Herbicide resistant common sowthistle (*Sonchus oleraceus* L.) and prickly lettuce (*Lactuca serriola* L.): management considerations in lentils

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April 2022

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Publications arising from this thesis

Journal Articles

Merriam AB, Malone J, Gill G, Preston C (2021) Can rotations improve management of herbicide-resistant annual sowthistle (Sonchus oleraceus) and prickly lettuce (Lactuca serriola) in lentil production systems of southern Australia?. Weed Technol. 35: 532–538. doi: 10.1017/wet.2020.134

Peer Reviewed Conference Papers

Merriam AB, Boutsalis P, Malone J, Gill G, Preston C (2018) Extent of herbicide resistant common sowthistle (Sonchus oleraceus) in southern Australia. 21st Australasian Weeds Conference. Sydney, Australia

Abstract

Common sowthistle and prickly lettuce are globally widespread weeds that have become increasingly common in annual cropping systems of southern Australia over recent decades. They are especially problematic in pulse crops and together are responsible for estimated revenue losses of AUD $1.5 million annually in southern Australia. Several factors complicate their management in these systems: herbicide resistance, prolific production of highly mobile seed, and a lack of effective herbicide options.

In Australia, resistance to the acetolactate synthase inhibiting herbicides is widespread in both species and cases of resistance to other herbicide groups are emerging. The extent of resistance in southern Australia has been quantified through repeated field surveys, and cases of ALS inhibitor resistance have been further characterised and mechanisms have been explored. In both species, a wide variety of mutations at the Proline-197 position of the ALS gene have differing consequences for cross resistance between chemical families within the group, further complicated in common sowthistle by a second genome and additional copy of the ALS gene.

The combination of prolific production of highly mobile seed and herbicide resistance genes have raised questions around gene flow and genetic diversity in these species as it relates to resistance management. Many different resistance-causing mutations were identified in both species, even on a small spatial scale, and there is some evidence of spatial clustering at a larger scale. Population genetic analysis performed using Genome by Sequencing (GBS) techniques revealed differences in genetic clustering and seed mobility between the two species.

While these species are more easily controlled in cereal and oilseed crops, management in pulses is a problem because very few post-emergence herbicides are registered for broadleaf weed control in these crops. Two management trials have explored different control tactics under the circumstances: a rotation trial comparing the carryover effect of management in a previous season’s cereal crop and a trial incorporating a novel lentil cultivar tolerant to photosystem II inhibiting herbicides (HRAC Group 5). Results of the rotation trial indicate that management practices used in the previous season can have a carryover benefit to the following year under some circumstances, but that colonization from outside the paddock is also a significant factor. The metribuzin tolerant breeding line of lentils showed promise for control of these broadleaf weeds and there was no evidence of herbicide damage impacting lentil yield, but breeding programs are still several years away from official release.

The complexities of managing these weeds in pulse crops necessitates an integrated weed management strategy which includes physical and cultural tactics in addition to chemical control. This requires a sound understanding of the biology and ecology of these weed species in cropping systems of southern Australia. This project has explored several aspects of these weeds and their role in the farming system and has also highlighted similarities and differences between the species that may be exploited in their management.

Thesis 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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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

14/03/2022

Alicia Merriam Date

PhD Candidate

Acknowledgements

I would like to begin by acknowledging the Traditional Owners of the land upon which this work was conducted. Research was conducted mainly on Kaurna Country, with a brief foray to Yuggera Country, and the thesis was finished on Wadawurrung Country. Field experiments were conducted on Narangga and Kaurna Country and weed populations for the experiments were sourced from the Country of many cultural groups including the Kaurna, Narangga, Nukunu, Ngadjuri, Peramangk, Meru, Ngarrindjeri, Ngargad, Bindjali, and Buandig People. I pay my respects to their Elders past and present.

This research was supported by an Australian Government Research Training Program Scholarship and a Grains Research and Development Corporation (GRDC) Grains Research Scholarship. The research undertaken as part of this project is made possible by the significant contributions of growers through both trial cooperation and the support of the GRDC.

I would like to thank my supervisors, Professor Christopher Preston, Associate Professor Gurjeet Gill and Dr. Jenna Malone for all their guidance, support, advice and patience. The diversity of experience and viewpoints that you all brought to the team broadened my horizons and encouraged me to think for myself. Jenna: you are a role model as a representative of the next generation of leadership in women in STEM - a successful researcher, a mum, and an advocate for EMCR advancement and success. When needed, I felt like I could speak with you candidly and that was invaluable. Gurjeet: your feedback has been some of the most thought provoking and constructive, and I appreciated the words of encouragement that always accompanied and balanced any necessary criticisms. Chris: firstly, thank you for hiring me in my first role in Australia, changing the course of my career and life. Thank you for taking me on as a student, your expertise along the way, and your patience at the end. Thank you also for the annual invitation to your Thanksgiving dinner – this always meant a lot during a time of year I was often missing family overseas.

Thanks are also due to all other members of the Weed Science Research Group: Postdoctoral fellows Dr. Peter Boutsalis, Dr. Fleur Dolman, Dr. Mahima Krishnan, Dr. Tijana Petrovic, and Dr Weihua Long provided valuable advice at many points along the way. Technical staff Dr. Geetha

Velappan, Dr. Sam Kleemann and Ruwan Lenorage provided valuable technical assistance. To the other PhD students, including Dr. Hue Dang, Dr. Patricia Adu-Yeboah, Rajesh Barua and Zarka Ramiz: your hard work and perseverance was inspiring. Finally, to the Roseworthy crew: Malinee Thongmee, Jerome Martin, Daniel Peterson, Hugh Cameron, and especially Ben Fleet – your technical assistance with field trials went above and beyond and I am so grateful.

I would like to thank all those from the University of Adelaide Waite Campus community who crossed my path during my studies. Special thanks are due to Dr. Judy Rathjen (for candid advice and enabling my houseplant addiction), Alyce Hayes-Dowling (truly a source of joy), Dr. John Carragher (for the lemon slice and potato ice cream), and Associate Professors Matt Denton and Glenn McDonald (for employing me as a tutor, a role I truly enjoyed). Thanks to the Biometry Hub, particularly Sam Rogers, who organized many workshops that helped me stumble through learning R.

Other industry partners also played important roles during my studies. Thanks to Chris Davey at YP Ag, not only for on-the-ground knowledge but also for liaison with growers to identify trial sites. Thanks to Dr. Michael Widderick, external advisor, who provided valued input at candidature milestones. Thanks to Dr. Navneet Aggarval and Dr. Penny Roberts of SARDI-Clare for the opportunity to collaborate. Thanks to Dr. James Hereward for significant guidance and support regarding the genotyping by sequencing studies and to Emeritus Professor Gimme Walter at the University of Queensland for hosting me for lab work relating to these studies.

I'd like to rewind a few years and acknowledge Julia Leeson, my supervisor from 2011 to 2015 at the Agriculture and Agri-Foods Canada Weed Ecology lab, which was my first foray into weed science. Julia is one of the most hard working and inspiring women in science I have ever had the opportunity to meet. I'm forever grateful to Julia for what I learned from her, the opportunities she gave me and the influence that she had on my career and course of my life. I'd also like to acknowledge Associate Professor Eric Lamb, my undergraduate thesis supervisor at the University of Saskatchewan. Eric set an amazing example of ethical leadership in academia. In addition to being a committed researcher and ecologist, he was a supportive and kind mentor, and truly a good human.

To my parents, Miranda Jones and Jim Merriam, who fostered scientific curiosity and a love of the outdoors from my earliest years: your unconditional love and support has played a pivotal role in every one of my achievements and I feel so privileged to be your daughter. An academic and an artist – you brought such different perspectives to parenting but both set an example of the value of being passionate about life's work – a fundamental lesson I will carry with me always. To my mum: you taught me to never shy away from an adventure or challenge! Your regular reminders to take care of what really matters – my physical and mental health – were of fundamental importance. To my dad: you overcame so much adversity to provide a better life for your children, and I admire you for that. Without your encouragement throughout my education, I doubt I would have gotten to this point.

And last, but most certainly not least, thank you to my partner, fellow (former) PhD student Dr. David Brunton. I could not have asked for a better companion on the PhD journey: you inspired me, encouraged me, helped me in the lab and accompanied me in the field, and pushed me to persevere during the difficult times. I truly believe I could not have done it without your support, companionship, and encouragement. You have been an inspiration to me as a dedicated student, a devoted son, and a passionate and skilled researcher. I am honoured to have had you as my partner for the past five years, and to now call you my fiancé. I cannot wait to marry you and see what the next chapter holds for us.

1 CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Annual sowthistle (*Sonchus oleraceus* L.) and prickly lettuce (*Lactuca serriola* L.) are common broadleaf weeds of disturbed areas, such as annually cropped fields (Hutchinson et al., 1984; Weaver and Downs, 2003). Both prickly lettuce and annual sowthistle are native to Eurasia, but today have a cosmopolitan distribution due to their wide adaptability and extremely successful dispersal (Hutchinson et al., 1984; Peerzada et al., 2019; Weaver and Downs, 2003). Both weeds are naturalised in all Australian states, with particular prominence in grain producing regions (Chadha et al., 2019; Peerzada et al., 2019), where they are considered an emerging threat (Llewellyn et al., 2016). They tend to be particularly problematic in weakly competitive crops and broadleaf crops that offer few selective herbicide options.

While they are not strongly competitive and generally were not thought to reduce crop yields (Amor, 1986b; Hutchinson et al., 1984), more recent research has demonstrated yield loss in certain situations (Manalil et al., 2020; Wu et al., 2019). Furthermore, annual sowthistle is an alternate host for pests and disease vectors (Hutchinson et al., 1984; Widderick, 2019), and prickly lettuce can cause problems at harvest by blocking equipment with the milky latex produced by its tissues (Amor, 1986b). Both species can affect grain quality by contributing to green matter and moisture levels at harvest (Amor, 1986b; Widderick, 2019).

In some situations these weeds have proven difficult to control, particularly because both species are prolific seed producers and have evolved resistance to several herbicides commonly used to control them (Heap, 2022).

1.1.1 Weed management in grain production

Conventional agriculture aims to maximise yield of a single crop by optimizing growing conditions and reducing yield losses due to weeds, pests and disease pressures. Weeds represent one of the most significant costs to grain growers in Australia due to both yield loss and expenditure on weed management (Llewellyn et al., 2016). Herbicides have revolutionised weed control since the middle of the 20th century and are increasingly relied upon for weed control, especially following the adoption of reduced tillage or conservation agriculture practices (Powles and Yu, 2010). The second half of the 20th century saw rapid advances in herbicide discovery and herbicides with a wide variety of physiological modes of action were released commercially (Kraehmer et al., 2014). Furthermore, the turn of the 21st century saw the release and popularisation of the first transgenic herbicide-tolerant crops, which greatly facilitated weed control, but saw an increase in the use of certain herbicides (Mortensen et al., 2012).

The selection pressure imposed by herbicides on weed populations has resulted in the evolution of herbicide resistance in many species (Powles and Yu, 2010). Currently there are 509 confirmed, unique cases of herbicide resistance (weed species x herbicide mode of action), encompassing 21 of the 31 known herbicide modes of action and found in 266 different weed species (Heap, 2022). Under continued selection pressure resistant biotypes proliferate and, as new cases arise and spread, control of many species becomes increasingly intractable. Simultaneously, discovery and commercialisation of novel herbicides and modes of action has slowed over recent decades (Kraehmer et al., 2014).

These issues have led to a push for more integrated weed management tactics or Integrated Weed Management (IWM). The basis of IWM is a diversity of weed control tactics: physical, cultural and biological control methods in addition to chemical herbicides (Shaner and Beckie, 2014). The development of successful IWM tactics for a given weed species relies on a sound understanding of the biology and ecology of the weed species, as well as its interactions with each crop of interest.

1.2 Annual sowthistle and prickly lettuce

1.2.1 Distribution and impact in Australia and around the world

Annual sowthistle and prickly lettuce are native to Mediterranean Europe, but have spread throughout arable areas of the world and have presented a challenge in Australia in particular (Chadha et al., 2019; Peerzada et al., 2019). In Australian grain cropping systems, they interfere both during the growing season and during summer fallow periods in northern Australia where they deplete stored summer rainfall (Peerzada et al., 2019; Wu et al., 2019). Their impact in Australia has increased over time as they have been favoured by the adoption of reduced tillage systems (Chauhan et al., 2006; Weaver and Downs, 2003; Widderick et al., 2010). It is estimated that annual sowthistle is responsible for annual revenue losses of A$4.2 million in northern Australian grain production, and in southern Australia, prickly lettuce and annual sowthistle are responsible for an additional A$1.5 million in revenue loss (Llewellyn et al., 2016).

Reported cases of herbicide resistance in annual sowthistle are so far confined to Australia, while resistant populations of prickly lettuce have been reported from Australia and the United States (Heap, 2022). Selective control of these weed species, particularly in broadleaf crops with fewer alternative selective options, is impacted by acetolactate synthase (ALS) inhibitor (HRAC Group 2) resistance. This is the most common form of herbicide resistance for these weeds, and is widespread in Australian populations of both species, as well as prickly lettuce populations in the United States (Heap, 2022). Resistance to 2,4-D (HRAC Group 4) is emerging in annual sowthistle in southern Australia (Preston et al., 2017) and in prickly lettuce in the Pacific North West in the United States (Burke et al., 2009). Glyphosate (HRAC Group 9) resistance in both species is also present. Glyphosate resistant annual sowthistle is so far confined to the northern cropping region of Australia (van der Meulen et al., 2016). A suspected glyphosate-resistant population of prickly lettuce was identified in southern Australia in 2015 (Heap, 2022), although prickly lettuce is noted to be naturally tolerant of glyphosate.

1.2.2 Biology and field ecology of annual sowthistle and prickly lettuce

Annual sowthistle and prickly lettuce are annuals in the Asteraceae family. Prickly lettuce is a diploid species (2n = 18) and annual sowthistle is an amphidiploid (2n = 32) (Swanson, 1956). Initially, annual sowthistle was thought to be the result of a hybridisation between *Sonchus asper* (L.) Hill (n=18) and *Sonchus tenerrimus* (n=14) (Stebbins, 1953); however, more recent research has suggested multiple ancient hybridisation events with *S. asper* as the maternal parent (Cho et al., 2019). Both species produce a large number of seeds, of which only a few may survive to maturity.

They have similar capitulate flower heads typical of the Asteraceae, with each head producing many small seeds with feathery, sail-like appendages (pappi) which aid in their dispersal by wind after seed has matured on the plant (Figure 1) (Hutchinson et al., 1984; Weaver and Downs, 2003).

Figure 1. Mature plants of annual sowthistle (left) and prickly lettuce (right) growing in a lentil crop (A), mature achene and pappus of annual sowthistle (B) and prickly lettuce (C).

A single plant of either species can produce a great quantity of seed, but seed production seems to be inversely related to competition (Amor, 1986b; Hutchinson et al., 1984; Weaver and Downs, 2003). Amor (1986b) reported up to 48 000 seeds per prickly lettuce plant in the absence of competition in Victoria, Australia, while Marks and Prince (1981) reported an average of about 900 seeds per plant when grown in association with natural vegetation in England. Weaver and Downs (2003) noted that the number of flower heads was proportional to plant height and produced estimates ranging from 100 to 200 000 seeds per plant for prickly lettuce grown in wheat stubble or soybean in Ontario, Canada. Annual sowthistle can produce up to 8000 seeds per plant (Chauhan et al., 2006).

Both species are mostly autogamous (Hutchinson et al., 1984; Mejias, 1994; Weaver and Downs, 2003), meaning that they are self-fertilised, so most gene flow occurs by seed dispersal and is dependent on wind. Seeds of both species tend to be short-lived and have little to no dormancy or after-ripening requirement (Chadha et al., 2019; Chauhan et al., 2006; Hutchinson et al., 1984; Weaver and Downs, 2003), as is common for small-seeded annuals (Grime et al., 1981). This means they can germinate immediately after dispersal if favourable conditions are present. They tend to have relatively short seedbank persistence, with most seed germinating in the year following maturity. However, if favourable conditions for germination are lacking, seed may persist up to several years under field conditions, especially if buried (Chadha et al., 2019; Chauhan et al., 2006; Hutchinson et al., 1984; Weaver and Downs, 2003).

1.3 Herbicide resistance in annual sowthistle and prickly lettuce

Both species have a similar profile of herbicide resistance, with cases confirmed to some of the herbicide mode of action groups most commonly used to control them. Resistance to the ALS inhibitors was the first reported and is most widespread for both species. Both have also evolved resistance to the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor glyphosate and various synthetic auxins (Heap, 2022). There is potential for new cases of resistance to evolve and resistance monitoring programs in Australia and around the world continue to screen for resistance to additional herbicide groups. The current herbicide resistance monitoring surveys in southern Australia regularly collect and screen annual sowthistle, but unfortunately prickly lettuce is not covered by these surveys due to its much later seed maturity (P. Boutsalis. Personal communication). Instead, resistance monitoring of prickly lettuce in southern Australia relies more on grower-submitted suspected cases.

1.3.1 ALS inhibitor resistance

Resistance in these species to ALS inhibitors was first discovered in 1987 for prickly lettuce in the United States (Mallory-Smith et al., 1990a) and in 1991 for annual sowthistle in Australia (Boutsalis and Powles, 1995b), both in annual cropping situations. These cases were identified very shortly after the commercial release of ALS-inhibiting herbicides in the early 1980s, and resistance has spread relatively quickly (Alcocer-Ruthling et al., 1992). Since then, resistant biotypes of prickly lettuce have been found in the American states of Idaho (1987), Washington and Oregon (1993), and in the Australian states of South Australia (1994) and Victoria (2009) (Heap, 2022). For annual sowthistle, cases have been confirmed from the Australian states of Queensland (1991), Victoria (2010) and South Australia (2015) (Heap, 2022).

Resistance monitoring surveys in the southern cropping region of Australia suggest that ALS resistance in annual sowthistle is widespread, with over 90% of populations from some areas of Victoria and South Australia classified as resistant to sulfonylurea herbicides (Merriam et al., 2018). Since screening was initiated, 78% of all populations collected from the southern cropping region were resistant to sulfonylureas and 68% were resistant to imidazolinones (Merriam et al., 2018).

Because prickly lettuce is not included in the regular program of southern region herbicide resistance monitoring surveys, there is less data available, but one previous study suggested that resistance is also widespread in prickly lettuce. This study screened populations form the Mid North and Yorke Peninsula of South Australia with chlorsulfuron only, and found that 66% of populations collected in 1999 and 82% of populations collected in 2004 contained resistant individuals (Lu et al., 2007).

ALS inhibitor resistance in prickly lettuce has been shown to be caused by a single point mutation in the ALS gene (Eberlein et al., 1997; Guttieri et al., 1992; Preston et al., 2006) and is inherited by a single nuclear gene with incomplete dominance (Mallory-Smith et al., 1990b). Several different mutations that confer resistance to the ALS inhibitors have been reported, and each can result in a unique profile of cross resistance to the different chemical families (sulfonylureas, imidazolinones, and triazolopyrimidines) within the ALS-inhibiting herbicides (Heap, 2022; Preston et al., 2006). Surveys conducted in South Australia in 1999 and 2004 identified individuals with Pro-197-Thr, Pro-197-His, Pro-197-Ser and Pro-197-Leu mutations (Lu et al., 2007).

There have been fewer reports on the mechanism of resistance of annual sowthistle to ALS-inhibiting herbicides, but evidence points to a single point mutation in the ALS gene (Boutsalis and Powles, 1995a). A study by Park et al. (2012) on spiny annual sowthistle (*Sonchus asper* (L.) Hill), a closely related species, confirms this with a report of a proline-197-leucine substitution conferring resistance to both sulfonylureas and imidazolinones. Boutsalis and Powles (1995a) found that resistance was inherited through a single nuclear gene with incomplete dominance. More recently, Petersen (2018) sequenced the ALS gene of field-collected annual sowthistle from the Yorke Peninsula of South Australia and found several different point mutations at the Pro-197 position: Pro-197-Ser, Pro-197-Arg, and Pro-197-Leu, Pro-197-Thr, and Pro-197-Ala.

Although only Pro-197 mutations have been reported in ALS inhibitor resistant populations of prickly lettuce and annual sowthistle, substitutions at a number of additional sites in the ALS gene have been shown to cause resistance, including Ala$_{122}$, Val$_{205}$, Asp$_{376}$, Arg$_{377}$, Trp$_{574}$, Ser$_{653}$ and Glyc$_{654}$ (Tranel et al., 2022; Yang et al., 2018). Furthermore, while target site resistance remains the most common mechanism of resistance to the ALS inhibitors, non-target site resistance has also been reported in several species (Yu and Powles, 2014).

1.3.2 Synthetic auxin resistance

The first cases of resistance to synthetic auxin herbicides in these weed species were reported from wheat crops: in 2007 from the United States (prickly lettuce) (Burke et al., 2009) and in 2015 from Australia (annual sowthistle) (Heap, 2022; Preston et al., 2017). Resistance monitoring in southern Australia has thus far detected synthetic auxin resistance only in a small number of populations of annual sowthistle from the southeast of South Australia (Preston et al., 2017), and the Eyre Peninsula of South Australia, and in southwest Victoria (Merriam et al., 2018). More recently suspected resistant populations were identified from the South Australian Mallee region. 2,4-D screening has only been included in the survey since 2013, and has shown a resistance rate of 2.4% across all populations screened (Merriam et al., 2018). There have been no reports of prickly lettuce resistant to synthetic auxin herbicides in Australia, but there have been confirmed cases in the United States, particularly in the Pacific North West (Burke et al., 2009). These biotypes were initially examined for resistance to 2,4-D, but results also indicated they may have reduced sensitivity to other synthetic auxin herbicides (Burke et al., 2009)

The mode of action of synthetic auxin herbicides is complex and has been the subject of considerable debate in the herbicide resistance research community (Christoffoleti et al., 2015; Grossmann, 2010; Todd et al., 2020). Because of this complexity, synthetic auxin resistance in weeds was generally thought to be through non-target-site mechanisms (Goggin et al., 2016), but more recently target-site mechanisms have been identified (LeClere et al., 2018). Target site mutations conferring synthetic auxin resistance have been identified in two genes involved in auxin signalling pathway: the $\mathrm{SCF}^{\mathrm{TIR1/AFB}}$ ubiquitination complex (Gleason et al., 2011; Walsh et al., 2006) and Aux/IAA co-receptors (LeClere et al., 2018), although the former has only been demonstrated in *Arabidopsis thaliana* mutants and not in field-evolved weed populations.

The two most common non-target-site mechanisms are detoxification or metabolism of the herbicide within the plant, and reduced absorption or translocation of the herbicide (Goggin et al., 2016). Detoxification of synthetic auxin herbicides through metabolism is responsible for conferring resistance in chickweed (*Stellaria media* (L.) Vill.) (Coupland et al., 1990), nodding thistle (*Carduus nutans*) (Harrington and Woolley, 2006) and Kochia (*Bassia scoparia* (L.) A.J.Scott) (Keith et al., 2011). Synthetic auxin resistance caused by reduced absorption or translocation of herbicide within the plant has been identified in hemp-nettle (*Galeopsis tetrahit* L.) (Weinberg et al., 2006), wild radish (*Raphanus raphanistrum* L.) (Goggin et al., 2016) and oriental mustard (*Sisymbrium orientale* L.) (Dang et al., 2017) and others. Other studies have suggested mechanisms such as exudation of herbicide through the roots (Jugulam et al., 2013) and herbicide perception by the plasma membrane and alteration to auxin-signalling pathways (Jugulam and Hall, 2005; Xu et al., 2013).

The mechanism of resistance in the first synthetic auxin resistant populations of prickly lettuce from the United States is reduced translocation, inherited through a single codominant gene (Riar et al., 2011). To date, the mechanism and mode of inheritance of annual sowthistle resistance to synthetic auxins has not yet been confirmed.

1.3.3 EPSPS inhibitor resistance

The first cases of resistance to the EPSPS inhibitor glyphosate in these weed species were discovered in fallow situations in Australia, and reported in 2014 for annual sowthistle (from New South Wales, Australia) (Cook et al., 2014) and in 2015 for prickly lettuce (from Victoria) (Heap, 2022). Random herbicide resistance monitoring surveys in the northern region have reported around 20% of annual sowthistle samples resistant to glyphosate, mostly in northern New South Wales (van der Meulen et al., 2016). Annual sowthistle from resistance monitoring surveys in southern Australia has been screened for glyphosate resistance since 2013, corresponding to a total of around 169 samples, but resistance has not yet been detected (Merriam et al., 2018). Further studies on the putative resistant prickly lettuce population have not been carried out, and no further resistant populations have been identified.

Although no work has yet been published on the mechanisms and inheritance of glyphosate resistance in annual sowthistle or prickly lettuce, recent studies have shown that glyphosate resistance in annual sowthistle appears to be mediated by plant growth stage at time or herbicide application (Cook et al., 2014), ambient temperature (Chauhan and Jha, 2020; Peerzada et al., 2021), and atmospheric carbon dioxide concentration (Mobli et al., 2020). From looking at other weed species, it is known that the mechanisms of resistance to the EPSPS inhibitor are highly diverse (Pline-Srnic, 2006; Shaner et al., 2012). These mechanisms include target-site resistance (mutation in the EPSPS gene), gene amplification (overexpression of EPSPS), and reduced translocation (Shaner et al., 2012).

Rigid ryegrass (*Lolium rigidum* Gaudin) was the first weed species for which glyphosate resistance was reported (Powles et al., 1998), and it was determined by Lorraine-Colwill et al. (2002) that resistance was conferred by reduced translocation of the herbicide molecule from treated leaves to the actively growing tissues where it is most active. Subsequently, this mechanism was also identified in a number of other weed species (Dinelli et al., 2008; Koger and Reddy, 2005; Perez-Jones et al., 2004). Target site resistance to EPSPS inhibitors was first identified in goosegrass (*Eleusine indica* (L.) Gaertn.) (Lee and Ngim, 2000) and characterised by Baerson et al. (2002b). Shortly afterwards, target-site resistant populations of rigid ryegrass (Wakelin and Preston, 2006) and

Italian ryegrass (Perez-Jones et al., 2007) were also identified. Moderately increased EPSPS expression was initially noted in resistant populations of rigid ryegrass (Baerson et al., 2002a) and *Erigeron* spp. (Dinelli et al., 2006). Both species also had other mechanisms present, so this factor was initially overlooked as a significant contributor (Shaner et al., 2012). However, in 2006 a glyphosate-resistant population of Palmer amaranth (*Amaranthus palmeri* S. Wats.) was reported (Culpepper et al., 2006) and later confirmed by Gaines et al. (2010) to be resistant due to EPSPS gene amplification (up to 160-fold more copies).

1.3.4 Other cases of resistance

Weed species are always evolving and there is always potential for the development of new cases of resistance to herbicides commonly used to control a given weed species. There have been preliminary reports of annual sowthistle resistance to Photosystem II (PSII) inhibitor (HRAC Group 5) simazine as identified by chlorophyll fluorescence analysis of leaves (Fraga and Tasende, 2003) and conferred by a target-site mutation and herbicide detoxification. However, these results were not backed up by whole-plant bioassays, as is the standard for herbicide-resistance screening (Beckie et al., 2000). Nevertheless, herbicide resistance monitoring surveys in southern Australia have screened annual sowthistle for resistance to PSII inhibitor atrazine in 2014 and 2015, although no resistance has been detected (53 total samples screened) (Peter Boutsalis. Unpublished data).

Resistance monitoring must also evolve in response to reports from industry partners. Recently, reports have emerged from agronomists in South Australia of reduced efficacy of PDS inhibitor (HRAC Group 12) diflufenican on annual sowthistle (C. Preston. Personal communication. 5 March 2018). This has not yet been investigated through the resistance monitoring surveys, but will be included in the screening of future collections.

1.4 Gene flow and population structure in annual sowthistle and prickly lettuce

Prickly lettuce and annual sowthistle have highly mobile seed well adapted to wind dispersal (Hutchinson et al., 1984; Weaver and Downs, 2003), so the emergence of glyphosate and 2,4-D resistance is very concerning. This is particularly so given the evidence of widespread and rapid expansion of ALS-inhibitor resistance in these species. While continued selection pressure presents the possibility of independent evolution of novel resistant populations, highly mobile resistance genes are an additional concern for growers and have implications for management and biosecurity. A better understanding of how ALS inhibitor resistance has evolved and spread within the southern cropping regions of Australia could help inform management to prevent the spread of emerging cases of resistance.

