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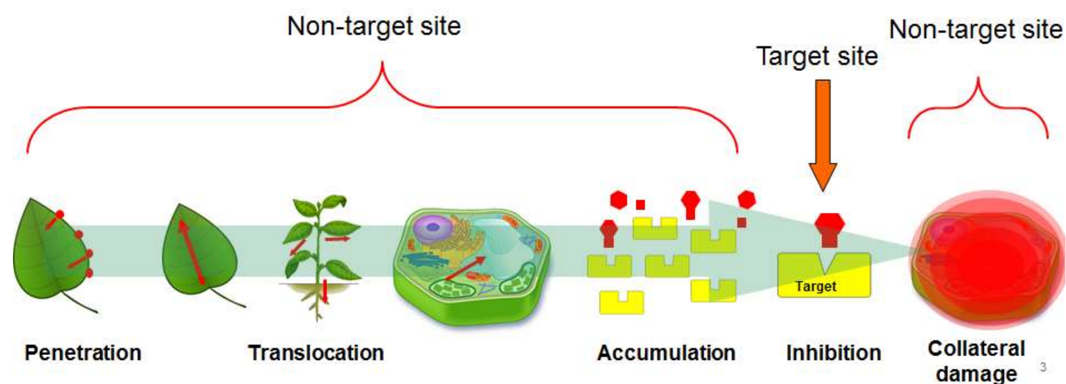
1.1 - Investigate the risks of evolution of non-target site resistance (NTSR) in cotton herbicides (knock-down and residual) in desktop study

Desktop Study:

Proposed Non-Target Site Resistance Risks in Australian Cotton Systems



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Executive Summary

This report provides an outline of what is known about non-target site resistance (NTSR), and what we suspect may be the implications for weed management in Australian cotton systems. In the current weed management environment (glyphosate-centric Roundup Ready Flex, with a small number of widely-distributed glyphosate-resistant species), highly specific mechanisms that provide resistance to one mode of action are of key importance. In the rapidly approaching near future (ExtendFlex cotton systems with three key herbicides and some existing resistance), cross-resistance and the accumulation of minor mechanisms (as provided by NTSR) are likely to be of increased importance.

The report describes the potential sites of action of NTSR mechanisms, which can be anywhere from the point of entry of the herbicide into plant tissues, through to the collateral effects of herbicide activity. Possible and known mechanisms at various points in the herbicidal pathway are detailed.

The genetic basis of NTSR is revealed to be more complex than for target-site mechanisms. This is due both to the fact that more than one NTSR mechanism might be present in a 'resistant' biotype, and the fact that mechanisms like vacuole sequestering are controlled by multiple genes. Since NTSR is the result of both regulatory processes (signal production, reception, and response) and protective processes of several kinds, they have the potential to interact together and accumulate, and possibly provide resistance across herbicide groups. The inheritance of these polygenic mechanisms is likewise complicated compared to target-site resistance. The regulatory/protective nature of NTSR is linked to their basis in plant stress response, and the link between stress and resistance is discussed in the report.

The current global situation for known NTSR mechanisms is summarised; at least five different modes of action used in Australian cotton farming are subject to NTSR in other parts of the world, and we can expect to find these here at some point. Of the four weed species in northern cotton systems that have been assessed to date; three have NTSR to glyphosate (barnyard grass, fleabane and windmill grass). A detailed analysis of glyphosate NTSR mechanisms is also provided as an example of the complexities of this problem.

It is suggested that detailed knowledge of NTSR mechanisms will require skills in population dynamics, genetics, direct weed toxicity experimentation, and biochemistry. Detection of these mechanisms is also discussed: populations with low-level resistance (which was commonly observed as 'rate creep') are likely to be a useful place to start looking for mechanisms that might eventually accumulate into highly resistant biotypes.

What is Non-target site resistance?

Non-target site resistance (NTSR) is resistance to a herbicide that is conferred by any mechanism other than a mutation at the target site. These mechanisms still have a genetic basis, and are heritable by progeny of the resistant plant, though the dynamics of inheritance and selection can be different from simple, single-gene target site mutations. The importance of NTSR mechanisms is only recently being recognised (Delye 2013). Even as recently as 2012, resistance science experts remained focused on target site mutations as the only critical indicator of relative levels of risk (Beckie & Tardif 2012).

NTSR can be due to any one or a combination of factors that prevents the herbicide reaching or affecting the target-site, in the target tissues, at a lethal dose (Delye 2013)

- Reduced herbicide penetration into leaf and stem tissue
- Reduced translocation
- Redirected translocation (ie away from critical meristematic tissues towards leaf tips, which can be sacrificed)
- Sequestering of herbicide molecules in cell vacuoles
- Enhanced herbicide degradation inside cells
- Protection against the collateral damage of herbicide action

(Note: More recent work suggests that the accumulation of many copies of an enzyme-coding gene that is the target of a particular herbicide mode of action (MOA) is responsible for some serious cases of herbicide resistance, especially to glyphosate. It is up for debate, however, whether or not 'target site amplification' constitutes a 'target site' mechanism.) This list reflects our current state of knowledge of NTSR mechanisms and is likely to expand considerably over the next decade.

Expression of NTSR mechanisms

NTSR mechanisms can be either constitutive (always 'switched on') or induced (genes switched on or upregulated only after some trigger affects the plant). In this, NTSR mechanisms are no different from other plant defence mechanisms. Different plant species (and, clearly, biotypes) make different investments in these defences, and vary between preferring constitutive or induced mechanisms, depending on the environment in which they evolved. There has been a great deal of study of plant defence mechanisms targeting insect predation and microbial infection (eg Sharma *et al.* 2012), and some on mechanisms to avoid environmental stress damage such as from heat (Timperio *et al.* 2008) or heavy metal contamination (Xiang & Oliver 1998). However, our understanding of similar mechanisms to avoid herbicide damage is only just beginning to improve. It does appear to be the case that some mechanisms are generalist in nature, which means they may be selected, or switched on and then selected, both in concert with environmental stresses, and between herbicide modes of action. Both of these possibilities are concerning.

