonariensis* in Queensland have been estimated to be 4.3 °C, 20 °C and 35 °C respectively (Wu et al., 2007). Timing of seedling emergence of *C. bonariensis* is difficult to predict as it produces large numbers of non-dormant seeds over extended periods of time which are able to disseminate widely and rapidly germinate when conditions are suitable (Zambrano-Navea et al., 2013).

The base water potential for germination was estimated to be -0.7 ± 0.151 MPa (Zambrano-Navea et al., 2013). Experiments testing emergence in response to rainfall and temperature showed that no seedlings emerged with rainfall levels of less than 10 mm; more seedlings emerged across all rainfall treatments over 10 mm where day/night temperatures were 30 °C/20 °C respectively in comparison to 25 °C/15 °C and the majority of germination occurred with rainfall of 20 to 30 mm (Keenan and Werth, 2012).

Seeds can survive for up to three years under typical field conditions in California (Shrestha et al., 2008a). Experiments carried out on *C. bonariensis* seed and soils sampled in Queensland showed that seed persistence initially declined rapidly from 80% to 50% within one month, then down to 10% within a year and 6% at three years. The rate of decline in seed viability was greatly influenced by soil type and burial. Of seed buried in a light sodosol, 8% remained viable at 24 months in comparison to

2% remaining viable in a heavy soil. Seeds buried at 5 to 10 cm retained greater viability (8.6% after 36 months) compared to 1.3% remaining viable at depths of 0 to 2 cm (Wu et al., 2007).

C. bonariensis is photoblastic, which means light is required for germination (Karlsson and Milberg, 2007; Wu, 2007). Seedling emergence from the soil surface was shown to be $23\pm 3\%$, dropping to $16\pm 3\%$ at 0.5 cm, $4\pm 1\%$ at 1 cm and no seedlings emerged from greater than 2 cm (Wu et al., 2007). Seedling emergence has been observed to occur from 3 days after sowing and after 30 days plants had four true leaves (Wu, 2007).

C. bonariensis is common in summer fallows with active growth starting in the spring or early summer. Seedlings typically require 11 weeks to reach the bolting stage (Wu, 2007) and a further three weeks to reach flowering stage (Thebaud and Abbott, 1995). *C. bonariensis* has been shown to emerge throughout the autumn, winter and spring, with the majority emerging in the autumn and early winter in Australia, forming a basal rosette stage to overwinter and then producing seed in the following spring or summer. A smaller percentage of seeds germinate in the spring and produce seed without overwintering (Wu et al., 2007). Overwintering seems to provide an ecological advantage, as seedlings actively grow despite the cold conditions, establishing a strong root system even though there appears to be little growth above ground (Wu, 2007).

C. bonariensis is a quantitative long day species (Wu, 2007). Seedling development is slow and rosette stage can be prolonged even under thermoperiods optimal for growth (27/22°C). When grown at 32/27°C, 27/22°C and 22/17°C stem elongation and flowering began earlier when days were longer (16 hours compared to 8 hours) (Zinzolker et al., 1985).

1.2.5. Importance and Impacts

C. bonariensis has recently been cited as one of the most problematic agricultural weeds globally. Weeds interfere with crop production in many different ways, including resource competition, allelopathy and their role as alternative hosts for crop pests (Bajwa et al. 2016).

There is little data on the impact of *C. bonariensis* on agricultural enterprises. Llewellyn et al. (2016) reported the impact of weeds on Australian grain production surveying 602 growers nationally. *Conyza* spp. was third most important weed in fallow nationally, infesting 2,793,252 ha and was responsible for \$43.2 million in lost revenue. *Conyza* species were ranked seventh nationally in terms of the area of grain crops infested (wheat, barley, canola, pulses and sorghum; 597,531 ha) and costing farmers \$1.3 million in lost revenue. In the Goondiwindi region, Queensland *C. bonariensis* infestation has increased fallow weed control cost by 100% (Thorn, 2004).

C. bonariensis causes significant yield decreases in sorghum as it competes for water and nutrients, particularly stored soil moisture in dryland areas. When compared to a weed free treatment, *C. bonariensis* caused a reduction in sorghum yield of 30% (Wu, 2007). In another study *C. bonariensis* reduced sorghum yield by 65 to 98% in the northeast grains region of Australia and the impact on wheat yield was dependent on row spacing (Wu et al., 2010). Similarly Ford et al. (2014) found that *C. canadensis* resulted in a 92% decrease in corn yields.

C. bonariensis can also seriously impact soybean yield (Silva et al., 2014; Trezzi et al., 2015). Soybean grain yields were reduced by an average of 25% as a result of 13.3 *C. bonariensis* plants m⁻² (Trezzi et al. 2013). At the lower densities, each *C. bonariensis* plant decreased soybean yield by 36%, 12% and 1.0%, when established at 81, 38 and 0 days before sowing respectively in experiments conducted in Brazil (Trezzi et al., 2015). The area had been disc ploughed followed by a tandem disc cultivation prior to commencement of the trial, no further cultivation took place after *C. bonariensis* establishment.

Weed management in fallows is critical in dryland grain production as summer rainfall makes a significant contribution to stored soil moisture reserves which are utilised by the following seasons winter crop (Hunt and Kirkegaard, 2011; Sadras et al., 2012; Hunt et al., 2013). *C. bonariensis* is a significant weed in fallows; it produces a deep tap root capable of establishing at depths greater than

35 cm and is able to survive drought conditions (Wu, 2007), suggesting that it has the potential to extract water to great depths. Experiments have shown that rainfall over the summer fallow period in drier seasons can increase winter crop yields by up to 50% (Sadras et al., 2012).

Agricultural Production Systems sIMulator (APSIM) modelling of 37 locations across southern Australia showed that summer rainfall contributes on average 1000kg ha⁻¹ or 33% of water limited attainable yield (Hunt and Kirkegaard, 2011). Field experiments demonstrated that controlling summer weeds increased subsequent crop yields by an average of 700 kg ha⁻¹ on sand soils and 900kg ha⁻¹ on clay soils in the low rainfall region of southern Australia (Hunt et al., 2013). In the 1970s in South Australia, it was shown that for every mm of stored soil moisture at sowing produced between 8 and 15kg ha⁻¹ wheat yield, with the largest increases being in fine textured soils. The variation in yield attributed to stored soil moisture ranges from 72% in dry years (200mm growing season rainfall) to 25% (350mm growing season rainfall) (French, 1978).

C. bonariensis acts as a wild host to a range of pests and diseases (Wu, 2007). *C. bonariensis* acts as a host to insect pests *Nysius graminicola* Kolenati and *N. cymoides* Spinola which damage summer fruit and vegetable crops, the formicid *Dorylus orientalis* Westwood a horticultural pest in China, and *Uroleucan bereticum* in Argentina. *C. bonariensis* also acts as a host to the witches' broom virus and tomato spotted wilt virus which both infect tomato crops and the lettuce mosaic virus in Brazil (Wu, 2007). *C. bonariensis* was also found to host *Nysius simulans* (Hemiptera; Lygaeidae) in soybean (Bajwa et al., 2016).

1.3. Herbicide Resistance

Herbicides are widely used globally to control weeds, and have revolutionised weed control over the last 65 years, contributing significantly to crop yields over this period (Heap, 2014a). Herbicide usage in the United States increased from 21,772 t of active ingredients in 1964, peaked at 195,045 t in 1982 and was estimated at 172,365 t in 2010 (Osteen and Fernandez-Cornejo, 2013). In the UK there was no discernible trend in the area where herbicides were applied to between 1970 and 1995,

sitting around 88% of the total area of arable crops, however the total spray area increased due to increasing number of applications applied each year (Ewald and Aebischer, 2000). New Zealand also has seen a trend of increasing herbicide sales over the 10 years prior to 2004 although this fluctuated with seasons (Manktelow et al., 2005) and Australia has shown a trend of increasing herbicide use between 1990 and 2006 (Figure 2). In 2006-07, 88.8% of agricultural businesses in Australia had reported conducting weed control activities and spending \$982 million on herbicides (Australian Bureau of Statistics, 2008b; Australian Bureau of Statistics, 2009).

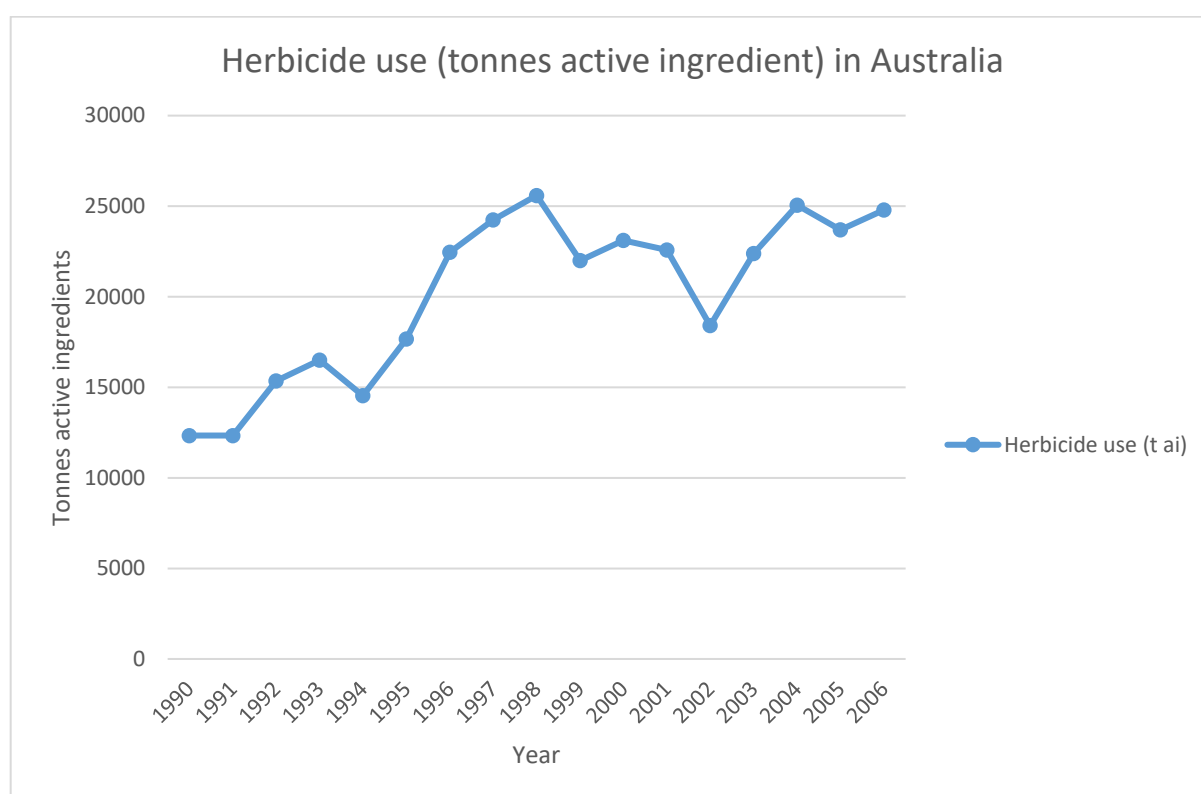


Figure 4: Herbicide use (tonnes active ingredients) in Australia between 1990 and 2006 (FAOSTAT, 2015)

Herbicide resistance in weeds such as *C. bonariensis* is driven by selection pressure (Heap, 2014a). Herbicide use eventually causes a shift in a weed population from a few resistant individuals in an otherwise susceptible population to a population dominated by resistant weeds. Development of herbicide resistance depends on selection pressure, initial frequency of resistance alleles within a

population, the inheritance and fitness characteristics of resistance alleles, the biology of the weed species and the herbicide mode of action (Preston, 2000).

Herbicide resistance is a global concern with a total of 479 reported cases in 69 countries, affecting 91 crops, 251 weed species (146 dicot and 105 monocots), 23 of 26 herbicide modes of action and 163 different herbicides. Australia accounts for 125 of the total reported cases affecting 47 weed species and 13 modes of action (Heap, 2017). Herbicide resistance is estimated to cost Australian grain growers an additional \$187 million in herbicide costs (Llewellyn et al., 2016).

There are five main mechanisms for herbicide resistance with a risk of either cross-resistance (where a single resistance mechanism results in resistance to more than one herbicide) or multi-resistance (where more than one resistance mechanism occurs within one plant) (Heap, 2014a). Target site resistance occurs due to mutations that alter the herbicide binding site preventing or reducing herbicide binding. Target site mutation was discovered to be the mechanism for acetolactate synthase (ALS) inhibitors in *Lactuca serriola* in 1987 and the mechanism most commonly responsible for resistance to ALS inhibitors, photosystem II (PSII) inhibitors and Acetyl Coenzyme A Carboxylase (ACCase) Inhibitors (Heap, 2014a). Enhanced metabolism is the increased ability of the plant to degrade the herbicide. Metabolic resistance was first reported to the ALS inhibitor chlorsulfuron in *L. rigidum* in Australia in 1986 and has since been found to be widespread in *Amaranthus tuberculatus* causing resistance to triazine herbicides (Heap, 2014a). Enhanced metabolic inactivation was found to confer cross-resistance to diclofop and ALS inhibitors in *L. rigidum* (Holt et al., 1993). Decreased absorption or translocation and sequestration into cell walls or into vacuoles reduces the ability of the herbicide to reach the target site and was the primary mechanism responsible for resistance to bipyridilliums (Heap, 2014a). Gene amplification or overexpression increased the production of the target site so a higher concentration of herbicide was needed to provide control. This was first discovered as the mechanism causing glyphosate resistance in *Amaranthus palmeri* in the USA (Gaines et al., 2010).

1.3.1. Resistance to EPSPS Inhibitors

Glyphosate is a unique broad-spectrum post emergence herbicide and is the only inhibitor of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) (Bradshaw et al., 1997; Preston, 2000). Originally released in 1974 it has grown to be a global herbicide registered in over 130 countries and approved for weed control in over 100 crops (Monsanto, n.d.).

EPSPS forms part of the shikimic acid pathway catalysing the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to produce EPSPS and inorganic phosphate (Pi) (Bradshaw et al., 1997). EPSPS is located primarily in plastids, although a cytoplasmic form also exists (Baylis, 2000).

Glyphosate has an adventitious allosteric interaction with EPSPS, so a significant part of the glyphosate molecule binds outside the EPSPS active site and a conformational change upon binding makes the active site unavailable to PEP (Baylis, 2000). Glyphosate also inhibits import of the EPSPS pre-protein into the chloroplast (Bradshaw et al., 1997). Inhibition of EPSPS results in a reduction in the synthesis of aromatic amino acids (tryptophan, tyrosine and phenylalanine) and an accumulation of shikimic and some hydroxybenzoic acids (de María et al., 2006).

Although EPSPS is the only known target for glyphosate, its action also affects physicochemical and physiological processes in plants, reducing photosynthesis, degrading chlorophyll, inhibiting auxin transport and enhancing auxin oxidation. This could be as a direct consequence of blocking the shikimate pathway (through which an estimated 30% of assimilated carbon passes) or due to feedback mechanisms (Baylis, 2000).

Glyphosate is rapidly transported through the phloem to metabolic sinks (de María et al., 2006). Although visible symptoms are slow to develop, physiological effects in treated plants can be detected much earlier. Large increases in shikimic acid occur within 24 hours of glyphosate application to *Betula papyrifera* (Baylis, 2000).

Resistance to glyphosate has been documented in 37 weed species, in 29 countries with the first documented case being recorded in 1997 in *L. rigidum* from Victoria, Australia (Heap, 2017). The first reported case of glyphosate resistance in a dicot species was in *C. canadensis* in 2001 (Yuan et al., 2010). The known glyphosate resistance mechanisms include target site mutation, target site gene duplication, active vacuole sequestration, limited cellular uptake and a rapid necrosis response (Sammons and Gaines, 2014).

1.3.1.1. Target Site Mutation

Target site mutations alter the binding site preventing or reducing herbicide binding (Beckie, 2011; Heap, 2014a). Target site resistance was found to be the mechanism of resistance in *Eleusine indica* from Malaysia, where a mutation of the EPSPS gene led to a proline-to-serine substitution at amino acid 106 (Pro106-Ser). Parallel studies also found that proline-to-threonine (Pro106-Thr) substitution causing glyphosate resistance in *E. indica* (Powles and Preston, 2006). Since then two further Pro106 substitutions have been discovered, Ala and Leu (Sammons and Gaines, 2014). Pro106 mutations have been found in *Amaranthus tubercalatus*, *Echinochloa colona*, *Digitaria insularis* and *Lolium* Spp. (Sammons and Gaines, 2014), *C. sumatrensis* (González-Torralva et al., 2014), *Leptochloa virgata* (Alcántara-de la Cruz et al., 2016b), and *Dibens pilosa* (Alcántara-de la Cruz et al., 2016a).

The first reported case of double amino acid substitution (Thr102-Ile + Pro106-Ser or TIPS mutation) was recorded in *E. indica* (Yu et al., 2015) and since then double mutation to Thr102-Ile followed by Pro106-Ser has been reported in *B. pilosa* alongside reduced translocation (Alcántara-de la Cruz et al., 2016a).

1.3.1.2. Target Site Gene Amplification

Target site gene amplification or over-expression increases the production of target sites available for herbicide binding, meaning a greater concentration of herbicide is needed to provide

control (Beckie, 2011; Heap, 2014a). The first reported case of gene amplification as a mechanism for glyphosate resistance was documented in *Amaranthus palmeri* from Georgia, USA. Genomes from resistant plants contained between 5 to over 160 more EPSPS gene copies than the genomes of susceptible plants (Gaines et al., 2010).

Since then gene amplification has been confirmed as the resistance mechanism for *A. palmeri* populations from another three USA states, and in the weed species *A. tuberculatus*, *A. spinosus*, *Lolium multiflorum*, and *Kochia scoparia* in the USA (Sammons and Gaines, 2014; Kumar et al., 2015; Salas et al., 2015), in *E. indica* populations from China (Chen et al., 2015), *Bromus diandrus* in Australia (Malone et al., 2016) and *Chloris truncata* in Australia (Ngo et al., 2017). EPSPS gene copy number varies with species and population (Table 2) and glyphosate resistance level appears to increase with higher EPSPS genomic copy number (Sammons and Gaines, 2014; Chatham et al., 2015; Salas et al., 2015).

Increased EPSPS mRNA alongside differential translocation were both present in *C. canadensis* populations from across the USA. Glyphosate-resistant (GR) biotypes had less downward translocation to roots than glyphosate susceptible (GS) biotypes and the relative EPSPS mRNA was 1.8 to 3.1 times higher than in GS biotypes (Dinelli et al., 2006).

ABC transporter genes M10 and M11 play a role in translocation, in GR *C. canadensis* populations from the US, Greece and Crete. The expression levels of the EPSPS genes were not significantly altered following glyphosate application, however both M10 and M11 were found to be highly upregulated in GR biotypes. This suggests that expression of ABC transporter genes may be playing a role in vacuolar sequestration in *C. canadensis* (Nol et al., 2012).

Tani et al. (2015) showed that synchronisation of ABC transporter genes M10, M11, M7 and P3 with the EPSPS gene was the cause of 13 fold glyphosate resistance. Experimentation on the effect of environmental conditions (temperature and light) on the expression of EPSPS and ABC

transporter genes M10 and M11 in *C. canadensis*, found that under normal environmental conditions (24°C) there was a six-fold upregulation of the EPSPS gene in the GR biotype at one day after treatment with glyphosate, there was also higher expression for M10 and M11 (Tani et al., 2016).

Table 1: Species, origin and EPSPS copy number for reported gene amplification in glyphosate-resistant weeds (Gaines et al., 2010; Sammons and Gaines, 2014; Chen et al., 2015, Kumar et al., 2015; Malone et al., 2016; Ngo et al., 2017)

Species	Origin	EPSPS genomic copy number
<i>Ameranthus palmeri</i>	Georgia, US	5-160
<i>A. palmeri</i>	North Carolina, US	20-60
<i>A. palmeri</i>	New Mexico, US	2-10
<i>A. palmeri</i>	Mississippi, US	33-59
<i>Ameraranthus tubersalatus</i>	Illinois, US	4
<i>Amaranthus spinosus</i>	Mississippi, US	26-37
<i>Bromus diadrus</i>	Australia	20
<i>Chloris tuncata</i>	Australia	32-48
<i>C. canadensis</i>	US (Multiple states)	1.8 – 3.1
<i>Eleusine indica</i>	China	28.3
<i>Kochia scoparia</i>	Colorado, US	3-9
<i>K. scoparia</i>	Montana, US	4-10
<i>Lolium multiflorum</i>	Arkansas, US	15-25
<i>L. multiflorum</i>	Arkansas, US	11-100

1.3.1.3. *Reduced Translocation and Active Vacuole Sequestration*

Glyphosate is a systemic herbicide and is translocated rapidly in most plants, following a similar distribution pattern as photo-assimilates with a source to sink relationship (Franz et al., 1997b). Glyphosate is poorly absorbed by leaves, but the herbicide that is absorbed is then rapidly and extensively transported out of the leaf through both the xylem and phloem (Franz et al., 1997a; Preston and Wakelin, 2008).