1.4.1 Dispersal of resistance genes in annual sowthistle and prickly lettuce

Genes may travel via pollen-, seed- and vegetative propagule-mediated gene flow. The first step involved is to determine to what extent each of these phenomena is present and whether they must be considered. For annual sowthistle and prickly lettuce, seed-mediated gene flow is of primary importance as both species are mainly autogamous and do not reproduce vegetatively (Hutchinson et al., 1984; Mejias, 1994; Weaver and Downs, 2003). However, insect visitation is often observed on these species (Hutchinson et al., 1984; Kitner et al., 2015), and some plant species are known to vary their reproductive strategy between populations or geographic regions (Rick et al., 1977; Stebbins, 1957; Vogler and Kalisz, 2001). For example, it is suggested that members of the *Lactuca* genus tend to be more allogamous towards their centre of origin (Kitner et al., 2015; Lindqvist, 1960).

Wind is the primary vector of seed dispersal in both species and is facilitated greatly by their seed morphology, although there is likely some contribution from other dispersal vectors. Weaver and Downs (2003) propose that water dispersal may be an important factor for prickly lettuce, citing the apparent concentrations of specimens along waterways in their examination of the distribution of this species in Canada. However, this could also be due to more suitable habitats being found in proximity to water and, therefore, greater chance for establishment in these areas by seeds dispersed there by wind, or by collection bias towards more easily-navigable watercourses in the herbarium specimens used in their study. Hutchinson et al. (1984) noted that animal vectors may play a minor role in annual sowthistle dispersal either through ingestion of seeds or attachment of the pappus to the coat of an animal. Achenes of various *Sonchus* species are known to form a part of the diet of some North American birds, and may remain viable after ingestion (Hutchinson et al., 1984). Attachment of achenes to the feathers of birds is a possible explanation for the presence of pappus-bearing species of Asteraceae on remote islands to which dispersal by wind alone would have been unlikely (Sheldon and Burrows, 1973). However, while both water-vectored and animal-vectored scenarios are entirely plausible for both annual sowthistle and prickly lettuce in some situations, their overall contribution is likely to be minor in comparison with wind dispersal.

1.4.2 Model-based and experimental methods of studying gene flow

Although wind-dispersal can be complicated, models of wind-dispersal are now more highly advanced than models for any other seed dispersal vector (Nathan et al., 2011). Even the most complicated of wind dispersal models are based on a basic ballistic function (Equation 1) where the dispersal distance D is determined by the seed release height h_t, the mean horizontal wind speed $\bar{u}$ and the terminal velocity of the seed V_t. More complex models build on and address some of the assumptions of this simplified model.

$$D = \frac{h_t \bar{u}}{V_t} \qquad \text{(Equation 1)}$$

Andersen (1993) experimentally measured seed terminal velocity of a number of wind-dispersed Asteraceae species and found values of 0.2816 ms^{-1} for annual sowthistle and 0.4336 ms^{-1} for prickly lettuce. The study also found a high correlation (r=0.9473) within these species between the seed terminal velocity and the square root of plume loading. Plume loading, equivalent to the concept of wing loading, is the ratio of a dispersal propagule’s weight to its area (Matlack, 1987). In the case of wind-dispersed members of Asteraceae it can be calculated using achene weight, or volume, in relation to the pappus area as in Andersen (1993). Křístková et al. (2014) noted significant variation in prickly lettuce achene morphology as a function of geographic location, resulting in different values of terminal velocity.

1.4.3 Molecular approaches to studying gene flow based on population structure

Due to the difficulties of experimentally tracking small-seeded, wind-dispersed species and the complexity involved in applying ballistic models of dispersal in highly variable environments, genetic studies of geographic population structure are increasingly being applied to make inferences about gene flow and dispersal (Bullock et al., 2006; Cruzan and Hendrickson, 2020; Nathan et al., 2003). Quantification of gene flow within a weed species can be achieved by using herbicide resistance as a molecular marker and considering the biology, reproductive strategy and dispersal mechanism of the species (Mallory-Smith et al., 2015). Assessments of population genetic structure using SNPs (Single Nucleotide Polymorphisms) can also be used to gain inferences on the evolutionary relationships within and between populations, and recent advances have made these methods accessible, scalable and applicable even for species without a reference genome available (Elshire et al., 2011; Peterson et al., 2012; Poland et al., 2012).

1.5 Management of annual sowthistle and prickly lettuce in broadacre agriculture

Management of these species can be difficult due to their prolific seed production, highly mobile wind-borne seed and ephemeral nature, and is also exacerbated by herbicide resistance. In southern Australia, ALS inhibitor resistance is very widespread, negating one of the main control options in broadleaf crops. Pulse crops, in particular, offer few herbicide options and relatively low crop competition (McDonald et al., 2007). Recently there has been heavy reliance on the use of imidazolinone herbicide tolerant crop technology in pulse crops in southern Australia.

1.6 Management in lentil crops

Lentils in particular pose a challenge, because they are a weakly competitive crop due to their short stature and low early vigour and have a long critical period of weed control (Fedoruk et al., 2011; McDonald et al., 2007). This provides favourable conditions for proliferation of prickly lettuce and annual sowthistle, as seed production is inversely related to competition (Amor, 1986b; Hutchinson et al., 1984; Weaver and Downs, 2003). Lentils also have little natural tolerance to herbicides and very few post-emergence options are available (McDonald et al., 2007), so the introduction of imidazolinone-tolerant varieties was a welcome development. However, this has led to concerns over the level of use of these herbicides, their propensity to select for resistance, and the extent of imidazolinone resistance present in populations of prickly lettuce and annual sowthistle. The lack of safe and effective post-emergence herbicide options in lentil crops places greater importance on controlling weeds effectively prior to sowing and employing non-herbicidal tactics.

1.6.1 Chemical control

Relatively few published studies have evaluated alternative herbicide regimes on annual sowthistle or prickly lettuce in southern Australia. One such study from Victoria examined the control of prickly lettuce in wheat and chickpeas using a variety of ALS inhibitors (in the wheat phase only), PSII inhibitors, PPO inhibitors (HRAC Group 14), and synthetic auxins (Amor, 1986a). In the wheat phase they found that only the ALS inhibitors and synthetic auxins provided a good balance of prickly lettuce control and crop safety (Amor, 1986a). Considering prickly lettuce has evolved resistance to both these groups of herbicides (Heap, 2022), they may no longer be effective recommendations. In chickpeas, several of the PSII inhibiting herbicides showed promise (Amor, 1986a). Widderick and Walker (2009) recommended synthetic auxins for control of sowthistle in wheat crops. These herbicides provide effective levels of control but emerging resistance in southern Australia is a concern.

Amjad and Hashem (2016) found that most fallow treatments tested provided highly effective control of annual sowthistle in Western Australia, but recommended a double knock of glyphosate and a PSI inhibitor (HRAC Group 22), such as paraquat, for its effectiveness on other weed species and reduced risk of resistance evolution. Wu et al. (2019) also found good levels of control in most fallow treatments, including those using ALS-inhibitors (in combination), glyphosate and synthetic auxins. However, to avoid resistance problems, treatments including herbicides with other modes of action, such as glufosinate, amitrole, and ammonium thiocyanate, should be used in rotation. Control of both weeds is greatly enhanced by treating young seedlings, which are more susceptible to herbicides (Widderick, 2019; Widderick and Walker, 2009; Wu et al., 2019).

1.6.2 Integrated weed management (IWM) approaches

Many recent recommendations from management trials on these species stress the importance of IWM strategies and caution against management choices that could increase the risk of resistance (Amjad and Hashem, 2016; Widderick and Walker, 2009; Wu et al., 2019). Selecting fields with low broadleaf weed seed burden is one of the most important considerations. Selection of more vigorous lentil cultivars and moderately increasing lentil planting density can provide some improvement of competition in this crop (McDonald et al., 2007).

Widderick and Walker (2009) stressed the importance of non-herbicidal tactics such as tillage of localised areas following significant flushes of germination, but cautioned against deep burial of seeds which can promote longer persistence of the seed. They also emphasise the importance of crop competition during the growing season.

Another complication in management is the wind-borne and highly mobile nature of the seed. Studies have demonstrated that prickly lettuce seed can move tens of kilometres from parent plants (Lu et al., 2007), and annual sowthistle is likely to have similar dispersal potential, meaning that management of these species requires a landscape-level approach in addition to control at the site level. Both species readily colonise roadsides and other uncropped, disturbed areas, serving as a source for seed dispersal (Hutchinson et al., 1984; Weaver and Downs, 2003). For these reasons, many growers view these species as somewhat out of their control. As such, little effort is put into long-term IWM practices and instead management is focused on within-season and within-field herbicidal control.

1.7 Conclusion

Annual sowthistle and prickly lettuce are difficult to control in lentil crops, particularly due to widespread ALS inhibitor resistance and the mobile nature of their seed. ALS inhibitors are some of the key herbicides used for control of broadleaf weeds, particularly in ALS-inhibitor-tolerant crop varieties. The frequency and levels of ALS inhibitor resistance in annual sowthistle and prickly lettuce in southern Australia are high enough that they pose a significant threat to sustainability of lentil production. Some details about ALS inhibitor herbicide resistance in these species remain unknown, such as the full spectrum of resistance-endowing mutations present in the landscape, their relative abundance, and implication for resistance levels to both the sulfonylurea and the imidazolinone herbicides. Furthermore, the implications of the amphidiploid nature of annual sowthistle as it relates to resistance genotype and phenotype are unknown. Genetic studies of populations structure can also help to understand patterns of gene flow and resistance evolution in these species, which may help to inform on the risks of evolution and spread of emerging cases of resistance, such as to the synthetic auxins and glyphosate. Finally, additional strategies and tools are required to improve management of these species, particularly in pulse crops such as lentils. The objectives of this project are:

- To test annual sowthistle and prickly lettuce populations in the region to quantify the extent of resistance to ALS inhibitors, synthetic auxins, glyphosate, and other groups.
- To determine the spectrum of ALS inhibitor resistance mutations present in lentil production areas of South Australia and determine their effect on level of resistance to the sulfonylurea and imidazolinone herbicides
- To study the genetic structure of populations of both weed species to make inferences on gene flow, and the evolution and spread of resistance.
- To examine the effect of an ALS gene duplication in amphidiploid annual sowthistle on resistance expression and implications for the evolution and spread of resistance
- To undertake management trials examining the effects of management in phases of rotation prior to lentils, given the highly mobile nature of the seed
- To evaluate herbicide treatments in a breeding line of metribuzin tolerant lentils under development.

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2 CHAPTER 2: POINT MUTATIONS INCLUDING A NOVEL PRO-197-PHE MUTATION CONFER CROSS RESISTANCE TO ACETOLACTATE SYNTHASE (ALS) INHIBITING HERBICIDES IN *LACTUCA SERRIOLA* IN AUSTRALIA

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Unsubmitted Manuscript

Statement of Authorship

Title of Paper	Several different point mutations including a novel Pro-197-Phe mutation confer cross resistance to acetolactate synthase (ALS) inhibiting herbicides in *Lactuca serriola* in Australia
Publication Status	Unpublished and unsubmitted work written in manuscript style
Publication Details	

Principal Author

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Contribution to the Paper	Participated in planning the study, conducted experiments, analysed and interpreted data, and wrote the manuscript		
Overall percentage	80%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/03/2022

Co-Author Contributions

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I. The candidate's stated contribution to the publication is accurate (as detailed above)
II. Permission is granted for the candidate to include the publication in the thesis; and
III. The sum of all co-author contributions is equal to 100% less the candidate's contribution

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Short title: Prickly lettuce ALS mutations

Point mutations including a novel Pro-197-Phe mutation confer cross resistance to acetolactate synthase (ALS) inhibiting herbicides in *Lactuca serriola* in Australia

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Abstract

Control of prickly lettuce has become increasingly difficult for lentil growers in southern Australia due to a lack of herbicide options, widespread herbicide resistant populations, and prolific production of highly mobile seed. This study aimed to quantify herbicide resistance in the Mid North (MN) and Yorke Peninsula (YP) of South Australia, identify and characterise the mechanisms of resistance, and investigate population structure and gene flow in this species. Seed of prickly lettuce was collected from across the study region and tissue samples were collected from a single field on the Yorke Peninsula. Seed samples were subjected to resistance screening at field rates of chlorsulfuron and imazamox + imazapyr and resistance was found in all populations, with average survival of 92% to chlorsulfuron and 95% to imazamox + imazapyr. Five different amino acid substitutions were found at the Proline-197 position of the ALS gene, with one (Pro-197-Phe) not yet reported in any other weed species. There was no significant difference between mutations in LD_{50} to sulfonylurea, but imidazolinone resistance was higher in plants with the phenylalanine mutation and lower in plants with the serine mutation. Population structure based on *snps* provided evidence both for the independent evolution of the same mutation in different populations, as well as frequent short to medium distance dispersal accompanied by occasional long-distance dispersal events, resulting in mobile resistance genes. Continued use of the imidazolinone herbicides will likely select for mutations conferring higher levels of resistance such as the Pro-197-Phe mutation and lead to further spread of resistance.

Key words: Prickly lettuce, ALS inhibitor resistance, target site mutations, genotyping-by-sequencing, population genetic structure, herbicide resistance evolution

2.1 Introduction

Prickly lettuce (*Lactuca serriola*) is becoming increasingly difficult to control in southern Australian pulse crops, with lentils presenting particular concern. This is due to production of abundant and highly mobile seed (Weaver and Downs, 2003), and to a lack of safe and effective herbicide options in lentils (McDonald et al., 2007). Lentil growers in southern Australia have recently begun to use imidazolinone-tolerant varieties to provide control of broadleaf weeds, including prickly lettuce. However, prickly lettuce populations already have widespread resistance to the sulfonylurea class of ALS-inhibiting herbicides (HRAC Group 2) (Lu et al., 2007).

The ALS inhibiting herbicides have the highest susceptibility to resistance evolution of any group of herbicides, comprising roughly a third of all documented cases of resistance covering 104 dicot and 65 monocot weed species (Heap, 2022). Upon their release, these herbicides quickly became popular due to their high efficacy, even at extremely low doses (Tranel and Wright, 2002). However, a high degree of efficacy also provides strong selection pressure for resistance evolution. This, combined with their widespread use, raised early concerns about their propensity to select for herbicide resistance. The first case of resistance to ALS-inhibiting herbicides was in a population of rigid ryegrass (*Lolium rigidum*) from Australia detected very shortly after commercial release in the early 1980s (Heap and Knight, 1986). Within fifteen years, resistance had been documented in more than 60 weed species from every continent except Antarctica (Tranel et al., 2022).

ALS inhibiting herbicides have a high propensity for resistance evolution because resistance to this group of herbicides is controlled by a single, dominant, nuclear gene and is associated with little or no fitness penalty (Tranel and Wright, 2002). Furthermore, over the years it has become apparent that a great number of possible point mutations at a variety of sites in the ALS gene can confer resistance (Tranel and Wright, 2002; Tranel et al., 2022; Yu and Powles, 2014).

While non-target site resistance to the ALS inhibitors has been reported in a number of species, target site resistance is the most common resistance mechanism (Yu and Powles, 2014). Point mutations at eight different sites on the ALS gene have been recorded in resistant individuals of various species to date (Tranel et al., 2022). The most common is the Proline-197 position (numbering based on the ALS sequence of *Arabidopsis thaliana*), with eleven different amino acid substitutions reported across 40 different weed species (Tranel et al., 2022). Different amino acid substitutions at each of the sites have been shown to cause differing profiles of cross resistance across chemical families within the ALS-inhibitor group of herbicides, and the resistance phenotype of a given substitution at a given position can vary between species (Tranel et al., 2022; Yu and Powles, 2014).

Thus far, resistance-causing amino acid substitutions of the ALS gene have been reported only at the Proline-197 site in prickly lettuce (Tranel et al., 2022). ALS inhibitor resistance in prickly lettuce was first detected in 1987 in the United States, in a population from Idaho which was found to contain a histidine substitution conferring broad resistance to the sulfonylureas and weaker, varying resistance to the imidazolinones (Guttieri et al., 1992; Mallory-Smith et al., 1990). Shortly thereafter, the first Australian case was documented in 1994 from two populations in South Australia that were found to have a threonine substitution with a similar resistance profile (Preston et al., 2006). Prickly lettuce resistance monitoring surveys conducted in South Australia in 1999 and 2004 identified populations with both the previously reported histidine and threonine substitutions, as well as two new substitutions: serine and leucine (Lu et al., 2007). These surveys established that resistance to the sulfonylureas was widespread in prickly lettuce with 66% of populations collected in 1999 and 82% of populations collected in 2004 containing resistant individuals.

Prickly lettuce produces prolific numbers of highly mobile seeds. Although seed production is variable and highly dependent on resource availability, estimates of maximum seed production range from 48,000 to 200,000 seeds per plant in the absence of crop competition (Amor, 1986; Weaver and Downs, 2003). Each of these small seeds is highly adapted to wind dispersal due to the presence of sail-like pappi that allow the seed to be carried away from the parent plant (Weaver and Downs, 2003). As such, resistance genes are expected to be highly mobile and resistance is likely to spread quickly. The extent to which seed dispersal drives the spread of resistance in this species and how often the same resistance-causing mutations have evolved independently remains unknown. Due to the difficulties of experimentally tracking small-seeded, wind-dispersed species, genetic studies of geographic population structure are increasingly being applied to make inferences about gene flow and dispersal (Bullock et al., 2006; Cruzan and Hendrickson, 2020; Nathan et al., 2003).

The purpose of this study was to investigate the extent of imidazolinone resistance in prickly lettuce in South Australia, characterise the mutations present and their effect on resistance phenotype, and combine this data with an analysis of the population structure of prickly lettuce to understand the evolution and spread of resistance across the landscape.

2.2 Materials and methods

2.2.1 Sources of plant material

The Mid North (MN) and Yorke Peninsula (YP) regions of South Australia are landscapes dominated by annual winter crop production systems including cereal, oilseed and pulse crops. These regions have a Mediterranean climate characterised by hot, dry summers and cool, wet winters. Plant material was sampled at two different spatial scales: from a single field near Port Clinton on the YP (YP-PC) to study the distribution of mutations at the site level, and across the MN and YP regions to examine mutations at a regional level and gauge levels of herbicide resistance across the regions.

A lentil field (YP-PC) with a large population of prickly lettuce was sampled in September 2018. Tissue samples were collected from individuals (n=191) within the field and from the edges of neighbouring fields. The location of each individual was recorded using a handheld GPS and individuals were chosen at intervals of no less than 10 m, with sampling spread out to cover the field.

Seed collection of prickly lettuce for resistance screening and regional scale analyses was conducted at seed maturity in January 2019. Seed was collected from cropping fields chosen at random and surveyed as described in Boutsalis et al. (2012). GPS locations and crop type were recorded at each sample site. Mature seed was pooled from individuals within the crop (>40 m from the fence line) to form a representative sample of the population. In addition, if individuals were present along the field edge (<20 m from the fence line) or adjacent roadside, seed was collected from these plants separately and treated as a separate "population" associated with the same GPS location. Samples were then air dried and stored at ambient temperature until screening.

Samples from other regions were also included to provide a broader geographic perspective in the population genetic analysis. Six samples each collected from the Adelaide Hills (AH) and the South East of South Australia (SE) were included. The Adelaide Hills are characterised by grazing, viticulture and small-scale horticulture and have very little history of use of ALS-inhibiting herbicides. The South East is characterised by mixed cropping and grazing, and lentils are rarely grown.

2.2.2 Herbicide resistance screening

Plants were grown outdoors during the 2019 winter growing season (May-July) at the Waite Campus of the University of Adelaide in Urrbrae, SA, Australia (34.97°S, 138.64°E) in a coco-peat potting mix (Boutsalis et al., 2012) and watered as needed. A small sample of seed of each population was sown on the surface of 0.9 L punnet pots (Masrac Plastics, Adelaide, Australia) for germination, transplanted at the one-leaf stage for a density of six plants per 0.9 L pot, and herbicide was applied at the 4-5 leaf stage. A laboratory moving boom cabinet sprayer equipped with Teejet flat fan nozzles (TeeJet 110015, TeeJet Technologies Australia) was used to apply herbicides (Table 1) at a water output of 118 L ha^{-1} at 300 kPa and 1 m s^{-1} to two pots (12 plants) per population. Herbicide screening rates are approximate to the recommended field rate for each herbicide.

The number of survivors was assessed 28 days after herbicide application, with plants remaining alive and actively growing classified as survivors or resistant individuals, and dead plants classified as susceptible. Data analysis of herbicide screening data was conducted using R Statistics Package (R Core Team, 2022). A Chi-square test of independence was used to test for interactions between average survival and crop type and sample location (crop, edge, road).

Table 1. Herbicide active ingredients used in the experiments, including rate used in the resistance screening experiments, trade names and manufacturers.

Active ingredients	Screening rate g ai ha^{-1}	Trade name	Manufacturer
Chlorsulfuron	15	Glean®	Dupont Pty Ltd, Australia
Metsulfuron-methyl	n/a*	Ally®	Dupont Pty Ltd, Australia
Imazamox + imazapyr	24.75 + 11.25	Intervix®	BASF Pty Ltd, Australia
Glyphosate	540	Roundup PowerMAX®	Nufarm Pty Ltd, Australia
2,4-D	455	Amicide Advance®	Nufarm Pty Ltd, Australia

*Metsulfuron-methyl was used in dose response experiments only

2.2.3 Identification of ALS gene mutations

Tissue samples of the youngest green leaf (approximately 50-100 mg) were taken from plants growing at YP-PC to investigate resistance mutations within a site, and from survivors of herbicide resistance screening to investigate resistance across the landscape. Tissue was stored at -80°C for DNA extraction, ALS gene amplification and sequencing. DNA extraction was completed using an Isolate II Plant DNA kit (Bioline, Eveleigh, NSW, Australia) as per the manufacturer's instructions. A random selection of samples was quantified using a spectrophotometric NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and extracted DNA was stored at -20°C.

Polymerase chain reaction (PCR) was used to amplify the target region of the ALS gene using primers that were designed from the genome sequence of annual sowthistle (J. Hereward, unpublished data) and described in Petersen (2018) (Table 2). These primers were also used for prickly lettuce. Two sets of forward and reverse primer pairs were used to cover the entire target region (~1.5 kb) of the ALS gene known to contain resistance-causing mutations.

PCR reactions comprised 1 µl of extracted DNA solution (containing between 5-100 ng DNA), 7.5 µl of 2X MyFi mix (Bioline, Eveleigh, NSW, Australia), 0.6 µl each of forward and reverse primer, and were made up to 15µl using sterile water. The shorter fragment (covering known mutation sites Ala 122, Pro 197 and Ala 205) was amplified in a Thermocycler using the following conditions: 1 min denaturing at 95°C, 35 cycles of 15 s elongation at 95°C, 15 s of annealing at 58°C, and 15 s of elongation at 72°C. For the longer fragment (covering known mutation sites Asp 376, Arg 377, Trp 575, Ser 653 and Gly 654), elongation time was increased to 20s. PCR products were checked by gel electrophoresis against an EasyLadder DNA molecular weight marker (Bioline, Eveleigh, NSW, Australia) and a negative control was included for each set of reactions to control for contamination. Successful samples were sent for Sanger sequencing by the Australian Genome Research Facility (AGRF, Adelaide, SA, Australia) using the relevant sets of primers (Table 2).

Table 2. Sequences of primers used to amplify the ALS gene from genomic DNA of annual sowthistle and prickly lettuce individuals. Two pairs of primers were used and the amino acids covered (from analogous *Arabidopsis thaliana* ALS gene sequences) and total fragment length are listed. Primer sequences were sourced from Petersen (2018).

Coverage of the ALS gene				
Amino acids*	Mutation sites	Product Length	Direction	Primer sequence 5’ – 3’
87 to 248	Ala 122, Pro 197, Ala 205	483	Forward	CTTTTGTCTCCCGCTTCGCCC
			Reverse	CCAGGTCGGCCGGAAGTTGC
349 to 670	Asp 376, Arg 377, Trp 575, Ser 653, Gly 654	963	Forward	GCTCGGAATGCATGGAACTGTTTAY
			Reverse	GCATACATTAMACAACAAGAAACGAAATTC

*Numbering is based on the sequence of *Arabidopsis thaliana*

ALS gene sequences were aligned with an annotated reference sequence of the ALS gene of *Arabidopsis thaliana*, a sequence from the genome of annual sowthistle (J. Hereward, The University of Queensland, unpublished data) and a sequence from a known susceptible population of prickly lettuce (a line commonly used as a standard susceptible control) using Geneious (Biomatters Ltd, Auckland, New Zealand). All sites known to confer resistance to ALS inhibiting herbicides were checked and the amino acid residue was recorded for each individual. Individuals were mapped according to their mutation and GPS location using ArcMap 10.5.1 (ESRI, Redlands, CA, USA).

2.2.4 Dose response experiments

Individuals with specific mutations were planted in larger pots for seed bulking. Seeds were collected from each individual plant separately, stored in paper bags and air-dried at room temperature. Seed of each line, as well as a known susceptible population, was sown onto the surface of trays containing a coco peat potting mixture and transplanted at the early one leaf stage into punnet pots (Masrac Plastics, Adelaide, Australia) at a density of five plants per pot. Plants were grown outdoors at the Waite Campus and watered as needed. Herbicide was applied at the 4-5 leaf stage using a laboratory moving boom cabinet sprayer as described above. Metsulfuron-methyl, a sulfonylurea herbicide and imazamox + imazapyr, an imidazolinone herbicide, were applied at a range of rates to four replicates per population. Metsulfuron-methyl (SU) was applied at 0, 0.09375, 0.1875, 0.375, 0.75, and 1.5 g ai ha^{-1} to standard susceptible controls, and at 0, 3, 6, 12, 24, and 48 g ai ha^{-1} to lines containing mutations. Imazamox + imazapyr (IMI) was applied at 0, 3, 6, 12, 24, and 48 g ai ha^{-1} to standard susceptible controls, and 0, 24, 48, 96, 192, and 384 g ai ha^{-1} to lines containing mutations. Survival was assessed 28 days later according to criteria above, and two experimental runs were completed. Dose response data were analysed using PriProbit (Sakuma, 1998).

2.2.5 Experimental test of outcrossing

The rate of outcrossing was estimated using a cross pollination experiment in which five pairs of resistant (R) and susceptible (S) plants were grown in proximity at the Waite Campus of the University of Adelaide in Urrbrae, SA (34.97°S, 138.64°E). An ALS-susceptible and ALS-resistant biotype was selected so that genetic transfer could be easily verified at the molecular level. Several flower heads were bagged on the S parent plant to exclude pollen and serve as a control to measure any background resistance inherent in the population. Seed from bagged and unbagged flowers on S parent plants was collected and screened for resistance.

A dose response experiment was conducted on the R and S parental lines to select an appropriate discriminating dose of chlorsulfuron for screening. Based on these results, a discriminating dose of 30 g a.i. ha^{-1} was used for prickly lettuce. Herbicide was applied at the 4-5 leaf stage using a laboratory moving boom cabinet sprayer equipped with Teejet nozzles (TeeJet 110015, TeeJet Technologies Australia) calibrated to a water output of 118 L ha^{-1} at 300 kPa and 1 m s^{-1}. Survivors were assessed 28 days after herbicide application (as either dead or alive) and tissue samples of surviving plants, as well as the R and S parents, were collected for ALS gene sequencing. Cross pollination experiment data were analysed using R Statistics Package (R Core Team, 2022). Survival proportions of bagged and unbagged flowers were compared using a t-test.

2.2.6 GBS library preparation and population genetic analysis

Before library preparation, extracted DNA samples were normalised to 2 ng µl^{-1} using a Quant-iT™ PicoGreen® dsDNA kit (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia) and a BioMark machine (Fluidigm Corporation, San Francisco, CA, USA). Samples from six 96-well plates were multiplexed using DNA barcodes: 96 forward adaptors based on Poland et al. (2012), which use 12bp Golay barcodes from Caporaso et al. (2012), provided a unique barcode set within each plate. Twelve reverse adaptors based on Poland et al. (2012) with the addition of variable length (8-12bp) reverse barcodes (one per row, offset by two rows between each plate to provide unique forward/reverse adaptor combinations) were used to internally index the samples. Each of the six libraries also had a unique Truseq-style i7 index. The library preparation protocol was developed based on Elshire et al. (2011) with modifications as described in Hereward et al. (2020). The pooled library was submitted for Illumina sequencing at Novogene (NovogeneAIT Genomics, Singapore).

GBS sequencing outputs were demultiplexed, de-novo assembled and *snps* were called using Stacks using the settings -m4 -M3 and -n3 (Catchen et al., 2013). A variant call format (vcf) file was produced using the populations command in Stacks (Catchen et al., 2013). The vcf files were then filtered using VCFtools (Danecek et al., 2011) based on the methods described in Etebari et al. (2021) and Hereward et al. (2020). First, the data were filtered to a minor allele count of 3 (one homozygote and one heterozygote) which allows likely erroneous singleton *snps* to be removed while retaining rare alleles (Linck and Battey, 2019). Minimum depth was set to 5 and only biallelic *snps* were kept. Then files were filtered for missing data using the following steps: markers missing more than 50% of data were removed (i.e. >50% of individuals were not genotyped at that marker). Then, individuals with missing data at more than 50% of markers was removed, to exclude individuals with bad quality genotyping. Finally, any marker missing more than 5% of data was removed, resulting in a dataset with relatively little missing data. Data were then imported to R Statistics Package (R Core Team, 2022) using vcfR (Knaus and Grunwald, 2017). Principal component analysis (PCA) was carried out using the packages adegenet (Jombart, 2008) and Rcpp (Eddelbuettel and Francois, 2011).