'Rate creep'

Before glyphosate resistance was seriously acknowledged as a problem, the phenomenon of 'rate creep' was commonly observed in farming systems relying on herbicides. (eg Gressel 1997; Hartzler 2005; Prairie Greens 2010) Rate creep – the gradual increase in rate required to provide adequate control of populations – has led to both label and formulation changes, especially in glyphosate, over the last decade or so. It was usually explained as a loss of very susceptible individuals from the population; in effect, a proposed levelling-out of

the susceptibility of populations. With our emerging understanding of NTSR mechanisms, it seems much more likely that 'rate creep' is in fact the evolution and accumulation of minor resistance traits. If we continue to allow rate creep to occur and view it as somehow benign compared to 'proper' herbicide resistance, we may find that important weeds will continue to accumulate minor resistance mechanisms, potentially adding up to high levels of resistance overall. The prevalence of the phenomenon of rate creep across species, however, suggests that this process may be inevitable.

The genetic basis of NTSR

Because there are many separate NTSR mechanisms, our understanding of their genetic basis is limited compared to that of relatively straightforward target site mutations. However, there are other reasons for our limited knowledge. Firstly, more than one NTSR mechanism may be present in any plant, making direct proteomic/transcriptomic analysis to find out what genes are responsible difficult. Secondly, some of those mechanisms may provide only very weak resistance on their own, so isolating them in isogenic plant lines would be difficult, as would finding a discriminating herbicide dose to detect the gene's presence directly. Thirdly, and most importantly, many NTSR mechanisms rely on a cascade of genetically-coded effects or events, especially in the case of induced mechanisms. In some of these cases, some of the steps have been researched genetically, and some have not. The sequestration of herbicide molecules in vacuoles, for example, is able to be demonstrated by direct experimentation. However, understanding how it works, and how many genes might be involved, is difficult because the vacuoles of plant cells are complex (Martinoia *et al.* 2007) and the identity of any individual tonoplast transporter for a particular molecule can be quite specific (Conte & Lloyd 2011). Because these are mechanisms that apply to all sorts of cell chemicals, understanding how they provide herbicide resistance can require a very broad biochemical knowledge. Indeed, Schützendübel & Polle (2002) suggest that micorrhizal association might be important in plant responses to heavy metal contamination – if such associations are also part of NTSR responses to herbicides, the basis for control of these mechanisms becomes even more complex.

As we can see, many instances of NTSR are polygenic, either because the mechanism itself is controlled by multiple genes in a cascading process, or because a multitude of small resistances are conferred by multiple genes acting at the same time. When the mechanism relies on a cascade of multiple genes, the inheritance of the herbicide resistance trait can still be monogenic, however, because the affinity of a single protein in the cascade for the specific herbicide could be caused by a point mutation. Our ability to detect the genetic basis of a trait is directly related to the numbers of genes that contribute to that trait. For example, many GWAS (genome wide association studies) of human disease find hundreds of markers of low effect, and the statistical significance of each of these is generally low. Investigations on the inheritance of NTSR using F1, F2 and Backcross families have generally found that resistance is either monogenic or polygenic and controlled by relatively few loci (e.g. Busi, Neve & Powles 2013, Rosenhauer *et al.* 2015). In such a study, however, genes that exist in a "recombination block" will be detected as a single locus, so the number of loci implicated should be taken to be the minimum rather than the absolute.

NTSR-enabled plants survive herbicide doses because their NTSR mechanisms are triggered rapidly, and the reaction to the trigger occurs quickly enough and with sufficient effectiveness to prevent herbicide damage to their cells. This process depends on the

correct and timely regulation of the alleles that are directly responsible for the reduction in the efficacy of herbicide action ('protectors')

- Signal transduction and gene regulation pathways are involved
- <u>Example</u>: *Alopecurus myosuroides* – differences in sensitivity of plants to ACCase inhibitor have been related to the dynamics and intensity of an increase in expression of glutathione-S-transferases
- Part of this mechanism is constitutive and part is induced

NTSR is a result of both 'protectors' and regulators. There are three levels of regulation of plant gene expression, and regulation of the expression of a particular gene can occur at several levels:

- Transcription level (genomic DNA into mRNA) – involves a number of transcription factors – proteins controlling the expression of gene clusters
- Post-transcriptional level – modification of mRNA stability, alternative splicing and/or mRNA editing
    - Here there is an important role of a range of small (19 – 40 bases) non-coding RNAs that mediate post-transcriptional silencing of gene expression
- Post-translational – modification of the activity of the protein. Involves an array of protein-activating proteins (kinases, phosphatases) which are part of signalling cascades. For plants to function effectively, post-translational regulation of all kinds of biochemical processes occurs. It should not be surprising that some of these mechanisms can be used by plants to change their cellular-level behaviour in order to mitigate, to some extent, the presence of herbicide molecules and damage caused by them.

Again, because these different kinds of processes all have the potential to affect plant function, there are different possibilities for the ways NTSR mechanisms might work. In the first two stages listed above, changes to gene expression and splicing can be detected by sequencing all the RNA in a given sample at a given time, this approach is termed transcriptomics. Post translational effects can only be determined through the examination of a specific proteins activity in combination with a transcriptomic approach.