Lorraine-Colwill et al. (2002) demonstrated differences in the translocation of glyphosate in *L. rigidum* with resistant populations accumulating more glyphosate in the leaf tips and less in the roots than susceptible populations. Reduced translocation has since been demonstrated to be the resistance mechanism in *L. multiflorum*, *C. canadensis* (Preston and Wakelin, 2008) and in *Chloris elata* (Brunharo et al., 2016). In *Lessingia virgata* and *Bidens pilosa* reduced translocation alongside target site mutations were recorded as the mechanisms for glyphosate resistance (Alcántara-de la Cruz et al., 2016a; Alcántara-de la Cruz et al., 2016b).

³¹P nuclear magnetic resonance (NMR) was used to observe glyphosate *in vivo* entering cells and cellular compartments identifying two mechanisms restricting glyphosate translocation. In *C. canadensis* the difference in vacuole sequestration of glyphosate between resistant and susceptible populations was ten-fold (Ge et al., 2010; Sammons and Gaines, 2014). *Lolium* spp. from Australia, Brazil, Chile, and Italy showed differing levels of glyphosate resistance were examined by ³¹P NMR. All GR variants showed glyphosate sequestration within the cell vacuole, whereas there was minimal or no vacuole sequestration in the GS variants. The extent of vacuole sequestration correlated qualitatively with the level of resistance (Ge et al., 2012).

1.3.1.4. *Metabolism*

Enhanced metabolism resistance results from the plant's ability to degrade the herbicide more rapidly (Beckie, 2011; Heap, 2014a). *C. canadensis* from Spain had similar shikimate accumulation in

both GR and GS biotypes until 72 hours after treatment (HAT), after that shikimate continued to increase in the GS biotype but then decreased by 40% in the GR biotype. It was found that glyphosate was metabolised faster in the GR biotype, disappearing completely by conversion into glyoxylate, sarcosine and aminomethylphosphonic acid 96 HAT. Glyphosate remained in the GS biotype until 120 HAT and glyoxylate was the only non-toxic metabolite detected in the susceptible biotype. Enhanced metabolism was working alongside reduced translocation in this population (González-Torralva et al., 2012).

1.3.2. Herbicide-Resistant *Conyza bonariensis* (L.) Cronquist

The first reported case of herbicide resistance in *C. bonariensis* was to paraquat in Egypt in 1985 (Shaaltiel and Gressel, 1985). Globally there have been 19 unique cases of herbicide-resistant *C. bonariensis* reported in 13 countries to nine active ingredients. These include resistance to EPSPS inhibitor glyphosate; PSI electron divertors paraquat and diquat; PSII inhibitors atrazine and simazine; and ALS inhibitors chlorsulfuron, pyriithiobac-sodium, sulfometuron-methyl and imazapyr (Matzrafi et al., 2015; Heap, 2017). Multiple-resistance to glyphosate and paraquat has been reported in California (Moretti et al., 2013) and to PSII inhibitors atrazine and metribuzin and ALS inhibitors pyriithiobac sodium, sulfometuron-methyl and imazapyr in Israel (Matzrafi et al., 2015). The majority of reported herbicide resistance cases are related to the EPSPS inhibitor glyphosate. Broad genetic variability is a factor that can contribute to the evolution of herbicide resistance in weeds and high genetic variability in *C. bonariensis* populations may be one of the main factors contributing to the selection of resistant biotypes (Soares et al., 2015).

1.3.2.1. Glyphosate Resistance in *Conyza bonariensis* (L.) Cronquist

Glyphosate resistance in *C. bonariensis* was documented in South Africa in 2003 (Heap, 2017) and since then has been found in Spain (Urbano et al., 2007; Dinelli et al., 2008), Brazil (Moreira et al., 2007), USA (Shrestha et al., 2008b), Greece (Travlos and Chachalis, 2010), Israel, China (Powles, 2008), Colombia and Portugal (Heap, 2017). Surveys in Australia have found

glyphosate-resistant *C. bonariensis* populations in Queensland, New South Wales and South Australia (Walker et al., 2011; Cook, 2013; Preston, 2014). Surveys conducted in Western Australia did not find glyphosate resistance (Owen et al., 2009). A national survey, including 14 samples collected from across Victoria did not find resistance in the state (Malone et al., 2012). Despite extensive surveying for glyphosate resistance in *C. bonariensis*, no surveys have focused on the northeast Victoria which is a productive and agriculturally diverse region.

Reduced translocation is a common mechanism for glyphosate resistance in *C. bonariensis*. Populations collected from RoundUp Ready (RR) soybean farms in Brazil had differential translocation; 90% of ¹⁴C-glyphosate absorbed remained in the treated leaf 72 HAT in the GR biotype in comparison to 70% remaining in the treated leaf of the GS biotype. The GS biotype had greater translocation efficiency with leaves, stem and roots showing greater glyphosate concentrations (Ferreira et al., 2008). In multi-resistant *C. bonariensis* from California, the mechanisms associated with GR and glyphosate-paraquat resistance (GPR) was reduced translocation. All biotypes absorbed between 52.9 to 58.3% of glyphosate applied, however the GR and GPR biotypes translocated less glyphosate out of the treated leaf than glyphosate-paraquat susceptible (GPS) biotypes (Moretti and Hanson, 2017).

Reduced translocation is often one of two or more mechanisms within an individual plant. Reduced translocation and increased EPSPS expression were found to be the resistance mechanisms responsible for glyphosate resistance in Spanish orchards. The populations had resistance indices (RI) between 2.9 and 5.6. The main difference between GR and GS populations was the mobility of glyphosate, with translocation from treated leaves to culm and root being less than the GS population. In two of the four populations, EPSPS mRNA prior to treatment was double that of GS and the other two GR biotypes. The highest RI correlated with plants having both reduced translocation and high basal EPSPS transcript levels (Dinelli et al., 2008).

Kleinman and Rubin (2017) found high shikimate levels in the roots and young leaves of GS *C. bonariensis* plants, regardless of the site of application, whereas in GR plants, shikimate accumulated mainly in treated young leaves. ¹⁴C-glyphosate studies, however, revealed the expected source-to-sink translocation pattern in both GS and GR plants suggesting resistance is due to altered subcellular distribution of glyphosate, keeping glyphosate sequestered away from the EPSPS target site in the chloroplast.

Target site mutations conferring resistance to glyphosate are yet to be confirmed in *C. bonariensis*. Populations collected from central and southern Greece did not have any EPSPS mutations that conferred resistance (Mylonas et al., 2014). Kleinman and Rubin (2017) sequencing of EPSPS DNA fragments of GR and GS plants revealed no alteration at the Pro106 position in populations from Israel. The low accumulation of shikimate in *C. bonariensis* from Brazil by the GR biotype suggests lower sensitivity of the EPSPS enzyme to glyphosate (Kaspary et al., 2016), however, the experiment did not investigate whether this was as a result of target site mutation.

GR *C. bonariensis* studied in Greece appeared to have no fitness penalty associated with resistance (Travlos and Chachalis, 2013). Under non-competitive conditions, growth and seed production of GR and GS biotypes were similar. The relative crowding coefficient (RCC) between the biotypes was close to one, however, the RCC values increased with time indicating GR had a small competitive advantage as plants matured (Travlos and Chachalis, 2013). In the USA a comparison of phenological development rates and biomass of susceptible and resistant plants revealed no differences (Shrestha et al., 2014). Glyphosate-resistant *C. bonariensis* from Brazil developed more rapidly than the susceptible biotype, having earlier bolting, flowering and seed set. In both years seed production per plant was higher in the resistant biotype and germination was 80% in comparison to 66.5% in the susceptible biotype (Kaspary et al., 2017). The lack of fitness penalty associated with glyphosate resistance in *C. bonariensis* suggests that resistance is likely to persist in areas where it has evolved.

Despite glyphosate resistance being confirmed in New South Wales, Queensland and New South Wales determination of the mechanisms associated with glyphosate resistance has not been attempted in GR *C. bonariensis* populations in Australia.

1.3.2.2. *PSI inhibitor resistance in Conyza spp.*

First identified in 1955 and extensively used since the 1960's (Heap, 2014a), paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a fast-acting contact herbicide that works within the chloroplast by diverting electrons from photosystem I (PSI) (Hawkes, 2014). There are currently 32 species with resistance to bipyridilliums (paraquat and diaquat) from 17 countries (Heap, 2017). The first reported cases of paraquat resistance in *Conyza* spp. were in 1980 when it was discovered in *C. canadensis* in Japan and *C. sumatrensis* in Taiwan (Heap, 2017). There are now 15 unique cases of paraquat resistance in *Conyza* spp. including 5 in *C. bonariensis* and multiple resistance to paraquat and glyphosate in both *C. bonariensis* and *C. canadensis* (Heap, 2017). There is only one reported case of paraquat resistance in *C. bonariensis* in Australia which was found in a New South Wales vineyard in 2016 (Heap, 2017).

Paraquat concentration required for 50% inhibition of paraquat resistant (PR) *C. bonariensis* biotype collected in Egypt was dependent on plant age. After germination, it was 30 times more resistant than the paraquat susceptible (PS) biotype, this increased to >300 times at 10 weeks of growth and then levelled out at 20-fold resistance. Levels of plasmid superoxide dismutase and of glutathione reductase were highest when paraquat resistance was at its peak, but were not significantly different from the PS biotype at times of lower paraquat resistance. Photoinhibition tolerance was highest when the relative amounts of enzymes were highest. It is possible that these enzymes play a role in defence against photooxidants (Amsellem et al., 1993). Ye and Gressel (1994) found a similar resistance pattern for *C. bonariensis* seeds collected in the same regions of Egypt with 30-fold resistance at one to eight weeks, 315 fold resistance at nine to 12 weeks and 34.5-fold resistance from 13 to 25 weeks.

Multi-resistant glyphosate-paraquat populations (GPR) of *C. bonariensis* found in a Californian orchard had 34-fold paraquat resistance in comparison to a glyphosate-paraquat susceptible (GPS) biotype, however, this was dependant on the season. The paraquat resistance index (RI) was 34 in the summer, 23 in the fall and 6.6 in the winter. The level of glyphosate resistance in the resistant biotypes was also dependent on the season, with GR populations having a RI of 5.2 in the summer but was similar to GS in the winter (Moretti et al., 2013).

GPR populations collected in California displayed reduced translocation as the mechanism of resistance to both herbicides. Glyphosate applied to GR and GPR populations translocated less out of the treated leaves than the GPS biotype. GPR populations translocated 3% or less of paraquat absorbed compared to 20% and above in GPS and GR biotypes (Moretti and Hanson, 2017).

1.3.2.3. PSII inhibitor resistance in Conyza spp.

There are 26 photosystem II (PSII) inhibitor herbicides belonging to six chemical classes (triazines, triazinones, triazolinone, uracils, pyridazinones and phenyl-carbamates) that have been commercialized globally, the most prominent being atrazine (Heap, 2014a). Triazine herbicides inhibit photosynthesis by competing with plastoquinone at its binding site which is located on the D1 protein in the photosystem two complex in chloroplasts (Heap, 2014b). Resistance to PSII inhibitors has been reported for *C. bonariensis* (L.) Cronquist in Spain and Israel and in 10 countries for *C. canadensis* (Heap, 2017; Pölös et al., 1988). There are some cases of triazine resistance due to enhanced metabolism, however, the majority of triazine resistance cases are due to a mutation (Ser 64 to Gly) in the psbA gene, which codes for the D1 protein and reduces the binding of triazine herbicides to the thylakoid membrane in chloroplasts (Heap, 2014b).

C. canadensis from a vineyard in Hungary was found to have resistance to both paraquat and atrazine, with RI's of 170 to paraquat and 300 to atrazine (Pölös et al., 1988). *C. canadensis* collected from orchards in Poland had an RI of 33.68 to atrazine (Gadamski et al., 2000). Plants of two *C.*

canadensis populations did not show significant injuries even when treated with 4.0 kg ha⁻¹ of atrazine (8X). It should be noted that the ED₅₀ of the susceptible population was quite high (768 g ha⁻¹) and above the recommended rate. In PSII-resistant populations, nucleotide (790) substitution was from A to G, resulting in a Ser264 to Gly substitution (Matzrafi et al., 2015).

1.3.2.4. ALS Inhibitor resistance in *Conyza* spp.

Herbicides that target the acetolactate synthase (ALS) are among the most widely used weed control chemicals since their introduction into the marketplace in the early 1980s. There are five classes of ALS-inhibitors including, sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinylthio-benzoates (PTBs) and sulfonylamino-carbonyltriazolinones (SCTs) (Zhou et al., 2007; Zheng et al., 2011). Herbicides from these classes inhibit acetolactate synthase, the first enzyme in the biosynthesis of the branched chain amino acids valine, leucine and isoleucine (Osuna and De Prado, 2003; Zheng et al., 2011).

The first reported case of resistance to ALS-inhibitors in *Conyza* spp. was *C. albida* in Seville, Spain in 2002. The population was found to have a resistance factor of 300 to imazapyr and cross-resistance to amidosulfuron, imazethapyr and nicosulfuron. There were no differences in absorption and translocation of ¹⁴C imazapyr between the R and S biotypes and the metabolism pattern was similar. The data suggested resistance was primarily a result of altered target site (Osuna and De Prado, 2003).

Three *C. canadensis* populations found to have ALS-resistance in Indiana, two populations displayed uniform resistance (>98% surviving 8.8 g a.i. ha⁻¹ cloransulam) and the third which contained 85% resistant individuals. All three populations were cross resistant to chlorimuron and bispyribac, one was also cross resistant to imazethapyr. Three different amino acid substitutions account for this resistance; a Pro to Ser substitution at position 197, a Pro to Ala substitution at position 197 and an Asp to Glu substitution at position 376 (Zheng et al., 2011).

ALS resistance testing of *C. bonariensis* populations collected in Israel revealed that 90% of populations were resistant to PTB herbicides, 43% were resistant to SU herbicides and 11% to IMI herbicides. *C. canadensis* populations collected as part of the same survey exhibited stronger and more consistent resistance to ALS inhibitors, which is assumed to be as a result of greater homozygosity (Matzrafi et al., 2015).

C. canadensis populations in Indiana and Ohio have been found to be resistant to glyphosate, ALS-inhibitors and multi-resistant to glyphosate and ALS-inhibitors. These populations produce similar amounts of biomass and seed compared to susceptible populations. The aboveground shoot mass and seed production did not differ between treated and untreated plants; therefore resistant plants have a growth and seed production advantage over susceptible biotypes after exposure to herbicide label rates (Davis et al., 2009).

1.4. Management of *Conyza bonariensis* (L.) Cronquist

C. bonariensis is one of the most difficult weeds to control in the sub-tropical grain cropping regions of Australia and therefore threatens sustainability of farming practices. No single control tactic is completely effective on *C. bonariensis*, and successful control can only be achieved by using a combination of management tools as part of an integrated weed management (IWM) strategy (Widderick et al. 2012).

1.4.1. Controlling *Conyza* species with herbicides

C. bonariensis plants tolerate high levels of herbicide applications due to high trichome density, high cuticle thickness in the adaxial side and low stomatal density of the adaxial side (Procopio et al 2013). Plant age plays an important factor in the ability to control *C. bonariensis* using herbicides; most consistent results have been achieved when treated prior to the 14 leaf stage, once at the rosette stage, regrowth often occurs following post emergent herbicides (Shrestha et al., 2008a). When herbicide rates were not adjusted for plant age, efficacy was reduced only by an

average of 1% when two month old weeds were treated compared to one month old weeds. However, when applied to three month old weeds, efficacy of treatments was significantly ($P < 0.001$) reduced by 3 to 30% (Walker et al. 2012).

The glyphosate response of both GR and GS *C. bonariensis* populations was dependant on phenological stages with the rate required for control increasing as a function of plant age. GS population was controlled by 560 and 1,120 g ha⁻¹ at the seedling and rosette stage, respectively. At the tillering and flowering stages, complete control was obtained by 2,240 g ha⁻¹. This data can explain some glyphosate failures in GS populations observed under field conditions. For the GR population, 4,480 g ha⁻¹ provided complete control at the seedling stage, but the rate did not provide any control of plants at the rosette or later growth stages (Urbano et al., 2007).

Experiments conducted on six *Conyza* populations found a linear correlation ($R^2 = 0.92-0.98$) between plant age and glyphosate damage. Sensitivity to glyphosate reduced with age for both *C. canadensis* and *C. bonariensis* (Kleinman et al. 2016). Compounding this problem, later timed herbicides in Australian summer fallows are often applied when plants are under stress, due to the hot, dry conditions, which results in closed stomata and reduced systemic activity, further reducing the efficacy of products applied (Ruiter and Meinen 1998).

Extensive research has been carried out to find effective herbicide control options for *C. bonariensis* in fallows both internationally and in Australia. Herbicides that have been found to control *C. bonariensis* in California include aminopyralid, atrazine, bromacil, chlorsulfuron, cycloate, hexazinone, isoxaben, metam, prometryn, pyrazon, rimsulfuron and simazine pre-emergence and aminopyralid, dicamba, diquat, glufosinate, glyphosate (if plants are susceptible), MCPA, paraquat, triclopyr, 2,4-D and 2,4-DB post-emergence (Shrestha et al., 2008a). The addition of amitrole, clopyralid, flazasulfuron, fluroxypyr, glufosinate or MCPA to glyphosate improved control in perennial crops in Spain (Sansom et al. 2013). In Brazil best control was provided by using a double knock

technique, which is sequential applications of herbicides from different mode of action groups. Best results were gained from glyphosate plus 2,4-D or chlorimuron-ethyl followed by paraquat plus diuron (Lamego et al. 2013).

Application of a single herbicide, even knockdown herbicides such as glyphosate, paraquat and diquat, do not provide adequate control of *C. bonariensis* in Australia, therefore combinations using different modes of action are required (Wu et al. 2008). Currently the most widely adopted control strategy employed over the summer fallow period in Australia's northern region is the double knock technique. The most common double knock strategy is glyphosate plus 2,4-D as the first application followed up by paraquat or paraquat and diquat seven days later. Other effective double knock strategies include glyphosate plus 2,4-D and picloram followed by paraquat plus diquat, glyphosate plus 2,4-D followed by amitrole plus paraquat or 2,4-D followed by paraquat plus diquat (Widderick et al. 2012). Earlier studies also highlighted tank mixes of glyphosate plus 2,4-D and picloram or 2,4-D ester plus amitrole + ammonium thiocyanate as providing good control of *C. bonariensis* (Wu et al. 2008). Residual herbicides that provided good long-term control of *C. bonariensis* include atrazine or atrazine plus metolachlor (Wu et al. 2008). Trials in 2009 showed that glyphosate plus 2,4-D and picloram followed by paraquat plus diquat with either atrazine or isoxaflutole provided the best control up to six months after application (Widderick et al. 2012).

Field experimentation investigating pre-plant options for control of GR *C. canadensis* in the USA found that spring applied saflufenacil at 100 g a.i. ha⁻¹ provided greater than 90% control for twelve weeks. Early spring applied saflufenacil at 50 g a.i. ha⁻¹ provided eight weeks of greater than 90% residual control, and early spring-applied simazine provided six weeks of greater than 90% control. When applied in late spring, saflufenacil was the only herbicide treatment that reduced *C. canadensis* densities by greater than 90% compared to 2,4-D + glyphosate (Davis et al., 2010). Eubank et al. (2008) found that glyphosate at 0.86 kg ae ha⁻¹ provided 60 to 65% control of GR *C. canadensis* four weeks after treatment and that increasing the dose to 1.25 kg ha⁻¹ increased control

to 73 to 74%. Glyphosate at 0.86 kg ha⁻¹ plus 0.84 kg ae ha⁻¹ 2,4-D at or dicamba at 0.28 ae ha⁻¹ provided ≥90% control. Paraquat alone at 0.84 kg a.i. ha⁻¹ ranged from 55 to 63% and control did not improve by increasing the rate to 0.98 kg ha⁻¹. Addition of 2,4-D or dicamba to paraquat maximized control both years (78 to 89%).