2.3 Results

2.3.1 Herbicide resistance screening

Resistance to both sulfonylurea and imidazolinone herbicides was widespread throughout the MN and YP regions, and survival of prickly lettuce following treatment was generally very high. Every population screened contained resistant individuals and the lowest survival recorded was 50% to chlorsulfuron and 60% to imazamox + imazapyr treatment. Several populations showed 100% survival: 37% of populations screened with chlorsulfuron and 70% of populations screened with imazamox + imazapyr. There was no resistance to glyphosate or 2,4-D confirmed in any population.

Average survival varied with crop type and collection location (crop, edge, or road side) (Table 3). Imidazolinone resistance in prickly lettuce was significantly affected by crop type, with survival levels of populations collected from wheat crops significantly lower than those collected in lentils, chick peas or field peas. Chlorsulfuron resistance was not associated with crop type and was uniformly high, ranging from 86 to 100% survival. Collection location (crop, edge, roadside) had a significant effect on imidazolinone resistance only. Samples collected in crop had lower average survival than those collected from crop edges, with roadside samples exhibiting intermediate survival.

Table 3. The number of prickly lettuce populations screened (n) to chlorsulfuron and imazamox + imazapyr, and the average survival across all individuals are presented as an overall total and separated by crop type and collection location.

	Chlorsulfuron		Imazamox + Imazapyr	
	Populations (n)	Survival (%)	Populations (n)	Survival (%)
Total	27	92	23	95
Crop type[b]				
Field peas	4	95 a	4	97 b
Chickpeas	6	90 a	3	100 b
Faba beans	2	88 a	-	-
Wheat	2	96 a	3	73 a
Lentils	4	86 a	2	100 b
Location				
Edge	10	87 a	9	100 b
Crop	8	94 a	7	88 a
Road side	9	93 a	7	97 ab

In each section of the table, values within a column followed by the same letter are not significantly different at $p = 0.05$.

[b] Crop types with fewer than two populations or 15 individuals were excluded from the analysis.

2.3.2 *Mutation frequency, distribution and resistance phenotype*

Mutations were found only at the Proline-197 site, but several different substitutions were present (Figure 1). All amino acid substitutions detected across the region were also present at YP-PC. Of 191 prickly lettuce samples collected at YP-PC, 169 yielded DNA sequences of acceptable quality for sequencing and a Proline-197 mutation was found in all of them. At the regional scale, DNA samples were obtained from 80 individuals at approximately 30 sample locations. Tissue was collected from survivors of ALS-inhibitor screening and all individuals had a Proline-197 mutation.

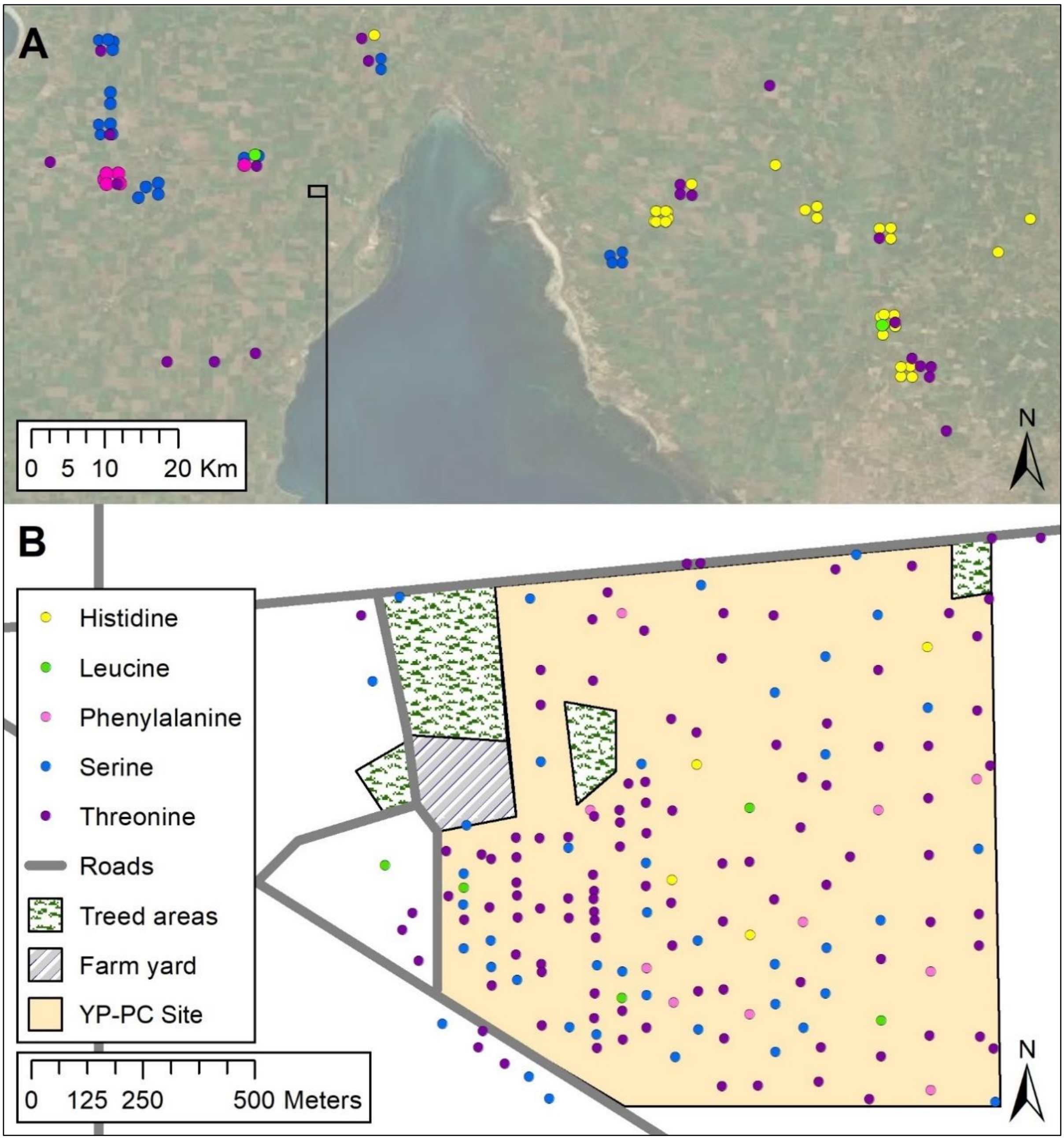

Figure 1. Proline-197 substitutions found in prickly lettuce sampled across the Mid North (MN) and Yorke Peninsula (YP) regions (A), and at the YP-PC site (B). On the regional map (A), several sample locations had multiple individuals tested, so labels have been dispersed to avoid overlap.

There were some regional differences in allele frequency between MN and YP. Pro-197-Ser mutations were much more common in YP, while Pro-197-His mutations were more common in MN. Pro-197-Phe mutations were only found at three sites in YP (including at YP-PC) (Figure 1). Allele frequencies observed at YP-PC were similar to those observed in the wider YP region (Table 4). Pro-

197-Thr mutations were most common, followed by Pro-197-Ser, with Pro-197-Phe, Pro-197-His and Pro-197-Leu also present at lower levels. The additional samples from SE and AH were also examined. Out of the six additional samples collected from SE, four had resistance mutations (all Pro-197-Ser), and out of six from the Adelaide Hills, one had a resistance mutation (Pro-197-His).

Table 4. Proportion of ALS mutations in prickly lettuce at regional and site-level spatial scales and the effect of mutations on LD_{50}s and resistance indices (RI) for metsulfuron methyl and imazamox + imazapyr.

		Percentage of samples				Metsulfuron-methyl		Imazamox + imazapyr	
Amino acid*		MN	YP	YP-PC	Line	LD_{50}	RI	LD_{50}	RI
			%			g ai ha^{-1}		g ai ha^{-1}	
ACC	Pro-197-Thr	35	42	64	Thr-1	--	--	179 (113, 365)	12.6
CAC	Pro-197-His	57	4	2	His-1	34.4 (26.2, 45.4)	217	245 (181, 336)	17.2
					His-2	40.0 (30.2, 53.5)	253	237 (175, 325)	16.7
					His-3	45.5 (34.0, 61.9)	288	188 (137, 261)	13.3
					His-4	47.5 (35.9, 67.1)	300	261 (192, 361)	18.4
					His-5	56.9 (41.1, 80.7)	359	333 (242, 476)	23.5
TCC	Pro-197-Ser	4	38	25	Ser-1	--	--	48.8 (48.8, 48.8)	3.40
TTC	Pro-197-Phe	0	13	6	Phe-1	--	--	523 (304, 2200)	36.8
					Phe-2	26.3 (16.9, 48.9)	166	481 (300, 1750)	33.8
					Phe-3	52.3 (31.1, 126)	330	856 (439, 6410)	60.2
CTC	Pro-197-Leu	4	4	3	Leu-1	34.8 (25.3, 56.5)	220	381 (276, 1890)	26.8
CCC	WT	--	--	0	WT-1	0.145 (0.106, 0.195)		11.1 (5.80, 21.3)	
					WT-2	0.168 (0.125, 0.224)		15.2 (7.80, 29.5)	
					WT-3	0.161 (0.120, 0.214)		16.3 (8.70, 31.6)	

Numbers in parentheses represent upper and lower limits of 95% confidence intervals.

*Underlined bases denote changes from cysteine (C) in the wild-type proline (CCC) at Pro-197

Dose response experiments revealed some variation in resistance phenotype based on amino acid substitution at the proline-197 position, but relative consistency within mutations (Table 4). Although biotypes with the same mutation had some variability, there were no significant differences in LD_{50} within groups containing the same mutation. Populations carrying the wild-type proline (WT) were highly susceptible to metsulfuron-methyl, and all other amino acid substitutions resulted in strong resistance. Resistance index (RI) ranged from 166 times the LD_{50} rate in the susceptible, to 359 times higher. However, confidence intervals for all biotypes tested had some overlap.

Resistance to imazamox + imazapyr was more variable. Populations carrying the wild-type proline were highly susceptible, but the resistance index for the various mutations ranged from 3.4 to 60. The Pro-197-Ser mutation provided the lowest level of resistance, Pro-197-Leu, Pro-197-Thr and Pro-197-His mutations were intermediate and Pro-197-Phe mutations were associated with the strongest resistance. The LD_{50} for the Pro-197-Ser mutation was significantly different to the wild type, but only 3.4 times higher, and had an LD_{50} similar to the field rate of 48 g ai ha^{-1}. The Pro-197-Phe mutation had RI ranging from 33.8 to 60.2, and individuals with this mutation had significantly higher LD_{50} than individuals with the Pro-197-Ser or Pro-197-Thr mutations. The one biotype with the Pro-197-Leu mutation that was tested had a slightly lower LD_{50}, but a very wide confidence interval.

2.3.3 Gene flow, genetic diversity and population structure

In the cross-pollination experiment, comparing the frequency of resistance phenotypes detected in the offspring of S parent plants under pollen exclusion and open pollination indicated that outcrossing is rare. No resistance was detected under pollen exclusion, and while some evidence of outcrossing was detected under open pollination (0.143%), this frequency was not significantly different from 0 ($p = 0.391$). ALS gene sequencing of parental plants revealed a wild-type proline at the Pro-197 position in the S parent, and a threonine substitution at the Pro-197 position in the R parent. Offspring of S parent plants that survived screening were sampled and also found to have a threonine substitution at the Pro-197 position demonstrating that cross pollination had occurred.

Following filtering of the sequenced GBS library data, 271 individuals with 701 variant sites were retained in the dataset. The overall inbreeding coefficient (F_{IS}) was 0.5174, indicating an intermediate level of outcrossing even though the cross-pollination experiment failed to demonstrate significant resistance gene transfer between pollen-excluded and open-pollinated flowers. Principal component analysis showed some genetic clustering among the individuals in the study (Figure 2).

A significant proportion of the variance is explained by Principal Component 1 (45.29%), with 9.7% explained by PC2. When individuals are coloured by geographic region of collection (Figure 2A), individuals from the same region show a tendency to be genetically clustered in the PCA, but outliers are also common. An example of this is the individuals collected from YP-PC: while the vast majority are very closely related as one might expect (most are overlapping in the cluster at the bottom left of the plot), approximately six individuals within the field are from different genetic clusters.

When individuals are coloured based on the amino acid residue at the proline-197 position (Figure 2B), individuals with the same mutation did tend to cluster together genetically, but there were outliers present, likely indicating independent evolution of the same resistance mutation in those cases. All mutations except the newly reported Pro-197-Phe mutation feature in more than one genetic cluster. The tight cluster in the bottom left of the plot, representing the majority of YP-PC samples, is dominated by Pro-197-Ser and Pro-197-Thre substitutions, but also contains all the Pro-197-Phe substitutions as well as a small number of Pro-197-Leu and Pro-197-His substitutions.

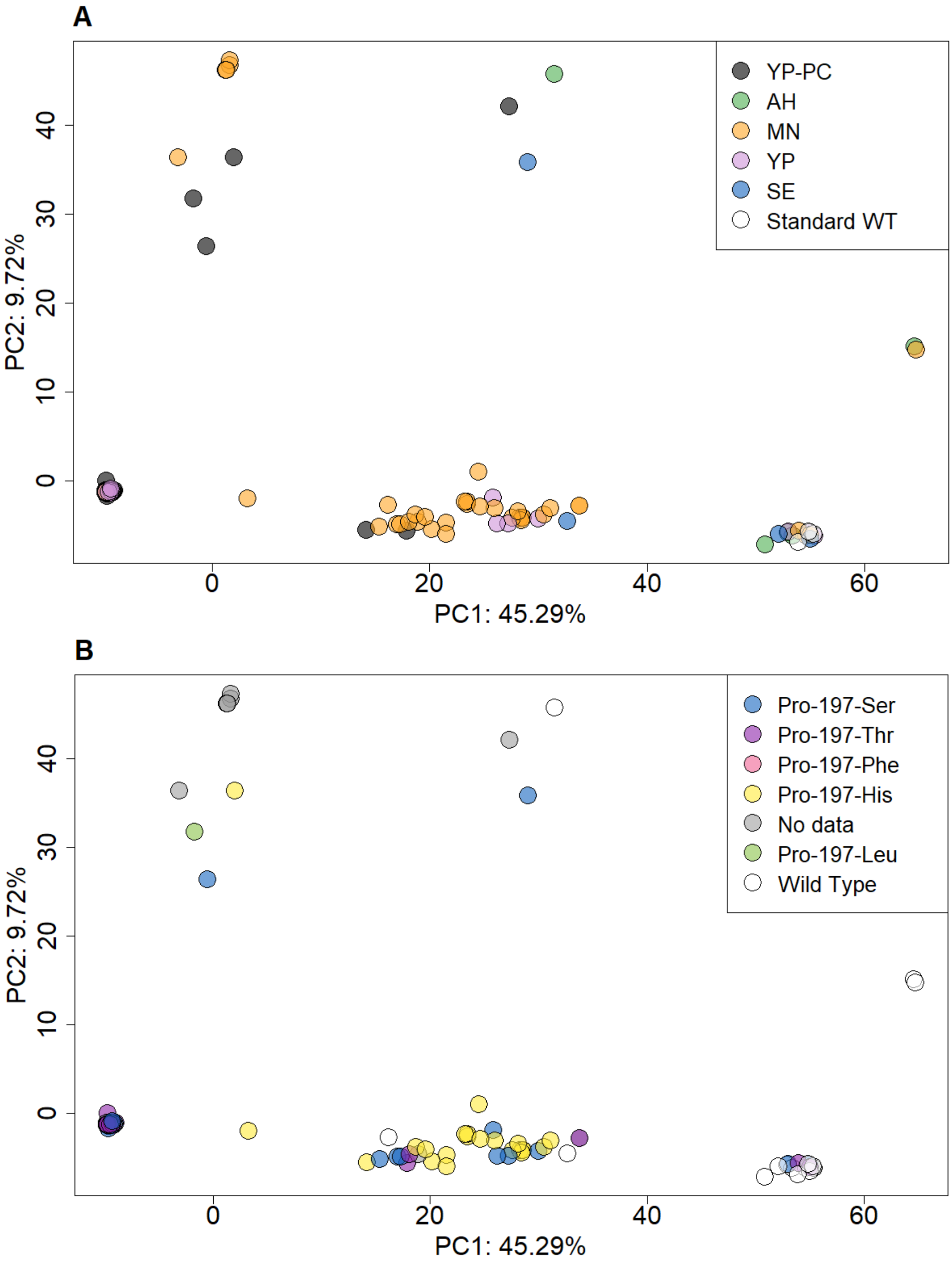

Figure 2. Principal component analysis plot of 271 prickly lettuce individuals on the basis of 701 variant sites, with individuals clustered by genetic similarity. The figures are based on identical genetic data but points are coloured by geographic location of collection (A), and amino acid residue at the Proline-197 position (B). Individuals indicated by ‘no data’ in (B) did not yield ALS gene sequences of acceptable quality for residue at the Pro-197 position to be assessed. MN = Mid North, YP = Yorke Peninsula, WT = wild type, SE = South East South Australia.

2.4 Discussion

Lower average survival to imidazolinone herbicides was observed in prickly lettuce populations collected from wheat crops compared with those found in pulse crops, although the sample size was small. Resistance was also lower in samples collected from within a crop as opposed to the crop edge (Table 3). Imidazolinone-tolerant varieties of both wheat and lentils are popular in these regions, and these crops would have been equally likely to have had a recent in-crop application of ALS inhibiting herbicides. In contrast, chickpeas and field peas have little imidazolinone herbicide use. Lower survival of in-crop samples is also a surprising result given that ALS inhibitors don't tend to be used on roadsides, and crop edges would have received the same treatment as the rest of the crop. Closer analysis suggests these results may be driven by a single population collected in a wheat crop that had low survival to the imidazolinones compared to other populations. This population comprised a single localised patch in an otherwise weed-free field, so is likely to represent the progeny of only a small number of plants.

Previous surveys of prickly lettuce from this region of South Australia have reported the proportion of populations containing sulfonylurea-resistant individuals at 66% in 1999 and 82% in 2004 (Lu et al., 2007), but the 2019 survey in this study found resistant individuals in all populations. As such, the frequency of resistance appears to have increased over time, along with the diversity of mutations resulting in resistance. Shortly following the initial discovery of a Pro-197-Thr substitution in ALS inhibitor-resistant prickly lettuce in the region in 1994 (Preston et al., 2006), a survey in 1999 found Pro-197-Ser, Pro-197-His and Pro-197-Leu substitutions (Lu et al., 2007), and the present study adds a Pro-197-Phe substitution to this list.

In the time since these earlier studies were conducted, imidazolinone herbicides have been heavily used due to the introduction of imidazolinone-tolerant varieties of canola, wheat, barley and lentils. Although the previous studies did not include screening with imidazolinones, this study has found resistance levels in prickly lettuce to be similar to the sulfonylureas (Table 3). This is in stark contrast to the closely related weed annual sowthistle, which shares many biological characteristics with prickly lettuce and is equally problematic for lentil growers in southern Australia. Annual sowthistle also has a long history of resistance to the ALS inhibitors, but only 78% of populations were classified as resistant to the sulfonylureas and 68% resistant to the imidazolinones (Merriam et al., 2018).

Pro-197-His, Pro-197-Thr, Pro-197-Ser and Pro-197-Leu substitutions have previously been reported in ALS inhibitor-resistant prickly lettuce (Guttieri et al., 1992; Lu et al., 2007; Mallory-Smith et al., 1990; Preston et al., 2006). The Pro-197-Phe substitution, however, has not been reported before in any weed species and constitutes a two base-pair change from the wild-type proline (CCC). As such, the Pro-197-Phe (TTC) mutation is likely to have arisen as a further mutation within an individual already carrying the Pro-197-Ser (TCC) or Pro-197-Leu (CTC) mutation. While the Pro-197-Leu mutation is rare across the survey region, the Pro-197-Ser mutation is especially common in YP, where the Pro-197-Phe mutation was found in this study (Figure 1). Pro-197-Ser mutations resulted in an LD_{50} approximate to the field rate of imazamox + imazapyr (Table 4). As such, individuals with this mutation would be only partially controlled by imidazolinone herbicides in the field, providing a selection pressure that is the likely driver of the evolution of the Pro-197-Phe mutation. This highlights how continued used of herbicides on resistant populations can have further consequences. Specifically, in this instance, the Pro-197-Phe mutation has been shown to result in stronger resistance to the imidazolinone herbicides than most of the other mutations present.

None of the mutations encountered resulted in significant differences in LD_{50} to the sulfonylureas (Table 4), which were the first ALS-inhibiting herbicides to be widely used in southern Australia. The 1999 survey reported by Lu et al. (2007) was conducted prior to the release of imidazolinone-tolerant crops in Australia and reflects the selection pressure imposed by the sulfonylureas alone. At that time, all the mutations encountered in that study would have likely provided equal fitness to sulfonylurea treatment, and it wasn’t until the introduction and widespread adoption of imidazolinone-tolerant crops that the increased sensitivity of the Pro-197-Ser mutation to the imidazolinones became important and led to the evolution of the Pro-197-Phe mutation.

Unfortunately crop rotation and recent herbicide use history was not available for the paddocks from which samples were collected, and this is a limitation of the study. This information would have provided further support for the link between evolution of resistance and herbicide use practices. However, due to the seed mobility of this species, over time regional trends would likely have a stronger effect than the herbicide use patterns in a specific paddock.

Individuals with the Pro-197-Phe mutation were genetically clustered with several other individuals having the Pro-197-Ser mutation, as well as some carrying the Pro-197-Leu mutation (Figure 2B), supporting the idea that it could have arisen from an additional single nucleotide change in an individual already carrying the Pro-197-Ser or the Pro-197-Leu mutation. The presence of multiple different resistance mutations in this genetic cluster could indicate that each mutation has arisen in situ within the population, rather than being transported from distant populations. Considered together with the relatively low rate of outcrossing in this species, and the finding of the same resistance mutations in different genetic clusters in the PCR, this suggests that populations have independently evolved multiple different resistance mutations, and that some specific mutations have evolved more than once in this region.

Colouring individuals by geographic provenance showed that individuals from the same area tended to be clustered together, but that some outliers were also present. This pattern is consistent with dispersal patterns dominated by short and medium distance dispersal, along with the occasional long-distance dispersal event. This is consistent with a theory of multiple independent resistance evolution events as a main driver behind the diversity of mutations in the landscape. It also indicates that while resistance mutations can spread through seed dispersal in this species, good herbicide resistance stewardship is still an important line of defence.

The consequences of the stronger resistance caused by the phenylalanine substitution remain to be fully explored. Since most other substitutions already cause resistance levels far exceeding the field rate, the additional consequences of this stronger mutation may not be reflected in the proportion of survivors in the field following herbicide application. However, stronger levels of resistance could potentially be reflected in reduced herbicide injury and, subsequently, greater potential seed production. The effect of the phenylalanine substitution on seed production is a possible future direction for research.

2.5 Conclusion

The results of this study indicate that the vast majority of prickly lettuce populations in this region are resistant to ALS-inhibiting herbicides and resistance is conferred by several different mutations. A new amino acid substitution with elevated levels of resistance to imidazolinone herbicides was also identified, demonstrating the ability of these weeds to acquire new mutations with additive effects on resistance when exposed to continued selection pressure. While the distribution of these mutations show some spatial structure, they are widely distributed and highly variable even at the scale of a single field. This reflects the prolific production of highly mobile seed in this species and supports the theory of both local proliferation of resistance mutations and the independent evolution of the same mutation at multiple times and places. In summary, ALS-inhibiting herbicides have a high propensity for selection of resistance, and their continued use may provide additional difficulties for managing prickly lettuce, as well as other weeds.

2.6 Acknowledgements

This research is supported by an Australian Government Research Training Program Scholarship and a Grains Research and Development Corporation (GRDC) Grains Research Scholarship (project UOA1801-003RSX). Thanks are also extended to David Brunton, Jess Nichols, and Tijana Petrovic for additional field and lab assistance. Special thanks are due to Emeritus Professor Gimme Walter and the Walter Lab at the University of Queensland for hosting lab work in relation to DNA library prep.

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3 CHAPTER 3: POPULATION STRUCTURE OF HERBICIDE RESISTANT *SONCHUS OLERACEUS* IN SOUTH AUSTRALIA: A VARIETY OF PRO-197 MUTATIONS ON TWO COPIES OF THE ACETOLACTATE SYNTHASE GENE CONFER CROSS RESISTANCE IN THIS HIGHLY MOBILE, AMPHIDIPLOID SPECIES

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Unsubmitted Manuscript

Statement of Authorship

Title of Paper	Population structure of herbicide resistant *Sonchus oleraceus* in South Australia: six different Pro-197 mutations on two copies of the acetolactate synthase gene confer cross resistance in this highly mobile, amphidiploid species
Publication Status	Unpublished and unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author	Alicia Merriam		
Contribution to the Paper	Participated in planning the study, conducted experiments, analysed and interpreted data, and wrote the manuscript		
Overall percentage	80%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/03/2022

Co-Author Contributions

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

I. The candidate's stated contribution to the publication is accurate (as detailed above)
II. Permission is granted for the candidate to include the publication in the thesis; and
III. The sum of all co-author contributions is equal to 100% less the candidate's contribution

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Signature		Date	21/02/2022

Short title: Sowthistle ALS mutations

Population structure of herbicide resistant *Sonchus oleraceus* in South Australia: six different Pro-197 mutations on two copies of the acetolactate synthase gene confer cross resistance in this highly mobile, amphidiploid species

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Abstract

Annual sowthistle is an annual weed that is difficult to control in lentil crops in southern Australia due to a lack of herbicide options, widespread herbicide resistance and prolific production of highly mobile seed. This study investigates herbicide resistance in the Mid North (MN) and Yorke Peninsula (YP) of South Australia, identifies and characterises the mechanisms of ALS inhibitor resistance in this amphidiploid species, and combines this with analyses of population structure and gene flow. Seed samples collected across the study region during several years of resistance monitoring surveys were subjected to resistance screening at field rates of chlorsulfuron, imazamox + imazapyr, glyphosate and 2,4-D. No resistance to 2,4-D or glyphosate was detected, however, ALS-inhibitor resistance was widespread and associated with a variety of Proline-197 mutations of the ALS gene, including leucine, alanine, arginine, serine, threonine, and histidine. These mutations were found in different combinations on either of the two copies of the ALS gene. An additional 200 tissue samples were collected from across a single field on the YP and the ALS gene was sequenced for all these individuals. Evidence was found of differing ALS inhibitor resistance profiles between mutation combinations and within mutation combinations, possibly mediated by assortment of the mutations to the ALS gene copies or altered gene expression. Population genetic studies showed evidence of frequent long-distance dispersal, resulting in highly mobile resistance genes, and multiple instances of resistance mutation evolution. Effective control of annual sowthistle needs to rely on reducing seed set and dispersal, particularly in the face of 2,4-D and glyphosate resistance emerging in other regions.

Key Words: ALS inhibitor resistance, amphidiploid, annual sowthistle, genotyping-by-sequencing, population genetic structure, herbicide resistance evolution

3.1 Introduction

Annual sowthistle (*Sonchus oleraceaus* L.) is a globally widespread weed of the Asteraceae family and is common in annual cropping systems around the world (Hutchinson et al., 1984). It has long been problematic in the northern cropping regions of Australia, and more recently has become more common in the southern cropping region of Australia, particularly in lentil crops. This is due to prolific production of highly mobile seed (Hutchinson et al., 1984), a lack of safe and effective herbicide options in lentils in particular (McDonald et al., 2007), and increasingly intractable herbicide resistance issues (Merriam et al., 2018). Cases of resistance to the auxin herbicides have been found from four different areas in the southern cropping region (around 4% of 278 populations, unpublished data) and glyphosate resistance is present in the northern cropping region of Australia (Cook et al., 2014; van der Meulen et al., 2016). In the southern cropping region herbicide resistance problems are largely a result of resistance to the acetolactate-synthase (ALS) inhibiting class of herbicides (HRAC Group 2) (Merriam et al., 2018). This is of particular concern to lentil growers, who are heavily reliant on imidazolinone-tolerant varieties to provide control of broadleaf weeds.

The ALS inhibiting herbicides are highly susceptible to herbicide resistance evolution in weeds, comprising roughly a third of all documented cases of herbicide resistance (Heap, 2022). The evolution of ALS inhibiting herbicide resistance has been documented in 104 dicot and 65 monocot weed species (Heap, 2022). These herbicides became very popular following their release due to their high efficacy at low doses, resulting in cost effective weed management (Tranel and Wright, 2002). However, their potency also provides a strong selection pressure for resistance evolution, which combined with their widespread use led to detection of resistance shortly after their commercial release. The first case of resistance to ALS-inhibiting herbicides was in the early 1980s in a population of rigid ryegrass (*Lolium rigidum* Gaud.) from Australia (Heap and Knight, 1986).