The phenomenon of crop safeners is a useful and direct example of the effects of regulation. These safeners provide enhanced metabolism of some types of herbicide molecules (Hatzioz & Burgos 2004), but not only through the introduction of metabolising molecules or substrates for the production of those molecules—they have also been found to upregulate genes that lead to herbicide metabolism (Riechers *et al.* 2010). Similar cascades of effects are entirely possible in field situations through endogenous plant mechanisms, in plants where such mechanisms are present and sufficiently strong.

- Expression of stress-associated regulators can also be regulated by exposure of the plant to stress
- Plant response to stress is controlled by regulation cascades initiated by stress sensors (limited known on this topic)

NTSR mechanisms are also associated with environmental stresses – Delye (2013) explains this as the co-opting of existing pathways for dealing with abiotic stress for a new type of abiotic stress. Therefore, the expression of genetic regulators (at any of the three levels explained above, or even involving other organisms as described in Schützendübel & Polle 2002) in response to exposure to stress could perhaps predispose plants to surviving

herbicide doses greater than those survived by plants without the capacity to up-regulate these abiotic stress responses. For example, glutathione compounds and related processes, which are known to be important for herbicide metabolism (Coleman *et al.* 1997; Cummins *et al.* 1999, among many similar studies), are also found in the pathways of responses to heavy metal ions (Schützendübel & Polle 2002) and oxidative stress generally (Tausz *et al.* 2004).

In such cases, it appears that stress-sensing processes involving hormones and other compounds leads to the production of signaling compounds, which trigger the production of regulatory processes that can prevent some level of cellular damage, including through regulation of redox processes. That these sensors, signals and regulation-controlling compounds are shared across multiple types of stress (Foyer *et al.* 1997; Song *et al.* 2004) suggests that they are also likely to be active in plant responses to herbicides. These and other studies have found that responses vary between species and between plants of the same species with different genes, meaning that selection of any part or parts of these protective response cascades is possible, though likely complex.

Whether genetic change is predominantly mediated through changes in gene-coding regions or in regulatory regions of the genome has been a hot topic of debate in evolution for some time. Recently, through the genomic analysis of multiple transitions from saltwater to freshwater by stickleback fish, it has been shown that 80% of the genomic regions under selection were non-coding rather than gene coding (Jones *et al.* 2012). This indicates that a large proportion of the genetic changes that confer NTSR are likely to be found in non-coding regions of weed genomes. This makes the discovery of specific mutations more difficult because we understand the code of gene coding regions very well, but our understanding of regulatory DNA is still in its infancy.

Epigenetic processes such as genomic DNA methylation and/or histone protein modifications are also involved in the regulation of expression of genes involved in stress responses. These are not modifications in the plant DNA sequence, but can still be transmitted to the progeny of the plant.

- A ‘stress imprint’ of mother plants can be directly transmitted to the progeny of plants as a preregulated expression pattern, without the need for mutations in regulator genes. That is, progeny plants can be more ready to activate (some of) their existing stress tolerance mechanisms, if their parent plant responds to and survives some stressor – perhaps including a herbicide. The level of resistance to field rates of herbicides of this phenomenon is unknown.

Inheritance of polygenic resistances is complex compared to single-gene mechanisms.

Where single genes control a resistance mechanism, the evolution of a population from relative susceptibility to relative resistance is affected by the interaction of a few factors: the initial frequency of the resistance gene, the frequency of application of the selection agent, the frequency of pre-reproduction death from other factors (eg other herbicides, natural seed mortality), gene movement into and out of the population, level of seed production, and environmental effects on the above. Where multiple genes contribute to the level of resistance to a herbicide, potentially in more than one distinct mechanism, the number of factors and their interactions increase, including epigenetic interactions, differential selection of different mechanisms at different effective doses, gene chromosomal linkages, and complete and incomplete heritability of all the parts of a specific mechanism together. Renton *et al.* (2011) demonstrates through modelling that evolution rate varies between genetically different types of resistance mechanism under similar conditions. The evolution of polygenic mechanisms is predicted to be more rapid under lower effective herbicide rates, showing that accumulation of small resistance traits can rapidly add up to serious problems.

NTSR and plant/weed stress

A plant's response to herbicide stress can be compared to other stresses (Figure 1; Delye 2013) A few key points emerge:

- Herbicide stress response is promoted in all genotypes, whatever their sensitivity to the herbicide. That is, stress detection and signalling processes are rapid enough to occur before death occurs even in susceptible plants.
- Stress response pathways did not emerge after the development of herbicides. The pathways evolved to deal with other common abiotic stresses, and are triggered by herbicide molecules' presence or the damage they cause. Thus, it is possible (even likely) that triggering a protective pathway with one herbicide could provide elevated levels of protection against another herbicide, and that some non-herbicide stresses induce the activation of mechanisms that protect against herbicides. This means that stress-mediated NTSR pathways could provide some level of cross-resistance to multiple modes of action. It also means spraying stressed plants could be risking increased likelihood of selecting NTSR mechanisms.
- Only genotypes that have enough elements in the response pathways triggered by herbicide application will be able to survive – though this is clearly very dose-responsive. Low effective doses (ie low applied dose or poor application conditions) are a high risk for allowing weakly resistant plants to survive. This phenomenon has been strongly observed by recent work from AHRI on low-dose selection of polygenetic (likely metabolic) resistance in annual ryegrass (Busi & Powles 2009, 2011; Manalil *et al.* 2011).