A study investigating residual herbicides to be applied pre-plant for cotton in the US found fluometuron, oxyfluorfen, and norflurazon were the most consistent herbicides evaluated, providing at least 80% residual control of GR *C. canadensis* through 8 weeks after treatment. Glufosinate alone applied in early and late March (8 and 10 weeks before cotton sowing) usually provided less than complete control, resulting in *C. canadensis* regrowth and subsequent seedling emergence. Addition of dicamba in a tank-mix with glufosinate generally improved *C. canadensis* control in addition to providing some residual suppression of further emergence. *C. canadensis* did not emerge over a 10- to 12-week period in plots treated with glufosinate plus dicamba plus flumioxazin (Norsworthy et al., 2009).

Glufosinate applied alone at 0.47 kg a.i. ha⁻¹ resulted in at least 88% control of *C. canadensis* and maximized soybean yield. Saflufenacil alone or in combination with glyphosate was effective against paraquat-resistant biotypes of *C. bonariensis* (Bajwa, 2016).

In experiments evaluating herbicide control of *C. bonariensis* in wheat and sorghum, a pre-plant application of chlorsulfuron at 15 g a.i. ha⁻¹ in wheat controlled *C. bonariensis* ≥90%. The efficacy of early post-emergent applications of metsulfuron–methyl at 4.2 g a.i. ha⁻¹ was variable at 57% in 2004 and 98% in 2005. Control at 85% was provided by metsulfuron–methyl at 4.2 g a.i. ha⁻¹ plus MCPA at 420 g ae ha⁻¹ plus picloram at 26 g ae ha⁻¹, or metsulfuron–methyl followed by late post-emergent 2,4-D amine at 300 g ae ha⁻¹. In sorghum, *C. bonariensis* reduced sorghum yield 65 to 98% if not controlled. A pre-plant application of glyphosate at 900 g ae ha⁻¹ plus 2,4-D amine at 900 g ae ha⁻¹ or dicamba at 500 g ae ha⁻¹ at one month prior to sorghum planting provided ≥95% control. Pre-

plant atrazine at 2,000 g a.i. ha⁻¹ controlled *C. bonariensis* 83 to 100% in sorghum. At planting atrazine at 2,000 or 1,000 g a.i. ha⁻¹ can be applied to control new emergence of *C. bonariensis* and grasses, depending on the weed pressure and spectrum (Wu et al., 2010)

Herbicide control options for mature *C. bonariensis* plants in lucerne pastures showed there are limited double knock treatments that gave good results. These included 2,4-DB ± flumetsulam (or ± a PSII inhibitor) followed by paraquat ± atrazine (or simazine). Many of these herbicides caused significant crop damage (Wu and Koetz 2012).

1.4.2. Non-chemical management tools for *Conyza bonariensis* (L.) Cronquist

Maintenance of diversity in weed management systems is critical for avoiding herbicide resistance evolution. Diversity will vary according to region, ecosystem, enterprises, economics and many other factors but include rotation of crops and herbicides, herbicide combinations for different modes of action at robust rates and the use of non-herbicide weed control tools (Powles, 2008).

The increased incidence of glyphosate-resistant and difficult to control weeds in the sub-tropical grain region of Australia have led industry to consider applying strategic tillage in zero till systems for improved weed control. A field trial was established to assess the impact of different types of strategic tillage on emergence of *C. bonariensis* and conducted on a vertisol in southern Darling Downs, Queensland. Across four weed emergence flushes, cumulative emergence density in zero till was 696 *C. bonariensis* plants m⁻² and tillage reduced emergence by 91 to 99% for implements including harrow, gyral, offset discs and one-way discs (McLean et al., 2012). In row cultivation equipment such as the how-plow, in-row roto-tiller, spring hoe weeders and berm rakes provided effective control in certain orchard and vineyard systems. To achieve maximum efficiency, it was important to use these implements while *C. bonariensis* plants were small (Shretha et al., 2008a).

Crop competition is a valuable integrated weed management tool. Field experimentation in southern Queensland found that on average, *C. bonariensis* density decreased by 28% as wheat

population increased from 50 to 100 plants m⁻² and by 44% as row spacing decreased from 50 to 25 cm. Seed production was reduced from an average 1250 seed heads plant⁻¹ in wheat grown at 50 cm row spacing and density of 75 plants m⁻² to 120 seed heads plant⁻¹ at 25 cm row spacing and 100 plants m⁻² (Widderick et al., 2012). *C. bonariensis* populations are larger in areas maintained as fallow than in areas planted with wheat or oats during the winter. Wheat and oats were found to exert a suppressive effect on *C. bonariensis*, providing greater ease of control with herbicides before seeding (Paula et al., 2011).

Grazing is an integral part of weed management strategy on many farms, and some growers use sheep to improve fallow weed control, whereas others use weeds as an alternative feed when other sources are scarce over the summer (Felton et al. 1994). *C. bonariensis* is innocuous to stock although it imparts a taint to milk reducing milk quality (Wu 2007). Feed testing on *C. bonariensis* plants showed them to contain 8.3 MJ kg⁻¹ DM metabolisable energy, 8.7% water soluble carbohydrates and 12-17% crude protein (Wu and Koetz 2012). Feed testing carried out as part of an experiment investigating the possibility of partial substitution of barley grain and soybean meal with *C. bonariensis* in the diet of lambs showed 89.6% organic matter, 15% crude protein, 62% neutral detergent fiber (NDF), 38% acid detergent fiber (ADF) and 10% lignin. The dry matter intake and feed conversion ratio was significantly impacted by the inclusion of 5%, 10% and 15% *C. bonariensis* in lamb diets, at the higher levels of inclusion feed intake was reduced but they had the highest conversion ratios (6.6 and 6.7 kg feed / kg body weight gain). For most of the parameters tested by the study, results for lambs fed *C. bonariensis* were similar or better than in control lambs (Abo Omar and Omar 2012).

At the flowering stage *C. bonariensis* plants have an average dry weight of 90.3 g per plant and a leaf to stem dry weight ratio of 1:1. The plant has a re-sprouting characteristic where approximately 4 to 6 buds at the top of the tap root enables regeneration after top removal. Therefore, 45 days after top removal at the flowering stage plants regrew and produced 67 g per plant of dry

matter (Wu 2007). This re-growth ability means that grazing does not provide control, but instead slows the development of *C. bonariensis*. (Wu 2007). Experiments investigating the impact of herbivory of *C. bonariensis* showed that increased herbivory decreased final biomass. Net fecundity of *C. bonariensis* also decreased due to herbivory and the severity of herbivory impacted on reproductive effort (Prieur-Richard et al. 2002). As seed production is the sole propagation method of *C. bonariensis* (Wu 2007), defoliation strategies that prevent or reduce seed set could potentially be a useful integrated weed management tool in mixed farming situations.

Limited research has been conducted on the use of defoliation in combination with herbicides as an IWM strategy and results from previous studies have varied. Shrestha et al., (2008a) stated that mowing stimulated branching and hardened plants making control with herbicides more difficult. However, de Vargas Pereira et al. (2016) found that herbicide treatments including glufosinate, diquat, bentazon and glyphosate+ saflufenacil were more efficient at controlling *C. bonariensis* when applied to regrowth than to pre-flowering plants.

This project is focused in northeast Victoria where no-till and reduced tillage cropping and mixed livestock-cropping farming is common. State-wide statistics for Victoria showed there were 2567 mixed farming businesses (running a mix of crops plus sheep and/or cattle) and 2513 cropping only businesses in 2007 (Australian Bureau of Statistics, 2008a). Riverine Plains, a farmer grower group focused on crop production carried out a survey of its members across north east Victoria and southern New South Wales in 2012, despite this survey being targeted at producers interested in crop production, 65% of farmers had livestock on their farms (Fiona Hart, Riverine Plains Inc, personal communication). Finding management solutions for *C. bonariensis* that integrate with the livestock component of businesses is important for the sustainability of mixed farming systems. There is currently little understanding of management systems that will allow the defoliation of fallows. Investigating management strategies that include defoliation will provide a greater diversity of options to farmers not just in the northeast of Victoria, but in all mixed farming regions.

1.4.3. Control of *Conyza Bonariensis* (L.) Cronquist on Channel Banks

Irrigation channel banks are an important source of weed infestation (Charles 1991) and controlling *C. bonariensis* in these areas is critical for reducing seed dispersal through wind and water. There has been limited research conducted to date on weed control on channel banks with respect to management of *C. bonariensis*. Herbicide mixes applied to channel banks in New South Wales cotton regions in 1989 were mainly atrazine, diuron and chlorsulfuron sprayed in combination after the last irrigation and glyphosate and dicamba used when weeds escaped residuals (Charles 1991). Currently registered herbicide active ingredients for use on channel banks include amitrole, amitrole + ammonium thiocyanate, diuron, fluometuron, glyphosate, imazapyr + glyphosate, pendimethalin, propyzamide and simazine (Australian Pesticides and Veterinary Medicines Authority 2017).

In 2015 a third of agricultural businesses in Australia were irrigating with 43% of all water being delivered via channels or pipelines (Australian Bureau of Statistics 2016). This highlights the vast network of irrigation channels and the importance of effectively managing weeds in these areas. Investigating management strategies that address these problems will provide a greater diversity of options not just in the northeast of Victoria, but in all mixed farming regions.

1.5. Research Questions

Currently, limited information is available regarding the herbicide resistance status of *C. bonariensis* in Victoria, Australia. Previous surveys have focused on Western Australia, Queensland, New South Wales and South Australia (Owen et al., 2009; Walker et al., 2011; Cook, 2013; Preston, 2014) but not northeast Victoria where it is becoming an increasingly important management issue. An initial objective of this research project is therefore to determine the level of herbicide resistance in *C. bonariensis* populations to EPSPS inhibitors, ALS inhibitors, synthetic auxins and PSI inhibitors across northeast Victoria.

Glyphosate resistance has been confirmed both globally and in other Australian states (Heap, 2017). Research has been conducted into the glyphosate resistance mechanisms present in *C. bonariensis* in Brazil, California, Greece, Israel and Spain (Dinelli et al., 2008; Ferreira et al., 2008; Mylonas et al., 2014; Kaspary et al., 2016; Kleinman and Rubin, 2017; Moretti and Hanson, 2017); however, no studies have investigated the glyphosate resistance mechanisms present in populations of Australian *C. bonariensis*. This research project aims to determine the glyphosate resistance mechanisms present in GR *C. bonariensis* populations identified in northeast Victoria.

Extensive research has been conducted into herbicide weed control options for *C. bonariensis*. The combination of defoliation and herbicide strategies to control *C. bonariensis* in grazed fallows has been relatively unexplored to date, therefore this study will investigate both defoliation and herbicide double-knock strategies for management.

Channel banks are an important source of weed infestation (Charles, 1991) and little work has been done to investigate control strategies in this area. The final focus of this research project is to determine what cost-effective options exist to successfully manage *C. bonariensis* on channel banks.

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Chapter 2: Multiple-resistance to EPSPS and ALS inhibitors in northeast Victorian populations of *Conyza bonariensis* (L.) Cronquist

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Abstract

Conyza bonariensis (L.) Cronquist is a difficult to control summer annual or biennial weed species in the cropping belt of southeastern Australia. Resistance to the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) inhibitor glyphosate has been identified in several populations in Queensland, New South Wales and South Australia. However, resistance has not yet been identified in Victoria. A survey was therefore conducted in the agricultural region of northeast Victoria to evaluate the prevalence of resistance to selected herbicides from diverse chemical families in populations of *C. bonariensis*. Of the populations surveyed, 40% proved resistant to glyphosate at a rate of 1080 g a.e. ha⁻¹, there was no correlation found between predominant land use and glyphosate-resistance. The acetolactate synthase (ALS) inhibitor chlorsulfuron failed to control any of the surveyed populations. Further research identified multiple herbicide resistance to glyphosate and ALS inhibitors chlorsulfuron, metsulfuron-methyl and sulfometuron-methyl in five of nine populations fully characterised. Resistance was not found to plant cell growth disruptors (synthetic auxins) 2,4-D and clopyralid or to the photosystem I (PSI) inhibitor paraquat. The high frequency of resistance to EPSPS and ALS inhibitors could explain the difficulty experienced in control of *C. bonariensis* across northeast Victoria and the findings demonstrate the importance of Integrated Weed Management (IWM) in decreasing the risk of herbicide resistance development.

2.1. Introduction

Herbicides have played an important role in agricultural weed control since the discovery of MCPA and 2,4-D in the 1940s (Kudsk and Streibig, 2003). Herbicide usage continues to increase worldwide (Ewald and Aebischer, 2000) and in the United States has increased from 21,772 tonnes of active ingredients in 1964 to 172,365 tonnes in 2010 (Osteen and Fernandez-Cornejo, 2013). Herbicide use in Australia has doubled from 12,337 tonnes of active ingredient in 1990 to 24,789 tonnes active ingredient in 2006 (FAOSTAT, 2015) and in 2006-07 agricultural businesses in Australia spent nearly \$1 billion on herbicides (Australian Bureau of Statistics, 2008).

Herbicide resistance is a global phenomenon with 479 reported unique cases affecting 251 weed species (146 dicot and 105 monocots), 23 of the 26 modes of action, 163 different herbicides and 91 crops in 69 countries (Heap, 2017). Herbicide resistance is often the result of repeated use of herbicides with little or no diversity in weed management practices (Preston, 2000; Beckie, 2011). Resistance is typically driven by selection pressure with repeated herbicide use causing a shift from a few resistant individuals in an otherwise susceptible population to one dominated by resistant individuals. Herbicide resistance occurs more readily in some herbicide families (classified by modes of action) and in some weed species than others. Development of herbicide resistance is dependent on the level of selection pressure, the initial frequency of resistance alleles within a population, the inheritance and fitness characteristics of resistance alleles and the biology of the weed species (Preston, 2000).

Conyza bonariensis (L.) Cronquist is a member of the Asteraceae family believed to have originated in South America (Wu, 2007). It is a global weed occurring in more than 40 crops in 70 countries (Zambrano-Navea et al., 2013). *C. bonariensis* is one of the most common summer annual or biennial weeds in Australia's northern grain growing region (Cook, 2013) and recently has become a problem weed throughout the cropping belt of southeastern Australia (Wu and Zhu, 2014).

Globally there have been 19 unique cases of herbicide-resistant *C. bonariensis* reported in 13 countries to nine active ingredients. These include the 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) inhibitor glyphosate; photosystem I (PSI) electron diverters paraquat and diquat; photosystem II (PSII) inhibitors atrazine and simazine; and acetolactate synthase (ALS) inhibitors chlorsulfuron, pyriproxyfen-sodium, sulfometuron-methyl and imazapyr (Matzrafi et al., 2015, Heap, 2017). There has also been multiple-resistance reported to glyphosate and paraquat in *C. bonariensis* biotypes from California (Moretti et al., 2013) and to PSII inhibitors and ALS inhibitors in Israel (Matzrafi et al., 2015).

The majority of reported cases of herbicide resistance in *C. bonariensis* are to the EPSPS inhibitor glyphosate. The first case was confirmed in South Africa in 2003 (Heap, 2017) and more have been found in Spain (Urbano et al., 2007; Dinelli et al., 2008), Brazil (Moreira, et al. 2007), USA (Shrestha et al., 2008), Greece (Travlos and Chachalis, 2010), Israel, China (Powles, 2008), Colombia and Portugal (Heap, 2017). In Australia, glyphosate-resistant *C. bonariensis* has been found in Queensland, New South Wales and South Australia (Walker et al., 2011; Cook, 2013; Preston, 2016). However, surveys conducted in Western Australia did not detect any resistant populations (Owen et al., 2009) and a national survey that included Victoria did not find resistance in the 14 samples collected across the state (Malone et al., 2012). Currently listed cases of glyphosate-resistant *C. bonariensis* on the national register include 38 from New South Wales, 13 from Queensland and 7 from South Australia (Preston, 2016)

In Australia, there are currently 26 active ingredients approved for the control of *C. bonariensis* (Australian Pesticides and Veterinary Medicines Authority, 2016); however, the modes of action commonly used in the management of weeds over the summer fallow period in the southern region include ALS inhibitors, synthetic auxins, PSI inhibitors and EPSPS inhibitors. Herbicide resistance has been confirmed to ALS inhibitors, EPSPS inhibitors, PSI inhibitors and photosystem II (PSII) inhibitors in *C. bonariensis* populations globally (Heap, 2017). In Australia *C. bonariensis* populations there are

confirmed cases of resistance to glyphosate and paraquat (Heap, 2017). A survey across the northern cropping region of Australia of fleabane seeds from surviving plants in chemical fallow did not find resistance to the synthetic auxin 2,4-D (Cook, 2013).

Cases of glyphosate-resistant *C. bonariensis* recorded on the national glyphosate register show the majority of cases were found in non-cropped areas (34 from roadsides, 10 from irrigation channels, 3 from railways and 1 from around buildings). There have been 16 cases observed in chemical fallows and one in a vineyard (Preston, 2016). The correlation between dominant land use activity and glyphosate resistance has not been investigated. The high seed production of *C. bonariensis*; estimated at 119,100 seeds per plant (Wu et al., 2007), and its wind-borne dispersal with low settling velocities of $0.2911\text{m}\cdot\text{sec}^{-1}$ (Andersen, 1992), support its ability to move from location to location. *C. bonariensis* can successfully colonise up to 1842 m from the parent population with up to 5% of seed landing more than 100 m away (Borger et al., 2010). These findings suggest that intensive glyphosate use on lands adjacent to agricultural fields may result in colonisation by glyphosate-resistant *C. bonariensis*.

Northeast Victoria is an agricultural region producing high value commodities including fruit and dairy. Other significant industries include grain cropping, meat production, tomatoes and grapes (Department of Environment and Primary Industry 2014a, Department of Environment and Primary Industry 2014b). Despite extensive weed surveys carried out in Australia, none have focused on northeast Victoria where *C. bonariensis* is becoming an increasingly problematic management issue over the summer. The diversity of farming systems present across this region makes it an ideal location to determine if dominant land use is a driver of herbicide resistance in a wind dispersed weed species like *C. bonariensis*. This study aimed to determine if resistance to commonly used herbicides including EPSPS inhibitors, ALS inhibitors, synthetic auxins or PSI inhibitors exists in northeast Victoria and to determine if there is a correlation between dominant land use and herbicide resistance in this species.

2.2. Materials and Methods

2.2.1. Physical Survey

Sample sites were randomly selected from *C. bonariensis* infested areas greater than 5km apart, geo-referenced and a description of the area including prominent land use types was recorded. Seeds were sampled from between 1 and 10 individual plants at a sample location and bulked. A total of 89 populations were collected in late April to early May 2014. Populations were either classified as occurring in areas dominated by dryland grain and pasture production (DL) or irrigated grain, pasture, orchards and vineyards (IR) (Table 2).

Table 2: Distribution of sample populations by predominant land use, dryland grain and pasture (DL), irrigated grain, pasture orchards and vineyards (IR) and sample location

Location Type	Dryland (DL)	Irrigated (IR)
Roadside	35	29
Irrigation channels	0	17
In field	2	2
Fence line / fire break	3	--
Railway line	1	--

2.2.2. Screening for resistance to EPSPS inhibitors

The populations sampled in the survey were screened for glyphosate resistance in environmentally controlled glasshouses at Charles Sturt University (CSU) in Wagga Wagga, NSW (GPS coordinates -35.053429, 147.350875). Seed was sown into six 5.5 litre pots filled with a garden loam mix containing two parts loam to one part sand, pots were then covered with 2 mm of sand. Pots were initially placed in a glasshouse with a minimum temperature setting of 15°C and a maximum of

30°C. Once well established (2 to 4 leaves), pots were moved to an outdoor netted area and plants were thinned to 10 per pot. Pots were hand watered daily throughout the experiment and fertiliser (Miracle Gro® All Purpose soluble 24 N – 8 P – 16 K) applied as required. Glyphosate treatments were applied as populations reached the 4 to 8 leaf stage. Treated pots were sprayed with a twin nozzle cabinet sprayer with 110-011 even nozzles travelling at 5 km hr⁻¹ 50 cm above the pots. Glyphosate (Roundup Powermax, Monsanto) was applied at 1080 g a.e. ha⁻¹ in 84.1 L ha⁻¹ water. Populations were assessed for survival three weeks post treatment and classed as resistant if more than 20% survived. Survival was determined by visual assessment three weeks after treatment application. Plants were classified as dead if the growing points were dead and majority of shoot tissue was browned or necrotic.