Target site resistance is the most common mechanism of resistance to the ALS inhibitors, although non-target site resistance has also been reported in several species (Yu and Powles, 2014). These herbicides are thought to be particularly susceptible to the evolution of target-site resistance because resistance is controlled by a single, dominant, nuclear gene and is associated with little or no fitness penalty (Tranel and Wright, 2002). Furthermore, point mutations at eight different sites in the ALS gene can confer resistance (Tranel and Wright, 2002; Tranel et al., 2022; Yu and Powles, 2014). The first site reported was Proline-197 (position numbers based on the ALS sequence of *Arabidopsis thaliana* (L.) Heynh.), which still accounts for the greatest number of cases and has eleven different amino acid substitutions reported across 40 weed species (Tranel et al., 2022). Amino acid substitutions at each of the sites have been shown to cause differing profiles of cross resistance to the chemical families within the ALS-inhibitors, and the effect of a given substitution at a given position can even vary between species (Tranel et al., 2022; Yu and Powles, 2014).

The first case of ALS inhibitor resistant annual sowthistle was detected in Australia in 1990 (Boutsalis and Powles, 1995), and surveys of annual sowthistle conducted between 2010 and 2016 across South Australia and Victoria found that 78% of populations were resistant to the sulfonylurea herbicides and 68% were resistant to the imidazolinone herbicides (Merriam et al., 2018). Thus far, resistance-causing amino acid substitutions of the ALS gene have been reported only at the Proline-197 site for annual sowthistle (Petersen, 2018; Tranel et al., 2022). Surveys of annual sowthistle in the Mid North and Yorke Peninsula regions of South Australia in 2017 and 2018 revealed a diversity of amino acid substitutions: serine, leucine, arginine, threonine, and alanine (Petersen, 2018).

Annual sowthistle is an amphidiploid species, where hybridisation of two species resulted in a duplication of chromosome numbers. It was initially proposed that annual sowthistle (n=32) is the result of a hybridisation between *Sonchus asper* (L.) Hill (n=18) and *Sonchus tenerrimus* L. (n=14) (Stebbins, 1953); however, more recent research has provided evidence for multiple ancient hybridisation events with *S. asper* as the maternal parent (Cho et al., 2019). Understanding target-site resistance in this species is complicated by the polyploid nature of annual sowthistle, because it results in two different copies of the ALS gene (one from each putative parental species). This creates the possibility of more than one resistance mutation at the target site. The consequences of multiple gene copies with target site mutations are varied in their effects on resistance expression (Delye et al., 2016; Iwakami et al., 2020; Panozzo et al., 2021), but no studies have examined this relationship in annual sowthistle.

The purpose of this study was to identify ALS mutations present in populations of annual sowthistle in cropping regions of southern Australia and characterise the effect of these mutations on resistance expression, particularly considering the amphidiploid nature of annual sowthistle. The resistance data was then combined with analysis of the population structure of annual sowthistle to better understand the evolution and spread of resistance across the landscape.

3.2 Materials and methods

3.2.1 Sources of plant material

The Mid North (MN) and Yorke Peninsula (YP) cropping regions of South Australia are dominated by annual winter cereal, oilseed, and pulse crops. These regions have a Mediterranean climate characterised by hot, dry summers and cool, wet winters. Plant material was sampled at two different spatial scales for use in experiments: seed was collected from across the MN and YP to examine mutations at a regional level, gauge levels of resistance across the regions, and to characterise the resistance genotype and phenotype of individuals. In addition, tissue samples were taken from a single field near Port Clinton on the YP (YP-PC) to investigate the genetics and distribution of mutations within a site.

Annual sowthistle samples were collected as part of herbicide resistance monitoring surveys conducted in the MN and YP in 2013, 2017 and 2018. Surveys were conducted shortly before harvest, between October and December of each year depending on the region. Seed was collected from cropping fields selected at random and surveyed as described in Boutsalis et al. (2012). GPS locations and crop type were recorded at each sample site. Mature seed of each species was pooled from individuals within the crop (>40 m from the fence line) to form a representative sample of the population. If individuals were present along the field edge (<20 m from the fence line) or roadside, seed was collected from these plants separately and treated as a discrete “population” associated with the same GPS location. Samples were then air dried and stored at ambient temperature until screening.

A lentil field (YP-PC) with a large and evenly distributed population of annual sowthistle was sampled in late September 2018. Tissue samples were collected from individuals (~200) within the field and from the edges of neighbouring fields. The location of each individual was recorded using a handheld GPS at intervals of no less than 10 m, with sampling spread out to cover the whole field.

Some additional samples from geographically distant regions were also included to provide a broader geographic perspective in the population genetic analysis. Five samples were collected from the South East (SE) of South Australia, along with six from New South Wales (NSW). The SE is characterised by mixed cropping and grazing, but lentil crops are grown rarely, and these samples were sourced from over 300 km away from the MN and YP samples. Samples sourced from NSW are glyphosate-resistant biotypes (Cook et al., 2014) from cotton cropping systems roughly 1,200 km away from the MN and YP.

3.2.2 Herbicide resistance screening

Herbicide resistance screening was conducted at the Waite Campus of the University of Adelaide (34.97°S, 138.64°E) during the winter growing season immediately following each survey. Plants were grown outdoors using a coco-peat potting mix (Boutsalis et al., 2012) and watered as needed. For each population a small sample of seed of each population (approximately 100-200 mg) was sown on the surface of 0.9 L punnet pots (Masrac Plastics, Adelaide, Australia) for germination. Plants were transplanted at the one-leaf stage at a density of six plants per 0.9 L pot, and herbicide was applied at the 4-5 leaf stage. Each herbicide (Table 1) was applied to two pots from each population. A laboratory moving boom cabinet sprayer equipped with Teejet flat fan nozzles (TeeJet 110015, TeeJet Technologies Australia) was used to apply herbicides at a calibrated water output of 118 L ha^{-1} at 300 kPa and 1 m s^{-1}. Herbicide screening rates used were within the range of recommended field rates for each herbicide.

The number of survivors was assessed 28 days after herbicide application, with plants remaining alive and actively growing classified as survivors or resistant individuals, and dead plants classified as susceptible. Herbicide screening data was analysed using R Statistics Package (R Core Team, 2022). A Chi-square test of independence was used to test for interactions between average survival and survey year, crop type and sample location (crop, edge, road).

Table 2. Herbicide active ingredients used in the experiments, including rate used in the resistance screening experiments, trade names and manufacturers.

Active ingredients	Screening rate g ai ha^{-1}	Trade Name	Manufacturer
Chlorsulfuron	15	Glean®	Dupont Pty Ltd, Australia
Metsulfuron-methyl	n/a*	Ally®	Dupont Pty Ltd, Australia
Imazamox + imazapyr	24.75 + 11.25	Intervix®	BASF Pty Ltd, Australia
Glyphosate	540	Roundup PowerMAX®	Nufarm Pty Ltd, Australia
2,4-D	455	Amicide Advance®	Nufarm Pty Ltd, Australia

*Metsulfuron-methyl was used in dose response experiments only

3.2.3 *Identification of ALS gene mutations*

Tissue samples of the youngest green leaf (approximately 50-100 mg) were taken from plants growing at YP-PC to investigate resistance mutations within a site, and from survivors of herbicide resistance screening to investigate resistance across the landscape. Tissue was stored at -80°C for DNA extraction, ALS gene amplification and sequencing. DNA extraction was completed using an Isolate II Plant DNA kit (Bioline, Eveleigh, NSW, Australia) as per the manufacturer's instructions. A random selection of samples was quantified using a spectrophotometric NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and extracted DNA was stored at -20°C.

Polymerase chain reaction (PCR) was used to amplify the target region of the ALS gene using primers that were designed from the genome sequence of annual sowthistle (J. Hereward, The University of Queensland, unpublished data) and described in Petersen (2018) (Table 2). Two sets of forward and reverse primer pairs were used to cover the entire region (~1.5 kb) of the ALS gene known to contain resistance-causing mutations. PCR reactions comprised 1 µl of extracted DNA (containing between 5-100 ng DNA), 7.5 µl of 2X MyFi mix (Bioline, Eveleigh, NSW, Australia), 0.6 µl each of forward and reverse primer, and were made up to 15µl using sterile water. The shorter fragment (covering known mutation sites Ala 122, Pro 197 and Ala 205) was amplified in a Thermocycler using the following conditions: 1 min denaturing at 95°C, 35 cycles of 15 s elongation at 95°C, 15 s of annealing at 58°C, and 15 s of elongation at 72°C. For the longer fragment (covering known mutation sites Asp 376, Arg 377, Trp 575, Ser 653 and Gly 654), elongation time was increased to 20s. PCR products were checked by gel electrophoresis against an EasyLadder DNA molecular weight marker (Bioline, Eveleigh, NSW, Australia) and a negative control was included for each set of reactions to control for contamination. Successful samples were submitted for Sanger sequencing by the Australian Genome Research Facility (AGRF, Adelaide, SA, Australia) using the relevant sets of primers (Table 2).

Table 2. Sequences of primers designed from the genome sequence of annual sowthistle used to amplify the ALS gene. Two pairs of primers were used and the domain and amino acids covered (from analogous *Arabidopsis thaliana* ALS gene sequences) are listed. Primer sequences were originally described in Petersen (2018).

Coverage of the ALS gene				
Domain	Amino acids*	Length	Direction	Primer sequence 5’ – 3’
A, C, D	87 to 248	483	Forward	CTTTTGTCTCCCGCTTCGCCC
			Reverse	CCAGGTCGGCCGGAAGTTGC
B, D	349 to 670	963	Forward	GCTCGGAATGCATGGAACTGTTTAY
			Reverse	GCATACATTAMACAACAAGAAACGAAATTC

*Numbering is based on the sequence of *Arabidopsis thaliana*

ALS gene sequences were aligned with an annotated reference sequence of the ALS gene of *Arabidopsis thaliana*, a sequence from the genome of annual sowthistle (J. Hereward, The University of Queensland, unpublished data) and a known susceptible (a line commonly used as a standard susceptible control) using Geneious (Biomatters Ltd, Auckland, New Zealand). All sites known to confer resistance to ALS inhibiting herbicides were checked in the sequences and the amino acid residue was recorded for each individual. Individuals were mapped according to their mutation and GPS location using ArcMap 10.5.1 (ESRI, Redlands, CA, USA).

3.2.4 Dose response experiments

After identifying all ALS-gene mutations present, several mutations, or combinations of mutations of interest were selected and individuals with those genotypes were planted in larger pots for seed bulking. Seeds were collected from each individual plant separately to ensure a pure line containing the mutation of interest. Seed samples of each line were stored in paper bags and air-dried at room temperature. Seed of each line, as well as a standard susceptible control, was sown onto the surface of trays containing a coco peat potting mixture and transplanted at the early one leaf stage into punnet pots (Masrac Plastics, Adelaide, Australia) at a density of five plants per pot. Plants were grown outdoors at the Waite Campus and watered as needed. Herbicide was applied at the 4-5 leaf stage using a laboratory moving boom cabinet sprayer as described above. A sulfonylurea (SU) and an imidazolinone (IMI) ALS-inhibiting herbicide was applied at a range of rates to four reps per population. Metsulfuron-methyl (SU) was applied at 0, 0.09375, 0.1875, 0.375, 0.75, and 1.5 g ai ha^{-1} to standard susceptible controls, and at 0, 1.5, 3, 6, 12, and 24 g ai ha^{-1} to lines containing mutations. Imazamox + imazapyr (IMI) was applied at 0, 3, 6, 12, 24, and 48 g ai ha^{-1} to standard susceptible controls, and at 0, 12, 24, 48, 96, and 192 g ai ha^{-1} to lines containing mutations. Survival was assessed 28 days later according to criteria above, and two experimental runs were completed. Dose response data were analysed using PriProbit (Sakuma, 1998).

3.2.5 Genome assortment and expression

Cloning studies were undertaken for annual sowthistle to determine if assortment of mutations onto each of its two genomes could explain the different expression of herbicide resistance phenotypes observed in dose response experiments. Several mutation combinations exhibiting differential expression among the lines tested were selected for further analysis. Within each mutation combination selected, a highly-resistant and less resistant line were compared. PCR-amplified ALS-gene fragments from each line were cloned into an *E. coli* plasmid vector (TOPO TA Cloning Kit, Thermo Fisher Scientific Australia Pty Ltd, Australia) according to kit instructions and approximately 20 colonies from each line were sampled. DNA was extracted using an Isolate II Plasmid Mini Kit (Bioline, Eveleigh, NSW, Australia) and sequenced using the primers provided with the cloning kit. ALS gene regions were compared with references for ALS from each of the two genomes in annual sowthistle (James Hereward, unpublished data) using Geneious (Biomatters Ltd, Auckland, New Zealand) and the sequences within each individual were allocated to one copy or the other. The two ALS reference sequences were classified as originating from *Sonchus asper* and (putatively) *Sonchus tenerrimus* by comparing with RNAseq data for *S. asper* (Genbank accession number SRX2544259).

Gene expression studies were carried out on two lines with the same mutations, but different genome assortment to determine whether the ALS gene from one genome was expressed more strongly than the other. RNA was extracted from each of the lines using an Isolate II RNA Plant Kit (Bioline, Eveleigh, NSW, Australia) and then used for complementary DNA (cDNA) synthesis using a Tetro™ cDNA Synthesis Kit (Bioline, Eveleigh, NSW, Australia). The ALS gene from these cDNA samples was then amplified by PCR using protocols listed above, and subjected to plasmid cloning as described above. More colonies were selected in this instance (43 from one line and 52 from the other) and DNA was extracted using an Isolate II Plasmid Mini Kit (Bioline, Eveleigh, NSW, Australia) and sequenced using the primers provided with the cloning kit. ALS gene regions from each clone were again assigned to references for the two genomes in annual sowthistle and assessed for uneven expression of the genomes or amino acid substitutions and compared using a two-proportion z-test in R (R Core Team, 2022).

3.2.6 Experimental test of outcrossing

A cross pollination experiment was conducted to measure the rate of outcrossing in five pairs of resistant (R) and susceptible (S) plants each grown in proximity at the Waite Campus of the University of Adelaide in Urrbrae, SA (34.97°S, 138.64°E). Some flower heads were bagged on the S parent to exclude pollen and serve as controls for any background resistance inherent in the biotype. A dose response experiment was conducted on the R and S parental lines to select an appropriate discriminating dose of chlorsulfuron for screening. Based on these results, a discriminating dose of 15 g ai ha^{-1} was selected for annual sowthistle. Finally, tissue samples were taken from the R and S parent in each pair to verify the amino acid at the Pro-197 position to be used as a marker.

After each plant pair had flowered, seed was collected from bagged and unbagged flowers on the S parent plants was collected and screened for resistance. Herbicide was applied at the 4-5 leaf stage as described above. Survivors were assessed 28 days after herbicide application (as either dead or alive) and tissue samples of surviving plants were collected for ALS gene sequencing as described above. Survival proportions of plants originating from seed from bagged and unbagged flowers were compared using a t-test R (R Core Team, 2022).

3.2.7 GBS library preparation and population genetic analysis

DNA samples from both local and landscape level studies were normalised to 2 ng µ^{-1} before library preparation using a Quant-iT™ PicoGreen® dsDNA kit (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia) and a BioMark machine (Fluidigm Corporation, San Francisco, CA, USA). Samples from six 96-well plates were multiplexed using DNA barcodes as described in Poland et al. (2012) and using 12 bp Golay barcodes from Caporaso et al. (2012). Each of the six pools of 96 also had a unique Truseq-style i7 index. The library preparation protocol was developed based on Elshire et al. (2011) and is described in Hereward et al. (2020). Pooled libraries were submitted for Illumina sequencing at Novogene (NovogeneAIT Genomics Singapore PTE LTD).

GBS sequencing outputs were demultiplexed, de-novo assembled and *snps* were called using Stacks using the settings -m4 -M3 and -n3 (Catchen et al., 2013). A variant call format (vcf) file was produced using the populations command in Stacks (Catchen et al., 2013). The vcf files were then filtered using VCFtools (Danecek et al., 2011) based on methods described in Etebari et al. (2021) and Hereward et al. (2020). First the data were filtered to a minor allele count of 3 (one homozygote and one heterozygote) which allows likely erroneous singleton *snps* to be removed while retaining rare alleles (Linck and Battey, 2019). Minimum depth was set to 5 and only biallelic *snps* were kept. Then files were filtered for missing data using the following steps: markers missing more than 50% of data were removed (i.e. >50% of individuals were not genotyped at that marker). Then, individuals with missing data at more than 50% of markers was removed, to exclude individuals with bad quality genotyping. Finally, any marker missing more than 5% of data was removed, resulting in a dataset with relatively little missing data. Data were then imported to R Statistics Package (R Core Team, 2022) using vcfR (Knaus and Grunwald, 2017). Principal component analysis (PCA) was carried out using the packages adegenet (Jombart, 2008) and Rcpp (Eddelbuettel and Francois, 2011).

3.3 Results

3.3.1 Herbicide resistance screening

Resistance to sulfonylurea and imidazolinone herbicides was widespread throughout the MN and YP regions, but there was no resistance to glyphosate or 2,4-D detected in any population. Survival of the annual sowthistle populations following treatment was generally high, with slightly more variation in response to the imidazolinone than the sulfonylurea herbicides. Only 7% of annual sowthistle populations were completely controlled by chlorsulfuron and 13% by imazamox + imazapyr. Previous surveys in the region have classified populations as resistant when they exhibit greater than 20% survival (Boutsalis et al., 2012), and under these measures 90% of MN and 88% of YP populations were resistant to chlorsulfuron, and 84% of MN and 67% of YP populations were resistant to imazamox + imazapyr.

Average survival to herbicides varied with survey year, crop type and sample location (crop, edge, or road) (Table 3). Annual sowthistle populations collected during the 2017 survey exhibited greater survival to chlorsulfuron than other years, but lower survival to imazamox + imazapyr compared to the 2018 survey. Crop type had a significant effect on chlorsulfuron resistance only. Lentil crops were associated with higher survival levels to chlorsulfuron than other pulse crops (field peas, chickpeas and faba beans), with annual sowthistle from barley crops exhibiting similarly high levels of resistance and annual sowthistle from wheat having intermediate levels. There was no significant interaction between crop type and imidazolinone resistance in annual sowthistle, with populations exhibiting a range of survival levels within each crop. Collection location (crop, edge, road) was significant for both herbicides, but the trend was different. Chlorsulfuron resistance was highest among roadside samples and lowest among crop edge samples, while imidazolinone resistance was lowest in roadside samples and highest in crop.

Table 3. Annual sowthistle populations were screened with a sulfonylurea (chlorsulfuron) and an imidazolinone (imazamox + imazapyr) treatment. The number of populations screened (n), and the average survival across all individuals are presented for each survey year, crop type and collection location. Annual sowthistle collected in 2013 was screened with chlorsulfuron only.

	Chlorsulfuron		Imazamox + Imazapyr	
	Populations	Survival (%)	Populations	Survival (%)
Survey				
2013	49	75 a	-	-
2017[a]	32	91 b	29	42 a
2018	45	76 a	41	69 b
Crop type[b]				
Field peas	10	66 a	7	60 a
Chickpeas	10	79 a	9	51 a
Faba beans	2	80 a	-	-
Wheat	36	83 ab	13	60 a
Barley	8	88 b	-	-
Lentils	33	88 b	25	68 a
Location				
Edge	53	72 a	32	52 b
Crop	52	85 b	31	62 c
Road	12	99 c	7	42 a

In each section of the table, values within a column followed by the same letter are not significantly different at $p = 0.05$

[a] The 2017 survey sampled annual sowthistle from pulse crops only (lentils, field peas, and chickpeas)

[b] Crop types with fewer than two populations or 15 individuals were excluded from the analysis.

3.3.2 Mutation frequency, distribution and resistance phenotype

Mutations were found only at the Pro-197 site, although several different amino acid substitution combinations were identified (Figure 1). Of 200 annual sowthistle samples collected at YP-PC, 187 yielded DNA samples of acceptable quality for sequencing and at least one Pro-197 mutation was found in all but one sample. At the landscape scale across MN and YP, DNA samples were obtained from 120 individuals from approximately 80 sample locations. Tissue was collected from survivors of ALS inhibitor screening and all individuals had a mutation at the Pro-197 site in at least one copy of the ALS gene. Some individuals had a mutation in both copies of the ALS gene, and some had a mutation in only one copy. Where two mutations were present, different mutations were often found in each copy of the gene, although there were three individuals within YP-PC and one individual at the regional scale with a serine mutation in both copies.

There were some differences in allele frequency observed between MN and YP across the landscape and at YP-PC (Table 4). At the landscape-level scale, the wild-type allele (WT) was most common in both the MN and the YP, but third most common at YP-PC. The Pro-197-Arg mutant allele had the second highest frequency in the MN and Pro-197-Ser allele was second most frequent in the YP. Within the YP-PC site, Pro-197-Leu allele was most frequent followed by Pro-197-Ala. There was little concordance in allele frequency patterns between the YP-PC site and the YP region, despite YP-PC being located within the YP (Figure 1). Pro-197-His was the least common allele in all areas, found in only one individual in the MN and one in YP-PC.

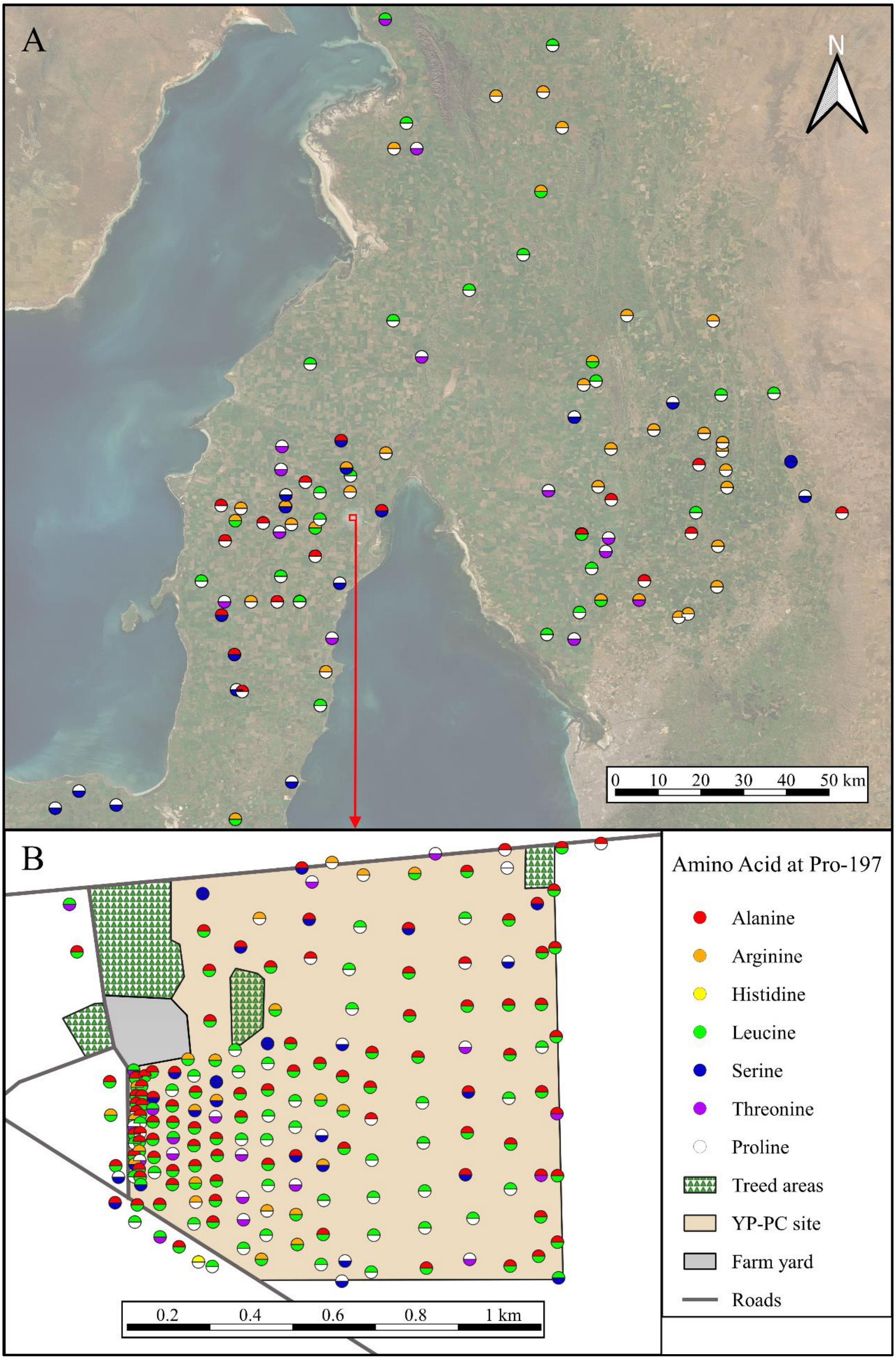

Figure 1. Proline-197 amino acid substitution combinations found in annual sowthistle sampled across the Mid North (MN) and Yorke Peninsula (YP) regions (A), and from within the YP-PC site (B). Each half of the circle represents the amino acid coded on one copy of the ALS gene.

Table 4. Allele and substitution combination frequency at the Proline-197 position of the annual sowthistle ALS gene within each region (MN and YP) and at the single field site (YP-PC). WT = Wild Type, MN = Mid North, YP = Yorke Peninsula.

	Percentage of samples			
Alleles	MN	YP	YP-PC	Overall
		%		
Pro-197-Leu	17	12	37	28
WT	44	36	19	27
Pro-197-Ala	5	13	26	19
Pro-197-Arg	22	14	6	11
Pro-197-Ser	4	16	7	8
Pro-197-Thr	8	9	5	6
Pro-197-His	<1	--	<1	<1
Substitution combinations				
Pro-197-Ala/Pro-197-Leu	2	3	44	26
WT/Pro-197-Leu	25	10	22	20
WT/Pro-197-Arg	34	10	3	11
WT/Pro-197-Thr	12	10	7	9
WT/Pro-197-Ala	9	12	4	7
Pro-197-Arg/Pro-197-Leu	6	5	8	7
WT/Pro-197-Ser	5	13	4	6
Pro-197-Ala/Pro-197-Ser	--	6	6	5
Pro-197-Arg/Pro-197-Ser	2	6	2	3
Pro-197-Leu/Pro-197-Thr	2	2	3	2
Pro-197-Ser/Pro-197-Ser	2	--	1	1
Pro-197-Arg/Pro-197-Thr	2	2	--	<1
WT/Pro-197-His	2	--	<1	<1
Pro-197-Ala/Pro-197-Thr	--	--	1	<1
Pro-197-Leu/Pro-197-Ser	--	--	1	<1
Pro-197-Ser/Pro-197-Thr	--	--	<1	<1
WT/WT	--	--	<1	<1

Substitution combination frequency also varied between MN and YP, and between YP-PC and the landscape-level study (Table 4). YP-PC was strongly dominated by individuals with Pro-197-Ala/Pro-197-Leu allele combination, while individuals at the landscape scale showed more variation. Individuals carrying a mutation on both copies of the ALS gene were also much more common in YP-PC (66%) than at the landscape scale (16% in MN and 24% in YP). Most of the substitution combinations were found in both the field and across the landscape, with only the more uncommon combinations (≤ 1%) found in only one or the other. Pro-197-Arg/Pro-197-Thr allele combinations represented 2% of samples in both the MN and the YP, but was not found at YP-PC. Conversely, several combinations found at YP-PC (at frequencies of ≤ 1%) were not found across the rest of the landscape. Overall, YP-PC contained the greatest diversity of substitution combinations despite being dominated by the most common combination of Pro-197-Ala/Pro-197-Leu. Individuals from the YP encompassed less diversity, but the frequency of each substitution combination was more evenly spread between mutations.

The dose response experiments also revealed differences in resistance phenotype across different allele combinations for both metsulfuron-methyl and imazamox + imazapyr (Table 5). All individuals carrying a mutation on at least one copy of the ALS gene had significantly higher LD_{50} to the sulfonylurea (SU) herbicide metsulfuron-methyl than the wild type. For the imidazolinone (IMI) imazamox + imazapyr, some of the substitution combinations had overlapping confidence intervals with one or more of the WT lines. This occurred for all three WT/Pro-197-Ser lines and one of the WT/Pro-197-Arg lines, as well as line WT/Arg-1. In general, substitution combinations conferring strong resistance to the SU herbicides also conferred strong resistance to the IMI herbicides, with one exception. The WT/Pro-197-Leu combination resulted in the second lowest average LD_{50} to the SU herbicide, but the second highest average LD_{50} to the IMI herbicide, although LD_{50} was quite variable between the six lines with that combination.