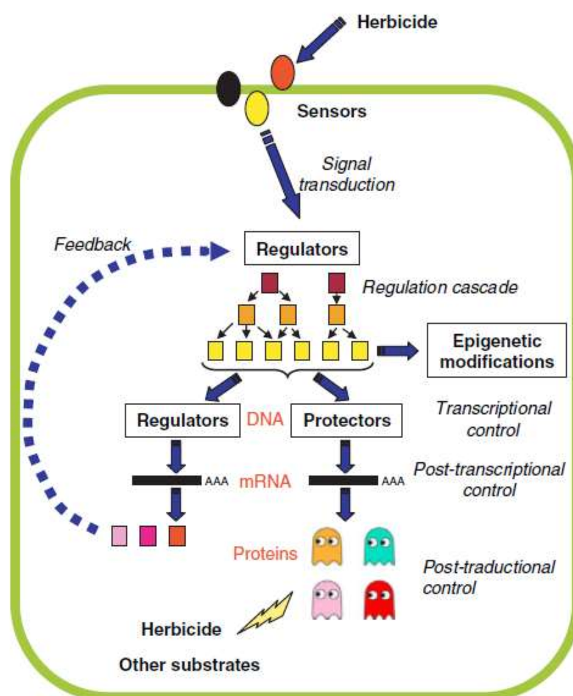

Figure 1. Overall model of NTSR. Plant cell receives herbicide stress signal via sensors. The herbicide stress signal is transducted to regulators and triggers regulation cascades(s) that modulate the expression of 'protectors' at the DNA, mRNA and/or protein level, and can generate herbicide 'stress imprint' by epigenetic modifications. The herbicide signal also triggers the modulation of regulators via feedback, enabling the adjustment of NTSR over time (Delye 2013)

How NTSR Mechanisms Work

Non-target herbicide resistance can be caused by mitigation processes that follow four phases (Yuan *et al.* 2007, Figure 2). To this we can add a fifth stage before the herbicide molecules enter the plant. This is one model of NTSR, and it is likely that there are as yet uncharacterised mechanisms that do not follow this schema.

Phase 0 – exclusion. Plants naturally attempt to control the entry of foreign molecules, through leaf surface characteristics such as hairs, wax, and stomatal density and activity. Greater investment in these characteristics is likely to provide some (though small) level of resistance to herbicides through lowering the effective dose entering the plant.

Phase I – detoxification, where the herbicide molecules are activated so that certain functional groups can be exposed for phase II enzymes i.e. oxidation by P450 monoxygenases or mixed function oxidases.

Phase II – conjugation of a bulky hydrophilic molecule to the activated molecule using thiols or sugars. This allows the end product to be recognized by phase III transporters, i.e. Glutathione *S*-transferases (GSTs) and glycosyltransferases.

Phase III – transportation of the conjugated molecule into the vacuole or extracellular space by active transport i.e. ABC (ATP-binding cassette) transporters, or preferential transport into sacrificial non-essential tissues such as the leaf tips.

Phase IV – further degradation of conjugated molecule in the vacuole or extracellular space. Could involve many plant detoxifying proteins.

To date, the genetic basis of non-target herbicide resistance has been established in four gene families: P450s, GSTs, glycosyltransferases and ABC transporters.

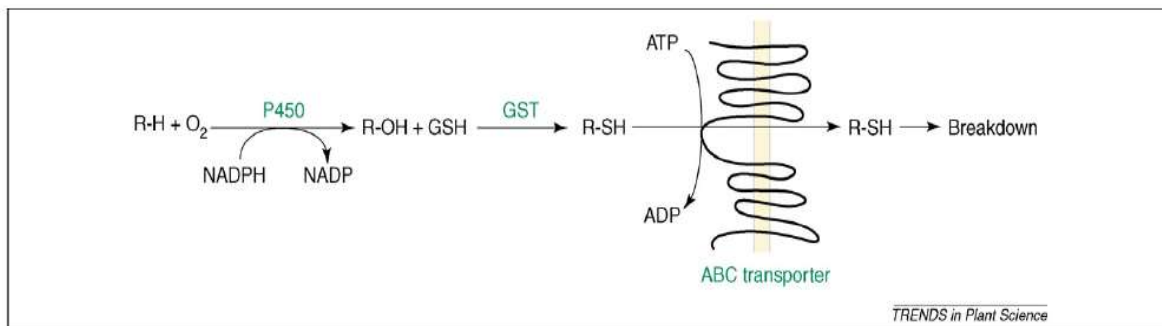

Figure 2. Scheme of P450, GST, glycosyltransferase and ABC transporter gene-encoded resistance activities. The scheme falls into a four-step detoxification process, the monooxygenase activity for P450 genes, the GST conjugation reaction for GST genes, the ATP-dependant transport of small molecules via ABC transporters, followed by subsequent detoxification in vacuoles (Yuan *et al.* 2007).

Cytochrome P450 Monooygenases (P450s)

Plant P450s are bound to the endoplasmic reticulum and are involved in the synthesis of hormones, sterols and fatty acid derivatives. Their role in herbicide conversion is usually hydroxylation or dealkylation (activation and insertion of an atom from molecular oxygen to form a more reactive product using electrons from NADPH (NADPH P450 reductase). P450s will metabolise certain herbicides to products with reduced or modified phytotoxicity that are then further activated, often by conjugation to glucose and subsequently transported into the vacuole (well known in crops such as wheat and maize which tolerate several modes of action). To date, P450 resistance has been reported mostly in grass species. This may be either due to grasses having more P450 genes than dicots, or most research has focused on target-site resistance. A number of herbicides used in Australia have weed species worldwide where enhanced rates of P450 metabolism have been found. These are listed in Table 1.