2.2.3. Dose Response to EPSPS inhibitors

Of the seed collected, three susceptible (GS) and 6 resistant (GR) populations were randomly selected for further characterization by dose response; DL3 (GR), DL4 (GS), DL13 (GR), DL19 (GR), IR5 (GR), IR7 (GS), IR10 (GR), IR11 (GS), and IR14 (GR). An initial dose response experiment was conducted in an environmentally controlled glasshouse at CSU. Seeds were germinated in trays containing potting mix (RichGro® All Purpose) and 10 plants were transplanted into 5.5 L pots filled with a 2 parts loam to 1 part sand. Glasshouse temperatures were set to a minimum of 15°C and a maximum of 30°C, pots were watered by hand daily and fertiliser applied as required. Pots were arranged on glasshouse benches in a randomized complete block design. Treatments were applied at 6 to 8 leaf stage using a twin nozzle cabinet sprayer with 110-011 even nozzles travelling at 5 km hr⁻¹ 50 cm above the pots. Water volume was 84.1 L ha⁻¹.

Susceptible populations were treated with glyphosate at 0, 135, 270, 540, 810, 1080, 1620 and 2160 g a.i. ha⁻¹, while resistant populations received doses of 0, 540, 1080, 1620, 2160, 4320,

6480 and 8640 g a.i. ha⁻¹. Treatments were replicated four times. Survival was assessed at 21 days after glyphosate application by visual assessment.

The results from this initial experiment demonstrated there was survival up to 2160 g a.i. ha⁻¹ for resistant populations and 540 g a.i. ha⁻¹ in susceptible populations (data not presented).

Glyphosate rates for further dose response experiments were therefore reduced to 0, 67.5, 135, 202.5, 270, 540, 675 and 810 g a.i. ha⁻¹ for susceptible populations and 0, 270, 540, 810, 1080, 1620, 2160 and 4320 g a.i. ha⁻¹ for resistant populations.

The second and third dose response experiments were conducted at two locations including the CSU site at Wagga Wagga and the University of Melbourne, Dookie Campus (Dookie), Victoria, GPS coordinates -36.384038, 145.707053. The methodology was the same as for the initial dose response with modifications. Pot size was decreased to 2L, plants per pot reduced to six and replicates increased to six. At Dookie, materials were locally sourced; soil used was a basic lawn sand purchased from A1 Landscaping in Shepparton and fertiliser was Thrive® All Purpose Water Soluble Fertiliser (25 N – 5 P – 8.8 K). The glasshouse was set to the same temperature parameters as CSU and glyphosate treatments were applied in 83.5 L ha⁻¹ water using a battery operated knapsack sprayer fitted with 110-015VP nozzles travelling at 6 km h⁻¹ at 50 cm above the pots.

2.2.4. Screening for resistance to ALS inhibitors, synthetic auxins and PSI inhibitors

A total of 71 populations from the original 2014 survey were established in potting mix in the glasshouses at Dookie Campus. Six plants per pot were transplanted into 15 x 2 L pots containing the same locally sourced basic lawn sand and placed in an outdoor area at Dookie. Pots were watered by overhead sprinklers daily and fertilised as required. Five replicate pots were sprayed with 15 g a.i. ha⁻¹ chlorsulfuron, 875 g a.e. ha⁻¹ 2,4-D (Amicide 625, Nufarm Australia) or 400 g a.i. ha⁻¹ paraquat (Gramoxone, Syngenta) at the 4-8 leaf stage. Chlorsulfuron was applied to 71 populations, while both 2,4-D and paraquat treatments were applied to 60 populations as seedling numbers were limited by

poor germination. Treatments were applied using the knapsack spray application system described above. Populations were visually assessed for survival and any with survival of greater than 20% were classed as resistant. Plants were classified as dead if the growing points were necrotic.

Thirty pots representing the nine populations previously characterised by glyphosate dose response (DL3, DL4, DL13, DL19, IR5, IR7, IR10, IR11, and IR14) were transplanted and grown out as part of a larger screening experiment. Five replicates of each of these populations were treated with 3 g a.i. ha⁻¹ metsulfuron-methyl (Ally, DuPont), 600 g a.i. ha⁻¹ clopyralid (Lontrel, Dow AgroSciences), 15 g a.i. ha⁻¹ chlorsulfuron, 875 g a.e. ha⁻¹ 2,4-D or 400 g a.i. ha⁻¹ paraquat using the same knapsack sprayer as above at the 4-8 leaf stage. Five pots were left untreated.

A follow up ALS inhibitor screening experiment was conducted at CSU on the nine populations used for the glyphosate dose response described above. Plant growing conditions were as described for CSU EPSPS dose response experiments above. Five replicate pots of each population were sprayed with 12.75 g a.i. ha⁻¹ sulfometuron-methyl using the cabinet sprayer system described above. Sulfometuron-methyl was chosen to confirm ALS resistance as unlike chlorsulfuron, it is not readily metabolised by plants (Burnet et al., 1994; Owen et al., 2007). Populations were visually assessed for survival and classed as resistant if more than 20% survived.

2.2.5. Dose response to ALS inhibitors, synthetic auxins and PSI inhibitors

To determine if the survival observed in the screening experiment was a clear indicator of resistance, follow up dose response experiments were conducted at CSU. Experiment set up was as described above and growing conditions matched those for CSU EPSPS dose response experiments. Treatments were applied using the cabinet sprayer as described in 2.2. Survival was visually assessed five weeks after treatment application.

A metsulfuron-methyl dose response experiment was conducted with five replicates with populations DL3 and DL19 treated with 0, 0.75, 1.5, 2.25, 3, 4.5, 6 and 12 g a.i. ha⁻¹ of metsulfuron-

methyl and populations IR10 and DL4 treated with 0, 0.325, 0.75, 1.5, 2.25, 3, 4.5 and 6 g a.i. ha⁻¹. For the second experiment replicates were reduced to three due to poor seedling emergence and rates of metsulfuron-methyl applied to all four populations were 0, 2.25, 3, 4.5, 6 and 12 g a.i. ha⁻¹.

The initial 2,4-D experiment had five replicates and rates applied were 0, 218.75, 437.5, 656.25, 875, 1312.5, 1750 and 3500 g a.e. ha⁻¹ for populations IR10 and DL19 and 0, 109.38, 218.75, 437.5, 656.25, 875, 1312.5 and 1750 g a.e. ha⁻¹ for populations IR5 and DL4. In the second experiment replicates were reduced to three and rates applied to IR10 and DL19 were 0, 218.75, 437.5, 656.25, 875, 1312.5 g a.e. ha⁻¹ 2,4-D and IR5 and DL4 had 0, 109.38, 218.75, 437.5, 656.25, 875 g a.e. ha⁻¹ rates 2,4-D applied.

The chloryalid dose response experiment was performed with five replicates and rates included 0, 150, 300, 450, 600, 900, 1200 and 2400 g a.i. ha⁻¹ for populations IR5 and DL13. Populations DL4 and IR10 were treated with 0, 75, 150, 300, 450, 600, 900 and 1200 g a.i. ha⁻¹ chloryalid. A follow up experiment with three replicates and IR5 and DL13 were treated with 0, 150, 300, 450, 600 and 900 g a.i. ha⁻¹ clopyralid and DL4 and IR10 with 0 75, 150, 300, 450 and 600 g a.i. ha⁻¹

Five replicate pots of populations IR14 and DL19 were treated with 0, 100, 200, 300, 400, 600 and 800 g a.i. ha⁻¹ paraquat and populations IR10 and DL4 were treated with 0, 50, 100, 300, 400 and 600 g a.i. ha⁻¹. No follow up experiment was conducted as there was 100% mortality in all populations at all doses greater than 100g ha⁻¹ paraquat in this experiment and the populations were clearly susceptible to this herbicide.

2.2.6. Data Analysis

For all screening experiments percentage survival was calculated for each individual pot. The average of all pots for each population was then used to determine resistance.

Chi-square analysis was conducted in GraphPad Prism 7.02 (GraphPad Software, San Diego, CA, USA) on location data to determine if there was any difference in the frequency of glyphosate-resistant populations between dryland, irrigated cropping and horticulture/viticultural areas or between roadside and samples collected from other locations.

For glyphosate dose response experiments, plant number per pot prior to treatment and plant number 21 days after treatment were used to determine percentage survival. Data were analysed using 2-way ANOVA in GraphPad Prism 7.02 to determine if there was any statistical difference between glyphosate dose response experiments at CSU and Dookie. Although there was no significant difference between the two experiments there was an interaction between treatment and experiment so these experiments have been analysed separately. Survival data was used to calculate ED₅₀ values with 95% confidence intervals as well as dose response curves using the Finney Equivalent method in PriProbit (Sakuma, M., 1998).

For ALS inhibitor, synthetic auxin and PSI inhibitor dose response experiments, plant number per pot prior to treatment and plant number five weeks after treatment were used to determine percentage survival. For paraquat and chloryalid experiments, survival at the lowest doses applied was under 50% confirming susceptibility so no further analysis was conducted.

For metsulfuron-methyl and 2,4-D experiments there was sufficient survival for ED₅₀ values to be estimated. Percentage survival was calculated using plant numbers per pot counted prior to treatment application and those counted five weeks after treatment application. Two-way ANOVA was carried out in GraphPad Prism 7.02 to determine if there was any difference between the two dose response experiments for each herbicide. There was no significant difference between the results generated in metsulfuron-methyl experiments and data was thus combined. There was a significant difference in one population in the 2,4-D experiments so these experiments have been analysed separately. ED₅₀ was calculated in GraphPad Prism 7.02 using non-linear regression, inhibitor vs normalised response.

2.3. Results and Discussion

2.3.1. Resistance to EPSPS inhibitors

Populations collected during the physical survey in 2014 were classed as glyphosate-resistant (GR) if more than 20% of plants survived 1080 g ha⁻¹ glyphosate. Of the 88 populations tested, 37 (42%) were resistant, a further 30 populations (34%) had some level of survival below the threshold used with only 21 (23%) populations completely susceptible. Similar results were found in central California where 27% of the 90 populations tested were classed as resistant, 52% as intermediate and only 21% susceptible to glyphosate (Shrestha et al., 2014).

Across the landscapes, 44% of DL populations and 40% of IR populations were resistant to glyphosate. Chi-squared analysis showed there was no significant difference in the frequency of resistance between the two land use types. Samples were also recorded as either from roadsides, channel banks, firebreaks and fence lines or cropped fields. Again, chi-square analysis showed no significant difference in the frequency of resistance based on sample location. This suggests that the incidence of glyphosate resistance in *C. bonariensis* is similar across the region regardless of dominant land use. The high mobility of *Conyza* seeds within the environment may explain the consistent levels of resistance found across the region when populations have been endemic for a number of years. An alternative explanation is that all of the land use types have glyphosate applied, albeit at different frequencies. Therefore, selection for glyphosate resistance in *C. bonariensis* may be occurring throughout the region. Up to one third of populations classed as susceptible contained some resistant individuals. As resistance is driven by selection pressure (Preston, 2000), continued heavy reliance on glyphosate within the region may further increase in the already high levels of resistance found.

Results from the glyphosate dose response experiments showed differences in resistance indices between the populations. In both experiments IR7 was found to be the most susceptible biotype. In the experiment based at CSU the ED₅₀ of IR7 was 228 g a.i. ha⁻¹ and at Dookie it was 201

g a.i. ha⁻¹. The most resistant populations in both experiments were IR5, IR10 and DL19 with ED₅₀ values well above 1,000 g a.i. ha⁻¹ (Table 3). ED₅₀ values tended to be similar between the two experiments with the exception of the higher ED₅₀ values recorded at Dookie for IR5 and IR10.

Table 3: Estimated ED_{50} values for glyphosate for *C. bonariensis* populations from two separate dose response experiments at Charles Sturt University (CSU in Wagga Wagga) on plants 62 days of age and at Dookie (Victoria) on plants at 64 days of age.

	CSU		Dookie	
	ED_{50}	Confidence Intervals	ED_{50}	Confidence Intervals
	(g a.i. ha ⁻¹)	(g a.i. ha ⁻¹)	(g a.i. ha ⁻¹)	(g a.i. ha ⁻¹)
DL3	545	220 to 822	480	418 to 540
DL4	355	318 to 395	303	274 to 338
DL13	606	526 to 683	566	477 to 651
DL19	3165		3156	2455 to 4684
IR5	1359	871 to 2265	5916	2876 to 351837
IR11	384	279 to 525	284	256 to 317
IR14	608	467 to 742	457	291 to 599
IR7	228	158 to 321	201	178 to 227
IR10	1541	1267 to 1935	3670	

In both experiments, some of the populations showed large variation as noted by the large confidence intervals for the calculated ED_{50} values (Table 3). This could be a result of the seed collection methodology, seed from multiple plants in the same location was collected as a bulk sample. The high mobility of *C. bonariensis* seed could mean that populations from a single collection site may have been generated from different parent populations.

Results from glyphosate dose response experiments show a gradient of resistance, with Gr DL3, DL13 and IR14 having lower levels of resistance than GR IR5, IR10 and DL19. This suggests that there may be differential expression of resistance or varying mechanisms present across the region resulting in the range of ED₅₀ values observed. Potential glyphosate resistance mechanisms are numerous and now exceed those currently described for any other herbicide (Sammons and Gaines, 2014). Mechanisms of glyphosate resistance previously identified in *Conyza* spp. include reduced translocation (Feng et al., 2004; Dinelli et al., 2008; González-Torralva et al., 2014) and vacuolar sequestration (Ge et al., 2010); altered subcellular distribution patterns suggesting sequestration in the chloroplast (Kleinman and Rubin, 2017); increased expression of EPSPS mRNA (Dinelli et al., 2006; Dinelli et al., 2008); overexpression of ABC transporter genes (Nol et al., 2012); target site mutation (González-Torralva et al., 2014) and increased glyphosate metabolism to non-herbicidal forms (González-Torralva et al., 2012). In *Conyza* spp. there are many cases where multiple resistance mechanisms have been identified within an individual plant to confer higher level resistance (Dinelli et al., 2006; Dinelli et al., 2008; González-Torralva et al., 2012).

The widespread distribution of EPSPS inhibitor resistance demonstrated in the screening of populations collected in this survey is concerning as the lack of a fitness penalty associated with glyphosate resistance in *Conyza* spp. suggests resistance is likely to persist (Travlos and Chachalis, 2013; Shrestha et al., 2014).

2.3.2. Resistance to ALS inhibitors

Of the 71 populations screened with 15 g a.i. ha⁻¹ chlorsulfuron from northeast Victoria survey, all but one had greater than 65% survival. For the nine characterised populations screened with metsulfuron-methyl, four populations were susceptible, three populations had low level resistance (less than 50% survival) and two populations had survival >50% (Table 4).

To determine if the screening result for chlorsulfuron was due to ALS resistance, a follow up screening of the nine characterised populations with 12.75 g a.i. ha⁻¹ sulfometuron-methyl was conducted. Sulfometuron-methyl, unlike chlorsulfuron, is not readily metabolised by plants (Burnet et al., 1994; Owen et al., 2007) making it the ideal choice to determine if ALS target site resistance is likely. Results from screening with sulfometuron-methyl demonstrated that IR7 was the only susceptible population (Table 4). This confirmed that resistance to ALS-inhibiting herbicides was indeed widespread in *C. bonariensis* across northeast Victoria and that herbicide options available to control this weed are currently limited.

Table 4: Percentage survival for nine populations under glasshouse conditions selected for characterisation from a northeast Victoria field survey to labelled rates of chlorsulfuron, metulfuron-methyl, sulfometuron-methyl, 2,4-D, paraquat and, clopyralid. Treatment application 93 days after planting.

Herbicide	% Survival					
	Chlorsulfuron	Metsulfuron- methyl	Sulfometuron- methyl	2,4-D	Paraquat	Clopyralid
Rate (g a.i. ha ⁻¹)	15	3	12.75	875	400	600
DL13	89.7	30.6	41.7	3.3	0	60.7
DL19	86.7	70	95.8	24.1	7.1	0
DL3	87.5	75.3	90	3.2	0	0
DL4	77.8	0	52.5	0	3.3	0
IR11	100	31.1	100	2.9	0	53.1
IR14	87.5	12.5	A*	25.6	20	0
IR5	100	47.78	100	0	0	90
IR10	97.1	2.8	100	11.5	8	0
IR7	92.9	8.3	2.86	0	0	0

*NA – not available

Two dose response experiments were conducted with metsulfuron-methyl to determine the level of resistance present in four populations. Results from the two-way ANOVA conducted on these experiments showed there was no significant treatment by experiment interaction, therefore data was

combined for analysis. The labelled rate in fallow for metsulfuron-methyl is 3 to 4.2 g a.i. ha⁻¹ (DuPont 2009). DL4 is the only population characterised that was classed as susceptible with an ED₅₀ of 1.178 g a.i. ha⁻¹. DL3, IR10 and DL19 were resistant with high levels of survival at the highest rate (12 g a.i. ha⁻¹) included in the experiments and calculated ED₅₀ values greater than 20 g a.i. ha⁻¹ (Table 5).

Table 5: Response to metsulfuron-methyl of four C. bonariensis populations as ED₅₀ with 95% confidence intervals. Treatments applied at 54 days of age in first experiment and 65 in the second. Data from two experiments did not differ significantly and were combined.

Population	ED ₅₀ (g a.i. ha ⁻¹)	Confidence Intervals (g a.i. ha ⁻¹)
DL3	20.82	12.26 to 43.68
DL4	1.178	0.8427 to 1.612
DL19	44.14	31.42 to 68.78
IR10	35.66	23.77 to 64.74

In *C. bonariensis* ALS resistance has been documented in Israel to chlorsulfuron (Heap 2017), pyriithiobac-sodium, sulfometuron-methyl and imazapyr (Matzrafi et al., 2015). ALS resistance has also been documented in *C. canadensis* (Zheng et al., 2011; Heap, 2017), *C. albida* (Osuna and De Prado, 2003) and *C. sumatrensis* (Heap, 2017). Although the number of documented cases of ALS resistance in *Conyza* species is relatively low, it is logical to assume that there may be other unreported cases of ALS resistance in *C. bonariensis*, because the initial frequency of ALS mutations is generally high and this mode of action has been widely used over large areas, including Australia (Heap, 2014). These experiments clearly demonstrated a high level of sulfonylurea (SU) resistance in northeast Victoria.

Populations DL3, DL13, DL19, IR5 and IR10 were resistant to both glyphosate and all three ALS inhibitors tested. Two populations, IR10 and DL19, had high ED₅₀ values for both glyphosate and metsulfuron-methyl. This is the first example of multiple resistance to both EPSPS and ALS inhibitors reported in *C. bonariensis*, however, multiple resistance to EPSPS and ALS inhibitors has been reported in *C. canadensis* in Canada (Byker et al., 2013), Israel and America and in *C. sumatrensis* in Brazil (Heap, 2017). The detection of resistance present to two herbicide modes of action in the region suggests the chemical control options available are limited.

2.3.3. Synthetic auxin resistance

Of the 60 populations screened with 875 g a.e. ha⁻¹ 2,4-D, eight had survival between 20 and 30% and two of the nine characterised populations had survival between 20 and 30% following treatment with 875 g a.e. ha⁻¹ 2,4-D (Table 4).

Populations DL4, DL19, IR5 and IR10 were investigated in two dose response experiments. Two-way ANOVA found an interaction between experiment and treatment for population DL4, so two experiments have been analysed separately. The field rate of 2,4-D is 875 g a.e. ha⁻¹ (Nufarm Australia, 2011). All populations exhibited 0% survival at 875 g ha⁻¹ in both experiments and had low calculated ED₅₀ values, confirming that resistance was not present in these populations (Table 6).

Table 6: 2,4-D dose response experiments (1) and (2) conducted with populations of plants at 54 (1) and 65 (2) days of age. All populations were deemed susceptible to 2,4-D.