Table 5. LD_{50}s and resistance indices (RI) of annual sowthistle mutation combinations following treatment with metsulfuron methyl and imazamox + imazapyr, as well as genome assortment of amino acid residues in selected substitution combination pairs having differing LD_{50} in response to treatment with herbicides.

Substitution combination	Experimental line	Metsulfuron-methyl LD_{50}	Metsulfuron-methyl RI	Imazamox + imazapyr LD_{50}	Imazamox + imazapyr RI	Genome assortment *S. asper*	Genome assortment *S. tenerrimus*
		g ai ha^{-1}		g ai ha^{-1}			
Pro-197-Arg/Pro-197-Leu	Arg/Leu-1	18.1 (10.8, 41.4)	163	142 (94.0, 236)	14.3	Leu	Arg
	Arg/Leu-2	18.6 (10.9, 44.1)	168	109 (73.8, 174)	11		
	Arg/Leu-3	64.1 (29.0, 280)	577	142 (94.0, 238)	14.4	Leu	Arg
Pro-197-Leu/Pro-197-Thr	Leu/Thr-1	16.2 (8.89, 37.8)	146	97.5 (55.3, 203)	9.84		
	Leu/Thr-2	15.9 (9.04, 37.8)	143	78.6 (43.5, 166)	7.93		
	Leu/Thr-3	48.1 (21.5, 195)	433	110 (62.4, 228)	11.1		
WT/Pro-197-Thr	WT/Thr-1	24.3 (13.7, 64.6)	219	48.4 (33.9, 68.9)	4.88		
	WT/Thr-2	33.9 (18.1, 96.8)	305	55.9 (39.3, 80.2)	5.64		
	WT/Thr-3	19.4 (11.5, 44.1)	175	98.5 (68.2, 147)	9.93		
Pro-197-Arg/Pro-197-Ser	Arg/Ser-1	9.72 (5.96, 17.7)	87.5	27.4 (18.8, 39.3)	2.77		
	Arg/Ser-2	39.4 (22.3, 105)	355	50.4 (35.9, 70.6)	5.09		
	Arg/Ser-3	17.5 (11.6, 31.2)	158	36.6 (26.1, 51.1)	3.69		
WT/Pro-197-Arg	WT/Arg-1	21.4 (12.6, 45.5)	193	18.6 (12.9, 25.7)	1.87	WT	Arg
	WT/Arg-2	25.6 (14.3, 58.6)	231	24.0 (17.0, 33.4)	2.42		
	WT/Arg-3	9.39 (5.86, 16.4)	84.6	25.6 (18.5, 35.1)	2.58	WT	Arg
WT/Pro-197-Leu	WT/Leu-1	15.6 (9.93, 25.5)	140	194 (131, 301)	19.6		
	WT/Leu-2	10.5 (7.18, 15.5)	94.6	139 (96.7, 207)	14.1		
	WT/Leu-3	4.14 (2.81, 6.03)	37.3	57.5 (41.2, 80.5)	5.8	Leu	WT
	WT/Leu-4	8.63 (5.82, 12.9)	77.7	84.0 (59.7, 119)	8.48	Leu	WT
	WT/Leu-5	11.6 (7.67, 18.1)	104	106 (75.3, 152)	10.7		
	WT/Leu-6	22.6 (14.3, 37.8)	204	94.5 (66.7, 136)	9.53		
WT/Pro-197-Ser	WT/Ser-1	8.50 (5.85, 12.8)	76.6	19.8 (12.6, 29.3)	2	WT	Ser
	WT/Ser-2	5.00 (3.40, 7.31)	45	21.0 (12.8, 32.9)	2.12	Ser	WT
	WT/Ser-3	5.31 (3.59, 7.91)	47.8	24.6 (15.6, 36.6)	2.48		
WT/WT	WT/WT-1	0.0821 (0.0396, 0.136)		11.1 (7.28, 16.8)			
	WT/WT-2	0.126 (0.0768, 0.182)		10.4 (7.31, 14.8)			
	WT/WT-3	0.125 (0.0735, 0.184)		8.27 (5.80, 11.7)			

Numbers in parentheses represent upper and lower limits of 95% confidence intervals.

While LD_{50} did vary between individuals with the same mutation, in most cases this difference was not significant. In response to SU treatment, line Arg/Ser-1 had lower LD_{50} than line Arg/Ser-2, and line WT/Leu-3 had lower LD_{50} than line WT/Leu-1, line WT/Leu-2, line WT/Leu-5 and line WT/Leu-6. LD_{50} to the IMIs was also lower in line WT/Leu-3 compared to line WT/Leu-1 and line WT/Leu-2. Variation between substitution combinations was not consistently significant, but the line with strongest SU resistance, line Arg/Leu-3, had higher LD_{50} than line Arg/Ser-1, line WT/Arg-3, all WT/Pro-197-Leu lines other than line WT/Leu-6, and all WT/Pro-197-Ser lines. The line with strongest IMI resistance was line WT/Leu-1, with LD_{50} significantly higher than line WT/Leu-3, line WT/Leu-4, line WT/Thr-1, line WT/Thr-2 and all lines with the Pro-197-Arg/Ser, WT/Pro-197-Arg, and WT/Pro-197-Ser combinations.

3.3.3 Genome assortment and expression

Pairs of annual sowthistle lines with the same substitution combination but different LD_{50} did not have different genome assignment of substitutions in most cases (Table 5). Line WT/Ser-1 and line WT/Ser-2 had the serine mutation assigned to different genomes, with line WT/Ser1 having higher SU resistance and the serine mutation found on ALS2. All other pairs tested had the same genome assignment of mutations. However, despite some variation, overlapping confidence intervals (Table 5) between the members of all pairs indicate that genome assortment cannot be ruled out as an explanation for differing LD_{50}s between lines with the same combination.

The gene expression analysis of two lines of annual sowthistle with the same mutation combination, but different resistance phenotype identified no significant difference in ALS gene expression ($p = 0.471$). Following RNA extraction and vector cloning, line WT/Ser-1 had Ser represented on 60% of clones (n=47) while line WT/Ser-2 had Ser represented on 50% of clones (n=42).

3.3.4 Gene flow, genetic diversity, and population structure

The cross-pollination experiment indicated that outcrossing in annual sowthistle is rare. No resistance was detected in individuals sourced from bagged (pollen-excluded) flowers, and while some resistance was detected under open pollination (0.214%), the frequency was not significantly different from 0 (*p* = 0.301). Each S parent plant was wild-type at the Pro-197 position and the R parents had a leucine mutation at Pro-197. Offspring of S parent plants that survived screening were also found to have a leucine substitution at the Pro-197 position showing that cross-pollination had occurred

Following filtering of the sequenced GBS data, 238 individuals with 2324 variant sites were retained in the dataset. The overall inbreeding coefficient (F_{IS}) was 0.3189, indicating an intermediate level of outcrossing despite the fact that the cross-pollination experiment failed to demonstrate significant resistance gene transfer between pollen-excluded and open-pollinated flowers. Principal component analysis (PCA) indicated very little genetic clustering among the individuals in the study, with the first two Principal Components accounting for only a small amount of the overall variance (5.96% by PC1 and 4.87% by PC2) (Figure 2).

When individuals were sorted by geographic region and year of collection (Figure 2A) very little genetic clustering was evident across samples collected within South Australia. Some of the samples from NSW, over 1000 km away, and the standard wild type (WT) susceptible (which was originally collected from an island in the Port River (Adelaide, South Australia) but has been inbred at the Waite Campus for several generations) were placed in distinct clusters. The individuals collected from YP-PC encompass the same range of variation detected in individuals collected across the MN and YP regions. The cluster in the bottom left is composed of two MN 2013 individuals, three YP 2017/2018 individuals, and several individuals from YP-PC, whereas the cluster in the bottom right is made up of one YP 2013 individual and several individuals from YP-PC.

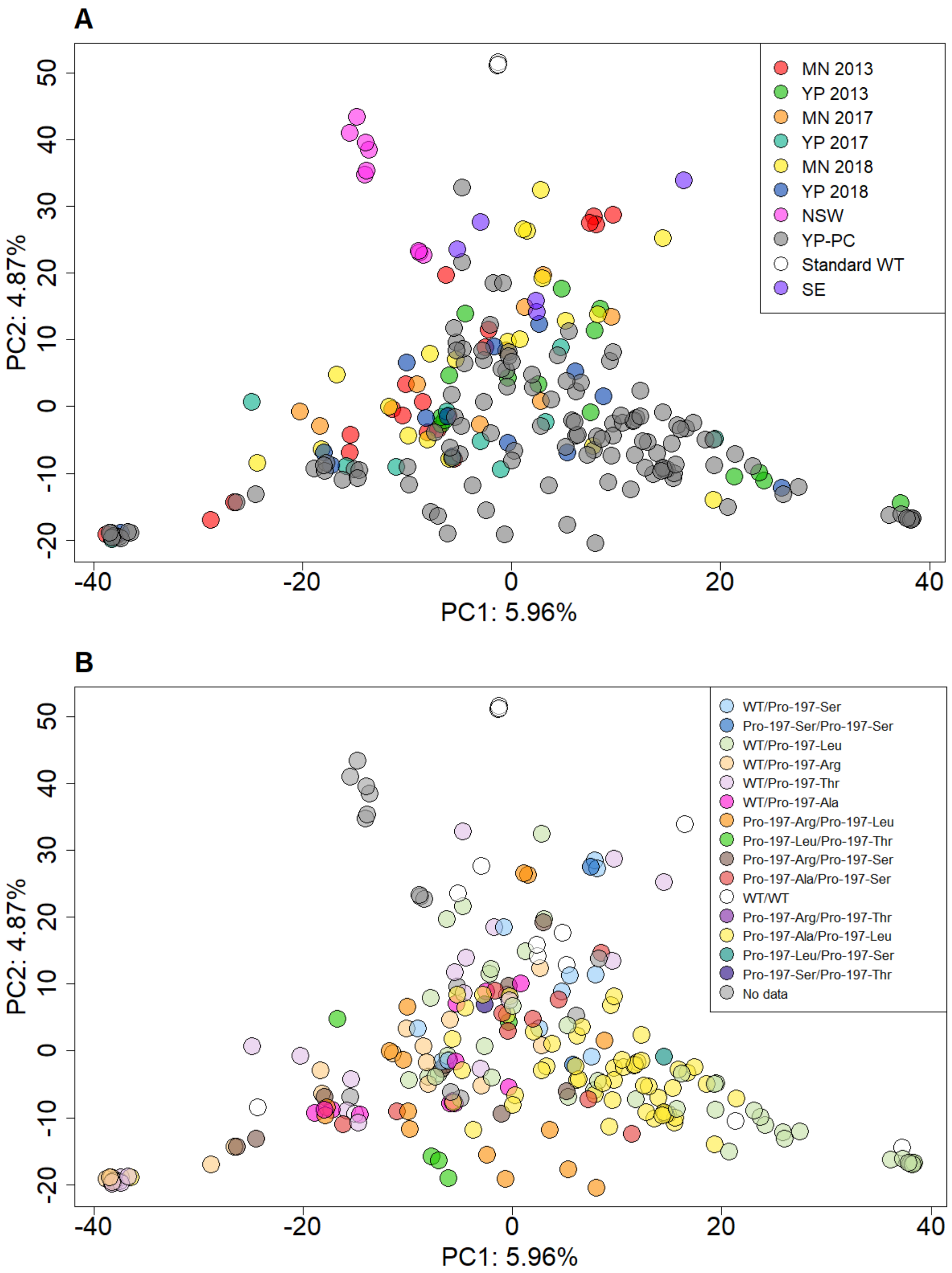

Figure 2. Principal component analysis plot of 238 annual sowthistle individuals based on 2,324 variant sites; clustering of individuals represents their genetic similarity. The two figures are based on identical genetic data, but points are colouredby geographic location/year of collection (A), and amino acid residue at the Proline-197 position on each copy of the ALS gene (B). Individuals indicated by ‘no data’ in (B) did not yield ALS gene sequences of acceptable quality for the residue at the Pro-197 position to be assessed. MN = Mid North, YP = Yorke Peninsula, NSW = New South Wales, WT = wild type, SE = South East South Australia.

When individuals were coloured based on the amino acid substitution combination at the Pro-197 position (Figure 2B), there is also limited evidence of individuals with the same combination clustering genetically. Several of the most frequent combinations (Table 4) encompass a relatively wide range of variation, such as Pro-197-Ala/Pro-197-Leu, WT/Pro-197-Leu, and WT/Pro-197-Thr. The uncommon Pro-197-Ser/Pro-197-Ser combination, found in just three individuals, clusters with some of the WT/Pro-197-Ser individuals. Some of the other less common combinations, such as Pro-197-Leu/Pro-197-Thr and Pro-197-Arg/Pro-197-Ser, appear to be present in more than one cluster. The cluster at the bottom left of the plot is mostly WT/Pro-197-Thr and WT/Pro-197-Arg, with one Pro-197-Arg/Pro-197-Thr (very closely clustered with WT/Pro-197-Thr and WT/Pro-197-Arg) and one Pro-197-Ala/Pro-197-Leu. The cluster at the bottom right is mainly made up of WT/Pro-197-Leu with one WT/WT and one individual without ALS gene sequence data.

3.4 Discussion

Resistance to chlorsulfuron and imazamox + imazapyr was very common across the study region. A previous study reported 78% of annual sowthistle populations resistant to chlorsulfuron from across southeastern Australia (Merriam et al., 2018). The current study found 90% of MN and 88% of YP populations resistant, indicating that these regions could have slightly above average levels of resistance compared to other regions in southeastern Australia. Merriam et al. (2018) reported imazamox + imazapyr resistance across southeastern Australia at 68%, but this study found 84% in the MN and 67% in the YP, representing above average and average values respectively. The only two populations that were completely controlled by both herbicides were collected from the edge of fields and may have experienced less selection pressure. At one of these locations, a sample was also collected from within the crop which had 100% survival to chlorsulfuron and 80% survival to imazamox + imazapyr.

The previous study on ALS-inhibitor resistant annual sowthistle in southeastern Australia also found that sulfonylurea resistance levels in annual sowthistle have not changed significantly within the timeframe of the survey program (since 2010), with all areas sampled twice over a 5-year interval showing no significant difference in the proportion of populations classified as resistant (Merriam et al., 2018). Further survey work presented in this paper confirms that while resistance is widespread, it does not appear to have increased between 2013 and 2018, with higher levels in 2017 likely being an artefact of sampling rather than a genuine trend (Table 3). Road side samples accounted for 22% of samples in 2017 (compared to 10% in 2013 and 0 in 2018), and across both years were associated with greater chlorsulfuron resistance and lower imazamox + imazapyr resistance (Table 3). Differences in average survival correlated to survey year, crop type and collection location are likely strongly influenced by small sample sizes in each of these categories.

The present study found six different Pro-197 substitutions, constituting the same set of substitutions reported from the region by Petersen (2018), plus the uncommon Pro-197-His substitution. Allele frequencies varied between the YP and the MN (Table 4), but there was also a difference in allele frequencies between YP-PC and the YP as a region, even though the YP-PC site was located within the YP. This implies that stochastic variation and the selective effects of local management could be more important in determining allele frequencies than stable regional trends. This would be consistent with the diversity of mutations and the prolific production of highly dispersive seed.

The closely related weed species prickly lettuce (*Lactuca serriola* L.), also a member of the Cichorieae tribe within the Asteraceae family, also has widespread resistance to the ALS inhibiting herbicides in the MN and YP regions, with a previous study reporting 66% sulfonylurea resistance in 1999 and 82% in 2004 (Lu et al., 2007). More recent work found 100% of populations resistant to both a sulfonylurea and an imidazolinone (Merriam, unpublished data). Although both species have been exposed to the herbicides for the same period of time, high levels of resistance to both the imidazolinones and the sulfonylureas appear in nearly all prickly lettuce populations (with >90% of individuals surviving in most populations) while resistance in annual sowthistle appears to have reached an equilibrium at around 80% of individuals resistant to the sulfonylureas and half to two thirds of individuals resistant to the imidazolinones, depending on the region in question.

The amphidiploid nature of annual sowthistle is one possible explanation for this, with the presence of a second, wild-type susceptible copy of the ALS gene tempering the effect of a mutation acquired in the other copy of the ALS gene. There was some evidence that individuals with mutations on both copies of the ALS gene were slightly more resistant to the herbicides, but the specific mutation involved is likely to be more important. This study found the Pro-197-Arg, Pro-197-Leu and Pro-197-Thr mutations provided stronger resistance to both the IMI and the SU herbicides, especially when combined with a second mutation. The Pro-197-Leu mutation alone conferred strong IMI resistance but weak SU resistance, and as such may have been favoured by the recent increased usage of IMI herbicides for broadleaf weed control in IMI tolerant crops.

A limitation of this study is that unfortunately crop rotation and recent herbicide use history was not available for the paddocks from which samples were collected. This information would have provided further support for the link between evolution of resistance and herbicide use practices and may have helped explain some of the differences observed between annual sowthistle and prickly lettuce. However, due to the seed mobility of this species, over time regional trends would likely have a stronger effect than the herbicide use patterns in a specific paddock.

Some lines with the same mutation combination showed differing LD_{50}s, with one possible explanation being a greater contribution of one or the other of the ALS gene homologs to resistance. Genome assortment of proline-197 mutations could not be ruled out as a cause for different resistance profiles in individuals having the same substitution combination. Results were not consistent across groups tested (line WT/Ser-1 and line WT/Ser-2 had different assortment, but all other pairs tested had the same assortment) (Table 5). Differential gene expression of the different copies could also not be ruled out, with both individuals tested showing similar expression of the serine mutation with respect to the wild-type proline, despite having each of these on different copies of the ALS gene. The reasons for the variation in response to herbicides of individuals carrying the same mutations remain to be identified, but non-target site resistance mechanisms may also be present.

The population genetic data was consistent with the highly mobile nature of this weed species and provides evidence for long distance dispersal and multiple evolution events of the same resistance mutation combination. Generally, little evidence of genetic clustering was found, with individuals collected within YP-PC encompassing a similar range of variation seen in all individuals across the

MN and YP regions (Figure 2). The lack of overall population genetic structure is indicative of high levels of gene flow and dispersal, and the fact that even outlier individuals from NSW failed to segregate could be indicative of frequent long distance dispersal.

Examining the genetic clustering of individual mutation combinations in the PCA might provide some clues regarding the provenance of some of these combinations. Several of the more uncommon mutation combinations, such as Pro-197-Ser/ Pro-197-Ser and Pro-197-Arg/ Pro-197-Thr, are found in clusters containing the more common single mutations, indicating that they likely arose through the acquisition of an additional mutation in the other copy of the ALS gene. Whether these rarer mutations are simply more recently evolved or affected by other selective pressures remains to be established and is another possible avenue for future research.

3.5 Conclusion

This study found high levels of ALS-inhibitor resistance present in annual sowthistle in the MN and the YP and revealed a great diversity of Pro-197 ALS gene mutations involved in conferring resistance. Different mutations and mutation combinations showed some evidence of differing effects on LD_{50} to the herbicides tested, but further work is required to confirm these differences and establish why different LD_{50} phenotypes were found between lines carrying the same mutation combination. It is possible that these effects are mediated by the assortment of mutations and different expression of the ALS genes from the two genomes in annual sowthistle, this could not be proved conclusively in this study. The population genetics data was consistent with high levels of dispersal in this species. While this is not likely to be an issue of significant concern to growers in terms of ALS inhibitor resistance due to its ubiquity, it is more concerning in light of the advent of emerging cases of 2,4-D resistance in southeastern Australia and glyphosate resistance in northern Australia as the high levels of dispersal will likely result in rapid spread of resistance.

3.6 Acknowledgements

This research is supported by an Australian Government Research Training Program Scholarship and a Grains Research and Development Corporation (GRDC) Grains Research

Scholarship (project UOA1801-003RSX). Special thanks to Peter Boutsalis and all participants in earlier years of the herbicide resistance monitoring surveys (UCS 00024) during which annual sowthistle was collected, including: Samuel Kleemann, Geetha Velappan, Ruwan Lenorage, Kathy Bergen, and Sarah Marshall. David Brunton and Jess Nichols provided additional field and lab assistance which was greatly appreciated. Special thanks are due to Emeritus Professor Gimme Walter and the Walter Lab at the University of Queensland for hosting lab work for DNA library prep.

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4 CHAPTER 4: CAN ROTATIONS IMPROVE MANAGEMENT OF HERBICIDE-RESISTANT ANNUAL SOWTHISTLE (*SONCHUS OLERACEUS*) AND PRICKLY LETTUCE *(LACTUCA SERRIOLA*) IN LENTIL PRODUCTION SYSTEMS OF SOUTHERN AUSTRALIA?

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Weed Technology: Published Manuscript

Statement of Authorship

Title of Paper	Can rotations improve management of herbicide-resistant annual sowthistle (*Sonchus oleraceus*) and prickly lettuce (*Lactuca serriola*) in lentil production systems of southern Australia?
Publication Status	Published
Publication Details	Merriam AB, Malone J, Gill G, Preston C (2021) Can rotations improve management of herbicide-resistant annual sowthistle (*Sonchus oleraceus*) and prickly lettuce (*Lactuca serriola*) in lentil production systems of southern Australia? Weed Technol. 35: 532–538. doi: 10.1017/wet.2020.134

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Overall percentage	85%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/03/2022

Co-Author Contributions

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

I. The candidate's stated contribution to the publication is accurate (as detailed above)
II. Permission is granted for the candidate to include the publication in the thesis; and
III. The sum of all co-author contributions is equal to 100% less the candidate's contribution

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Can rotations improve management of herbicide-resistant annual sowthistle (*Sonchus oleraceus*) and prickly lettuce (*Lactuca serriola*) in lentil production systems of southern Australia?

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Research Article

Cite this article: Merriam AB, Malone J, Gill G, Preston C (2021) Can rotations improve management of herbicide-resistant annual sowthistle (*Sonchus oleraceus*) and prickly lettuce (*Lactuca serriola*) in lentil production systems of southern Australia?. Weed Technol. **35**: 532–538. doi: 10.1017/wet.2020.134

Received: 30 July 2020
Revised: 13 November 2020
Accepted: 20 November 2020
First published online: 9 December 2020

Associate Editor:
Michael Walsh, University of Sydney

Nomenclature:
bromoxynil; metsulfuron-methyl; MCPA; picolinafen; annual sowthistle; *Sonchus oleraceus* L.; prickly lettuce *Lactuca serriola* L.; lentils; *Lens culinaris* L.; wheat; *Triticum aestivum* L.

Keywords:
Herbicide resistance; crop rotation; common sowthistle; crop competition

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Abstract

In southern Australia, annual sowthistle and prickly lettuce have become more prevalent following the adoption of reduced tillage cropping systems. They are especially problematic in lentil and other pulse crops, which are weakly competitive and have few herbicide options available for POST control of broadleaf weeds. This study aimed to evaluate the influence of management in a previous cereal crop on weed densities in a subsequent crop. At two field sites, crop seeding density and POST herbicide treatments (a conventional choice that included metsulfuron-methyl and MCPA; and a proactive choice that included bromoxynil, picolinafen, and MCPA) were applied to a wheat crop, and weed density was assessed at the beginning of the following season to measure for a legacy effect of the treatments. Study site populations were also screened for herbicide resistance and were found to have high (≥90% survival) ALS inhibitor resistance. Crop competition treatments had no effect on weed populations, and effects of herbicide treatment were significant at only one of the sites. At this site, both herbicide treatments had lower weed densities than the nontreated in the first year, but the legacy effect was only significant for annual sowthistle density in the proactive treatment. At both sites, even where weeds were extremely sparse or completely controlled following herbicide treatment in the first year, moderate densities were observed the following year, indicating that colonization from the seedbank or adjacent areas could be contributing to weed numbers. Weed density assessments and accurate knowledge of the herbicide resistance status of target weeds should guide herbicide selection to maximize control.

Introduction

Annual sowthistle and prickly lettuce are wind-dispersed annual weeds of the Asteraceae family with wide geographic distribution. These species are often found growing in association with each other and readily colonize open habitats such as roadsides, cropland, and other disturbed areas (Hutchinson et al. 1984; Weaver and Downs 2003). They have become more common in annual cropping systems in Australia since the widespread adoption of reduced tillage systems because the seeds remain close to the soil surface (Weaver and Downs 2003; Widderick et al. 1999), which favors germination of both species (Chadha et al. 2019; Chauhan et al. 2006). These weed species can affect yield in subsequent crops by depleting soil moisture in summer fallows (Widderick et al. 2010), and a recent study has demonstrated yield loss in wheat due to competition at high densities of annual sowthistle (Manalil et al. 2020). Both species reduce grain quality by increasing moisture levels and staining grain with their milky sap (Widderick 2019), with prickly lettuce having the potential to cause problems to mechanical equipment at harvest due to this milky sap (Amor 1986). Finally, annual sowthistle is an alternate host for crop pests and disease vectors between seasons (Hutchinson et al. 1984). Across all crops, annual sowthistle is estimated to cause annual revenue losses of A$4.2 million in grain production regions of northern Australia and annual sowthistle and prickly lettuce together are responsible for an additional A$1.5 million annually in revenue loss in grain production regions of southern Australia (Llewellyn et al. 2016).

Broadleaf weeds such as prickly lettuce and annual sowthistle are especially hard to control among pulse crops due to a lack of selective POST herbicide options and poor crop competition, with lentils being one of the least competitive pulse crops (McDonald et al. 2007). Control of these weeds has become an issue for many growers in the lentil-production regions of southern Australia. Due to the difficulty of in-crop control, recommendations often focus on controlling these weeds prior to sowing or crop emergence (GRDC GrowNotes™ 2018). Seed production of annual sowthistle and prickly lettuce is sensitive to competition, with production estimates under little or no competition being greater than 10,000 seeds per plant (Amor 1986; Hutchinson et al. 1984). Therefore, the weed seedbank within a field can increase considerably following a season in which a weakly competitive pulse crop has been grown. However, seedbank persistence in both species is short under field conditions, with little or no innate dormancy (Chadha et al. 2019; Chauhan et al. 2006). Provided cues of light and moisture, germination rates of greater than 80% have been reported in both species, whereas factors such as seed burial or drought conditions can increase the proportion of seed that persists in the seed bank beyond a season (Chadha et al. 2019; Chauhan et al. 2006).

The first herbicide-tolerant lentil varieties to be introduced in Australia have tolerance to the imidazolinone chemical family of the acetolactate synthase (ALS)-inhibiting herbicides (Weed Science Society of America [WSSA] Group 2). These varieties allow the use of imidazolinones for broadleaf weed control and provide some tolerance to sulfonylurea herbicide residues in the soil (Bruce et al. 2019; Pulse Breeding Australia 2011; Rodda et al. 2016). Consequently, these varieties have become very popular since their introduction, and they are now widely grown. However, ALS-inhibiting herbicides are considered high risk for the evolution of herbicide resistance. A single nuclear gene with a semidominant inheritance pattern frequently confers resistance via a large number of point mutations that have little or no fitness penalty (Tranel and Wright 2002). The increased use of imidazolinone herbicides is likely to select for resistance in these weeds and presents a significant risk to the sustainability of these production systems.

Annual sowthistle and prickly lettuce are similar in their spectrum of resistance to herbicides, with cases reported to the ALS inhibitors 2,4-D (WSSA Group 4, synthetic auxins), and glyphosate (WSSA Group 9, enolpyruvyl shikimate phosphate synthase inhibitors) in both species (Heap 2020). Cases of synthetic auxin and glyphosate resistance in Australia are still relatively recent and limited in number, but ALS inhibitor resistance is widespread in both species. ALS inhibitor-resistant annual sowthistle was first identified in Australia in 1991 (Boutsalis and Powles 1995). The first case of resistance in prickly lettuce was identified in the United States in 1987 (Mallory-Smith et al. 1990), and discovery of resistant populations in Australia occurred in 1994 (Heap 2020). According to data from resistance monitoring surveys in southern Australia, the frequency of resistance to the sulfonylurea herbicides in annual sowthistle is estimated at 78%, and at 68% to the imidazolinone herbicides across the region (Merriam et al. 2018). Earlier surveys of prickly lettuce conducted in lentil-production areas of South Australia in 1999 and 2004 reported the frequency of sulfonylurea resistance at 66% and 82%, respectively (Lu et al. 2007).