Glutathione S-Transferases (GSTs)

GSTs are families of multifunctional enzymes that catalyse conjugation of glutathione to a variety of electrophilic, hydrophobic substrates. They have a role as a protective mechanism against oxidative stress by interacting with active oxygen species. GSTs are involved in stress response, and some herbicides can be detoxified by glutathione conjugation. These glutathione-conjugated herbicides can be sequestered in the vacuole, or exuded via root tips. An example of this is maize which has a high GST activity and can conjugate triazines to glutathione. Evolved GST-mediated triazine resistance has been reported in *Abutilon theophrasti* – due either to increased GST activity, or a possible mutation in the GST gene to improve GST catalytic efficiency. Fenoxaprop resistance in *Echinochloa phyllopogon* is likely be due to glutathione-herbicide conjugation. A. myosuriodes with P450-catalyzed metabolism, also has higher GST activity, there could also be a secondary role in mitigating against oxidative stress. GSTs can play a direct role (herbicide conjugation) and an indirect role (stress response) in herbicide resistance

Table 1. Herbicides registered in Australia with weed species (worldwide) that have evolved enhanced rates of cytochrome P450-mediated herbicide metabolism (adapted from Powles and Yu 2010).

Herbicide	Herbicide group	Species	Crops used in
Clodinafop (Topik)	ACCase (A)	*Alopecurus mysuroides*	Wheat
Diclofop-methyl (Hoegrass)	ACCase (A)	*Alopecurus mysuroides* *Lolium rigidum* *Lolium multiflorum* *Avena sterilis*	Wheat, barley
Fenoxaprop-*p*-ethyl (Wildcat)	ACCase (A)	*Alopecurus mysuroides* *Echinochloa phyllopogon* *Digitaria sanguinalis*	Wheat
Haloxyfop (Verdict)	ACCase (A)	*Alopecurus mysuriodes*	Canola, chickpeas, faba beans, **cotton**, mung beans, soybeans, sunflowers, fallow
Propaquizafop (Shogun)	ACCase (A)	*Alopecurus mysuriodes*	Canola, chickpeas, faba beans, **cotton**, sunflowers
Tralkoxydim (Achieve)	ACCase (A)	*Lolium rigidum*	Wheat, barley
Chlorsulfuron (Glean)	AHAS (B)	*Alopecurus mysuriodes* *Lolium rigidum*	Wheat, barley
Imazethapyr (Spinnaker)	AHAS (B)	*Digitaria sanguinalis*	Chickpeas, faba beans, mung beans, soybeans
Atrazine	PSII (C)	*Lolium rigidum*	Canola, sorghum, maize, fallow
Diuron	PSII (C)	*Lolium rigidum*	**cotton**, chickpeas, faba beans
Metribuzin	PSII (C)	*Lolium rigidum*	Soybeans
Simazine	PSII (C)	*Lolium rigidum*	Canola, chickpeas, faba beans
Pendimethalin	DNAs (D)	*Lolium rigidum*	Wheat, barley, canola, chickpeas, faba beans, **cotton**, soybeans, mung beans, pigeon peas, sunflowers, maize

Glycosyltransferases

Glycosyltransferases comprise a large gene family in which proteins conjugate a sugar molecule to a wide range of lipophilic small molecule acceptors including plant hormones, secondary metabolites, and xenobiotics such as herbicides. Conjugation reactions enable glycosyltransferases to diversify the secondary metabolites via sugar attachments, to maintain cell homeostasis by quickly and precisely controlling plant hormone concentration, and detoxifying herbicides. First evidence of their role in NTSR was induced glycosyltransferase activity in multiple herbicide resistant *Alopecurus myosuroides* (Brazier *et al.* 2002).

ATP-binding-cassette Transporters

ABC transporters confer herbicide resistance by compartmenting herbicides and their metabolites. They are targeted to membranes and have one or two ATP binding cassettes for active transport using ATP hydrolysis. ABC transporters have been characterized for a wide range of functions including excretion of toxic compounds, sequestration of secondary metabolites, translocation of fatty acids and phospholipids, as well as transporting chlorophyll catabolites, auxins and heavy metals to maintain cell homeostasis. There is limited evidence to link ABC transporters with NTSR in weeds. However, ABC transporter activity toward herbicide metabolites has been well established in model species and crop plants.

Exclusion

Some plants may have the ability to limit the entry of foreign molecules through the leaf surface. An example of this was shown by (Michitte *et al.* 2007) who identified a *Lolium multiflorum* population with reduced absorption of glyphosate on the lower side of the leaf, and reduced spray retention on the leaf. Other studies have found altered or restricted

translocation of the herbicide throughout the plant. Wakelin *et al.* 2004 showed a population of *Lolium rigidum* where glyphosate was sent to the leaf tips and kept away from the meristematic tissue (this mechanism was previously mentioned in phase III). It appears that altered translocation can occur in the vascular tissues in this way, as well as within the cell such as sequestration into the vacuole as was shown by Ge *et al.* 2010 in a population of *Conyza canadensis*. One or both of these factors may also explain some paraquat resistance. There is some evidence to suggest that it may be sequestered into the vacuoles, though this mechanism appears to be independent of the mechanism for glyphosate (Powles and Yu 2010)

Coordination among detoxifying gene family proteins

Transgenic expression of a single gene in these families can induce herbicide resistance; this indicates that NTSR can be monogenic. Some work on annual ryegrass with altered translocation suggested that the mechanism (though unknown at the time) was monogenic. However, there is evidence that coordinated regulation of detoxifying genes confers resistance in weeds (Yuan *et al.* 2007).

In *A. myosuroides*, the conjugation of diclofop-methyl with glucose in a resistant biotype was found to be reduced upon treatment with a P450 inhibitor, indicating that P450-based oxidation might be a pre-requirement for glycosylation (Menendez and DePrado 1996). In one case, biotypes only lost resistance upon treatment with a P450 inhibitor and a GST inhibitor (Letouze and Gasquez 2003).