Population	ED ₅₀ (g ae ha ⁻¹)	Confidence Interval (g ae ha ⁻¹)
DL4 (1)	71.56	42.41 to 109.2
DL4 (2)	208.7	138 to 308.2
DL19 (1)	139.7	86.73 to 206.2
DL19 (2)	84.94	31.97 to 156.9
IR5 (1)	106.3	59.33 to 165.1
IR5 (2)	241.9	134.7 to 416.9
IR10 (1)	198.2	132 to 287.7
IR10 (2)	90.59	45.67 to 147.6

Three of the nine characterised populations showed potential resistance to 600 g a.e. ha⁻¹ clopyralid in the initial screen (Table 4). The two follow up dose response experiments with populations DL4, DL13, IR5 and IR10 resulted in 100% mortality at all chlopyralid doses applied down to 75 g a.i. ha⁻¹ (data not shown). This data strongly suggests that chlopyralid resistance is not present in the region, or that broader surveying is required to observe existing resistance within regional populations.

To date there have been no reported cases globally of synthetic auxin resistance in *C. bonariensis* (Heap, 2017), and a previous survey conducted in Australia's northern grains region also showed no resistance to 2,4-D (Cook, 2013). In the current study, synthetic auxin resistance was not

observed in northeast Victoria. However, herbicide resistance is driven by selection pressure and 2,4-D is a commonly used herbicide in fallow situations. Should there be continued intensive use of this mode of action, it is possible that selection pressure for resistance development in *C. bonariensis* populations may result in eventual resistance.

2.3.4. PS1 inhibitor resistance

Paraquat resistance in *C. bonariensis* has been confirmed in Egypt, Japan, South Africa and Australia and multiple resistance to glyphosate and paraquat in California, USA (Heap, 2017). Of the 60 populations screened with 400 g a.i. ha⁻¹ paraquat, only one had 20% survival and for the nine populations characterised for glyphosate resistance one population also showed 20% survival to paraquat at this rate (Table 4).

Four populations selected for dose responses studies had 100% mortality at dose rates of 200 g ha⁻¹ and above. Percentage mortality ranged from 20 to 60% at 100 g ha⁻¹. All populations were classed as susceptible as the labelled use rate in Australia ranges from 300 g ha⁻¹ to 600 g ha⁻¹ depending on time of the year and region of use (Syngenta Crop Protection, 2016). As *C. bonariensis* has developed paraquat resistance elsewhere in Australia (Heap, 2017), high selection pressure may potentially result in evolved herbicide resistance in northeast Victoria. IWM needs to be an integral part of managing *C. bonariensis* to reduce the risk of developing resistance to this important knock-down herbicide.

2.4. Conclusions

Of the randomly collected populations of *C. bonariensis* from northeast Victoria, only 23% of populations tested were completely susceptible to 1080 g ha⁻¹ glyphosate. Predominant land use, irrigated or dryland areas; or the collection location, roadsides, fields, irrigation channels or fence lines; did not influence the frequency of glyphosate resistance observed. In addition, widespread resistance to ALS inhibitor herbicides was observed, with chlorsulfuron failing to give adequate control

any population tested. Considerable resistance was also found to metsulfuron-methyl and sulfometuron-methyl in these populations. Multiple-resistance to glyphosate and all three ALS inhibitors occurred in five of the nine populations characterised with all herbicides. In contrast, no resistance was detected to the synthetic auxin herbicides 2,4-D and clopyralid or to the PSI inhibitor paraquat, suggesting that these particular herbicides could still be used for the effective control of this species.

The high frequency of resistance to EPSPS and ALS herbicides present in this weed could explain the recent difficulty experienced in its control in the region. Results from these comprehensive screening studies show that IWM is critical for effective management of *C. bonariensis* to reduce the risk of resistance to glyphosate, ALS inhibitors and potentially other herbicide mode of actions.

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Chapter 3: Exploring mechanisms of glyphosate resistance in *Conyza bonariensis* (L.) Cronquist populations from northeast Victoria

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Abstract

C. bonariensis (L.) Cronquist is a global weed and is a significant problem across Australia's grain growing regions. Nine populations collected as part of a resistance survey conducted across northeast Victoria showed varying levels of glyphosate resistance and have been characterised into three levels of resistance. Low level resistance (Gr) biotypes DL3, DL13 and IR14 have resistance Indices (RI's) between 2.3 and 2.8 and high level resistance (GR) biotypes DL19, IR5 and IR10 with RI's over 6, were compared with glyphosate susceptible (GS) biotypes DL4, IR7 and IR11. These populations exhibited differential accumulation of shikimate suggesting insensitive 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS). Genomic DNA sequencing identified both Pro106-Thr and Pro106-Ser mutations, with mutations occurring in all three resistance groups, suggesting that (an)other mechanism(s) must be contributing to resistance. Further research into expression of EPSPS and ABC transporter genes may shed more light on the mechanisms conferring resistance in *C. bonariensis* populations from northeast Victoria.

3.1. Introduction

Conyza bonariensis (L.) Cronquist is an annual or biennial member of the Asteraceae family, which is believed to have originated in South America (Wu, 2007). It is now a globally invasive weed

occurring in more than 40 crops in 70 countries (Zambrano-Navea et al., 2013). *C. bonariensis* is the most widespread weed in Australia's northern region (Cook, 2013) and has more recently become a problem in the cropping belt of southeast Australia (Wu and Zhu, 2014).

C. bonariensis is notoriously difficult to control and is one of the six most costly weeds to manage in fallow nationally and is ranked third most significant weed by southern grain growers in fallows by area as well as yield and revenue loss in subsequent crops (Llewellyn et al., 2016). *C. bonariensis* was the second most common weed species found in 63% of sample locations in a 2012 survey of the northern-region (Widderick et al., 2014). In the Goondiwindi region, Queensland, it has increased fallow weed control costs by 100% (Thorn, 2004).

Glyphosate is a unique broad-spectrum post emergence herbicide and the only herbicide that inhibits 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) (Bradshaw et al., 1997; Preston, 2000). EPSPS catalyses the sixth step in the seven step shikimic acid pathway where shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) react to produce 5-enolpyruvylshikimate 3-phosphate (EPSP) and inorganic phosphate (Herrmann and Weaver, 1999). Inhibition of EPSPS results in a reduction in the synthesis of the aromatic amino acids (tryptophan, tyrosine and phenylalanine) and the accumulation of shikimic and some hydroxybenzoic acids (de María et al., 2006).

Glyphosate resistance has been documented in 36 weed species, in 27 countries (Heap, 2017). Glyphosate-resistant (GR) *C. canadensis* has been reported in 13 countries including 25 different USA states and GR *C. sumatrensis* has been reported in 4 countries (Heap, 2017). GR *C. bonariensis* has been found in Spain, Brazil, the US, Greece, Israel, South Africa, Colombia, Portugal (Heap, 2017) and China (Powles, 2008). In Australia, surveying has identified glyphosate-resistant *C. bonariensis* in Queensland, New South Wales and South Australia (Walker et al., 2011; Cook, 2013; Preston, 2014).

Reduced translocation is the most common resistance mechanism of resistance to glyphosate in *Conyza* species. *C. canadensis* in the United States and Spain (Feng et al., 2004; Koger and Reddy, 2005; Dinelli et al., 2006; González-Torralva et al., 2012), *C. sumatrensis* in Spain (Gonzalez-Torralva et al., 2014) and *C. bonariensis* from Brazil, California and Spain (Dinelli et al., 2008; Ferreira et al., 2008; Moretti and Hanson, 2017) all displayed differential translocation of ¹⁴C glyphosate in resistant biotypes. Restriction of translocation as a result of sequestration of glyphosate in the vacuole was found to be the resistance mechanism in *C. canadensis* (Ge et al., 2010; Sammons and Gaines, 2014). *C. bonariensis* in Israel had altered subcellular distribution of glyphosate discovered through differential shikimate accumulation suggesting sequestration in the chloroplast. Translocation studies showed the standard source-to-sink pattern of glyphosate movement (Kleinman and Rubin, 2017).

There are two pathways for metabolism of glyphosate in plants which create aminomethylphosphonic acid (AMPA), glyoxylate and sarcosine, AMPA is further degraded into methylamine and then to formaldehyde (de Carvalho et al., 2012). Metabolism of glyphosate is rare having been reported in *Equisetum arvense* (Marshall et al., 1987) and *Digitaria insularis* (de Carvalho et al., 2012). GR *C. canadensis* from Spain was reported to have both reduced translocation and increased metabolism of glyphosate into AMPA, glyoxylate and sarcosine as the resistance mechanisms (González-Torralva et al., 2012).

Reduced translocation of glyphosate plus increased levels of EPSPS mRNA have been reported for GR *C. bonariensis* and *C. canadensis* (Dinelli et al., 2008). Decreased translocation, increased EPSPS synthase transcript levels and increased branching were also reported in GR *C. canadensis* from USA (Dinelli et al., 2006).

Over-expression of ABC transporter genes, which play a role in translocation, has been reported as the resistance mechanism in *C. canadensis* populations from the USA, Greece and Crete. (Nol et al., 2012). Synchronisation of ABC transporter genes (M10, M11, M7 and P3) and EPSPS

gene was found to correlate with 13-fold glyphosate resistance in a *C. canadensis* population from Greece (Tani et al., 2015; Tani et al., 2016).

Target site mutation is less common, having only been reported in *C. sumatrensis*. The GR biotype of this species had a proline to threonine substitution on EPSPS2 (Gonzalez-Torralva et al., 2014). Mylonas et al. (2014) and Kleinman and Rubin (2017) did not find Pro106 mutations in *C. bonariensis*.

A resistance survey conducted across northeast Victoria found 40% of the 89 populations collected were resistant to 1080 g a.e. ha⁻¹ glyphosate (Chapter 2: section 3.1). Nine of these populations were characterised by dose response and results identified three groups of resistance, glyphosate susceptible (GS) biotypes DL4, IR7 and IR11; Gr biotypes DL3, DL13 and IR14 which had low level resistance (Resistance Indexes (RI's) between 2.3 and 2.8); and GR biotypes DL19, IR5 and IR10 with high level resistance (RI's between 6 and 30). To date there have been no studies identifying the resistance mechanisms present in Australian GR *C. bonariensis* populations. This chapter aims to determine the resistance mechanisms that may be associated with observed levels of glyphosate resistance found in northeast Victoria.

3.2. Materials and Methods

3.2.1. Seed source

Seeds were collected from self-pollinated plants grown out from the first dose response experiments (as detailed in Chapter 2). Seed for GS biotypes (DL4, IR7 and IR11) were taken from untreated controls; seed for Gr (DL3, DL13, IR14) and GR (DL19, IR5, IR10) populations were taken from populations surviving low dose glyphosate application of 540 g ae ha⁻¹.

3.2.2. Glyphosate Absorption and Translocation

Investigation of glyphosate absorption and translocation was based on the methods described by Wakelin et al. (2004) with some modifications. An initial experiment was conducted with *C.*

bonariensis biotypes listed above and sampling times of 24, 48 and 72 hours after treatment (HAT). This experiment showed that absorption and translocation had stabilised at 48 HAT so this timing was used for sampling in subsequent experiments.

Populations DL3, DL13, DL19, IR5, IR7, IR10 and IR11 (DL4 and IR14 had insufficient plants established to be included in experiment) were grown in potting mix in the growth rooms at the University of Adelaide, Waite Campus (GPS Coordinates: 34°58'5.78"S / 138°38'11.10"E) and 10 plants were transplanted at the 2-4 leaf stage into a black plastic container (26 x 19 x 9 cm) in a hydroponic medium consisting of Hoagland's nutrient solution and black polypropylene beads. At the 4-6 leaf stage plants received an application of 225 g a.e. ha⁻¹ glyphosate (Roundup PowerMax, Nufarm, Victoria, Australia) in 118 L ha⁻¹ water. This was followed within 15 minutes with a 1 µl droplet of ¹⁴C glyphosate (approximately 227 Bq, with 12.53 mM glyphosate) applied to the adaxial surface of a marked leaf.

At 48 HAT plants were harvested and roots, shoot and treated leaf separated. Treated leaves were gently shaken in 5 ml 0.1% Triton wash solution (Sigma-Aldrich, NSW, Australia) to remove unabsorbed ¹⁴C glyphosate and then removed. Wash solutions had 8 ml Ultima Gold XR scintillation solution (PerkinElmer, Waltham, MA) added for scintillation counting.

Each plant section (washed treated leaf, shoot and roots) were placed into a combusto-cone paper receptacle (PerkinElmer, Waltham, MA) and dried at room temperature for at least a week. These samples were combusted individually using a PerkinElmer Oxidizer 307, at a burn time of 30 seconds. The ¹⁴CO₂ released was trapped and counted in 14 ml of scintillation mixture containing Permafluor E+ / Carbosorb (1:1, V/V) (PerkinElmer, Shelton, CT).

Radioactivity of both the wash solutions and combusted plant samples was determined by liquid scintillation counting (LSC) using a tri-carb 2100TR Liquid Scintillation Analyzer (Packard Bioscience Company, Meriden, CT). Standards used were: S1- 1µl ¹⁴C glyphosate into Permafluor /

Carbosorb mix (empty burn), S2- empty cone combusted and S3 -1 μ l 14 C glyphosate into cone, combusted.

Efficiency of radioactivity recovery was calculated using the equation: $\text{efficiency} = 100 \times (S3 - S2) / (S1 - S2)$. This was then used to correct scintillation readings to account for efficiency using the equation: $\text{recovered radiation} = (\text{Scintillation reading} - S2) / (\text{efficiency}/100)$. Percentage absorption was then calculated from corrected readings using the equation: $\% \text{ absorption} = 100 - (100 \times \text{radiation wash} / \text{total radiation})$. Percentage radioactivity translocated to individual plant sections was calculated as: $\% \text{ translocation} = 100 \times \text{radioactivity in section} / \text{radioactivity absorbed}$.

The experiment was repeated. A two-way ANOVA was conducted in GraphPad Prism 7.02 (Graphpad Software, San Diego, CA, USA) to determine if there were significant differences between experiments one and two. This determined there was no difference between experiments, but that there was an interaction between experiment and treatment. As a result, the two experiments have been analysed separately. One-way ANOVA was conducted in GraphPad Prism 7.02 to determine if there were any differences between biotypes in absorption or translocation of glyphosate.

3.2.3. Shikimate assays

Shikimate assays were conducted using the method detailed in Shaner et al. (2005) with modifications. Nine populations (DL3, DL4, DL13, DL19, IR5, IR7, IR10, IR11 and IR14) were grown in a glasshouse at The University of Adelaide, Waite Campus. At the rosette stage, ten discs were cut from a single fully extended leaf and treated with one of five glyphosate rates. Two discs were placed into a single well of a 96-well microplate containing 200 μ l solution of 10 mM phosphate buffer (pH7) and glyphosate to the final concentrations of 0, 25, 50, 100, 200 μ M. There were five replicate leaves from 5 replicate plants for assays. The plates were then incubated at room temperature under lamps at 65 μ mol m $^{-2}$ s $^{-1}$ for 16 hours (Fluval LED A3981, Rolf C. Hagen Corp, Mansfield, MA, USA). After incubation 50 μ l 0.25 M HCl was added to each sample and then the samples were frozen at -20°C for 30 minutes. The frozen plates were then incubated at 60°C for 15 minutes.

An aliquot of 25 μl from each well was then transferred into fresh microplates and standards were created of 0, 1, 2.5, 5, 10, 25 and 50 μM shikimate acid. 100 μl of 0.25% periodic acid / 0.25% sodium metaperiodate solution was added. Samples were then incubated at room temperature for 1 hour and then 100 μl of a quench solution containing 0.6 M NaOH plus 0.22 M NaSO₃ was added to terminate the reaction. A total of 150 μL of each sample was taken to measure absorbance at 380 nm using a double-beam Cintra10 UV spectrometer (GBC Scientific Equipment, Braeside, Victoria, Australia).

The experiment was repeated twice, results were analysed by two-way ANOVA in GraphPad Prism 7.02 and as there was no significant difference between experiments the results of the two experiments were combined.

Standards were used to generate a standard curve and this was used to convert absorbance to concentration of shikimate using the equation: nmol shikimic acid cm^{-2} of leaf tissue = absorbance divided by slope -2.512×10 (volume) $\times 7.9577$ (area). The data was analysed using non-linear regression, one-phase association in GraphPad Prism 7.02.

3.2.4. Target Site Mutation

Genomic DNA (gDNA) was extracted from 5 replicate plants from the same 9 populations using an ISOLATE II Plant DNA kit (Bioline, Alexandria, NSW, Australia) in accordance with manufacturer instructions.

Polymerase chain reaction (PCR) amplification was conducted using a number of different kit and primer combinations, including MyFi (Bioline, Alexandria, NSW, Australia), DreamTaq, Platinum Taq and Phire PCR Kit (Thermo Fisher Scientific Australia Pty Ltd), in combinations four different primer sets including AW1 F (AACAGTGAGGAYGTYCACTACATGCT) and AW1 R (CGAACAGGAGGGCMTCACTGCCAAG), EPSPS F (GTTGCGGGACAAGCA) and EPSPS R (AGGGCAACCACAGCAA), cbEPSPS F (CAGTGAYGATGTTTATTACATGCT) and R (CTCTACGTCTCCAGTGAAA) and cbEPSPS2 F (GTTTATTACATGCTTGGAGCTTT) and R

(ATAGATCCTGACAATTTACCTTTC). The only combination resulting in bands was a 20 μ L PCR reaction containing 100ng DNA, 1X Phire Plant PCR Buffer, 0.5 μ M cbEPSPS-F primer, 0.5 μ M cbEPSPS-R primer, 0.4 μ L Phire Hot Start II DNA polymerase. The reaction was conducted in an Eppendorf Mastercycler Gradient automated DNA thermal cycler (Eppendorf, Hamburg, Germany). An annealing temperature of 58°C was selected following the results of a gradient PCR reaction, all other temperatures followed the manufacturer's recommended cycling protocol: 5 minute initial denaturation at 98°C, 40 cycles of 98°C denaturation, 58°C annealing and 72°C extension, followed by 1 minute final extension at 72°C. This experiment was replicated on the gDNA extracted from 4 individuals from each biotype.

PCR products were examined on 1.5% agarose gels stained with 1x SYBR Safe DNA gel stain (Life Technologies, Mulgrave, Victoria, Australia). Gels were electrophoresed in 1xTAE Buffer at 110V for 35 minutes and photographed under UV light. EasyLadder (Biolone, Alexandria, NSW, Australia) was used to compare sample DNA fragment sizes, with the fragment size amplified approximately 1000 bp.

Sanger sequencing of PCR product with cbEPSPS primers used for amplification was conducted by the Australian Genome Research Facility (AGRF) at Adelaide. Sequencing data was aligned using ClustalW and compared against the known EPSPS susceptible sequences for *Eleusine indica* using Geneious 8.13 (Biomatters Ltd, Auckland, NZ).

To confirm the mutations seen in sequencing of gDNA and to determine the frequency of codons at Pro106, PCR fragments from gDNA were cloned using the Topo TA cloning kit (Thermo Fisher Scientific Australia Pty Ltd). Prior to conducting the cloning reaction, 10 μ L PCR product was A-tailed with 1x buffer, 10mM deoxynucleotide (dNTP) solution, 25nM MgCl₂ and 5U Platinum Taq (Thermo Fisher Scientific Australia Pty Ltd) was incubated at 94°C for 2 minutes followed by 74°C for 30 minutes. A-tailed product, 2 μ L, was mixed with 1 μ L salt solution (1.3 M NaCl + 0.06 M MgCl₂

supplied in kit), 2 μL H_2O plus 1 μL of the TOPO Vector and incubated at room temperature for 5 minutes before being placed on ice. The cloning reaction was then transformed using the One Shot chemical transformation protocol where 2 μL of the cloning reaction was added to One Shot chemically competent *Escherichia coli* and incubated on ice for 30 minutes. Cells were heat-shocked for 30 seconds at 42°C and immediately transferred back to ice. S.O.C. medium, 250 μL , was added and the tubes were then shaken horizontally (200rpm) at 37°C for an hour. Samples of 100 μL and 150 μL from each transformation was spread onto Luria-Bertani (LB) plates containing $50 \mu\text{g mL}^{-1}$ kanamycin and incubated overnight at 37°C .

Individual colonies were then picked from the LB plates using a pipette tip, the entire tip was placed into 10 mL tubes containing 4 mL LB medium containing $50 \mu\text{g mL}^{-1}$ kanamycin. A total of 30 colonies were selected from each biotype. Tubes were shaken horizontally overnight at 37°C . Plasmid DNA was then isolated from *E. coli* cultures using an Isolate II Plasmid Mini Kit (Biolone, Alexandria, NSW, Australia) as per manufacturer's instructions.