Using a diverse crop rotation can help in managing weeds by providing control opportunities and varying selection pressures applied to the weed community (Derksen et al. 2002) while also providing a yield benefit (Angus et al. 2015; Hunt et al. 2019; Zhao et al. 2020). Pulse crops are commonly grown as a break-crop in a rotation focused on cereals, and many studies have evaluated their benefits for management of weeds and other agronomic issues in the cereal phase (Legere and Stevenson 2002; Moyer et al. 2005; Seymour et al. 2012). In particular, inclusion of break crops in cereal-based rotations can help to alleviate management issues involving intractable grass weeds (Colbach et al. 2010; Seymour et al. 2012). However, rotations with a high frequency of broadleaf crops can result in similar issues around management of difficult-to-control broadleaf weeds (Koocheki et al. 2009; Stevenson and Johnston 1999), and fewer studies have focused on the reciprocal benefit provided by a cereal phase to weed management issues in the break crop phase (Hegewald et al. 2018; Miller et al. 2003).

Given the lack of effective herbicide options available for broadleaf weed control among lentil crops, exploiting management opportunities provided by a prior cereal phase is appealing, but seed mobility of annual sowthistle and prickly lettuce is a complicating factor. This study aims to determine whether specific management practices applied to a prior cereal crop can have a legacy effect on the density of these broadleaf weeds in the following crop, despite seed mobility.

Materials and Methods

Experimental Design and Sampling

Two trial sites approximately 100 km apart were established in 2018 in lentil-production areas of South Australia: one near Kulpara (KYP) on the Yorke Peninsula and one near Roseworthy (RS2) in the Mid-North cropping region (Table 1). The soil type at KYP is a calcareous gradational clay loam, while the soil at RS2 is loam over poorly structured red clay (Government of South Australia Department for Environment and Water 2016) and both are in landscapes dominated by annual winter crop production systems that incorporate a mix of oilseed, pulse, and cereal crops. Both sites have a Mediterranean climate characterized by cool, wet winters and hot, dry summers. Lentils had been grown in both selected sites the previous year and the trial was run for 2 yr over the 2018 and 2019 seasons. The sites received below average growing season rainfall in both years of the trial (Table 1).

Prior to seeding wheat in 2018, 648 g ai ha^{-1} of glyphosate (Roundup PowerMAX®, Nufarm Pty Ltd, Australia) and 100 g ai ha^{-1} of pyroxasulfone (Sakura® 850 WG, Bayer Crop Science Pty Ltd, Australia) were applied to both trial areas to control existing weeds and to provide residual control of grass weeds. Treatments were applied during the 2018 season in a split-plot design with four replicates incorporating, in factorial arrangement, two levels of crop competition applied to the whole plots and three POST herbicide treatments (including a control) applied to the subplots. Experimental designs for each site were generated using the AGRICOLAE package in R (de Mendiburu 2020). Crop competition treatments were applied using two seeding rates (60 and 90 kg ha^{-1}) of wheat [cv. Chief] with a knife-point seeder on 25-cm row spacings, and monoammonium phosphate fertilizer was applied at 80 kg ha^{-1} (10 kg ha^{-1} N and 21.9 kg ha^{-1} P) at the time of seeding. Plot width was set at the width of the seeder available at KYP and RS2 at 16 m and 10 m, respectively, and lengths were set to establish plots of equal area at each site (160 m^2).

Herbicide treatments included a conventional choice, including an ALS inhibitor and a synthetic auxin; and a proactive choice that included a photosystem II inhibitor (WSSA Group 6), a phytoene desaturase inhibitor (WSSA Group 12) and a synthetic auxin (Table 2). Herbicide treatments were applied approximately 10 wk after sowing using a quad-bike boom sprayer equipped with flat-fan nozzles (TeeJet 110015, TeeJet Technologies Australia) spaced 50 cm apart and operating at 10 km h^{-1} and 200 kPa for an output of 57.6 L ha^{-1}. GPS coordinates and detailed measurements were taken at each site at the end of 2018 before harvest in order to be able to superimpose the trial in the same area in 2019,

Table 1. Geographic location of the trial sites, growing season rainfall during the trial years and long-term average, dates of treatment application in 2018 and crop rotation in the trial years and the year prior.[a]

		Growing season rainfall[b]			Treatment dates, 2018		
	Coordinates	2018	2019	Average	Seeding	POST herbicides	Crop rotation, 2017–2019
		———mm———					
KYP	34.08°S, 138.00°E	186	182	291	May 12	July 12	Lentils-Wheat-Barley
RS2	34.54°S, 138.70°E	201	209	280	May 18	August 1	Lentils-Wheat-Lentils

[a] Abbreviations: AWS, Automatic Weather Station; KYP, Kulpara trial site; RS2, Roseworthy trial site.

[b] In temperate Australia, growing season rainfall is that received between April and October. Rainfall data are from the nearest available weather station: Port Clinton (Yararoo) for KYP and Roseworthy AWS for RS2 (Australian Government Bureau of Meteorology, 2019). Averages are based on years 1919–2019.

Table 2. Herbicides applied in 2018 in the field trials at KYP and RS2, and in herbicide resistance screening of trial site populations of annual sowthistle and prickly lettuce. Active ingredients, trade names, rates and manufacturers are presented.[a]

Active ingredients	Trade name	Rate	Manufacturer
		g ai ha^{-1}	
Field trials			
Metsulfuron-methyl + MCPA	Ally®; MCPA 750®	3 + 675	FMC Pty Ltd, Australia; Nufarm Pty Ltd, Australia
Bromoxynil + Picolinafen + MCPA	Flight EC®	151 + 5 + 252	Nufarm Pty Ltd, Australia
Herbicide resistance screening			
Chlorsulfuron	Glean®	15	Dupont Pty Ltd, Australia
Imazamox + imazapyr	Intervix®	24.75 + 11.25	BASF Pty Ltd, Australia
Glyphosate	Roundup PowerMAX®	540	Nufarm Pty Ltd, Australia
2,4-D	Amicide Advance®	455	Nufarm Pty Ltd, Australia

[a] Abbreviations: KYP, Kulpara trial site; RSW, Roseworthy trial site.

and this was verified using satellite imagery where available. The 2019 crop at RS2 was lentils ('Hurricane'), with barley ('Spartacus') sown at KYP, and uniform management was applied across each trial site.

Crop establishment counts were taken 5 wk after sowing at both sites in 2018. Counts of prickly lettuce and annual sowthistle were completed prior to herbicide treatment application, approximately 10 wk after sowing, to establish initial weed densities. Post-treatment weed counts were completed at both sites approximately 7 wk after herbicide application in 2018. Sowthistle capitula counts were taken at KYP prior to harvest in November 2018 to estimate seed production. Mature weed plants in each plot were identified, and the number of capitula were recorded to generate an estimate of the average number of capitula per plant in each plot: if fewer than five plants were found in a plot, the number of capitula on each was recorded, and if more than five plants were identified, five were randomly selected from the plot for capitula counts. These numbers were multiplied by the number of plants per plot from the earlier post-treatment weed counts and an estimated average of 170 seeds per capitula based on the literature (Mobli et al. 2020; Ollivier et al. 2020). Due to low weed densities in 2018 at RS2, an extra weed density assessment was completed before presowing herbicide treatments in 2019. Legacy effect was assessed by counts taken at both sites following 2019 crop emergence but prior to POST herbicide treatments. Weed density assessments in 2018 were conducted by counting weeds present in the entire plot due to low numbers, and in 2019 counts were carried out using 0.25-m^2 quadrats.

Herbicide Resistance Screening

Mature seed samples of annual sowthistle and prickly lettuce were collected from the trial sites at KYP and RS2 for herbicide resistance screening. Fields were surveyed as described by Boutsalis et al. (2012), with mature seed of each species pooled to make a representative sample of the population, air-dried in paper bags and stored at ambient temperature until screening. Annual sowthistle samples were collected in late 2018 and prickly lettuce was sampled in early 2019.

Herbicide resistance screening was completed at the Waite Campus of the University of Adelaide in Urrbrae, SA (34.97°S, 138.64°E) as described by Merriam et al. (2018) with two experimental runs in spring 2020. Seeds from each study site population and a standard susceptible control population of each species were sown on the soil surface in punnet pots (Masrac Plastics, Adelaide, Australia) containing a coco-peat potting mix (Boutsalis et al. 2012) and transplanted at the one-leaf growth stage into 0.2-L punnet pots at one plant per pot. A total of 10 seedlings per population were screened with each herbicide, and a nontreated control for each population was also included. Seedlings were grown outdoors with watering as required. Herbicide application occurred at the four- to five-leaf stage using a laboratory moving boom cabinet sprayer equipped with TeeJet flat-fan nozzles (110015) calibrated to an output of 118 L ha^{-1} at 300 kPa and 1 m s^{-1}. Herbicide screening rates (Table 2) were within the label-recommended field rate range in Australia for each herbicide. Survival was assessed 4 wk after herbicide application as the percentage of living plants remaining in each treatment. Mortality was classified as dead leaf tissue with no further production of new growth.

Data Analysis

All statistical analyses were performed using R Statistics Package (R Core Team 2018). Herbicide resistance screening data were analyzed using a two-proportion Z-test ($P = 0.05$) to compare each study site population with the relevant standard susceptible population of each species. Data were pooled between experimental runs because there was no significant difference between the runs. A factorial ANOVA of weed density assessments indicated that results differed between the trial sites, so data were analyzed separately for each site. Crop density and herbicide treatments were tested with a two-way ANOVA using ASReml statistical software

(Gilmour et al. 2009), which indicated that only herbicide treatments effects were significant. One-way ANOVA was then applied to compare herbicide treatments, and Tukey's multiple comparison test was then used to compare the treatment means at a significance level of $P = 0.05$.

Results and Discussion

Herbicide Resistance Screening

Seeds collected from plants growing at each site prior to the experiment starting were assessed for resistance to sulfonylurea, imidazolinone and auxin herbicides as well as glyphosate. The populations of both weed species sampled from each site were resistant to the sulfonylurea and imidazolinone herbicides, but susceptible to 2,4-D and glyphosate (Table 3). Sulfonylurea resistance was especially high, with all populations exhibiting at least 95% survival. This was expected to affect the performance of the conventional herbicide treatment, which includes a sulfonylurea component. The standard susceptible population included in screening was fully controlled (0% survival) by all screening herbicides and the nontreated control sample of each population had 100% survival.

Table 3. Percent survival of annual sowthistle and prickly lettuce populations from the trial sites to herbicides used in resistance screening.[a,b]

	Annual sowthistle		Prickly lettuce	
	KYP	RS2	KYP	RS2
	Percent survival			
Chlorsulfuron	95*	100*	100*	100*
Imazamox + imazapyr	75*	35*	100*	65*
Glyphosate	0	0	0	0
2,4-D	0	0	0	0

[a] Trial site populations were sampled in summer 2018–2019 and screened in spring 2020. Data from each experimental run were pooled. Values followed by an asterisk (*) are greater than survival in the standard susceptible for each species (at $P = 0.05$), which in all cases was completely controlled.

[b] Abbreviations: KYP, Kulpara trial site; RS2, Roseworthy trial site.

Crop Establishment and Density Effects

Wheat establishment was more variable at RS2 compared with that at KYP, but both sites had different ($P < 0.05$) crop plant density for the two sowing rate treatments applied (Table 4). Sowing guides for southern Australia often recommend densities of 100 to 150 plants m^{-2} for rainfed wheat production, and the Yield Prophet database, which collates input and yield data from across Australia, reports a mean sowing density of 145 (± 26 SD) plants m^{-2} in the southern grain production region (Hochman and Horan 2018). Therefore, the lower seeding density used in this trial represents a typical plant density, whereas the higher sowing rate is an above-average plant density.

There was no effect ($P > 0.05$) of crop density treatments on the assessed metrics of weed density or seed production at either site in 2018 or 2019. The benefits of increased crop density for weed control are well established and are effected through a reduction in weed biomass and fecundity rather than weed density (Bajwa et al. 2017; Lemerle et al. 2004; McDonald et al. 2007; van der Meulen and Chauhan 2017). Therefore, crop competition may not have affected weed density in 2018 but could become apparent in the following year by reducing biomass and reproductive success of the 2018 weed population. Since destructive weed biomass measurements in 2018 would have affected 2019 results, fecundity was estimated through seed production estimates at KYP at the end of 2018 (Supplementary Table 1), but no effect of crop density was found. The experimental densities represent only a typical and an above-average plant density, and wheat is a relatively competitive crop, particularly against broadleaf weeds (Mobli et al. 2020; Walsh 2019). Lower crop density or a less competitive crop could result in greater seed production.

Initial Weed Density and Herbicide Treatment Effects

Initial weed densities at each site were assessed after crop emergence and prior to herbicide treatment in 2018 (Table 4). Densities of both weeds were variable across the two experimental sites, but KYP had much higher densities of both species than RS2. Density of annual sowthistle was higher than prickly lettuce at both sites.

Trial Site RS2

There was no effect ($P > 0.05$) of herbicide treatment on weed density at RS2, possibly due to the low initial weed density and high variability across the site, so results are presented as averages across the site at each assessment (Table 4). Following herbicide treatment in 2018 (Posttreat), annual sowthistle density decreased compared with initial density assessments and prickly lettuce was not detected in the trial area. An extra weed density assessment was conducted at RS2 before sowing in 2019, when densities were at their highest, but there was still no treatment difference in weed populations (Supplementary Table 2). In the counts taken following crop emergence, but prior to POST herbicide application in 2019 (Pretreat), densities were lower than counts taken prior to sowing, but there was no difference between treatments.

Despite the low density of annual sowthistle and absence of prickly lettuce in the RS2 trial area following herbicide treatment in 2018, both weeds were present prior to sowing in 2019 (Supplementary Table 2) and after crop emergence (2019 Pretreat; Table 4). Possible reasons for this include prolific seed production by survivors in the study area (such as in nontreated plots), colonization from outside the study area, and contributions from the soil seedbank. The presence of prickly lettuce in 2019, despite being completely absent from the trial area following herbicide treatment in 2018, suggests that colonization and seedbank recruitment (of seed produced in 2017 prior to the trial) are important factors at this site. There were patches of prickly lettuce present along the edge of the RS2 site and around a large tree within the crop that could be the source of this colonization, in addition to seed dispersed from farther afield in the landscape. Seedbank recruitment is unlikely to be the major contributor since both species have little dormancy and most seed will germinate within the first year provided the necessary cues. Chauhan et al. (2006) reported >90% germination in annual sowthistle, and Chadha et al. (2019) reported >85% germination in prickly lettuce following exposure of seed to light and moisture, with germination and emergence declining with increasing osmotic potential and depth of seed burial. However, the lack of substantial rainfall events in 2018 and seed burial at seeding could have resulted in some persistence of viable seed produced in 2017 to the 2019 season.

Trial Site KYP

Sowthistle density in 2018 and 2019 at KYP was affected by the 2018 herbicide treatments (Table 4). At the 2018 Posttreat assessment the proactive treatment resulted in the lowest sowthistle density, with an 81% reduction in plant numbers compared to the nontreated control. The conventional treatment resulted in a

Table 4. Crop establishment, annual sowthistle, and prickly lettuce density at RS2 and KYP during the trial period.[a,b]

	Crop establishment		Annual sowthistle			Prickly lettuce		
	2018		2018		2019	2018		2019
Assessment	60 kg ha^{-1}	90 kg ha^{-1}	Pretreat	Posttreat	Pretreat	Pretreat	Posttreat	Pretreat
	plants m^{-2}							
RS2								
Plant density	142 (6)	201 (4)	0.014 (0.002)	0.010 (0.002)	0.38 (0.091)	0.002 (0.002)	0	0.17 (0.062)
KYP								
Plant density	145 (3)	225 (3)	0.29 (0.030)			0.033 (0.007)		0.97 (0.22)
Proactive				0.15 a	2.1 a		0.0023 a	
Conventional				0.43 b	6.8 b		0.0031 a	
Nontreated				0.79 c	5.6 b		0.019 b	

[a] Abbreviations: KYP, Kulpara trial site; RS2, Roseworthy trial site.

[b] Crop establishment is shown for the low (60 kg ha^{-1}) and high (90 kg ha^{-1}) seeding rates. Pretreat refers to counts taken following crop emergence but prior to POST herbicide application. Posttreat counts were taken following POST herbicide application. Where no significant treatment difference exists, counts have been averaged across the whole site area, and values are expressed as mean plant density with standard error in parentheses. Means within a column followed by the same letter are not significantly different at $P = 0.05$.

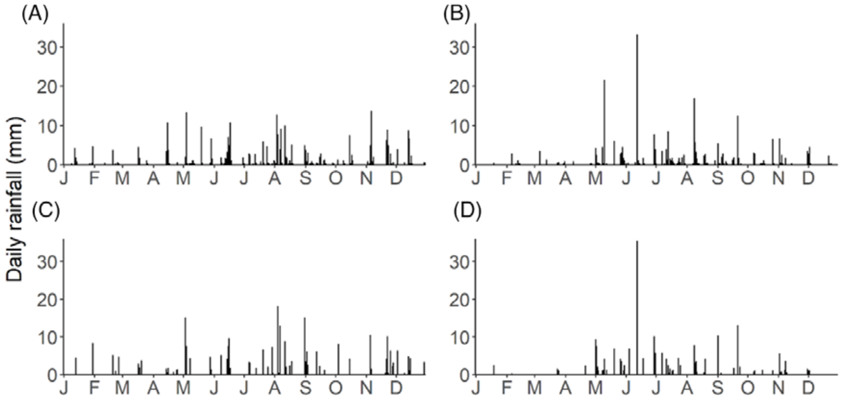

Figure 1. Daily rainfall totals at Roseworthy (RS2) in 2018 (A) and 2019 (B), and at Kulpara (KYP) in 2018 (C) and 2019 (D) (Australian Government Bureau of Meteorology 2019).

46% reduction in sowthistle plant densities. Annual sowthistle seed production estimates followed the same pattern, with the lowest seed production in the proactive treatment and the highest seed production in the nontreated plots (Supplementary Table 1). At the 2019 Pretreat assessment, the proactive treatment still had the lowest density of sowthistle plants (63% control), but there was no longer a statistically significant difference between the conventional treatment and nontreated plots. This difference from 2018 to 2019 may be because the population of annual sowthistle at KYP is resistant to sulfonylurea herbicides, with 100% survival at the field rate (Table 3). The residual component of the conventional herbicide treatment relies on a sulfonylurea herbicide, metsulfuron methyl, so this treatment would have provided no residual control after the initial immediate effects of the MCPA component. It could also be due to seed produced in the nontreated plots dispersing to other areas of the trial, which is an innate characteristic of these weed species.

Prickly lettuce density at KYP at the 2018 Posttreat assessment was higher in the nontreated plots, but there was no difference between the conventional and proactive herbicide treatments (Table 4), both achieving about 86% control with respect to the nontreated. Under both herbicide treatments, weed density was similar to the initial density across the site, whereas density in the nontreated plots increased. This suggests that new germinations may have occurred following the application of herbicide treatments. There was no effect ($P > 0.05$) of herbicide treatment at the 2019 Pretreat assessment and therefore no legacy effect observed for prickly lettuce.

Increase in Weed Density from 2018 to 2019

Weed densities at 2019 Pretreat assessments were higher for both species at both sites compared with the initial densities recorded in 2018 (Table 4). In both years, these assessments were completed at similar stages—following crop emergence but prior to POST herbicide application—so they represent an overall increase in density from 2018 to 2019. At RS2, this could be influenced by the crop selection in each year—the 2018 crop (wheat) was much more competitive than the 2019 crop (lentils; Bajwa et al. 2017). However, at KYP, the 2019 crop (barley) was more competitive than the 2018 crop (wheat), but density still increased. The more likely factor is the difference in rainfall between the two growing seasons. Growing season rainfall did not differ greatly between the two years at either site (Table 1), but there was a lack of substantial rainfall events in 2018 (Figure 1). The only daily rainfall totals greater than 20 mm during the entire trial period were in 2019 on May 10th (RS2), and June 12th (both sites; Australian Government Bureau of Meteorology 2019). These rainfall events occurred several weeks before the 2019 Presow assessment at RS2 and the 2019 Pretreat assessment at both sites. Germination in both weed species is highly moisture dependent and large rainfall events stimulate flushes of germination (Chadha et al. 2019;

Widderick et al. 2010), which could be responsible for the elevated numbers observed in 2019 assessments.

Implications for Management

The results of this trial suggest that legacy effects on weed density of herbicide treatment from one year to the next may be density dependent for annual sowthistle and prickly lettuce, with high initial densities in the first year increasing the chances of treatment differences in the following year. Significant treatment differences in the first year were observed only in the two situations with the highest initial weed density, sowthistle and prickly lettuce at KYP. Only sowthistle at KYP, with the highest initial density, had a legacy effect in the second year. Since the prices of herbicide treatments used in this study differed greatly with the proactive treatment costing roughly five times more than the conventional treatment, the initial density and resistance status of target weeds should be considered when employing this tactic. High densities of annual sowthistle or prickly lettuce may justify the extra expense, especially if ALS inhibitor resistance is present. In any case, earlier studies have shown that the incidence of annual sowthistle and prickly lettuce resistance to the ALS inhibitors is extremely widespread in southern Australia (Lu et al. 2007; Merriam et al. 2018), so this mode of action should not be relied on.

The data demonstrated the ability of these weed species to increase in abundance even after effective control the year prior. This increase could come from prolific seed production of surviving plants, colonization from outside the area, or contributions from the soil seed bank. Individuals of these species have the potential for prolific seed production, particularly when growing in uncropped areas where they may face less intense competition (Amor 1986; Hutchinson et al. 1984). These individuals have the potential to contribute substantially to weed numbers in an adjacent field. Although colonization from outside areas or dispersal between plots was not quantified in this study due to the practical difficulty of experimentally tracking dispersal in small seeded, wind-dispersed species (Bullock et al. 2006), these factors would certainly occur in the field. Both annual sowthistle and prickly lettuce have relatively short seedbank persistence but have the ability to persist beyond one growing season in the absence of suitable growing conditions (Chadha et al. 2019; Hutchinson et al. 1984; Weaver and Downs 2003; Widderick et al. 2010). Furthermore, both annual sowthistle and prickly lettuce germinate in a range of temperatures so germination can occur at any time of year in Australia provided sufficient moisture is available. Crop monitoring, particularly in the weeks following substantial rainfall events, can help to identify and control these flushes of germination.

The inconsistency of management benefits carrying over to the following year indicate that the strategy of targeting broadleaf weeds in a prior cereal crop is insufficient on its own when dealing with highly fecund, wind-dispersed weeds, although it can be a useful tool in some situations. Even if high levels of control are achieved in the cereal phase, these species could colonize from the soil seedbank, adjacent fields, fence lines and roadsides, or other uncropped areas. Reducing seed set, especially for individuals growing under reduced competition, should be a priority since both species produce large quantities of mobile seed.

Acknowledgments. This research is supported by an Australian Government Research Training Program Scholarship and a Grains Research and Development Corporation (GRDC) Grains Research Scholarship (project UOA1801-003RSX). Technical assistance was provided by David Brunton, Ben Fleet, and Jerome Martin from the University of Adelaide Weed Science Research group. We thank the grower and farm manager at each site for accommodating the trials on their property. No conflicts of interest are declared.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wet.2020.134

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5 CHAPTER 5: CONTROL OF COMMON SOWTHISTLE (*SONCHUS OLERACEUS*) AND PRICKLY LETTUCE (*LACTUCA SERRIOLA*) USING PSII-INHIBITING HERBICIDES IN A NOVEL BREEDING LINE OF METRIBUZIN-TOLERANT LENTIL (*LENS CULINARIS*)

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Unsubmitted Manuscript

Statement of Authorship

Title of Paper	Control of common sowthistle (*Sonchus oleraceus*) and prickly lettuce (*Lactuca serriola*) using PSII-inhibiting herbicides in a novel variety of metribuzin-tolerant lentil (*Lens culinaris*)
Publication Status	Unpublished and unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Joint first Author)	Alicia Merriam		
Contribution to the Paper	Conducted one of three field trials, analysed and interpreted data, wrote the manuscript		
Overall percentage	50%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am joint first author of this paper, with Navneet Aggarwal.		
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Co-Author Contributions

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

I. The candidate's stated contribution to the publication is accurate (as detailed above)
II. Permission is granted for the candidate to include the publication in the thesis; and
III. The sum of all co-author contributions is equal to 100% less the candidate's contribution

Name of Co-Author (Joint first author)	Navneet Aggarwal		
Contribution to the Paper	Planned the study, conducted two of three field trials, helped with data interpretation, and edited the manuscript.		
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Contribution to the Paper	Helped in planning and conducting two out of three field trials and edited the manuscript.		
Signature		Date	23/02/2022

Control of common sowthistle (*Sonchus oleraceus*) and prickly lettuce (*Lactuca serriola*) using PSII-inhibiting herbicides in a novel breeding line of metribuzin-tolerant lentil (*Lens culinaris*)

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Highlights:

- Metribuzin-tolerant *Lens culinaris* is a promising weed management tool for growers
- This breeding line has excellent crop safety at field rates of metribuzin
- Effective management of *Sonchus oleraceus* and *Lactuca serriola* was achieved
- This breeding line will take pressure off imidazolinone tolerant *Lens culinaris* varieties

Abstract

Control of *Sonchus oleraceus* L. and *Lactuca serriola* L. is difficult in pulse crops in southern Australia due to a lack of available post-emergence herbicide options and widespread resistance to the acetolactate synthase (ALS) inhibitor herbicides. The introduction of new herbicide tolerance traits to these crops is one possible avenue for improving control options. This study aims to evaluate control of ALS inhibitor resistant *S. oleraceus* and *L. serriola* in a novel breeding line of metribuzin-tolerant lentils (*Lens culinaris* Medik.) currently under development in Australian lentil breeding programs. Field experiments conducted over three site-years in South Australia evaluated different rates and application timings of metribuzin, with and without the addition of POST diflufenican, compared to PRE terbuthylazine applications. Herbicide treatments achieved significant levels of control of both *S. oleraceus* and *L. serriola* compared to the untreated control, and there was no negative effect of herbicide treatment on lentil establishment or yield despite two sites experiencing environmental conditions conducive to crop damage by the herbicides used in the experiment. Greater levels of weed control were observed at higher rates of metribuzin for PSPE application timings (for *L. serriola*) and with the POST application of diflufenican. Terbuthylazine treatments also performed well compared to the untreated control but were not as effective as the higher rates of metribuzin. These results indicate that the introduction of a metribuzin-tolerant lentil cultivar will provide an effective broadleaf weed control option in the crop while also avoiding some of the herbicide damage risks associated with currently available options.

Key Words: herbicide tolerance, triazinone, PSII inhibiting herbicides, broadleaf weeds

Abbreviations: ALS: acetolactate synthase, GSR: Growing season rainfall (April – October), PPI: Pre-plant incorporated (herbicide application), PSPE: Post-sowing pre-emergence (herbicide application), POST: Post-emergence (herbicide application), PSII: Photosystem II

5.1 Introduction

Weed control in lentil crops (*Lens culinaris* Medik.) is a challenge because it is inherently a weakly weed competitive crop with low early vigour and there are few post-emergence herbicides available for control of broadleaf weeds in particular (McDonald et al., 2007). Over the last several decades, the dicotyledonous annual weeds *Sonchus oleraceus* L. and *Lactuca serriola* L. have become more common in southern Australia because they are favoured by the adoption of no-till crop seeding practices (Weaver and Downs, 2003; Widderick et al., 1999). These weeds have proven especially problematic in lentil crops and are very common in areas where lentils are intensively grown. Due to demand for alternative, plant-based protein sources, and market price signals, lentils are an increasingly popular break crop in winter annual grain production regions in Australia, however, weed management issues are a barrier to its sustainability as an economically viable component in these systems.

S. oleraceus and *L. serriola* produce a milky sap and can cause mechanical problems at harvest, increase moisture levels in harvested grain and further affect grain quality by staining grains (Amor, 1986; Widderick, 2019). Their seed is readily dispersed by wind (Hutchinson et al., 1984; Weaver and Downs, 2003) and they are taller than the lentil crop, further facilitating dispersal. In addition, they are prolific seed producers, especially in the absence of competition (Amor, 1986; Hutchinson et al., 1984). Furthermore, both species have evolved widespread resistance to the sulfonylurea (SU) (Lu et al., 2007) and imidazolinone (IMI) chemical families of the ALS-inhibiting herbicides in Australia (Merriam et al., 2018). Cases of resistance to 2,4-D and glyphosate have also been reported in Australia for both species (Heap, 2022), but are much less common.

Due to their short stature and low early vigour, lentil plants are weakly competitive and have a long critical period of weed control (CPWC) before canopy closure is achieved (Fedoruk et al., 2011; McDonald et al., 2007). Furthermore, they have little natural tolerance to herbicides and very few post-emergence options are available (McDonald et al., 2007). Therefore, weed management focuses heavily on selecting fields with low weed burden and controlling weeds prior to crop seeding and emergence (GRDC GrowNotes™, 2018). However, weeds such as *S. oleraceus* and *L. serriola* are known to germinate year-round if conditions are favourable (Chadha et al., 2019; Chauhan et al., 2006), and can readily colonise fields from adjacent roadsides and uncropped areas due to their mobility (Hutchinson et al., 1984; Lu et al., 2007). The requirement for additional options to extend control through the season has made herbicide tolerance traits in lentils a priority for breeding programs.