For Phase III coordination, GSTs and glycosyltransferases normally first conjugate the toxin/herbicide and then the conjugated product can be transported into the vacuoles by an ABC transporter with specificity to the GSH or sugar conjugate, this has been shown for chlorminuron-ethyl.

The coordinated nature of these mechanisms suggests that polygenic resistance ‘systems’ based on metabolism and translocation can and do evolve. The evolution of these types of NTSR would be complex and unpredictable, and their specificity to particular herbicides is unknown. Even when these systems are specific to one chemistry, once in place and activated there may be relatively less evolutionary steps required for it to be co-opted for a different chemistry.

Summary of implications – for industry

The greatest risk that NTSR poses to industry, especially given the move to a herbicide stack, is the possibility that these mechanisms may confer cross resistance. Cross resistance is defined here as an inherited resistance to one herbicide that also allows a weed to survive different herbicide MOA's. Of particular relevance to the cotton industry in Australia is the likelihood that glyphosate exposure could endow NTSR mechanisms that confer resistance to Dicamba or Glufosinate. Given the current state of knowledge on NTSR mechanisms this risk is difficult to fully assess at this stage. Clearly, a NTSR that acts to exclude chemicals from leaf or stem entry (Phase 0, exclusion) is likely to confer cross resistance. Conversely, a mechanism for transporting a specific herbicide within a plant will likely involve a binding protein and the likelihood of cross resistance will depend on the substrate specificity of that protein. Increased activity of single transporter proteins has, however, been shown to confer multidrug resistance in both prokaryotes and eukaryotes (Balzi & Goffeau 1995).

The best worked examples of NTSR cross resistance to date involve herbicide metabolism mechanisms (mostly cytochrome P450's). The current understanding of glyphosate metabolism in plants is that it occurs in a few species via an uncharacterised GOX-like gene pathway, but there is no relationship between metabolism and resistance level in the species that have been investigated to date (Sammons & Gaines 2014). It is therefore relatively unlikely for metabolism based cross resistance between glyphosate and glufosinate/dicamba.

Currently, there are commonly used herbicides in cotton systems that have known NTSR mechanisms in weed species worldwide (Table 2). These are potential candidates (amongst others) where NTSR could evolve here. Assessing the risk of NTSR cross resistance in the Australian cotton system clearly requires an understanding of the resistance mechanism, which will in turn require genomic, transcriptomic, imaging and biochemical approaches. Currently we know that at least three glyphosate resistant species in cotton have a NTSR (fleabane, barnyard grass and windmill grass). Determining the NTSR mechanisms in these species is a priority, as is assessing further species for the presence of target/non target resistance.

Table 2. Herbicides used in Cotton systems with known NTSR populations (Worldwide).

Herbicide	Weed	Mechanism
Haloxyfop (Verdict)	*Alopecurus myosuroides*	P450
Propaquizafop (Shogun)	*Alopecurus myosuroides*	P450
Diuron	*Lolium rigidum*	P450
Pendimethalin	*Lolium rigidum*	P450
Glyphosate	*Conyza canadensis*	ABC transporter?
Glyphosate	*Lolium multiflorum*	Reduced absorption
Glyphosate	*Lolium rigidum*	Altered translocation
Paraquat	*Hordeum leporinum* *Hordeum glaucum* *Arctotheca calendula*	Restricted translocation

Summary of implications for project

This review highlights the various mechanisms of NTSR and the complexity of the underlying genetics. The critical questions regarding cross resistance highlighted in the previous section are considered priorities for project UQ1501. Specifically;

- What are the mechanisms of NTSR present in each of the three species discovered to date?
- Do different populations have different NTSR mechanisms?
- Are these mechanisms able to confer cross resistance to other chemistries that will be in the stack?
- How does plant stress affect the efficacy/activation of NTSR mechanisms?

These four questions will guide the experimental approaches which will include imaging analysis of labelled herbicides, population genetic outlier analyses, further transcriptome experiments including plant stress, and morphological characterisation of leaf traits.

Herbicides that will become more widely used in cotton with stacked traits such as glyphosate, glufosinate and dicamba will be the main focus of our research. Paraquat is another herbicide which also requires some attention due to its widespread use in cotton and grains systems. It is expected that resistance to group A herbicides is imminent, if not already present, however will not be the focus of this research as we endeavour to increase our knowledge of the implications regarding the other herbicides.

Glossary

Constitutive (gene expression) - a gene that is transcribed continually compared to a facultative gene which is only transcribed as needed.

Induced (gene expression) - a gene whose expression is either responsive to environmental change or dependent on the position of the cell cycle

Signal transduction - occurs when an extracellular signalling molecule activates a specific receptor located on the cell surface or inside the cell. In turn, this receptor triggers a biochemical chain of events inside the cell, creating a response. Depending on the cell, the response alters the cell's metabolism, shape, gene expression, or ability to divide. The signal can be amplified at any step. Thus, one signalling molecule can cause many responses.

Splicing - a modification of the nascent pre-messenger RNA (pre-mRNA) transcript in which introns are removed and exons are joined

Intron - any nucleotide sequence within a gene that is removed by RNA splicing while the final mature RNA product of a gene is being generated. The term intron refers to both the DNA sequence within a gene and the corresponding sequence in RNA transcripts

Exon - any nucleotide sequence encoded by a gene that remains present within the final mature RNA product of that gene after introns have been removed by RNA splicing. The term exon refers to both the DNA sequence within a gene and to the corresponding sequence in RNA transcripts. In RNA splicing, introns are removed and exons are covalently joined to one another as part of generating the mature messenger RNA.