Samples were tested for the presence of plasmid DNA (pDNA) using a FastDigest kit (Thermo Fisher Scientific Australia Pty Ltd). A 20 μL reaction containing 2 μL pDNA, 2 μL 10x FastDigest green buffer, 1 μL EcoRI enzyme and 15 μL H_2O was incubated at 37°C for 5 minutes. This was then run on 1.5% agarose gel as described above.

An 11 μL sample containing 4 μL pDNA with 1 μL M13 F (GTA AAA CGA CGG CCA G) or R (CAG GAA ACA GCT ATG AC) primers was sent to AGRF for Sanger sequencing. Sequencing data was aligned using ClustalW and compared against the known EPSPS susceptible sequences for *Eleusine indica* using Geneious 8.13.

Results from cloning experiments confirmed the mutations observed by sequencing of gDNA. As mutations were present in both resistant and susceptible biotypes, cDNA analysis was attempted to determine if these genes were being expressed.

RNA was extracted using three different protocols, the Qiagen RNeasy Mini Kit (Qiagen, Chadstone, Victoria, Australia), Isolate II RNA Mini Kit (Bioline, Alexandria, NSW, Australia) and lab based method using TRIzol. The Isolate II kit failed to yield any RNA. Both the Qiagen and the TRIzol method yielded RNA but had DNA contamination. This was removed from both using Ambion DNA-free kit (Thermo Fisher Scientific Australia Pty Ltd). cDNA was then synthesised using 2 different kits, the Tetro cDNA synthesis kit and the Superscript IV Reverse Transcriptase kit (Thermo Fisher Scientific Australia Pty Ltd).

cDNA from all four combinations was included a 20 µl PCR reaction containing 2 µL cDNA, 1X Phire Plant PCR Buffer, 0.5 µM cbEPSPS-F primer, 0.5 µM cbEPSPS-R primer, 0.4 µL Phire Hot Start II DNA polymerase. The reaction was conducted with an annealing temperature of 58°C, and subsequent protocol followed the manufacturer's recommended cycling protocol as described above.

PCR products examined on 1.5% agarose gels stained with 1x SYBR Safe DNA gel stain. Gels were electrophoresed in 1xTAE Buffer at 110V for 35 minutes and photographed under UV light. All four combinations failed to result in bands.

3.3. Results and Discussion

3.3.1. Absorption and Translocation of glyphosate

Absorption and translocation of ¹⁴C glyphosate was determined for two GS populations (IR7 and IR11), two Gr populations (DL3 and DL13) and three GR populations (DL19, IR5 and IR10). Translocation experiments were conducted twice and compared by two-way ANOVA in GraphPad Prism 7.02. Results from this analysis showed that although there was no significant difference between the two experiments, there was an interaction between experiment and treatment. The results from the two experiments were therefore analysed separately.

The amount of ¹⁴C glyphosate applied that was absorbed by plants ranged from 28 to 43% in experiment one and from 17% to 30% in experiment two. There was no significant difference in the

amount of absorption between biotypes (Table 7). Absorption totals were lower than documented in *C. bonariensis* from California where absorption ranged between 52.9 and 58.3% (Moretti and Hanson, 2017) and could be as a result of the growing conditions.

In experiment a, the only population that showed a significant difference in translocation was Gr biotype DL3. There was a slight increase in ¹⁴C-glyphosate remaining in the treated leaf of DL3 (67%) in comparison to GS biotype IR11 (48%) and GR biotypes DL19, IR5 and IR10 (48%, 52% and 48% respectively) and slightly less ¹⁴C-glyphosate in the shoots of DL3 (7%) than GR biotypes DL19 and IR5 (21% and 18% respectively). There was however, no significant differences recorded between biotypes in experiment b (Table 7). Efficiency of radiation recovery based on standards was 78.7% for experiment a and 75.8% for experiment b.

Table 7: Absorption and translocation of glyphosate in experiments 1 and 2 in seven populations of *C. bonariensis* seedlings. Analysis conducted in GraphPad Prism 7.02.

		Experiment 1						
		DL3	DL13	DL19	IR5	IR7	IR10	IR11
Wash	Average %	27.64a	35.53a	31.02a	32.06a	39.19a	43.15a	31.87a
	applied							
Treated	Average %	68.95ab	56.43a	47.65a	51.73a	58.37a	52.13a	48.04a
Leaf	absorbed							
Shoot	Average %	7.12ab	12.16a	21.07a	17.7a	13.61a	16.22a	15.7a
	absorbed							
Root	Average %	23.3a	31.41a	31.28a	30.57a	28.03a	31.66a	36.26a
	absorbed							
		Experiment 2						
Wash	Average %	29.22a	16.92a	22.4a	23.74a	29.6a	27.78a	30.11a
	applied							
Treated	Average %	43.94a	59.98a	47.22a	61.81a	54.05a	38.7a	40.02a
Leaf	absorbed							
Shoot	Average %	34.62a	25.13a	38.9a	19.87a	32.26a	39.76a	35.52a
	absorbed							
Root	Average %	21.45a	15.89a	13.88a	18.32a	13.69a	21.54a	24.46a
	absorbed							

Reduced translocation was previously reported to confer glyphosate resistance in a number of species (Lorraine-Colwill et al., 2002; Preston and Wakelin 2008; Alcántara-de la Cruz et al., 2016; Alcántara-de la Cruz et al., 2016, Brunharo et al. 2016) and is a common mechanism conferring resistance in *C. bonariensis*. In Brazil and Spain, reduced translocation was observed in resistant plants (Dinelli et al., 2008; Ferreira et al., 2008), this mechanism was attributed to multiple resistance to glyphosate and paraquat found in California (Moretti and Hanson, 2017). These authors observed a significant reduction in the amount of ¹⁴C-glyphosate translocated out of the treated leaf.

In this study, there was only a small difference in the distribution of ¹⁴C-glyphosate in Gr biotype DL3 with slightly more glyphosate present in the treated leaf, slightly less in the shoot and the same amount reaching the root in experiment one. This difference was not observed in the second experiment, therefore glyphosate resistance in both the Gr and GR populations tested here is probably not due to restricted translocation, however, further experimentation may be required to confirm this.

3.3.2. Shikimate Assays

When glyphosate effectively inhibits EPSPS, shikimate, the dephosphorylated shikimate-3-phosphate found as the substrate of the enzyme, accumulates. Shikimate accumulation following glyphosate treatment has previously been used to identify GR soybean, cotton and weeds with resistance due to alteration of EPSPS (Shaner et al., 2005).

The nine biotypes used in the assays were grouped into three levels of resistance GS (DL4, IR7 and IR11), Gr (DL3, DL13 and IR14) with RI's between 2.3 and 2.8 and GR (DL19, IR5 and IR10) with RI's over 6 (Chapter 2). Shikimate accumulated in all nine biotypes, however, the rate constant (k) was significantly different between biotypes meaning that differential shikimate accumulation was occurring. High levels of shikimate accumulated in susceptible populations at low glyphosate dose rates; IR7, IR11 and DL4 accumulated 224, 198 and 118 $\mu\text{mol cm}^{-2}$ shikimate respectively at a dose rate of 25 μM glyphosate. All three susceptible populations plateaued at 100 μM glyphosate, accumulating between 280 and 290 $\mu\text{mol cm}^{-2}$ shikimate and at a 200 μM glyphosate GS populations

accumulated just over 300 $\mu\text{mol cm}^{-2}$ shikimate. The most resistant populations IR10, IR5 and DL19 accumulated less than 10 $\mu\text{mol cm}^{-2}$ shikimate at a glyphosate dose rate of 25 μM and only accumulating 109, 183 and 229 $\mu\text{mol cm}^{-2}$ shikimate respectively at a dose rate of 200 μM glyphosate (Figure 5).

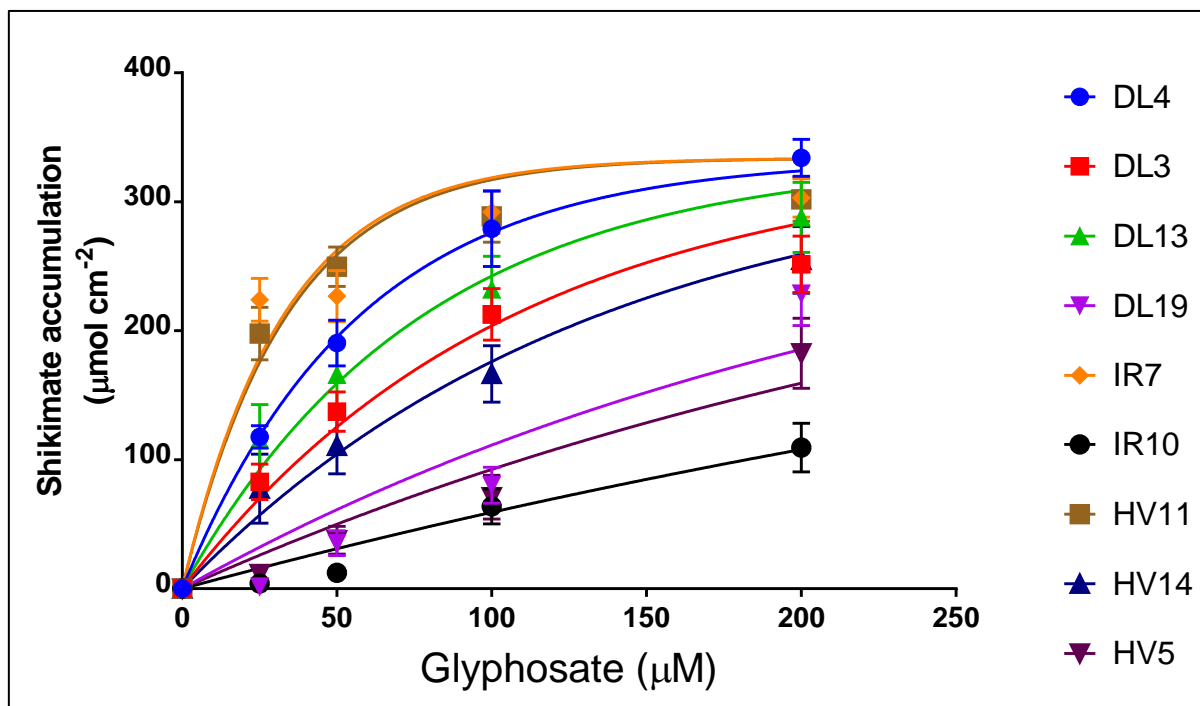


Figure 5: Shikimate accumulation ($\mu\text{mol cm}^{-2}$) in response to glyphosate treatment (μM) for nine *C. bonariensis* populations: populations DL4, IR7 and IR11 (GS); DL3, DL13 and IR14 (Gr); and DL19, HV5 and IR10 (GR) in seedlings. Model used: non-linear regression, one-phase association in GraphPad Prism 7.02.

Shikimate assays have been shown to differentiate GR and GS crops in which resistance is due to target site mutation (Shaner et al., 2005). Kaspary et al. (2016) found differential shikimate accumulation in *C. bonariensis* in Brazil but did not identify the resistance mechanism present. Koger et al. (2005) also found that shikimate accumulated in all biotypes of *C. canadensis* at high glyphosate concentrations, but at lower glyphosate concentrations the accumulation was less in GR biotypes than GS. The resistance in these populations was potentially a result of reduced translocation, not target site mutation, suggesting that shikimate assays are capable of detecting both target and non-target

site resistance. *C. canadensis* in which over-expression of ABC transporter genes was proposed to be the resistance mechanism conferring resistance also showed differential shikimate accumulation (Nol et al., 2012). As reduced translocation has not been observed in the current study, the resistance mechanism could potentially be a result of altered target site.

3.3.3. Target Site Mutations in EPSPS

Target site mutations at Pro-106 in EPSPS have been shown to confer resistance to glyphosate in 13 different weed species and substitutions at the Pro-106 codon include Pro to Ser, Ala, Thr or Leu (González-Torralva et al., 2014; Sammons and Gaines, 2014; Cross et al., 2015; Alcántara-de la Cruz et al., 2016; Bracamonte et al., 2016; Dominguez-Valenzuela et al., 2017; Ngo et al., 2017). EPSPS DNA fragments, ~1000 bp, from gDNA extracted from four individuals were sequenced for nine populations of *C. bonariensis*. This species is an allopolyploid (Thebaud and Abbott, 1995; Wu, 2007; Okada et al., 2015) and as a result there are multiple copies of the EPSPS gene. In the nine populations included in the experiment, one (HV11) failed to produce any reliable sequencing data. In the remaining eight there were Pro-106-Thr and Pro-106-Ser mutations present in six populations including in two of the GS biotypes. In the 3 most resistant populations (DL19, IR5 and IR10) both Thr and Ser substitutions were identified (Table 8).

Table 8: Sequencing data from gDNA at Pro-106 from eight *C. bonariensis* populations. As *C. bonariensis* is an allopolyploid, there were up to three peaks visible on the electropherogram at this position. These are presented below with the highest peak being listed first and the smallest peak listed last.

Population	Individual Number			
	1	2	3	4
DL3 (Gr)	Pro	Pro	Pro	Pro
DL4 (GS)	Pro	Pro	Pro	Thr
	Thr	Thr	Thr	Pro
DL13 (Gr)	Pro	Pro	Pro	Pro
DL19 (GR)	Pro	Pro	Ser	Thr
	Ser	Ser	Thr	Pro
	Thr	Thr	Pro	Ser
IR5 (GR)	Ser	Ser	Thr	Thr
	Pro	Thr	Ser	Pro
	Thr	Pro	Pro	Ser
IR7 (GS)	Pro	Pro	Thr	
	Thr	Thr	Pro	
IR10 (GR)	Ser	Pro	Thr	
	Pro	Ser	Pro	

	Thr	Thr	Ser	
IR14 (Gr)	Pro	Pro	Pro	Thr
	Thr	Thr	Thr	Pro

PCR fragments from gDNA were cloned and individually sequenced to determine the frequency of mutations at Pro106. The results from this experiment confirmed the mutations observed in the sequencing results from gDNA, with both Pro106-Thr and Pro106-Ser being found in the *C. bonariensis* populations from northeast Victoria. It again showed that mutations were present at all levels of resistance, including the susceptible populations. GS population IR11 and Gr population DL3 had no mutations present; GS IR7, Gr DL13 and IR14; and GR IR5 and IR10 all had Pro106-Thr mutations. Both GS biotype DL4 and GR DL19 had both Pro106-Thr and Pro106-Ser mutations (Table 9).

Table 9: Number of each variant of Pro 106 codon identified in clones of PCR products for the EPSPS gene in *C. bonariensis* populations.

Population	Pro (CCA)	Thr (ACA)	Ser (TCA)	TOTAL
DL3 (Gr)	19	0	0	19
DL4 (GS)	15	2	5	22
DL13 (Gr)	9	9	0	18
DL19 (GR)	7	11	10	28
IR5 (GR)	17	8	5	25
IR7 (GS)	11	8	0	19
IR10 (GR)	16	5	0	21
IR11 (GS)	18	0	0	18
IR14 (Gr)	15	13	0	28

Target site mutations are rare in *Conyza* spp. having thus far only been detected in *C. sumatrensis* (González-Torralva et al., 2014), comparison between the R and S proteins showed an amino acid substitution at position 182 (amino acid number based on the start codon (ATG) of the *A. thaliana* EPSPS sequence) consisting of a proline to threonine conversion in the R-biotype. Discovery of mutations at Pro-106 in *C. bonariensis* in this study, however, does not fully explain the resistance present as mutations at Pro-106 were found in all classifications of resistance: GS, Gr and GR. This suggests that the resistance in this case could be due to differential gene expression or to a combination of target site mutations and some other as yet unknown mechanism(s).

As *C. bonariensis* is a hexaploid species (Wu, 2007) gene dose could be having an influence on the resistance seen in biotypes investigated in this study. Investigation of hexaploid *Avena fatua* found that a single ACCase mutation provided relatively low-level resistance to the ACCase herbicides, but resistance levels were significantly increased where individual plants accumulated multiple ACCase mutations. Based on diclofop I₅₀ R/S ratios, individuals with two mutations had higher resistance than those with a single mutation (Yu et al., 2013). Although gene dose could be contributing to the resistance displayed by *C. bonariensis* populations included in this study it does not fully explain the resistance present. Populations with both Pro106 and the Pro106-Thr mutation included Gr DL13 and IR14 which had approximately 50:50 ratio between Pro106 and Pro106-Thr, GS population IR7 had a 60:40; GR population IR5 had an 70:30; and GR IR10 a 75:25 Pro:Thr ratio. If gene dose was the sole mechanism at play one would expect a higher proportion of Pro106-Thr in GR and Gr biotypes than in the GS biotype, but these populations do not follow that trend.

Reduced translocation has also been ruled out as the cause of resistance in *C. bonariensis* populations collected in northeast Victoria. Results from shikimate assays suggested reduced sensitivity of EPSPS and sequencing of both genomic and plasmid DNA has confirmed mutations at Pro106 that are known to confer resistance. As these mutations are occurring in both resistant and susceptible populations (an)other mechanism(s) must be contributing to the resistance found.

Over-expression of various genes has been reported as a resistance mechanism in *Conyza* spp. In *C. canadensis* over-expression of EPSPS mRNA has been found in GR populations from Spain and the USA (Dinelli et al., 2006) and in *C. bonariensis* (Dinelli et al., 2008). Over-expression of ABC transporter genes in *C. canadensis* (Nol et al., 2012) and also in *C. canadensis* synchronisation of ABC transporter and EPSPS were reported to confer resistance (Tani et al., 2015; Tani et al., 2016). Over expression of EPSPS or ABC transporter genes were not examined in this study; however, this would be a fruitful area for further research into resistance mechanisms in *C. bonariensis* populations from northeast Victoria.

3.4. Conclusion

From the herbicide resistance survey and supporting dose response experimentation discussed in Chapter 2, three levels of resistance to glyphosate were identified in *C. bonariensis* from north-east Victoria; glyphosate susceptible (GS) biotypes DL4, IR7 and IR11; Gr biotypes DL3, DL13 and IR14 which had low level resistance (RI between 2.3 and 2.8); and GR biotypes DL19, IR5 and IR10 with high level resistance (RI's over 6). Although translocation is a common glyphosate resistance mechanism present in *Conyza* spp., this was not identified as conferring resistance in any of these nine populations. The biotypes displayed differential accumulation of shikimate suggesting insensitive EPSPS may be involved in the resistance. Sequencing of both genomic DNA and individual sequence clones identified both Pro106-Thr and Pro106-Ser mutations. These mutations occurred all three groups of resistance, suggesting that some of these populations of *C. bonariensis* carry target site mutations which only confer low levels of resistance. The presence of these mutations and the effects of gene dose does not fully explain the different levels of resistance among the populations, so (an)other mechanism(s) must be contributing. Further work is needed to confirm the mechanism conferring resistance in these populations

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Chapter 4: Control of *Conyza bonariensis* (L.) Cronquist in mixed farming systems

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Abstract

Conyza bonariensis (L.) Cronquist is a global weed. It is one of the most widespread weeds in Australia's northern grains region and has more recently become a significant in the cropping belt of south eastern Australia. A successful ruderal invader, it is common in fallows and on irrigation channel banks. Field trials conducted at Oaklands and Goolgowi, New South Wales evaluated herbicide strategies for controlling *C. bonariensis* using products currently approved for use on irrigation channel banks in Australia. Treatments included both tank mixes and double knocks using approved herbicides glyphosate, amitrole + ammonium thiocyanate, diquat, diuron and saflufenacil, however none provided effective control on channel banks. Given the inability to gain successful control of this weed on channel banks there is continued risk of colonisation of *C. bonariensis* into surrounding lands.

Grazing is an important component of fallow management in southern Australia, however little is known about its potential as a strategic tool for managing *C. bonariensis* as part of an Integrated Weed Management (IWM) program. Field experiments were conducted at Dookie, Victoria and Goolgowi, New South Wales to investigate control options in grazed fallows. Double knocks that included defoliation and herbicide combinations using glyphosate, amitrole + ammonium thiocyanate, saflufenacil, MCPA, 2,4-D, paraquat + diquat, glufosinate, MCPA + dicamba, and / or clopyraid were

tested. Results from both experiments have shown that treatments providing the best level of control were paraquat + diquat (432 + 368 g a.i. ha⁻¹) applied 5-10 days after defoliation providing 98% control at both sites and MCPA + dicamba (1,768 + 416 g a.e. ha⁻¹) applied 8-9 days prior to defoliation providing 100% control.