The first herbicide-tolerant lentil variety in Australia was commercially released in 2011 and had tolerance to the IMI herbicides, which allowed growers to use these herbicides in-crop for broadleaf weed control (Bruce et al., 2019; Pulse Breeding Australia, 2011; Rodda et al., 2016). These varieties are now very popular and widely grown in lentil-production regions of Australia. However, the ALS-inhibiting herbicides have high propensity for resistance evolution (Tranel and Wright, 2002) and the availability of IMI tolerant varieties in many other crops (such as wheat, barley, and oats) has led to overuse of these herbicides. There are currently more than twenty different grass and broadleaf weed species with resistance to the ALS inhibiting herbicides reported from winter annual cropping systems in Australia (Heap, 2022).

Across southern Australia, *S. oleraceus* resistance is estimated at 78% to the SUs and 68% to the IMIs (Merriam et al., 2018), and surveys of *L. serriola* in 1999 and 2004 in South Australia estimated SU resistance at 66% and 82%, respectively (Lu et al., 2007). This highlights the need for alternative strategies, including new herbicide tolerance traits, to effectively control these weeds and take some of the pressure off the ALS-inhibitor herbicides. New lines of metribuzin-tolerant lentils have been discovered through mutagenesis and are currently under development and evaluation for commercial viability (McMurray et al., 2019a; McMurray et al., 2019b; McMurray et al., 2019c). Novel varieties developed through this pipeline could be a valuable tool to improve weed control options for lentil growers.

Metribuzin belongs to the PSII inhibiting class of herbicides, and although resistance has been reported in Australia, cases are currently limited to only three weed species in winter annual cropping systems and no resistance has been found in *S. oleraceus* and *L. serriola* (Heap, 2022). Resistance to the PSII-inhibiting herbicides is primarily conferred by a target-site mutation and associated with a significant fitness penalty, although cases of metabolic resistance have also been reported (Gronwald, 1997). Herbicide tolerance in the experimental lentil lines investigated here is also conferred by a target site mutation and accompanied by a fitness penalty, and this fitness penalty will continue to be a focus for breeding programs (McMurray et al., 2019a). However, the fitness penalty, if present in weeds, can be beneficial for their management, as it results in selection for susceptible biotypes in the absence of herbicide selection pressure (Keshtkar et al., 2019). Therefore, provided it is used in moderation as part of a diverse rotation, the metribuzin-tolerant crop technology has a slight benefit over ALS-inhibitor tolerant technology, which is associated with little to no fitness penalty in resistant weeds (Tranel and Wright, 2002).

Metribuzin is registered for control of *S. oleraceus* and *L. serriola* in Australia in several crops including lentils, but at the pre-emergence stage only (Anonymous, n.d.-b). Rates range from 135 to 285 g ai ha^{-1} depending on soil texture and the label also specifies a minimum sowing depth and warns of several conditions that can result in herbicide damage to the crop (Anonymous, n.d.-b). Other PSII-inhibiting herbicides such as terbuthylazine are also registered in lentils at the pre-emergence stage and can provide suppression of these weeds, but crop damage can occur (Anonymous, n.d.-c). Both herbicide labels warn that significant rainfall events shortly after application have the potential to wash the herbicide down to the crop seed zone and cause injury. These conditions occur regularly in crop production areas of southern Australia, which experience winter-dominant rainfall. Therefore, the use of these herbicides comes with some degree of inherent risk.

A lentil variety with high levels of tolerance to metribuzin that allows the use of metribuzin at higher rates would significantly improve control of broadleaf weeds such as *S. oleraceus* and *L. serriola* for lentil growers, and will reduce the risk of crop damage. The purpose of this study is to evaluate control of *S. oleraceus* and *L. serriola* and crop safety in novel metribuzin tolerant lentil germplasm line M043, under development by the South Australian Research and Development Institute (SARDI), using various metribuzin application rates and timings, supplemented with diflufenican, and compared to the current standard of terbuthylazine.

5.2 Materials and methods

5.2.1 Experimental design and sampling

Experimental sites were established in South Australia at Maitland in 2018 and 2019, and at Roseworthy in 2019 (Table 1). Both sites have a Mediterranean climate with hot, dry summers and cool, wet winters and are situated in landscapes dominated by annual winter crop production. Due to the strong relationship between activity of herbicides used in the experiment and rainfall, daily rainfall totals for each of the experimental sites were sourced from the nearest Bureau of Meteorology station and overlaid with herbicide application dates to aid in interpretation of results (Figure S1).

Table 1. Location, site characteristics, and key experimental management dates of the three site years included in the study.

Site characteristics	Maitland 2018	Maitland 2019	Roseworthy 2019
Location	34.37°S, 137.69°E	34.46°S, 137.71°E	-34.54°S, 138.70°E
Soil type	Calcareous gradational clay loam	Hard gradational clay loam	Loam over poorly structured red clay
Experiment year GSR[a] (mm)	326	253	209
Average GSR[a] (mm)	393	393	280
Previous crop	Wheat	Lentils	Wheat
Important management and assessment dates			
Sowing	22 June 2018	6 May 2019	4 June 2019
Crop establishment counts	25 July 2018	5 June 2019	4 July 2019
Weed density counts	30 October 2018	11 October 2019	13 Sept 2019
Harvest	20 December 2018	13 November 2019	18 November 2019

[a] GSR: Growing season rainfall. In temperate Australia, growing season rainfall is that received between April and October. Rainfall data are from the nearest available weather station: Maitland for the 2018 and 2019 Maitland sites and Roseworthy AWS for the 2019 Roseworthy site (Australian Government Bureau of Meteorology, 2019).

The experiment was sown at a seed rate 50 kg ha^{-1} for a target crop density of 120 plants m^{-2} with a 6-row plot seeder and fertiliser was applied at 80 kg ha^{-1} banded below the seed (monoammonium phosphate at Roseworthy and diammonium phosphate at Maitland). Soil moisture was low at sowing time: 15.5% at Maitland in 2018 and 14.3% at Maitland in 2019 at 2-10 cm soil depth. Weeds were controlled prior to sowing at all experimental sites with an application of 900 g ai ha^{-1} propyzamide and 1080 g ae ha^{-1} glyphosate, with the addition of 12 g ai ha^{-1} carfentrazone-ethyl at the Maitland sites (Table 2). The experiment areas were rolled shortly after crop emergence, and fungicide and insecticides were applied through the season as required. A POST (post-emergence) application of 240 g ai ha^{-1} Clethodim (Table 2) was applied across all experimental sites in July for control of grass weeds.

PPI (pre-plant incorporated) herbicide treatments were applied immediately pre-sowing, PSPE (post-sowing pre-emergence) treatments were completed within the following week and POST treatments were applied at the 5-node crop growth stage. All herbicide applications at Roseworthy were done using a hand-held CO_2-pressurised hand boom equipped with TeeJet TT110015 nozzles and operating at 150 kPa and 5 km h^{-1}, while at Maitland applications were done using a tractor-mounted shrouded sprayer equipped with Minidrift 015 nozzles operating at 200 kPa and 5.8 km h^{-1}. At both sites, nozzles were spaced 50 cm apart, 50 cm above the crop and total output was 100 L ha^{-1}.

Herbicide treatments were designed around two rates of metribuzin, applied PSPE or POST, and with or without a supplemental POST application of diflufenican. This treatment list was supplemented with two rates of POST terbuthylazine and a low rate of metribuzin PSPE plus diflufenican POST for comparison purposes. Herbicide products used and manufacturers are listed in Table 2 and the full treatment list can be found in Table 4.

In order to supplement background levels of the target weeds at Roseworthy, seeds of *S. oleraceus* and *L. serriola* from locally representative populations were evenly spread across the plots at a density of 120 seeds m^{-2} and 25 seeds m^{-2}, respectively. The Maitland experimental sites had sufficient background populations of these weeds. Densities of *S. oleraceus* and *L. serriola* were assessed at least 8 weeks following POST herbicide applications.

Table 2. Herbicides used in experimental treatments, site preparation, and experimental site management in the experiments. Active ingredients, trade names, formulations by weight or volume and manufacturers are listed.

Active ingredient	Trade name	Formulation	Manufacturer
Metribuzin	Metribuzin WG	750 g kg^{-1}	Genfarm Crop Protection Pty Ltd, Australia
Terbuthylazine	Terbyne® WG	750 g kg^{-1}	Sipcam Pty Ltd, Australia
Diflufenican	Brodal® Options	500 g L^{-1}	Bayer Crop Science, Pty Ltd, Australia
Propyzamide[a]	Rustler® WG	900 g kg^{-1}	FMC Pty Ltd, Australia
Glyphosate[a]	Roundup PowerMAX®	540 g L^{-1}	Nufarm Pty Ltd, Australia
Carfentrazone-ethyl[a]	Carfentrazone-ethyl EC	240 g L^{-1}	Imtrade Australia Pty Ltd, Australia
Clethodim[b]	Select® EC	240 g L^{-1}	Arysta LifeSciences Pty Ltd, Australia

[a]These herbicides were applied across the whole experimental area for site preparation.

[b]Applied POST across the whole experimental area for control of grass weeds which were not the focus of the experiment.

5.2.2 Data analysis

Data were analysed using R Statistics Package (R Core Team, 2022) along with the r packages ‘agricolae’ (de Mendiburu, 2020) and ‘ASReml’ (Gilmour et al., 2009). Two-way ANOVA found that site differences were present, so herbicide treatment data were analysed separately for each site. Herbicide treatment effects on weed density were found to be significant using ANOVA and multiple comparison was applied based on square root transformed data using Tukey’s HSD.

5.3 Results and discussion

5.3.1 Rainfall conditions at the sites

The optimal conditions for the application of both metribuzin and terbuthylazine depend on rainfall, as these herbicides are absorbed primarily by the roots and soil moisture levels affect mobility (Anonymous, n.d.-b; Anonymous, n.d.-c). The Australian labels for these herbicides recommend both to be applied to moist soils, and best results are achieved when followed by 6-12 mm of rain within 2 weeks for metribuzin, and 20-30 mm within 2-3 weeks for terbuthylazine (Anonymous, n.d.-b; Anonymous, n.d.-c). However, heavy rainfall events following application can wash the herbicide into the crop seed zone, causing crop damage and reducing the period of activity on weeds. Diflufenican is a contact herbicide so must be applied before canopy closure, less than eight weeks, but ideally between four and six weeks after sowing (Anonymous, n.d.-a). All three products function best when weeds are actively growing and unaffected by moisture stress.

Maitland in 2018 had above average growing season rainfall and this was distributed more evenly throughout the growing season than at the two sites in 2019 (Figure S1A). The crop was sown following 30 mm of rainfall in the previous week, providing adequate soil moisture. However, only 14 mm of rainfall was received in the three weeks following terbuthylazine application. Metribuzin was applied PSPE about a week after the PPI treatments, when no rainfall had been received in the previous 10 days and was followed by about 12 mm in the following two weeks. Applications of POST metribuzin and diflufenican were also applied in suitable conditions shortly after a rainfall event and followed by sufficient rainfall. This site had the longest interval between sowing and POST treatments at just under eight weeks, but these were still applied prior to canopy closure.

Maitland in 2019 had low growing season rainfall characterised by one large event (>30 mm), several medium events (>10 mm) and many smaller events throughout the season (Figure S1B). Approximately 25 mm were received in the week prior to sowing and PPI treatments, and over 30 mm in the three weeks following, providing optimal conditions for the activity of terbuthylazine. PSPE treatments of metribuzin were applied a week later and were also followed with sufficient rainfall. Metribuzin and diflufenican were applied POST about a week after the large rainfall event of the season, and nearly 30 mm were received in the following two weeks, also providing optimal conditions. The diflufenican treatments were applied 6 weeks after sowing, within the optimal window.

The 2019 Roseworthy site had the lowest growing season rainfall, and this was primarily concentrated in two large events with the rest consisting of small, sporadic rainfall events (Figure

S1C). Only 8 mm were received in the week prior to sowing meaning that less surface soil moisture was present at this site compared to the other sites. A large rainfall event of 33 mm occurred about a week after sowing. POST treatments were applied under more favourable conditions with sufficient rain in the weeks before and after, and approximately 6 weeks after sowing.

The patterns of rainfall experienced at each of the site-years (Figure S1) may have affected behaviour of herbicides and contributed to some of the differences in weed control between the sites.

5.3.2 Crop safety and yield

Herbicide treatment had no significant effect on crop establishment or yield in any of the site years, although results differed between site years (Table 3). The M043 lentil line used in this study was previously determined to have 33-fold tolerance to metribuzin compared to its conventional parent line PBA Flash, and GR_{50} values greater than the field rate of metribuzin (McMurray et al., 2019a). Some bleaching of lentil leaves was observed following application of diflufenican, which lasted for up to four weeks, however, this did not affect lentil yield. There was also no indirect positive effect on crop yield due to effective weed control achieved by the herbicide treatments, but the weeds studied in this experiment are not typically associated with competition-based yield reductions, except at extremely high weed densities (Manalil et al., 2020).

Crop establishment was higher at Maitland in both years than at Roseworthy (Table 3). Grain yield was measured at the 2018 Maitland and 2019 Roseworthy sites only, with Maitland having a significantly higher grain yield than Roseworthy. Poor crop establishment at Roseworthy could be related to environmental conditions such as soil moisture at sowing. The poor establishment was likely the main cause for lower yield, along with the lower growing season rainfall received at Roseworthy (209 mm compared with 326 mm at Maitland in 2018). All three sites experienced lower than average growing season rainfall, but the shortfall was greater at Roseworthy (25%) than at Maitland in 2018 (17%).

Table 3. Crop establishment in all three site years and yield and water use efficiency in 2018 at Maitland and 2019 at Roseworthy.

Site year	Crop establishment	Grain yield	Water use efficiency*
	plants m^{-2}	t ha^{-1}	kg $ha.mm^{-1}$
2018 Maitland	124 a	1.41 a	4.33 a
2019 Maitland	120 a	--	--
2019 Roseworthy	77 b	0.66 b	3.17 b
P value	<2e-16	9.952e-15	1.916e-06

Means within a column followed by the same letter are not significantly different at p=0.05.

*Water use efficiency has been calculated as yield per mm of growing season rainfall (rainfall received between April and October).

Water use efficiency has been approximated as yield per millimetre of GSR (rainfall received between April and October each year) and helps to standardise yields, which are highly dependent on rainfall as a limiting factor. The 2018 Maitland crop achieved greater water use efficiency than the 2019 Roseworthy crop (Table 3). This is likely due to weed pressure, as the Roseworthy site had a high population of annual ryegrass (*Lolium rigidum*) that escaped pre-sowing herbicide applications and was not controlled until later in the season. The literature reports lentil water use efficiencies ranging from 3.8 kg $ha.mm^{-1}$ in Syria (Zhang et al., 2000) to 6.7 kg $ha.mm^{-1}$ in Western Australia (Siddique et al., 2001), so WUE falls at the low end for both sites in this study.

Yield at Roseworthy was lower than the regional average of 1.3 t ha^{-1} for lentils in that year (Lewis, 2020). Yield at the Maitland site was equal to the regional average of 1.4 t ha^{-1} (Lewis, 2019). However, experiments on the Yorke Peninsula have previously demonstrated the yield penalty for this lentil cultivar resulting from the herbicide tolerance mutation (Roberts et al., 2020). Improving yield and water use efficiency in this cultivar will be a focus of breeding programs in order to prepare it for commercial release.

These results demonstrate the crop safety of the herbicides treatments on the M043 line in a field setting. In 2019, both sites experienced a significant rainfall event of over 30 mm within weeks after application of PPI and PSPE herbicides, which is a risk factor for herbicide injury to the crop (Anonymous, n.d.-b; Anonymous, n.d.-c), yet no crop damage was observed. Currently, growers are using terbuthylazine for weed control in lentils, but under certain environmental conditions crop damage can occur. The introduction of the M043 tolerant line would allow growers to use metribuzin at higher and more effective rates confidently in lentils.

5.3.3 Weed control

Significant herbicide treatment effects were observed for both *L. serriola* and *S. oleraceus* in all site-years (Table 4). *S. oleraceus* density was higher on average than *L. serriola* at Maitland in 2018 and Roseworthy in 2019, while at Maitland in 2019 *L. serriola* density was higher. Although the weed seed bank was artificially supplemented at the Roseworthy site, it still had lower weed density than both Maitland sites. Maitland in 2019 had similar combined weed density to Maitland in 2018, despite having significantly less rainfall, but differed in the dominant species present.

With the exception of *S. oleraceus* at Maitland in 2018, and *L. serriola* in Maitland in 2019, initial weed densities in the study were relatively low. This is a limitation of the study and does impact on the ability to differentiate between herbicide treatments particularly at Roseworthy in 2019. However, it should be noted that while crop yield impacts are only seen at very high densities of these weed species (Manalil et al., 2020), other issues can arise even at low weed density. For example, plants growing under low competition from a weakly competitive lentil crop are capable of producing very significant amounts of seed (Amor, 1986; Hutchinson et al., 1984) , and large plants can impact on grain quality by increasing moisture levels at harvest and staining grain (Amor, 1986; Widderick, 2019).

Table 4. Densities of *S. oleraceus* and *L. serriola* assessed at least 8 weeks following application of final POST herbicide treatments at Maitland in 2018 and 2019, and Roseworthy in 2019. Herbicide application abbreviations: PPI = pre-plant incorporated, PSPE = post-sowing pre-emergence, POST = post emergence.

		S. oleraceus			*L. serriola*		
	Rate	2018 Maitland	2019 Maitland	2019 Roseworthy	2018 Maitland	2019 Maitland	2019 Roseworthy
	g ai ha^{-1}	Plants m^{-2}					
Terbuthylazine (PPI)	750	3.90 b	1.33 abc	1.33 ab	0.43 b	1.67 cd	0 b
Terbuthylazine (PPI)	1050	2.67 bc	1.33 abc	0.67 b	0.43 b	3 bcd	0.67 ab
Metribuzin (PSPE)	270	3.10 bc	4.00 ab	1.00 ab	0 b	1.67 bcd	1.33 ab
Metribuzin (PSPE)	540	0.00 d	0.00 c	0.00 b	0 b	3 bcd	0.33 ab
Metribuzin (PSPE) + Diflufenican (POST)	210 + 100	-	0.33 c	1.33 ab	-	1.67 cd	0.33 ab
Metribuzin (PSPE) + Diflufenican (POST)	270 + 100	0.77 bcd	0.33 c	0.00 b	0 b	0 d	0 b
Metribuzin (PSPE) + Diflufenican (POST)	540 + 100	0.00 d	0.00 c	0.00 b	0 b	0 d	0.33 ab
Metribuzin (POST)	270	3.67 b	0.67 bc	1.67 ab	0 b	8 ab	0.33 ab
Metribuzin (POST)	540	0.43 cd	0.00 c	0.67 b	0.43 b	3.33 bcd	0 b
Metribuzin (POST) + Diflufenican (POST)	270 + 100	1.23 bcd	0.33 c	1.00 ab	0 b	5.33 bc	0.33 ab
Metribuzin (POST) + Diflufenican (POST)	540 + 100	0.00 d	0.00 c	0.67 b	0 b	6 bc	0 b
Untreated control	nil	25.67 a	5.67 a	4.67 a	4.90 a	17.67 a	2.67 a
P value		1.49E-09	8.64E-05	0.000947	1.33E-05	5.19E-07	0.00345

Means within a column followed by the same letter are not significantly different at *p*=0.05.

Multiple comparisons are based on square root transformed data but means have been back-transformed.

The herbicide treatments associated with the lowest densities of both weeds across all sites included metribuzin at 540 g ai ha^{-1} POST (with and without diflufenican), and PSPE (with and without diflufenican), and metribuzin at 270 g ai ha^{-1} applied PSPE (with diflufenican). In some cases, the *L. serriola* densities at Roseworthy in 2019 under these treatments were not significantly different to the control, but this site had very low, sporadic density of *L. serriola*. The treatments associated with higher weed densities (not significantly different to the untreated) of both weed species across all sites included the terbuthylazine treatments (particularly the lower rate), metribuzin at 270 g ai ha^{-1} applied PSPE (without diflufenican) and metribuzin at 270 g ai ha^{-1} applied POST (with and without diflufenican). Furthermore, when seed pods of *S. oleraceus* were counted at Maitland in 2019, terbuthylazine at 750 g ai ha^{-1} and metribuzin at 270 g ai ha^{-1} applied PSPE (without diflufenican) failed to achieve control of seed pod production significantly different to the untreated (Table S1**)**.

5.3.3.1 Effect of metribuzin rate

When looking at differences in metribuzin rate alone among otherwise analogous treatments, metribuzin performed better at the higher rate (540 g ai ha^{-1}) than at lower rates, especially on *S. oleraceus*. When metribuzin was applied PSPE without a follow-up application of diflufenican, the higher rate of metribuzin achieved significantly better control of *S. oleraceus* at Maitland in 2019. At Maitland in 2018 this difference between high and low metribuzin rate was significant only for POST applications of metribuzin without follow-up diflufenican.

Conventional lentils are damaged by POST metribuzin but the yield penalties are reduced at low rates (McMurray et al., 2019b). As such, some growers are willing to take the risk of application if broadleaf weed burden is high. Lower rates of metribuzin were included in this study for comparison purposes: 135 g ai ha^{-1} at Maitland in 2018 only at both application timings and with and without diflufenican (Table S1), and 210 g ai ha^{-1} at both 2019 sites applied PSPE with diflufenican only (Table 4). In some cases, the low rate treatments did provide some level of weed control over the untreated, however, they failed to consistently demonstrate the same level of control as higher rates.

At the 135 g ai ha^{-1} rate the difference is clear, especially for *S. oleraceus* (Table S1). When combined with POST diflufenican, the 135 g ai ha^{-1} rate achieved lower *S. oleraceus* control than both the higher rates at both application timings of metribuzin. Without POST diflufenican, the 135 g ai ha^{-1} rate performed worse than the highest rate, but not significantly differently to the intermediate rate at both application timings. For *L. serriola*, reduced efficacy of the 135 g ai ha^{-1} rate was observed for PSPE application of metribuzin alone, where it achieved lower levels of control than both the intermediate and high rates of metribuzin. In addition, without the addition of diflufenican, the lowest rate also failed to show any *L. serriola* control benefit over the untreated.

Therefore, although significant crop yield penalties may be avoided at low rates of metribuzin, this is a risky practice and fails to achieve the same levels of weed control as full rates of the herbicide. Metribuzin label amendments following the release of a tolerant lentil variety could help improve label compliance by providing higher levels of weed control without the crop safety risk. Although maximum label rates of metribuzin will not be limited by the herbicide tolerance of the M043 line, metribuzin is an important environmental pollutant (Dores et al., 2008; Pot et al., 2011), so recommended rates will have to balance weed control with environmental considerations.

5.3.3.2 Effect of application timing and supplementation with diflufenican

Pairwise comparison of treatments differing only in the application timing of metribuzin reveals a slight advantage of PSPE treatments. PSPE applications of metribuzin at both the 270 and 540 g ai ha^{-1} rates achieved significantly greater control of *L. serriola* at Maitland in 2019 than POST applications, but only when combined with POST application of diflufenican. This could be due to splitting the herbicide applications over two periods of exposure, allowing for better control of *L. serriola* seeds germinating at different times throughout the season. Although the metribuzin label does not specifically list *L. serriola*, it notes that for optimal control weeds should not be beyond the three-leaf stage. Metribuzin applied PSPE would provide control of early-season germinations, which may be beyond the optimal growth stage for control by the time POST application of metribuzin occurs.

Comparing analogous treatments differing only in the inclusion or exclusion of diflufenican showed an advantage to diflufenican use in combination with some of the treatments. The addition of diflufenican provided significantly better control of *S. oleraceus* at Maitland in 2019 when combined with the lower rate of metribuzin applied PSPE. As mentioned above, it seems the application of diflufenican is particularly complementary to the PSPE applications of metribuzin, as it increases the proportion of the weed population exposed to herbicide during the earlier stages of growth. The fact that a significant difference due to diflufenican addition was observed only for the lower rate of metribuzin and not the highest rate suggests that at this rate metribuzin is responsible for most of the control. *S. oleraceus* populations were reduced to 0 plants m^{-2} regardless of whether diflufenican is applied.

5.3.3.3 Comparison with terbuthylazine treatments

Increasing the rate of terbuthylazine (750 versus 1050 g ai ha^{-1}) did not improve control of either weed species in any site year. Terbuthylazine treatments performed similarly to the best metribuzin treatments on *L. serriola* in all site years, and on *S. oleraceus* at both 2019 sites. However, at Maitland in 2018 all the high-rate metribuzin treatments achieved significantly better control of *S. oleraceus* than the lower rate of terbuthylazine, and all but one (metribuzin 540 g ai ha^{-1} applied POST without diflufenican) outperformed the high rate of terbuthylazine. Terbuthylazine is currently registered for control of broadleaf weeds in conventional lentil crops, but can cause crop damage under certain conditions. Therefore, a metribuzin-tolerant lentil variety would allow improved weed control without the risk of crop safety.

5.3.4 Herbicide resistance management

Given the success of recently released imidazolinone-tolerant lentil varieties and subsequent increase in the frequency of use of imidazolinone herbicides, it is reasonable to expect that a new metribuzin tolerant variety could also be popular. While the PSII inhibiting herbicides have slightly lower propensity for resistance evolution than the ALS inhibiting herbicides, many cases have been reported, and in Australia several of these cases have arisen in triazine-tolerant canola crops (Heap, 2022).

ALS-inhibitor resistance in *S. oleraceus* and *L. serriola* is extremely common throughout the areas where the field experiments were conducted (Lu et al., 2007; Merriam et al., 2018), and is confirmed in the local population of both species at the Roseworthy experimental site (Merriam et al., 2021) and the *S. oleraceus* population at the Maitland 2018 site (50-55% survival to Intervix 500 ml ha^{-1} and imazapic 200 g ha^{-1}, respectively). The Maitland 2019 experimental site was selected following failure of Intervix to control both species the previous year. While this study shows imidazolinone-resistant biotypes are effectively controlled by high rates of metribuzin in a tolerant lentil crop, the development of multiple resistant biotypes is a risk that will need to be managed. Multiple resistance to the ALS and PSII inhibitors is reported in many weed species including *Poa annua* (Singh et al., 2021) and *Amaranthus palmeri* (Chaudhari et al., 2020) in the United States, *Amaranthus retroflexus* (Francischini et al., 2019) and *Bidens pilosa* (Takano et al., 2016) in Brazil, and *Lolium rigidum* in Australia (Ma et al., 2020).

Responsible use of the herbicide tolerant crop technology would see these varieties included as part of a diverse crop rotation along with cereals, oilseeds, and other pulses, and including conventional lentil varieties where appropriate to ensure a diverse herbicide rotation. Other triazine tolerant crops, such as triazine tolerant canola, should be used only sparingly in these rotations to reduce exposure to the PSII-inhibiting herbicides. Regular monitoring of crops for herbicide failures and suspected resistance can help to catch problems early, and seed-set reduction of any plants surviving to crop maturity can prevent problems carrying over into the following season. While the fitness penalty inherent in target-site resistance to the PSII inhibiting herbicides can be exploited to manage resistant weed populations (Keshtkar et al., 2019), this relies on removal of the herbicide selection pressure for several years to allow time for the susceptible biotype to outcompete the resistant biotype.

5.4 Conclusion

We conclude the novel metribuzin tolerant line has promise for weed control, however, further work to characterise the fitness penalty of the tolerance trait and improve yield through successive breeding is required for it to become a viable option for growers. Several treatments showed promise, particularly the higher rates of metribuzin. Both PSPE and POST application timings were effective, with PSPE applications outperforming POST applications on *L. serriola* at one site year, and in treatments including a POST application of diflufenican. The addition of diflufenican also provided some additional control of *S. oleraceus* when combined with the lower rate of metribuzin applied PSPE. The study demonstrated metribuzin treatments can provide a superior level of control to currently registered rates of terbuthylazine in conventional lentils. Responsible use of the herbicide tolerant crop technology will require a diverse crop rotation and conscientious management of herbicide applications to prevent the development of multiple resistance to the PSII and ALS inhibiting herbicides in lentil production areas of southern Australia. As improved weed control is the primary driver for development of this breeding line, further work prior to its commercial release should focus on other aspects of weed management, such as control of other broadleaf weeds known to be problematic in lentils, and tolerance to other PSII inhibiting herbicides.

5.5 Acknowledgements

This research is supported by an Australian Government Research Training Program Scholarship, a Grains Research and Development Corporation (GRDC) Grains Research Scholarship (Project UOA1801-003RSX), and strategic joint investment from GRDC and SARDI in Project DAS 00168BA. The metribuzin tolerant SARDI M043 lentil germplasm line was developed through GRDC-SARDI-University of Adelaide funded projects DAS 00107 and DAS00131, and authors appreciate the contribution of Larn McMurray, Dili Mao, Jeff Paul and Klaus Oldach for their efforts in its development. The research undertaken as part of this project is made possible by the significant contributions of growers through both trial cooperation and the support of the GRDC. Technical assistance provided by David Brunton, Ben Fleet and Jerome Martin of the University of Adelaide Weed Science Research Group, and Greg Walkley, John Nairn, Phil Rundle, Patrick Thomas and Jacob Nicolai of SARDI Clare Research Centre was highly appreciated.