Epigenetic - functionally relevant changes to the genome that do not involve a change in the nucleotide sequence

Cellular redox/respiration – oxidation of glucose to CO2 and the reduction of oxygen to water. Depends heavily on the reduction of NAD+ to NADH. Photosynthesis and cellular respiration are completmentary.

Redox state - often used to describe the balance of GSH/GSSG, NAD+/NADH and NADP+/NADPH in a biological system such as a cell or organ. The redox state is reflected in the balance of several sets of metabolites (e.g., lactate and pyruvate, beta-hydroxybutyrate, and acetoacetate), whose interconversion is dependent on these ratios. Redox proteins and their genes must be co-located for redox regulation according to the CoRR hypothesis for the function of DNA in mitochondria and chloroplasts.

Calvin cycle – most plants incorporate CO2 into sugars by means of a cycle of reactions called the Calvin cycle, which is driven by the ATP and NADPH produced in the light reactions but which can itself take place in the dark.

Hydroxylation – introduction of a hydroxyl group (-OH) onto an organic compound

Dealkylation – removal of an alkyl group (C_nH_{2n-1}) from a compound

Oxidative stress - associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defences, such as glutathione. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis

Apoptosis - the process of programmed cell death (PCD) that may occur in multicellular organisms

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1.2 - Improve the current SHeRA model for existing zonal management strategies for glyphosate-resistant weeds, taking into account additional risks with NTSR

Our approach to improving the SHeRA spatial model was planned to consist of adding some of the functionality developed in this project for the new Diversity model, making mechanistic improvements to the spatial realism aspects of the SHeRA model, and testing the accuracy of simulating other species spatially.

Specifically, we made changes to improve the accuracy and realism of pollen movement, and to assess in a generic way the agroecosystem outcomes of the inheritance of more genetically complex NTSR mechanisms.

Mechanistic changes to the underlying spatial model, allowing it to better simulate real speeds of weed dispersal, include improvements to seed and pollen distribution equations. We have adapted work by Fiona Evans and Art Diggle (Murdoch University/DAFWA) on the effectiveness of different statistical models that allow for relatively rare events, like long-distance pollination. SHeRa now includes Weibull and Cauchy distributions for pollen movement, which Evans and Diggle found were the most effective mathematical approaches to simulating this problem in an efficient way. Long-distance seed movement (in particular in soil on machinery) continues to be modelled as a bivariate statistic, as this seems able to reproduce field data on this problem.

Several new runs of previous simulations of patch management of glyphosate-resistant awnless barnyard grass were undertaken to test the new statistical methods. The new algorithms, in summary, seems to increase the variability in where new satellite patches appear. However, the greatest threat of patch expansion remains long-distance soil movement on machinery, not pollen movement. This is fortunate in that this kind of spatial spread is much more predictable (as it occurs in lines in the direction of travel of machinery) and therefore easier to manage. In previous work with SHeRA we suggested treating whole lengths of the cotton field where patches appear as intensive management zones for eradication, since this approach deals effectively with the most likely type of patch spread. This management strategy remains sound with the new simulations done with SHeRA in this project.

The multi-species, multi-gene, multi-herbicide functionality developed in working on the Diversity model have become quite complex over the course of this project. Our attempts to transfer this complexity directly to the SHeRA model were unsuccessful in the time available, so we elected to simplify our improvements. We added the capacity to simulate a range of NTSR mechanisms, but limited this to one mechanism in one herbicide at a time.

SHeRA is now also able to simulate annual ryegrass populations in a preliminary way. We hope to use the ryegrass version to test ideas about susceptible pollen flooding for resistance management, proposed by Sudheesh Manalil—this problem should also work as a good test for the updates to the model's pollen movement algorithm.

Some computational efficiency changes have made it possible to simulate much larger fields in SHeRA without long run times, making future results more immediately applicable. Although our modelling and decision support efforts in this project have focused on BYGUM, the Diversity model and the Weeds of Australian Cotton ID app, the SHeRA model remains a useful tool for developing weed management strategies, especially for patch management and eradication of resistant biotypes.

Further work with SHeRA would be valuable in the area of multiple accumulating resistance mechanisms, and new parameter sets for those broadleaf weeds that lend themselves to patch management through limited wind dispersal and seedbank longevity, such as the vines and

bladder ketmia. As always, adjusting these models to account for other herbicides as they inevitably become more important in the system (such as key residuals and group I herbicides) would also allow SHeRA to continue providing useful information.

1.3 - Develop a multi-species, multi-herbicide resistance model to determine risk in proposed stacked herbicide-tolerant cotton varieties

Because herbicide resistance evolves in very large populations over periods of many years, modelling is an important tool for investigating the dynamics of the problem. The Diversity model tracks the simultaneous evolution of resistance to multiple herbicides, using multiple genetic pathways, in several weed species at once. Tracking multiple species and simultaneous resistances is an important development in resistance modelling, and the Diversity model is a world first in this respect. We used the Diversity model to test weed management strategies for new Australian cotton varieties with multiple herbicide tolerances ('triple-stacked' varieties).

1.3.1 Summary of results: The diversity required for long-term control of the key weeds we tested (awnless barnyard grass, common sowthistle, flaxleaf fleabane) in Australian cotton goes beyond using three herbicides, especially given there is already a substantial background of existing resistance to one or more of these herbicides. Assuming some glyphosate resistance is already present, simulations showed that glyphosate-resistant summer grass populations reach 20,000 seeds/m^2 within only a few years using the triple-stack herbicides (glyphosate, glufosinate and dicamba) and a minimum of other tactics. Adding three pre-emergent modes of action plus cultivation to the system effectively controls these glyphosate-resistant grasses for over 30 years. In conditions where initial resistance genes for dicamba and glufosinate are as frequent as one in 100, however, highly fecund weeds such as fleabane can be hard to control beyond 15 years without highly diverse management.