4.1. Introduction

Conyza bonariensis (L.) Cronquist is an annual or biannual member of the Asteraceae family (Wu, 2007) and is a global weed occurring in more than 40 crops in 70 countries (Zambrano-Navea et al., 2013). *C. bonariensis* is the most widespread weed in Australia's northern region (Cook, 2013) and has recently become a problem weed throughout the cropping belt of south-eastern Australia (Wu and Zhu, 2014).

Conyza spp. are successful ruderal invaders (Alpen et al., 2014) infesting irrigation channels and fallows (Wu, 2007). Glyphosate-resistant *Conyza* infests hundreds of km of glyphosate-treated irrigation channel banks in California's central valley (Powles, 2008). A survey conducted in northeast Victoria included 18 populations collected from irrigation channel banks across the region; of these 39% were resistant to glyphosate (Chapter 2). Channel banks are a source of weed infestation (Charles 1991) and *C. bonariensis* has the ability to colonise up to 1842 m from the parent plant via wind dispersal (Borger et al., 2010). Irrigation channel banks are elevated in the landscape offering the potential for increasing the already high dispersal ability of this species. A survey of weed seeds in irrigation water in the Yakima Valley and Columbia River, Washington found 15.2 *C. canadensis* seeds with a germination percentage of 80% per 254 kL of water (Kelley and Bruns, 1975). This demonstrates the potential significance of irrigation water as a source of weed infestation and the importance of effectively managing weeds in this area.

There is a vast network of irrigation channels across northeast Victoria. In 2015 a third of agricultural businesses were irrigating, with 43% of all irrigation water being delivered via irrigation

channels or pipelines (Australian Bureau of Statistics, 2016). Due to the risk of infesting surrounding areas it is important to effectively manage weeds in these areas. Investigating management strategies that provide effective control of *C. bonariensis* will help to reduce the risk of further spreading herbicide-resistant seed into nearby fields and across neighbouring regions. This paper aims to investigate herbicide options to control *C. bonariensis* on irrigation channel banks.

C. bonariensis is a serious weed in fallows. In the Goondiwindi region of Queensland, the recent increase in populations of *C. bonariensis* in fallows has increased fallow weed control costs by 100% (Thorn, 2004). *Conyza* spp., including *C. bonariensis* are ranked third most significant weed in fallows by area, yield loss and revenue by grain producers in Australia's southern growing region, accounting for 73,177 tonnes of yield loss and \$17.5 million in lost revenue (Llewellyn et al., 2016).

Herbicides are the primary method of weed control in no-tillage farming systems (Locke et al., 2002). There have been a number of studies focusing on the chemical control of *C. bonariensis*. Synthetic auxins, photosystem I (PSI) inhibitors, photosystem II (PSII) inhibitors, ALS inhibitors and cellulose biosynthesis inhibitors were modes of action found to provide control of *C. bonariensis* in California (Shrestha et al., 2008). Tank mixing amitrole, clopyralid, flazasulfuron, fluoxypyr, glufosinate or MCPA with glyphosate improved control in Spain (Sansom et al., 2013). In Brazil control was provided by an application glyphosate plus 2,4-D or chlorimuron-ethyl followed by a sequential application of paraquat plus diuron (Lamego et al., 2013).

Experimentation in Australia has shown applications of a single herbicide do not provide adequate control of *C. bonariensis* and combinations using different modes of action are required to achieve good control (Wu et al., 2008). Studies have highlighted tank mixes of glyphosate plus 2,4-D + picloram or 2,4-D ester plus amitrole + ammonium thiocyanate as providing good control of *C. bonariensis* (Wu et al., 2008). Good long-term control can be achieved with the residual herbicides atrazine or an atrazine plus metolachlor (Wu et al., 2008). Trials in 2009 showed that glyphosate plus

2,4-D + picloram followed by paraquat + diquat plus either atrazine or isoxaflutole provided the best control up to six months after application (Widderick et al., 2012).

The double-knock technique (sequential applications of different weed management techniques, usually herbicides with different modes of action) has been a widely adopted control strategy in summer fallows across the northern region. The most common double-knock is glyphosate plus 2,4-D as the first application followed by paraquat or paraquat + diquat 7 days later. Other effective double-knock strategies include glyphosate plus 2,4-D + picloram followed by paraquat + diquat, glyphosate plus 2,4-D followed by amitrole + paraquat or 2,4-D followed by paraquat + diquat (Widderick et al., 2012).

Integrated weed management (IWM) and ensuring diversity of weed management strategies is key to the long-term sustainability of herbicides (Powles, 2008). *C. bonariensis* is difficult to manage with variable control delivered by herbicides (Wu et al., 2008). No single control strategy is completely effective on *C. bonariensis* and successful control is only achieved by using a combination of management tools as part of an IWM strategy (Widderick et al., 2012).

Although the term double-knock is generally applied to sequential herbicide applications from different mode of action groups, it can also include cultivation or non-herbicide tactics (Widderick et al., 2012). Despite this extensive work into herbicide management of *C. bonariensis*, so far, no studies have been conducted looking at defoliation as part of an IWM system or as one of the practices in a double-knock strategy.

Grazing is an integral part of weed management on many farms in southern Australia (Felton et al., 1994). Some growers use sheep to improve fallow weed control, whereas others use weeds as an alternative source of sheep feed when other sources are scarce over the summer. *C. bonariensis* is safe for stock to consume (Wu, 2007). A feed lot experiment investigating partial inclusion of *C. bonariensis* into lambs diets, showed that for most parameters tested lambs fed *C. bonariensis* had

results similar or better than control lambs fed a standard cereal – soybean meal ration (Abo Omar and Omar, 2012).

At the flowering stage *C. bonariensis* plants have an average dry weight of 90.3 g plant⁻¹. The plant has a re-sprouting characteristic where about 4 to 6 buds at the top of the tap root enable regeneration after top removal. At 45 days after top removal at the flowering stage, plants regrew and produced 67 g plant⁻¹ of dry matter (Wu, 2007).

The impact of grazing *C. bonariensis* on the ability to manage this weed is difficult to predict. Grazing does not kill *C. bonariensis*, but instead slows development (Wu, 2007). Experiments investigating the impact of herbivory of *C. bonariensis* showed net fecundity decreased due to herbivory and the severity of herbivory impacted on reproductive effort (Prieur-Richard et al., 2002), suggesting that although grazing does not control *C. bonariensis* it could be a valuable tool in reducing seed set.

There has been limited research conducted on the use of defoliation as part of an IWM program and the results in relation to *C. bonariensis* are conflicting. Shrestha et al. (2008) stated that mowing stimulated branching and hardened plants making control with herbicides more difficult. Employing grazing often delays herbicide applications past the optimum timing for *C. bonariensis* of seedling to rosette stage (Shrestha et al., 2008). *C. bonariensis* becomes harder to control with herbicides as the plant ages (Walker et al., 2012; Urbano et al., 2007). Conversely, de Vargas Pereira et al. (2016) found herbicides glufosinate, diquat, bentazon and glyphosate + saflufenacil provided greater control when applied to regrowth than to pre-flowering plants.

There is currently limited understanding of management systems that will allow the grazing of fallows without compromising *C. bonariensis* control. This paper therefore aims to investigate potential double knock dual herbicide applications that combine herbicide applications with defoliation to effectively manage *C. bonariensis* in grazed fallows.

4.2. Materials and Methods

4.2.1. Management on irrigation channel banks

To investigate the control options on channel banks replicated field trials were conducted in Oaklands, NSW (GPS coordinates: 35°22,56.61S 146°6,45.84E) in 2015/2016 and in Goolgowi, NSW (GPS coordinates: 34°01.724 / 145°34.955) in 2017. At Oaklands, plots were 3 x 5 m and treatments were replicated 3 times and in Goolgowi, plots were 3 x 8m and treatments replicated 4 times. Both trials were laid out in a randomized complete block design. Treatments (Table 10) were applied at late growth stages varying from stem elongation to flowering at Oaklands and from stem elongation to early bud formation at Goolgowi. Treatments were applied in a water volume of 83.5 l ha⁻¹ with a battery-operated knapsack sprayer fitted with 110-015VP nozzles travelling at 6km/hr at 50cm above the foliage. Three by 1m² *C. bonariensis* counts were conducted in marked quadrats in each plot prior to the first treatment and then again at the end of the trial.

Table 10: Treatment list for channel bank experiments conducted in Oaklands Victoria in 2015/2016 and Goolgowi Victoria in 2017

Active Ingredient	Application date	Application date
	Oaklands	Goolgowi
Untreated		
Glyphosate (1080 g a.e. ha ⁻¹)	18/12/2015	01/01/2017
Amitrole + Ammonium Thiocyanate (1,325 + 1,166 g a.i. ha ⁻¹)	18/12/2015	01/01/2017
Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha ⁻¹)		
Glyphosate (1,080 g a.e. ha ⁻¹)	18/12/2015	01/01/2017
Amitrole + Ammonium Thiocyanate (1,125 + 990 g a.i. ha ⁻¹)		
Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha ⁻¹)		
Diquat 200 (80 g a.i. ha ⁻¹)	18/12/2015	01/01/2017
Nonyl Phenol Etyhlene Oxide (102 g a.i. ha ⁻¹)		
Diuron (800 g a.i. ha ⁻¹)	18/12/2015	01/01/2017
Saflufenacil (23.8 g a.i. ha ⁻¹)	18/12/2015	01/01/2017
Glyphosate (264 g a.e. ha ⁻¹)		
Paraffin Oil (400 g a.i. ha ⁻¹)		
Glyphosate (1,080 g a.e. ha ⁻¹) f/b	18/12/2015	01/01/2017
Diquat (80 g a.i. ha ⁻¹)	24/12/2015	10/01/2017
Nonyl Phenol Etyhlene Oxide (102 g a.i. ha ⁻¹)		
Glyphosate (1,080 g a.e. ha ⁻¹)	18/12/2015	01/01/2017

Amitrole + Ammonium Thiocyanate (1,125 + 990 g a.i. ha⁻¹)

Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha⁻¹)

f/b

Diquat (80 g a.i. ha⁻¹)

24/12/2015

10/01/2017

Nonyl Phenol Etyhlene Oxide (102 g a.i. ha⁻¹)

4.2.2. Management in fallows

To investigate control options in fallows that include defoliation, a replicated trial was established at The University of Melbourne, Dookie Campus Victoria (GPS coordinates: -36.408400, 145.705246) in 2015. Plots were 3 x 15m and treatments (Table 11) were replicated 4 times and laid out in a randomized complete block design. Treatments were applied to naturally occurring *C. bonariensis* populations at later growth stages varying from stem elongation to early flowering using the sprayer system as discussed above.

As *C. bonariensis* numbers were low across the site, weed counts were conducted across the entire plot prior to and at 22 and 65 days after the first treatment. All *C. bonariensis* plants were cut by hand to 2 cm above ground height at the listed defoliation timings (Table 11). As there were other weeds present across the trial area, once the *C. bonariensis* defoliation cuts had been conducted the entire plot was mown. *C. bonariensis* was cut in all plots at the end of the experiment, 65 days after initial treatment again to 2 cm above ground level. All plants collected were dried in ovens at 65°C for 5 days to determine dry matter (g).

A second experiment was conducted in Goolgowi NSW (GPS coordinates: 34°01.724 / 145°34.955) in 2017. Plots were 3 x 8m, and treatments (Table 11) were replicated four times and laid

out in a randomised complete block design. The treatments were applied using the sprayer set up discussed above at development stages of stem elongation to early bud formation.

As the weed density was higher at this site, three 1 m² fixed *C. bonariensis* counts per plot were conducted at marked quadrats prior to the first treatment, and at 31 and 59 days after initial treatment. Dry matter cuts were taken from defoliation treatments at the same marked quadrats at listed defoliation timings (Table 11) and in all plots at the end of the experiment, 59 days after initial treatment. *C. bonariensis* plants were cut at 2 cm above ground level and oven dried at 65°C for 5 days to determine dry matter (g).

Table 11: Treatment list including herbicide and defoliation treatment application dates for experiments conducted at Dookie in 2015 and Goolgowi in 2017 (NA – treatment not applied)

Herbicide treatments and rates (g a.i. ha ⁻¹)	Dookie 2015		Goolgowi 2017	
	Herbicide	Defoliation	Herbicide	Defoliation
Untreated		Untreated		Untreated
Untreated		2/02/2015		31/12/2016
Untreated		2/02/2015		NA
Untreated		24/02/2015		NA
MCPA (700 g a.e. ha ⁻¹)	3/02/2015	11/02/2015	1/01/2017	10/01/2017
2,4-D (350 g a.e. ha ⁻¹)	3/02/2015	11/02/2015	1/01/2017	10/01/2017
Paraquat + Diquat (432 + 368 g a.i. ha ⁻¹)	7/02/2015	2/02/2015	10/01/2017	31/12/2016
Amitrole + Ammonium Thiocyanate (1,400 + 1,232 g a.i. ha ⁻¹)				
Glyphosate (658 g a.e. ha ⁻¹)	7/02/2015	2/02/2015	10/01/2017	31/12/2016
Soyal Phospholipids + Propionic Acid (75 + 75 g a.i. ha ⁻¹)				
Glufosinate (750 g a.i. ha ⁻¹)	7/02/2015	2/02/2015	10/01/2017	31/12/2016
MCPA + Dicamba (1,768 + 416 g a.e. ha ⁻¹)	3/02/2015	11/02/2015	1/01/2017	10/01/2017

2,4-D (1,000 g a.i. ha ⁻¹)				
Paraquat + Diquat (432 + 368 g a.i. ha ⁻¹)	7/02/2015	2/02/2015	10/01/2017	31/12/2016
Glyphosate (940 g a.i. ha ⁻¹)	7/02/2015	2/02/2015	10/01/2017	31/12/2016
Saflufenacil (23.8 g a.i. ha ⁻¹)				
Glyphosate (1080 g a.i. ha ⁻¹)	NA	NA	10/01/2017	31/12/2016
Paraffin Oil (400 g a.i. ha ⁻¹)				
Paraquat + Diquat (162 + 138 g a.i. ha ⁻¹)	7/02/2015	2/02/2015	10/01/2017	31/12/2016
Clopyralid (45 g a.i. ha ⁻¹)				
Glyphosate (658 g a.i. ha ⁻¹)	7/02/2015	2/02/2015	10/01/2017	31/12/2016
2,4-D (750 g a.e. ha ⁻¹)				
Amitrole + Ammonium Thiocyanate (1,400 + 1,232 g a.i. ha ⁻¹)	NA	NA	10/01/2017	31/12/2016
2,4-D (875 g a.e. ha ⁻¹)				

4.2.3. Data analysis

C. bonariensis plant numbers in the channel bank experiment conducted at Oaklands had a large amount of variation across the trial area. Quadrant counts for plant numbers were performed at the start and end of the trial and were used to calculate percentage control. This data was analysed by one-way ANOVA in GraphPad Prism 7.02 (Graphpad Software, San Diego, CA, USA) to determine if there was any difference between treatments.

As the plant numbers across the Goolgowi trial area were more consistent the final counts conducted 59 days after initial treatment were used for analysis. The count data was left

untransformed and compared using Fishers Protected Least Significant Difference (LSD) in GenStat (VSN International, Hemel Hempstead, UK).

For the fallow experiments two way ANOVA conducted in GraphPad prism 7.02 demonstrated there was an interaction between experiments at Dookie and Goolgowi and treatments, the trials were analysed separately. Plant counts and dry matter (DM) cuts carried out 65 days after initial treatment (DAT) were used in data analysis. Data collected at Dookie was transformed by square root for plant counts and by log for DM. Data collected at Goolgowi was transformed by square root for counts and DM data was left untransformed. Plant numbers and dry matter left at the end of the experiments where compared using Fishers LSD in GenStat.

4.3. Results and discussion

4.3.1. *Management on irrigation channel banks*

The site at Oaklands had a high degree of variability; populations varied from 5 – 54 plants m⁻² in counts conducted across the area prior to initial treatment. To manage this variability, counts were conducted in the same spot each time from marked sampling locations. Counts demonstrated a reduction in plant numbers at the end of the trial period across all treatments, with the exception of the untreated plot. There was however, no significant differences recorded in Oaklands due to the variation present within the trial (Table 12).

Table 12: Summary results from irrigation channel bank trial conducted at Oaklands Victoria.

Percentage control calculated using the counts done prior to treatment and those conducted at 65

DAT. Results from one-way ANOVA conducted in GraphPad Prism 7.02 showed no significant

differences between treatments.

Treatment	Percentage	
	Control	
Untreated	-0.97403	a
Glyphosate (1080 g a.e. ha ⁻¹)	6.451881	a
Amitrole + Ammonium Thiocyanate (1,325 + 1,166 g a.i. ha ⁻¹)		
Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha ⁻¹)	27.22222	a
Glyphosate (1,080 g a.i. ha ⁻¹)		
Amitrole + Ammonium Thiocyanate (1,125 + 990 g a.i. ha ⁻¹)		
Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha ⁻¹)	35.60618	a
Diquat 200 (80 g a.i. ha ⁻¹)		
Nonyl Phenol Eythlene Oxide (102 g a.i. ha ⁻¹)	31.57407	a
Diuron (800 g a.i. ha ⁻¹)	53.2381	a
Saflufenacil (23.8 g a.i. ha ⁻¹)		
Glyphosate (264 g a.e. ha ⁻¹)		
Paraffin Oil (400 g a.i. ha ⁻¹)	51.85185	a
Glyphosate (1,080 g a.e. ha ⁻¹) f/b	40.42553	a

Diquat (80 g a.i. ha⁻¹)

Nonyl Phenol Ethylene Oxide (102 g a.i. ha⁻¹)

Glyphosate (1,080 g a.e. ha⁻¹)

Amitrole + Ammonium Thiocyanate (1,125 + 990 g a.i. ha⁻¹)

Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha⁻¹) f/b

Diquat (80 g a.i. ha⁻¹)

Nonyl Phenol Ethylene Oxide (102 g a.i. ha⁻¹)

42.50441

a

At Goolgowi plant density was generally high with 32 plants m⁻² in the untreated control and herbicide treatments provided significant control. Amitrole + ammonium thiocyanate; 1080 g a.i. ha⁻¹ glyphosate with amitrole + ammonium thiocyanate; and 80 g a.i. ha⁻¹ Diquat provided the highest levels of control at 63 to 71% (Table 13). Although the control provided by these treatments is significant, it is far from providing an adequate solution to managing *C. bonariensis* on irrigation channel banks.

Table 13: Plants m⁻² and percentage control (compared to untreated control) at Goolgowi Victoria 59 days after initial treatment application. Analysed using Fishers Protected LSD in GenStat.

Active Ingredient	Plants m ⁻²		% Control
Untreated	32.42	c	0
Glyphosate (1080 g a.e. ha ⁻¹)	12.58	ab	61.2
Amitrole + Ammonium Thiocyanate (1,325 + 1,166 g a.i. ha ⁻¹)	9.33	a	71.2
Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha ⁻¹)			
Glyphosate (1,080 g a.e. ha ⁻¹)	11.75	a	63.8
Amitrole + Ammonium Thiocyanate (1,125 + 990 g a.i. ha ⁻¹)			
Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha ⁻¹)			
Diquat 200 (80 g a.i. ha ⁻¹)	12	a	63
Nonyl Phenol Etyhlene Oxide (102 g a.i. ha ⁻¹)			
Diuron (800 g a.i. ha ⁻¹)	14.83	ab	54.3
Saflufenacil (23.8 g a.i. ha ⁻¹)	21.08	abc	35
Glyphosate (264 g a.e. ha ⁻¹)			
Paraffin Oil (400 g a.i. ha ⁻¹)			
Glyphosate (1,080 g a.e. ha ⁻¹) f/b	14.08	ab	56.6
Diquat (80 g a.i. ha ⁻¹)			
Nonyl Phenol Etyhlene Oxide (102 g a.i. ha ⁻¹)			
Glyphosate (1,080 g a.e. ha ⁻¹)	25.67	bc	20.8
Amitrole + Ammonium Thiocyanate (1,125 + 990 g a.i. ha ⁻¹)			
Soyal Phospholipids + Propionic Acid (30 + 30 g a.i. ha ⁻¹) f/b			
Diquat (80 g a.i. ha ⁻¹)			
Nonyl Phenol Etyhlene Oxide (102 g a.i. ha ⁻¹)			

Effective weed control on irrigation channel banks is vital as they provide a source of infestation. The elevated sides of channel banks above the surrounding fields gives *C. bonariensis* plants an increased likelihood of updraughts and cross winds that influence seed release and therefore increases the potential dispersal distance (Borger et al., 2012). Irrigation water within the channels transports seed providing a source of potential infestation (Kelley and Bruns, 1975; Charles, 1991).