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5.7 Supplemental data

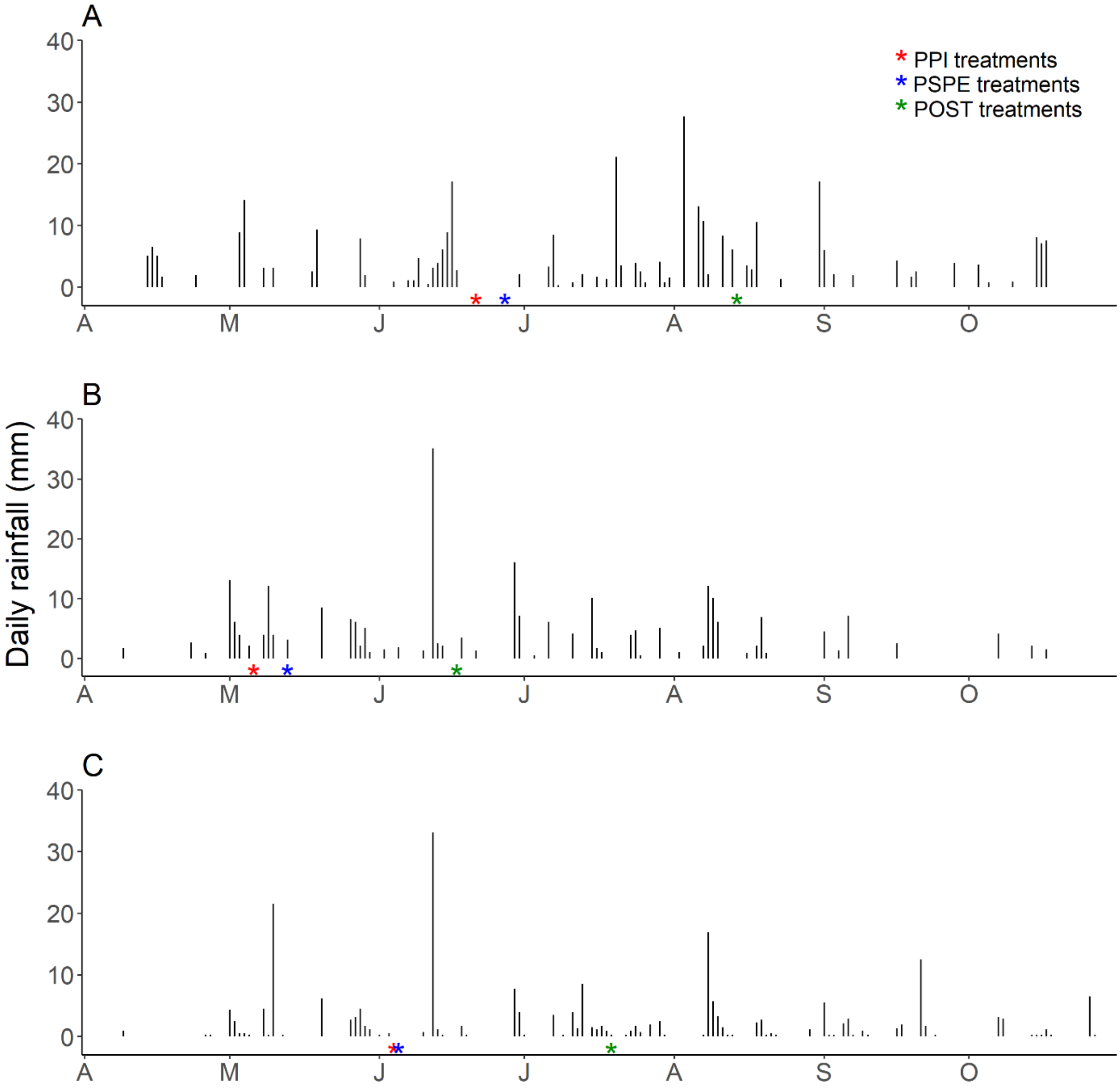

Figure S1**.** Daily rainfall totals and important experimental management dates during the growing season (April to October) at each of the site-years of the experiment: A) Maitland 2018, B) Maitland 2019, C) Roseworthy 2019. Herbicide treatments are indicated by red asterisks (pre-plant incorporated (PPI) applications), blue asterisks (post-sowing pre-emergence (PSPE) applications) and green asterisks (post emergence (POST) applications). Rainfall data are from the nearest available weather station: Maitland for the 2018 and 2019 Maitland sites (2 km and 5 km from the experimental sites, respectively) and Roseworthy AWS for the 2019 Roseworthy site (4 km from the experimental site) (Australian Government Bureau of Meteorology, 2019).

Table S1**.** Additional data collected at only one site-year including weed densities under the lowest metribuzin rate treatments and lentil seed weight at Maitland in 2018, and seed production of *S. oleraceus* at Maitland in 2019. Herbicide application abbreviations: PPI = pre-plant incorporated, PSPE = post-sowing pre-emergence, POST = post emergence.

		Maitland 2018		Maitland 2019
		Weed density		
Herbicide and application timing	Rate	*S. oleraceus*	*L. serriola*	*S. oleraceus* pod production
	g ai ha^{-1}	Plants m^{-2}		Capitula m^{-2}
Terbyne (PPI)	750	3.90 bcd	0.43 bc	71.67 ab
Terbyne (PPI)	1050	2.67 bcde	0.43 bc	28.00 b
Metribuzin (PSPE)	135	6.33 b	2.23 ab	-
Metribuzin (PSPE)	270	3.10 bcd	0 c	97.00 ab
Metribuzin (PSPE)	540	0 e	0 c	0.00 b
Metribuzin (PSPE) + Diflufenican (POST)	135 + 100	6.13 bc	0 c	-
Metribuzin (PSPE) + Diflufenican (POST)	270 + 100	0.77 de	0 c	24.33 b
Metribuzin (PSPE) + Diflufenican (POST)	540 + 100	0 e	0 c	0.00 b
Metribuzin (POST)	135	6.10 bc	1.33 abc	-
Metribuzin (POST)	270	3.67 bcd	0 c	19.33 b
Metribuzin (POST)	540	0.43 de	0.43 bc	0.00 b
Metribuzin (POST) + Diflufenican (POST)	135 + 100	8.00 b	0.43 bc	-
Metribuzin (POST) + Diflufenican (POST)	270 + 100	1.23 cde	0 c	1.67 b
Metribuzin (POST) + Diflufenican (POST)	540 + 100	0 e	0 c	0.00 b
Unsprayed control	nil	25.67 a	4.90 a	250.33 a
P value		5.96E-11	6.67E-06	8.732e-05

Means within a column followed by the same letter are not significantly different at *p*=0.05.

Multiple comparisons of weed density data are based on square root transformed data but means have been back-transformed.

6 GENERAL DISCUSSION AND CONCLUSION

6.1 Introduction

This research has demonstrated that ALS inhibitor resistance in both annual sowthistle and prickly lettuce is widespread throughout the areas where surveys were conducted. In annual sowthistle, imidazolinone resistance is less frequent and not as strong as resistance to the sulfonylureas, but is still sufficient to impede control of weeds in imidazolinone-tolerant crops. Glyphosate and synthetic auxin resistance are either not present or not widespread in either species in southern Australia, but pose a potential future threat. Analyses of populations structure indicated that both dispersal of resistant biotypes and multiple independent resistance evolution events likely contribute to the mosaic of resistance seen across the landscape and highlighted both similarities and differences between the two species. Finally, while annual sowthistle and prickly lettuce continue to pose management challenges in lentil, this project explored management tactics that address this issue through management in other phases of rotation and the use of herbicide tolerant crop technology.

6.2 Herbicide resistance in prickly lettuce and annual sowthistle

Widespread resistance to the ALS inhibiting herbicides is responsible for most resistance related management issues in prickly lettuce and annual sowthistle and has been for some time. Glyphosate and synthetic auxin resistance are not yet a widespread problem in either weed species in southern Australia, but probably represent a future threat due to increased reliance on these modes of action in the face of widespread ALS inhibitor resistance.

Confirmed cases of sowthistle resistant to glyphosate in northern Australia (van der Meulen et al., 2016), and to synthetic auxins in parts of southwestern Australia (Merriam et al., 2018; Preston et al., 2017) pose an ongoing threat due to the mobility of seed and evidence of long-distance dispersal in this species. Resistance screening with these herbicide groups should be maintained as a priority in both southern and northern Australia to monitor the spread of resistance.

While widespread resistance of prickly lettuce to glyphosate and synthetic auxins are not known to be present in Australia at this time, prickly lettuce has demonstrated the ability to evolve synthetic auxin resistance in the United States (Burke et al., 2009). Wind dispersal over such a large distance is highly improbable, so provided Australia's biosecurity regulations prevent human-vectored dispersal of resistant biotypes, an independent evolution event would be the most likely source of Australian cases of synthetic auxin resistance in prickly lettuce. Because the synthetic auxins represent an important line of defence against broadleaf weeds such as these in cereal crops, these herbicides should be used responsibly and weed populations should be monitored diligently for signs of emerging resistance.

6.2.1 ALS inhibitor resistance in prickly lettuce

ALS inhibitor resistance in prickly lettuce was investigated in detail in Chapter 2, where a 2019 survey found sulfonylurea and imidazolinone resistant individuals in every population screened, with average survival of 92% to the sulfonylureas and 95% to the imidazolinones. This contrasts with earlier surveys of the same region in which Lu et al. (2007) found sulfonylurea resistant individuals in only 66% of populations in 1999 and 82% of populations in 2004. While sample sizes are too small to prove that the frequency of resistance has increased over time, this is certainly a possibility given the continued and expanded use of ALS inhibiting herbicides, particularly following the adoption of imidazolinone tolerant crop technology.

Molecular work from earlier surveys in the region found Pro-197-Thr, Pro-197-Ser, Pro-197-His and Pro-197-Leu substitutions (Lu et al., 2007; Preston et al., 2006) endowing ALS inhibitor resistance, while the present study found a novel, uncommon Pro-197-Phe substitution in addition to the former list (Chapter 2: Figure 1). This novel substitution constitutes a two base-pair change and may have arisen from a further mutation within an individual already carrying a Pro-197-Leu or Pro-197-Ser substitution. Because Pro-197-Ser substitutions are far more common in the landscape than Pro-197-Leu substitutions, this would appear to be the more likely candidate. All substitutions studied were shown to confer similar levels of resistance to the sulfonylureas, but differences emerged when screening with the imidazolinones. The Pro-197-Ser substitution resulted in lower imidazolinone resistance (LD_{50} approximate to the field rate), while the Pro-197-Phe substitution resulted in higher levels of imidazolinone resistance than other substitutions (Chapter 2: Table 4).

Imidazolinone-tolerant crops were first released in Australia around the turn of the century and early concerns were raised about their propensity to select for resistance (Preston and Rieger, 2000). The appearance of a novel Pro-197-Phe substitution with elevated imidazolinone resistance following the adoption of imidazolinone tolerant crops could be an indication that the subsequent increased use of imidazolinone herbicides provided the selection pressure for this novel substitution to arise. Since the Pro-197-Ser substitution has an LD_{50} approximate to the field rate of one of the most popular and widely used imidazolinone products in Australia, it would have been a possible candidate for this selection pressure to act upon. Furthermore, genetic analysis showed that the individuals with the novel Pro-197-Phe substitution clustered with individuals carrying both the Pro-197-Leu and the Pro-197-Ser substitutions. Because the other substitutions (with the exception of the Pro-197-Ser mutation) already conferred imidazolinone resistance levels well in excess of the field rate, it is unclear whether the stronger Pro-197-Phe substitution will cause additional management problems. However, it still demonstrates the ability of continued selection pressure to cause further resistance evolution in already resistant populations.

6.2.2 ALS inhibitor resistance in annual sowthistle

ALS inhibitor resistance in annual sowthistle was examined in Chapter 3. Screening of annual sowthistle showed that ALS inhibitor resistance levels in the lentil-producing areas of the Mid North and the Yorke Peninsula may be higher than the overall average for south eastern Australia. Sulfonylurea resistance was found to affect 90% of Mid North and 88% of Yorke Peninsula populations, compared to 78% across south eastern Australia (Merriam et al., 2018), while imidazolinone resistance was found in 84% of Mid North populations and 67% of Yorke Peninsula populations, compared to 68% across south eastern Australia. These studies also found no evidence of an increasing trend in the proportion of sulfonylurea resistant populations since surveying began in 2010, indicating that an equilibrium level of resistance had already been reached prior to that point.

The present study found six different substitutions at Pro-197, including Pro-197-Leu, Pro-197-Ala, Pro-197-Arg, Pro-197-Ser, and Pro-197-Thr as reported from the region by Petersen (2018), plus a very uncommon Pro-197-His substitution (Chapter 3, Figure 1). Because annual sowthistle has two genomes and two copies of the ALS gene (Cho et al., 2019; Stebbins, 1953) these substitutions appeared in various combinations within an individual, either with each other or the wild type proline. Both the Mid North region and the extensively surveyed single field, YP-PC, were dominated by a single combination (WT/Pro-197-Arg and Pro-197-Ala/Pro-197-Leu, respectively), while the Yorke Peninsula region showed a greater spread among combinations.

Resistance phenotype also showed some variation according to the substitution combination (Chapter 3: Table 5). Although a mutation on only one copy of the ALS gene was sufficient to confer significantly higher sulfonylurea resistance compared to the wild type, lines with mutations on both copies tended to have higher resistance. For the imidazolinones, some of the lines with single mutations had overlapping confidence intervals with the wild type lines and generally lines with two mutations were more resistant. However, this effect was not consistent, and it appeared that the specific substitutions in question were of greater importance. The Pro-197-Arg and Pro-197-Thr mutations provided the highest levels of resistance to both the imidazolinone and sulfonylurea herbicides, especially when combined with a second mutation. The Pro-197-Leu mutation seemed to provide higher levels of imidazolinone resistance, but lower levels of sulfonylurea resistance (Chapter 3: Table 5).

Another phenomenon observed for annual sowthistle was that different lines with the same mutation combination exhibited different levels of resistance following herbicide treatment. It was hypothesised that this could be due to the specific ALS copy to which each mutation was assorted, or subsequently to differences in expression between the two ALS copies. Genome assortment was investigated for 4 pairs of lines each with the same mutation combination, but different levels of resistance. Only one of these pairs had the mutation assorted to a different copy of the ALS gene. This pair was further investigated for differences in gene expression between the two copies, but differences were not significant. As a result, these investigations are inconclusive and other factors may contribute to the observed differences in resistance.

6.2.3 Similarities and differences in herbicide resistance patterns between annual sowthistle and prickly lettuce

Although prickly lettuce and annual sowthistle are taxonomically related and ecologically similar (Hutchinson et al., 1984; Weaver and Downs, 2003), investigations of ALS inhibitor resistance in this project revealed some differences between the two species. While both species have been exposed to ALS inhibiting herbicides for a similar length of time and under similar intensity, resistance seems to be stronger and more widespread in prickly lettuce (Chapter 2: Table 3, Chapter 3: Table 3). Furthermore, imidazolinone resistance was less common than sulfonylurea resistance in annual sowthistle, but prickly lettuce showed a similar frequency of resistance to both herbicide families (Chapter 2: Table 3, Chapter 3: Table 3). This leads to the question of whether the amphidiploid status of annual sowthistle and the possibility of wild type and mutated copies of ALS occurring simultaneously within an individual could be providing a tempering effect on ALS inhibitor resistance in annual sowthistle. This could have implications for evolution of other types of target-site resistance in annual sowthistle compared to its diploid counterparts.

In this study, mutations were found only at the Pro-197 position of the ALS gene in both prickly lettuce and annual sowthistle. This was the first identified and remains the most common mutation site conferring resistance to the ALS-inhibiting herbicides, also encompassing the greatest number of reported substitutions (Guttieri et al., 1992; Tranel et al., 2022; Yu and Powles, 2014). The Pro-197-Ser substitution is the most commonly reported at this position (Yu and Powles, 2014), and was found in both species, although was not the most common in either species (second after pro-197-Thr in prickly lettuce, and fourth after Pro-197-Leu, -Ala, and -Arg in annual sowthistle).

Both species had a similar array of possible mutations, with Pro-197-His, Pro-197-Leu, Pro-197-Ser and Pro-197-Thr in common. Only annual sowthistle had Pro-197-Arg or Pro-197-Ala mutations and the novel Pro-197-Phe mutation was found only in prickly lettuce. The latter mutation was the only mutation not previously reported in any weed species (Tranel et al., 2022), and represents a two base-pair change from the wild type proline. This type of substitution has been shown to be far less common across weed species and substitution sites (Yu and Powles, 2014). The lack of significant variation in LD_{50} between prickly lettuce mutations makes comparison of the effects of specific mutations difficult between species, however, the Pro-197-Ser mutation did appear to provide weaker imidazolinone resistance in both species and weaker sulfonylurea resistance in annual sowthistle.

6.3 Population structure and gene flow in prickly lettuce and annual sowthistle

Both species showed high levels of autogamy in both experimental tests of outcrossing and population genetic analysis, although both experiments indicated outcrossing may be more common in annual sowthistle. The cross-pollination experiment indicated 0.21% resistance gene transfer in annual sowthistle and 0.14% in prickly lettuce, although neither of these figures were significantly different to the pollen-excluded control (p= 0.301 and p = 0.391, respectively). Population genetic studies based on multi-locus SNP analysis indicated an overall inbreeding coefficient (F_{IS}) of 0.318 in annual sowthistle and 0.517 in prickly lettuce. The inbreeding coefficient F_{IS} can be used to generate an estimate of the selfing rate s of a population if the population is at inbreeding equilibrium using Equation 1 (Allard et al., 1968). If inbreeding equilibrium were to be assumed in the populations in this study this would result in an estimated selfing rate of 0.48 for annual sowthistle and 0.68 for prickly lettuce.

$$s = \frac{2F_{\text{IS}}}{1 + F_{\text{IS}}} \qquad \text{(Equation 1)}$$

Principal component analysis of SNP data also revealed differences between the two species, with prickly lettuce showing more evidence of population structure and genetic clustering. The first two principal components explained only 10.8% of variation for annual sowthistle (Chapter 3: Figure 2) , compared with 55% for prickly lettuce (Chapter 2: Figure 2). This is an indication of less overall population structure, which is also evident in the distribution patterns of individuals within the PCA. In prickly lettuce there was more evidence of clustering both with respect to geographic region of origin and substitution at the Pro-197 position. The annual sowthistle samples showed very little geographic population structure, with samples from geographically distant areas failing to show significant segregation. Notably, the annual sowthistle samples collected from within the single field, YP-PC, encompassed the same range of variation observed in samples from across the landscape. This phenomenon was not observed in prickly lettuce.

For prickly lettuce, the clustering of all individuals with the Pro-197-Phe mutation with individuals carrying the Pro-197-Ser and Pro-197-Leu mutation provides some support for the idea that this uncommon new mutation arose from an additional mutation within an individual already carrying a mutated ALS gene, in keeping with the trend of two base-pair changes in ALS mutations being less common (Yu and Powles, 2014). So far, this mutation appears in only one cluster and thus there is no evidence it has evolved more than once up to this point. All other Pro-197 mutations of prickly lettuce, present since at least the late 1990s (Lu et al., 2007), do show evidence of having evolved more than once.

Overall, PCAs for the two species provided evidence of both occasional long-distance dispersal and multiple instances of the independent evolution of the same resistance mutation. The lack of population genetic structure for annual sowthistle, even when including samples of distant geographical origin, is indicative of high levels of gene flow and dispersal, including evidence of relatively frequent long-distance dispersal. Therefore, from the PCAs it can be inferred that annual sowthistle seed is more highly dispersive then prickly lettuce, traveling further on average and experiencing more frequent long distance dispersal events.

This is also supported by differences in the seed terminal velocity and seed morphology of the two species. Because annual sowthistle seed has a lower terminal velocity than prickly lettuce seed (0.28 ms^{-1} vs 0.43 ms^{-1}) (Andersen, 1993), under a simple ballistic model it will achieve a greater dispersal distance given the same release height and wind speed. Seed terminal velocity is largely determined by plume loading: the ratio of a dispersal propagule's weight to its area (Matlack, 1987). In wind-dispersed Asteraceae species this essentially refers to achene weight in relation to pappus area (Andersen, 1993). While both prickly lettuce and annual sowthistle have similar seed size, the seed of prickly lettuce is heavier (Chadha and Florentine, 2021; Chauhan et al., 2006) and the pappus of annual sowthistle has a greater cross-sectional area (Chapter 1: Figure 1), resulting in lower plume loading and terminal velocity.

6.4 Management of prickly lettuce and annual sowthistle

Control of annual sowthistle and prickly lettuce in lentil crops remains difficult with currently available herbicide and crop breeding technology. This is due to the poor competitive ability of lentils with weeds and a lack of safe and effective post-emergence herbicides (McDonald et al., 2007). Effective management, therefore, relies heavily on selecting fields with low weed burden. For this reason there is interest among growers for more competitive varieties and herbicide tolerant crop technology. This project undertook field experiments to explore aspects of prickly lettuce and annual sowthistle management from each of these two approaches.

6.4.1 Legacy effect of control in the cereal phase

One study examined the effect of management decisions in a wheat crop on weed numbers in a subsequent crop to determine whether a legacy effect of management would be significant between cropping seasons despite the dispersal abilities of these species. Crop competition (via wheat seeding density) and herbicide treatments were examined, but only herbicide treatments showed any significant legacy effect. The two crop seeding rates were selected representing an average and a slightly above average sowing density for the area. However, this may not have been enough contrast to generate a measurable effect, particularly given that wheat is already a relatively competitive crop against broadleaf weeds (Mobli et al., 2020; Walsh, 2019).

Legacy effects due to herbicide treatments were observed at only one of the two sites and only for annual sowthistle (Chapter 4: Table 4). It was hypothesised that this could be because annual sowthistle at this site had the highest initial weed density and, therefore, greater opportunity for the herbicide treatments to generate measurable differences. Another phenomenon observed was the reappearance of prickly lettuce at one of the sites despite being eliminated within the trial area following herbicide treatment the previous year. These results indicate that management tactics employed should be reflective of weed densities at the site in question. For heavy infestations, the choice of a highly effective broadleaf herbicide can have a legacy effect in the following crop, but when weed densities are low within the field, attention should be focused on seed sources from outside.

The herbicide treatments employed in the experiment included a “conventional” application of metsulfuron-methyl (ALS inhibitor) and MCPA (synthetic auxin), and a “proactive” treatment including bromoxynil (PSII inhibitor, HRAC group 5), picolinafen (PDS inhibitor, HRAC group 12) and MCPA. The populations of both annual sowthistle and prickly lettuce at the trial sites were shown to have resistance to ALS inhibitors (Chapter 4: Table 3), so the herbicide treatment selection was designed in part to show the reduced efficacy of a herbicide regime impaired by herbicide resistance.

No resistance to the groups used in the “proactive” treatment is known in either species from the study area, but concerns exist to some degree about each of the groups used. In annual sowthistle, synthetic auxin resistance is emerging in nearby regions (Preston et al., 2017), PSII inhibitor resistance has been demonstrated (Fraga and Tasende, 2003), and anecdotal reports of reduced efficacy of PDS inhibitor diflufenican have come from agronomists in South Australia (C. Preston. Personal communication 5 March 2018). All three groups are relied on for control of annual sowthistle, prickly lettuce, and other broadleaf weeds in cereal crops, so their use should follow responsible herbicide stewardship practices, such as a diverse rotation of herbicide mode of action groups, and herbicide resistance monitoring.

6.4.2 Evaluation of weed control in a novel breeding line of PSII-inhibitor-tolerant lentils

Lentil breeding programs in southern Australia have recently undertaken development of a novel lentil breeding line (germplasm line M043) tolerant to PSII inhibitor metribuzin (McMurray et al., 2019a; McMurray et al., 2019b). This could provide an important tool for weed control of ALS inhibitor resistant broadleaf weeds in lentils, and experiments were conducted to evaluate control of annual sowthistle and prickly lettuce in this breeding line using a variety of rates and application timings of metribuzin, testing the effect of an additional POST application of diflufenican, and comparing these treatments with currently registered rates of terbuthylazine.

The M043 line has been shown to have 33-fold tolerance to metribuzin compared to its conventional parent line PBA 'Flash' (McMurray et al., 2019a), so was expected to have excellent crop safety. No impacts on grain yield were observed due to any of the herbicide treatments (Chapter 5, Table 3), although diflufenican did cause some transient bleaching. Grain yield and water use efficiency varied between trial sites and in some cases were below regional or expected averages (Lewis, 2019; Lewis, 2020; Siddique et al., 2001). This is possibly due to the yield penalty of this breeding line that has been demonstrated (Roberts et al., 2020). Grain yield and water use efficiency will be areas of focus for breeding programs before a cultivar is commercialised from this parental line, but the high level of crop safety and weed control benefits help to justify these efforts.

Good levels of control were observed for both weed species at higher rates of metribuzin (540 g ai ha^{-1}), regardless of application timing or the addition of POST diflufenican. At intermediate rates (270 g ai ha^{-1} metribuzin), PSPE applications accompanied by diflufenican performed the best. Both terbuthylazine treatments failed to achieve levels of sowthistle control significantly different to the untreated in at least one of the site years and, in general, did not outperform metribuzin treatments. Terbuthylazine is currently registered for use in conventional lentil, but carries a high risk of crop damage depending on environmental conditions. The introduction of a metribuzin tolerant variety would eliminate the risk of crop safety while allowing the use of more effective metribuzin treatments.

As with any herbicide tolerant crop technology, the possibility of increased reliance on the herbicide group to which tolerance is provided, and subsequently the risk of increased selection pressure and herbicide resistance evolution, must be understood and managed (Preston and Rieger, 2000). Previous chapters have discussed the role of introduction of imidazolinone tolerant lentil varieties in the evolution of imidazolinone resistance in prickly lettuce and annual sowthistle. While PSII inhibitors do not carry the same level of risk for resistance evolution as the ALS inhibitors, resistance is relatively common and many cases in Australian weed species have arisen in triazine tolerant canola crops (Heap, 2022).

Because baseline levels of ALS inhibitor resistance in this region are already so high in both annual sowthistle and prickly lettuce in this region, the risk of multiple resistance to the ALS and PSII inhibitors is a concern, as has been demonstrated in Australian populations of *Lolium rigidum* (Ma et al., 2020). This lentil breeding line should therefore be used in a diverse crop rotation including conventional lentils in addition to other oilseed and cereal crops, and sparing use of other PSII inhibitor tolerant crops, such as triazine tolerant canola. If diligent resistance monitoring is maintained and resistant populations are identified early and seed set reduced, the inherent fitness penalty of target-site mutations conferring PSII inhibitor resistance may be able to be exploited to delay the onset of resistance (Keshtkar et al., 2019).

6.5 Conclusion

Although this study has examined aspects of the biology and ecology of these two weed species and their management in lentil, weed control is likely to remain problematic in this crop. While the possibility of a metribuzin tolerant variety of lentil would be welcomed by growers to improve options for POST control, this technology should be adopted cautiously to avoid a similar outcome to the current situation with the ALS inhibiting herbicides. Responsible stewardship of herbicides, incorporation of non-chemical control methods, and additional focus on seed set reduction will all be necessary. This project highlighted both similarities and differences in the two weed species, many of which have implications for management. While both species currently have similar resistance issues, future resistance threats differ. In annual sowthistle, the risk of synthetic auxin and glyphosate resistance is due mainly to dispersal from isolated or distant populations, while in prickly lettuce, independent evolution of novel cases is a more likely scenario. While ALS inhibiting herbicides can no longer be relied on for control of either weed species, studying such an advanced case of resistance has led to inferences on how resistance may evolve and spread in these species.

Further research could be applied to learn more about the novel Pro-197-Phe mutation in prickly lettuce and its implications for ALS inhibitor resistance. There is evidence from the present study that this mutation is at a relatively early stage of evolution, so it could serve as a valuable baseline to track the spread of this mutation. Further work is also required to elucidate the effect of annual sowthistle ALS inhibitor resistance genotype on resistance phenotype to determine the role of the two copies of the ALS gene. More targeted experiments with the lines used in this study could help to conclusively prove or disprove the involvement of differential gene expression, but non target site mechanisms should also be investigated. Management trials focusing on seed set reduction via both chemical and cultural means, along with more research on non-chemical control methods in general, will be valuable to increase the sustainability and longevity of the few effective post-emergence herbicide options available in lentil and other broadleaf crops.

6.6 References

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