Herbicide mixes applied to channel banks in New South Wales cotton regions in 1989 were mainly atrazine, diuron and chlorsulfuron, applied in combination after the last irrigation and glyphosate and dicamba used when weeds escaped the early residual herbicides (Charles, 1991). Currently registered herbicide active ingredients for use on irrigation channel banks include amitrole, amitrole + ammonium thiocyanate, diuron, fluometuron, glyphosate, imazapyr + glyphosate, pendimethalin, propyzamide and simazine (Australian Pesticides and Veterinary Medicines Authority, 2017).

The herbicide strategies using products currently approved for use on irrigation channel banks was investigated in these experiments, results indicate that even when applied using the double knock technique effective control of *C. bonariensis* was not achieved. This poor control increases the likelihood of herbicide resistance evolution.

The favourable dispersal conditions from channel banks and the lack of good control options ensures an ongoing source of seed infestation restricting the ability to get on top of this weed in adjacent areas. Growers in 1989 reported using regular mechanical cultivation on their irrigation channels to manage weeds (Charles, 1991), however, this is an unfavourable option due to its negative impact on irrigation water quality.

4.3.2. Management in grazed fallows

At Dookie, there were 0.78 *C. bonariensis* plants m⁻². The defoliation treatments had no significant effect on plant numbers in this trial. Several herbicide treatments in combination with defoliation reduced plant numbers by about 50%, including 700 g a.e. ha⁻¹ MCPA and 350 g a.e. ha⁻¹ 2,4-D both applied 8-9 days prior to defoliation; and 750 g a.i. ha⁻¹ glufosinate; 940 g a.e. ha⁻¹ glyphosate; 45 g a.i. ha⁻¹ clopyralid with paraquat + diquat; and 656 g a.i. ha⁻¹ glyphosate plus 750 g a.i. ha⁻¹ 2,4-D all applied 5-10 days after defoliation. Treatments providing the best control included paraquat + diquat applied 5-10 days after defoliation (98% control) and MCPA + dicamba applied 8-9 days prior to defoliation (100% control). Amitrole + ammonium thiocyanate applied with 658 g a.i. ha⁻¹ glyphosate with an adjuvant applied 5-10 days after defoliation (85% control) and 1000 g a.i. ha⁻¹ 2,4-D applied with 432 paraquat + diquat also 5-10 days after defoliation (89% control) provided a similar level of control (Table 14).

At Dookie the total dry matter (DM) production of *C. bonariensis* was 9 g m⁻². All treatments significantly reduced the amount of dry matter remaining at the end of the trial period with a single defoliation reducing DM left at the end of the trial period by 67%. Treatments that resulted in the largest reductions in shoot dry matter (g m⁻²) included MCPA + dicamba (1768 + 416 g a.e. ha⁻¹) applied 8-9 days prior to defoliation, paraquat + diquat (432 + 368 g a.i. ha⁻¹) applied 5-10 after defoliation and amitrole + ammonium thiocyanate (1400 + 1232 g a.i. ha⁻¹) applied with 658 g a.e. ha⁻¹ glyphosate plus an adjuvant applied 5-10 days after defoliation (Table 14). These were also three of the four treatments providing the biggest reductions in plant numbers.

Table 14: Summary results from Dookie field trial performed in 2016, showing plant counts and dry matter (DM) remaining at the end of the experiment (65 DAT). Defoliation timings were either 8-9 day after herbicide application (post) or 5-10 prior to herbicide application (prior). Percentage control and reduction compared with untreated control. Results from Fishers Protected Least Significant Difference (LSD) in GenStat shown.

Herbicide treatment	Defoliation	Plants m ⁻²		Shoot DM		% Reduction	
				Control	(g m ⁻²)		
Untreated	Untreated	0.78	e	0	8.92	h	0
Untreated	At 1 st Herbicide timing	0.56	de	29	2.96	g	67
Untreated	At 1 st herbicide timing 2 nd Cut: 22 days later	0.53	cde	32	0.64	bcd	93
MCPA	8-9 days post	0.39	cd	50	1.98	fg	78
2,4-D	8-9 days post	0.38	cd	51	1.53	defg	83
Paraquat + Diquat	5-10 days prior	0.02	a	98	0	ab	100
Amitrole + Ammonium Thiocyanate; Glyphosate; Soyal Phospholipids + Propionic Acid	5-10 days prior	0.11	ab	85	0.29	ab	97
Glufosinate	5-10 days prior	0.28	bc	64	1.09	cde	88
MCPA + Dicamba	8-9 days post	0	a	100	0	a	100

2,4-D; Paraquat + Diquat	5-10 days prior	0.08	ab	89	0.43	abc	95
Glyphosate	5-10 days prior	0.42	cd	47	0.98	cdef	89
Paraquat + Diquat; Clopyralid	5-10 days prior	0.41	cd	48	1.76	efg	80
Glyphosate; 2,4-D	5-10 days prior	0.41	cd	48	0.98	cdef	89

Plant populations were higher at Goolgowi at 32.4 plants m⁻² and all treatments had a significant impact, including a single defoliation. All treatments with the exception of one defoliation and 940 g a.e. ha⁻¹ glyphosate applied 5 to 10 days after defoliation provided over 85% control (Table 15). 940 g a.e. ha⁻¹ glyphosate applied 5-10 days after defoliation did not improve control over defoliation alone, suggesting that there may be resistance to glyphosate present at this site.

The better control gained at Goolgowi is most likely a result of the application of herbicide treatments to less mature *C. bonariensis* plants than those at Dookie. Development stages encountered included stem elongation to early flowering at Dookie and stem elongation to early bud formation at Goolgowi. This situation again highlights the importance of managing *C. bonariensis* early at the seedling stage, as it is more difficult to control when mature (Urbano et al., 2007; Walker et al., 2012). In addition, the number of herbicide options when using a defoliation double-knock strategy increase when there is the opportunity to apply herbicides earlier.

The total DM production was 32.4 g m⁻² or <1 g plant⁻¹. The amount of DM produced over the area was much greater than at Dookie; however, the plants were much smaller producing only 10% of the final dry matter of that at Dookie (11.5 g plant⁻¹). Eight of the double knock treatments provided large reductions in the amount of shoot dry matter remaining at the end of the trial (Table 15). Again,

all these treatments align with those that had given the largest reductions in plant numbers and include the three treatments that provided the largest reductions in dry matter in the trial at Dookie.

Table 15: Plants m⁻² and percentage control 59 days after initial treatment application at Goolgowi.

Percentage control and reduction compared with untreated control. Results from Fishers Protected

Least Significant Difference (LSD) in GenStat shown.

Herbicide Treatment	Defoliation	Plants m ⁻²		Shoot DM		% Reduction	
				Control	(g / m ²)		
Untreated	None	32.4	e	0	37.7	f	0
Untreated	At 1 st Herbicide timing	14.3	cd	56	17.1	f	55
MCPA	8-9 days post	0.4	a	99	0.9	abc	98
2,4-D	8-9 days post	1.9	a	94	0.9	abc	98
Paraquat + Diquat	5-10 days prior	0.5	a	98	0.3	abc	99
Amitrole + Ammonium Thiocyanate;	5-10 days prior	3.6	a	89	2.7	bcd	93
Glyphosate; Soyal							
Phospholipids + Propionic Acid							
Glufosinate	5-10 days prior	0.3	a	99	0.5	ab	99
MCPA + Dicamba	8-9 days post	0	a	100	0	a	100
Paraquat + Diquat; 2,4-D	5-10 days prior	1.3	a	96	1.2	abc	97
Glyphosate	5-10 days prior	11.3	bc	65	9.4	e	75
Saflufenacil; Glyphosate;	5-10 days prior	5.2	ab	84	4.9	de	87
Paraffin Oil							
Paraquat + Diquat;	5-10 days prior	3.0	ab	91	2.8	cd	92
Clopyralid							

Glyphosate; 2,4-	5-10 days prior	0.3	a	99	0.1	a	100
Amitrole + Ammounium	5-10 days prior	0.8	a	97	0.3	abc	99
Thiocyanate; 2,4-D							

The results from these experiments demonstrate that relying on defoliation alone does not provide adequate control of *C. bonariensis*. Previous studies that focused on control with herbicides alone have also found that a single treatment does not adequately control *C. bonariensis* (Widderick et al., 2012). Although defoliation does not kill the plants, the reduced DM present at the end of the experiment indicates that intensive grazing could severely reduce *C. bonariensis* reproductive ability, especially when defoliations are repeated as at Dookie. Prieur-Richard et al. (2002) had found that herbivory of *C. bonariensis* reduced its net fecundity with severity of herbivory impacting on reproductive effort. As seed production is the only means of propagation for *C. bonariensis* (Wu, 2007) decreased reproductive effort could have a significant impact on the amount of seed entering the seedbank.

Good control can be achieved when combining defoliation with herbicides in a double knock strategy. The best performing defoliation double knocks in this experiment were paraquat + diquat applied 5-10 days after defoliation and MCPA + dicamba applied 8-9 days prior to defoliation, which achieved 98-100% control. This is similar to those achieved by chemical double knocks of glyphosate applied with 2,4-D followed by paraquat + diquat, which achieved 99-100% control (Werth et al., 2010) and 450 g a.e. ha⁻¹ glyphosate applied with 2,4-D + picloram (450 + 112 g a.e. ha⁻¹) followed by paraquat + diquat, which achieved 99% control on plants up to 3 months old (Widderick et al., 2012).

The high level of control achieved in these treatments is an important result as these were applied at Dookie to plants that had reached stem elongation to early flowering. Walker et al. (2012) found that spraying three month old *C. bonariensis* plants reduced herbicide efficacy by up to 30%

when compared to one month old plants. Having this option allows for defoliation to be included as an IWM strategy in the management of *C. bonariensis* in mixed farming systems.

4.4. Conclusions

C. bonariensis is a successful ruderal invader that is commonly encountered on irrigation channels. Management on channel banks is important to reduce the risk of infesting surrounding lands. The level of control provided by treatments currently available for use on irrigation channel banks is not sufficient to eliminate the risk of spreading seed. IWM of these areas is important as the poor level of control provided by the herbicides approved for use in this area increases the likelihood of herbicide resistance development. New IWM tactics are required for management of *C. bonariensis* on channel banks and these could include new herbicide registrations, or the development of alternative effective tactics. Cultivation has been used successfully in the past, but is not a desirable option due to the potential negative impact on water quality.

C. bonariensis is a significant weed in summer fallows and the use of grazing is common on mixed farming properties. There is widespread glyphosate and ALS resistance in *C. bonariensis* populations across the region (Chapter 2). Having strategies that utilise herbicide mixes and defoliation could help to manage this weed in a region where herbicide options are reducing.

Field experimentation showed that MCPA + dicamba applied 8-9 days prior to defoliation; paraquat + diquat applied 5-10 after defoliation and amitrole + ammonium thiocyanate applied with glyphosate and an adjuvant, 5-10 days after defoliation provided good levels of control even when applied to late growth stages. This demonstrates that there is potential for using defoliation as one of the knocks in a double-knock strategy. The optimum timing for herbicide application is seedling to rosette stage (Shrestha et al., 2008), however, in grazed fallows it is common for herbicides to be applied later and when plants are under stress. The field experiment at Goolgowi which had

treatments applied at earlier growth stages than Dookie demonstrated that the number of effective treatment options increase with less developed plants.

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Chapter 5: Conclusions and Recommendations

Conyza bonariensis (L.) Cronquist is a globally significant weed occurring in more than 40 crops in 70 countries (Zambrano-Navea et al., 2013), it is believed to have originated in South America and has been endemic in Australia since the 1840s (Wu, 2007). In Australia, it is ranked as the third most important weed in fallow nationally, infecting 2,793,252 hectares (ha) and responsible for \$43.2 million in lost revenue (Llewellyn et al., 2016).

Globally there have been 19 unique cases of herbicide-resistant *C. bonariensis* reported in 13 countries to nine active ingredients, including glyphosate, paraquat, diquat, atrazine, simazine, chlorsulfuron, pyriithiobac-sodium, sulfometuron-methyl and imazapyr (Matzrafi et al., 2015; Heap, 2017). There has been multiple-resistance reported to glyphosate and paraquat in California (Moretti et al., 2013) and to photosystem II (PSII) inhibitor's atrazine and metribuzin and acetolactate synthase (ALS) inhibitors pyriithiobac-sodium, sulfometuron-methyl and imazapyr in Israel (Matzrafi et al., 2015). *C. bonariensis* in Australia has been reported to be glyphosate-resistant in Queensland, New South Wales and South Australia, and paraquat resistant in New South Wales (Heap, 2017).

Surveying of *Conyza bonariensis* populations across northeast Victoria revealed that 42% were glyphosate-resistant having 20% or greater survivors to 1080 g a.e. ha⁻¹. Only 23% of populations had 100% mortality at this herbicide application rate. Predominant land use in the area where populations were collected did not influence the frequency of glyphosate resistance observed. The high mobility of *Conyza* seeds within the environment possibly explains the consistent levels of resistance found across the region, alternatively all land use types have glyphosate applied, albeit at different frequencies therefore selection for glyphosate resistance in *C. bonariensis* may be occurring throughout the region. Normally herbicide resistance management is left to individual growers, however for weeds that colonise up to 1842 meters from the parent population (Borger et al. 2010)

there may be merit in a community approach involving councils and growers from across the region working collaboratively.

Widespread resistance to ALS inhibitor herbicides was observed, with chlorsulfuron failing to control any population screened. Considerable resistance was also found to metsulfuron-methyl and sulfometuron-methyl. Multiple-resistance to glyphosate and all three ALS inhibitors occurred in five of the nine populations characterised with all herbicides. Multiple resistance to 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) and ALS inhibitors has not previously been reported in *C. bonariensis* populations but has been reported in *C. canadensis* populations from Ohio, Delaware and Canada (Heap, 2017). The high frequency of resistance to EPSP and ALS herbicides present in this weed could explain the difficulty experienced in its control in northeast Victoria. No resistance was detected to the synthetic auxins 2,4-D and clopyralid or to the PSI inhibitor paraquat, therefore these herbicides could still be used for the control of this weed species.

Dose response experiments identified three distinct groups of glyphosate resistance, glyphosate susceptible (GS) (DL4, IR7 and IR11); Gr (DL3, DL13 and IR14) which had low level resistance having resistance Indices (RI's) between 2.3 and 2.8; and GR (DL19, IR5 and IR10) with high level resistance showing RI's over 6. The differing levels of resistance between the GR and Gr biotypes suggest more than one mechanism of resistance to glyphosate may be present across the region.

Reduced translocation is a common glyphosate resistance mechanism present in *Conyza* spp. however, this was not identified in the nine populations characterised. Populations displayed differential accumulation of shikimate suggesting insensitive EPSPS may be contributing to resistance. Sequencing of both genomic and plasmid DNA identified Pro106-Thr and Pro106-Ser mutations; both of which have been shown to confer glyphosate resistance (Sammons and Gaines, 2014). The discovery of these mutations does not fully explain the resistance seen as mutations occurred in all

three groups. As *C. bonariensis* is a hexaploid species (Wu, 2007), the number of gene mutations could be influencing resistance levels. In the hexaploid *Avena fatua*, a single ACCase mutation provided relatively low-level resistance to the ACCase herbicides, but resistance levels were significantly increased where individual plants accumulated multiple ACCase mutations (Yu et al. 2013). Although the number of mutations within an individual could be contributing to the differing levels of resistance displayed by *C. bonariensis* populations in this study, it does not fully explain the resistance, biotypes did not follow the expected trend of a higher proportion of mutations in GR and Gr biotypes than in GS biotypes.

As there are differing levels of glyphosate resistance present with the populations tested there may be multiple resistance mechanisms present in some populations. There have been several reported cases of multiple resistance mechanisms conferring higher levels of glyphosate resistance in *Conyza* spp. than biotypes with a single mechanism. GR *C. canadensis* from Spain was reported to have both reduced translocation and increased metabolism of glyphosate into aminomethylphosphonic acid (AMPA), glyoxylate and sarcosine as the resistance mechanisms (González-Torralva et al., 2012). Reduced translocation of glyphosate plus increased levels of EPSPS mRNA have been reported for GR *C. bonariensis* and *C. canadensis* (Dinelli et al., 2008). Decreased translocation, increased EPSPS synthase transcript levels and increased branching were also reported in GR *C. canadensis* from USA (Dinelli et al., 2006).

Further research is needed to confirm the glyphosate resistance mechanisms present in *C. bonariensis* populations from northeast Victoria. Gene expression may be involved with some mutations not being expressed or an additional mechanism(s), such as EPSPS or ABC transporter overexpression, may playing a role in *C. bonariensis* from northeast Victoria. Over expression of EPSPS or ABC transporter genes were not examined in this study; however, research in this area may provide greater insight into resistance mechanisms in *C. bonariensis* populations from northeast Victoria.

Channel banks are an important source of weed infestations (Charles, 1991) so management of *C. bonariensis* along irrigation channel banks is critical to reduce infestation of the surrounding lands by wind or water dispersed seed. Field trials at Oaklands and Goolgowi, New South Wales identified that the herbicides registered for use on irrigation channel banks do not adequately control *C. bonariensis* even when applied using the double-knock strategy. The poor level of control provided by the herbicides approved for on channel banks increases the risk of herbicide resistance development, so new integrated weed management (IWM) strategies that work in this area are needed. Growers in 1989 reported using regular mechanical cultivation on their irrigation channels to manage weeds (Charles, 1991), this however is an unfavourable option due to its negative impact on irrigation water quality. Other options for non-chemical weed control would be a valuable area of further study.

Resistance evolution to EPSPS and ALS inhibitors in northeast Victoria means that the adoption of IWM techniques is required to successfully control *C. bonariensis*. Grazing of fallows is common across northeast Victoria as well as in many mixed dryland crop and stock regions, however, little is known about combining defoliation with herbicide applications to control *C. bonariensis*. Field experiments at Dookie, Victoria and Goolgowi, NSW demonstrated that defoliation could be used along with herbicides in a double-knock strategy to control *C. bonariensis*. The treatments that provided the best level of control were paraquat + diquat (432 + 368 g a.i. ha⁻¹) applied 5-10 days after defoliation providing 98% control at both sites and MCPA + dicamba (1,768 + 416 g a.e. ha⁻¹) applied 8-9 days prior to defoliation providing 100% control at both sites. This demonstrates that there is potential for using defoliation as one of the knocks in a double-knock strategy.

IWM is important in minimising the impacts of herbicide resistance so having strategies that utilise defoliation could help to control this weed in regions where herbicide options are reducing due to resistance evolution. Other IWM strategies that have been recommended for the control of *C. bonariensis* in fallows include the chemical doubleknock, which has been widely adopted with the

most common doubleknock used being glyphosate + 2,4-D as the first application, followed by a paraquat or paraquat + diquat based option as the second-knock (Widderick et al., 2012). Tillage reduced emergence of *Conyza* by >90% irrespective of tillage treatment compared to zero tillage, with the one-way disc treatment producing the greatest reduction in emergence (Widderick et al., 2014).

This project has demonstrated that there is considerable herbicide resistance present to both glyphosate and ALS inhibitors in *C. bonariensis* populations from across northeast Victoria and identified the first reported multiple resistance to EPSPS and ALS inhibitors in *C. bonariensis*. Research conducted into the glyphosate resistance mechanisms present in selected populations found Pro106-Thr and Pro106-Ser mutations present, however, as these mutations were present in both resistant and susceptible biotypes this does not fully explain the resistance present. Further investigation into EPSPS and ABC transporter genes may provide greater insight into the mechanisms conferring resistance in these *C. bonariensis* populations. Field experimentation into management of *C. bonariensis* identified that there are currently no herbicide options currently approved for use on channel banks that provide effective control. Further research into alternative strategies including new herbicides or non-chemical control options is required to minimise the risk of infesting surrounding areas from surviving *C. bonariensis*. Use of defoliation as one of the treatments in a doubleknock strategy found herbicide and defoliation could be used in conjunction to successfully control *C. bonariensis*. Further experimentation investigating whether grazing is a viable defoliation option is required.

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