Across a range of tested scenarios, the results from the model suggest that there is a greater immediate threat from existing glyphosate-resistant weeds being uncontrolled with the glyphosate/glufosinate/dicamba stack than there is for rapid evolution of resistance to other herbicides. The risk of resistance to other herbicides is certainly present when the background level of resistance to the tracked herbicides is assumed to be relatively high. While this may seem to be at odds with other model findings, the management strategies we have simulated have, with a few exceptions, much higher diversity than has usually been considered in past models. This is not to discount the risk of further evolution of resistance: should an already glyphosate-resistant species also develop resistance to dicamba or glufosinate, strategies centred on the 'triple-stack' and other low-diversity strategies would certainly fail in the field. However, the relatively infrequent appearance of new resistances predicted by the model here suggest that Xtendflex type varieties, plus the requirements of controlling existing glyphosate resistance, are leading to the creation of relatively robust systems.

Population sizes are predicted to increase relatively slowly, if inexorably, under the BMP strategy in crop and in fallow. Thus, high management diversity is predicted to offer substantial windows of opportunity for managing emerging problems: growers may have time to make temporary changes to drive down population numbers, or to adjust their weed control programs entirely. It also suggests that these new multiple-resistant cropping systems could have a useful lifespan of two or more decades even with the emergence of new resistance, provided a high diversity of other tactics is included. It is clear that Australian cotton weed management in new triple-stack varieties should look much more like the BMP scenarios than the triple-stack. It's also prudent that the tactics included in a BMP strategy should be harder to evolve resistance to—that is, tactics like strategic tillage should be used more than ACCase herbicides or other high-risk modes of action. Glufosinate could have a significant role, as used in these scenarios and outlined in section 2.2 of this report, in protecting grass selectives and other herbicides from resistance, as part of a double knock strategy.

Various simulations, including some test scenarios not shown here, suggest that the existing cotton weed resistance management strategy remains effective: glyphosate plus two other tactics in crop and two in fallow, with extra tactics to control any survivors, or 2+2+0. These

scenarios do, however, show that in mixed weed populations this may mean more than three herbicides will need to be applied per year. The +2 in fallow should be different from the +2 in crop, and both grasses and broadleafs need assured high-effectiveness options for each +2. The +0 part of the strategy is often simulated here as cultivation, as it was in earlier modelling projects. The effectiveness of this approach should not be underestimated or undersold, despite the natural desire to minimise soil disturbance.

The introduction of triple stack systems like Xtendflex offers another needed opportunity to change the discussion around weed management in Australian cropping. The dominance of glyphosate especially under Roundup Ready seems to have taught growers (probably in every industrialised cropping system) to expect low diversity in weed management. In an immediate sense it may be true that one or two broadly effective tactics are better than a wider variety of more specialised tactics, but resistance continually debunks the sustainability of this approach. In talking with growers and advisers about the higher diversity simulations we have done in this project, there has been some reluctance to accept the idea of applying five or six modes of action per year. Cropping industries worldwide still need to come to terms with the value of conserving herbicide susceptibility long term, and part of the value of systems like Xtendflex is that they encourage at least this multi-chemistry thinking. While dicamba and glufosinate are not the most effective parts of the systems we've analysed here, their inclusion potentially has value in shifting perceptions around weed management that contains five or more tactics per year.

1.3.2 Model background: Experts in herbicide resistance have advocated for increased diversity in weed management systems as the best response to herbicide resistance for several decades. Diverse systems call for multiple herbicide modes of action as well as the use of non-herbicide tactics for weed control. The question of how much diversity in weed management is 'sufficient' diversity is usually only measured with rules of thumb, since diversity isn't easy to address experimentally, for several reasons: 1) Determining whether a diverse system might produce new resistant biotypes requires very large populations and long time scales; 2) the level of diversity and the types of tactics able to be included in any system depend on the agronomic environment, and not all agronomic environments have an equal potential for high diversity; 3) the more diverse a system becomes, the more permutations there may be for sequences, mixtures and timings to influence the system's resistance management potential, and thus determining optimal or even effective diversity becomes a complex problem. Herbicide resistance models have grown in complexity over the last 15-20 years, but this is the first time one has been devised specifically to test and measure diversity of weed management. Australian cotton systems have both the potential for high diversity (with many MOAs allowed in and between crops) and a history of low diversity in many fields, with resistance evolution observed as a result.

Prior to the commercial introduction of new stacked herbicide technologies in Australia, it would be useful and timely to develop a better understanding of how the current resistance environment might interact with proposed new herbicide regimes, so that susceptibility to the herbicides being added to the system might best be protected. A useful way to look at this, in the context of the diversity question, is to ask whether it is possible (and useful) to prevent rapid selection of resistance to the 'new' OTT herbicides, and if so, how much diversity of weed management is enough? If we could develop a cotton production system in Australia that doesn't create substantial evolutionary pressure for resistance in any particular direction, what would such a system look like? The Diversity model was created with this question in mind.

1.3.3 Model construction: The overall dynamics of the Diversity model are similar to existing age- and stage-structured weed models. Like these previous models, the Diversity model represents several discrete plant life stages: a soil seed bank, germinated seedlings, and mature flowering plants. Individuals may leave each state either by death or by transition to

the next stage, and seeds may enter the seed bank pool either by seed set from existing plants, or by influx from outside of the simulated field. Mature flowering plants produce both pollen and egg cells, and genetic mixing occurs to produce new seeds. A diagrammatic view of the model structure is shown below.

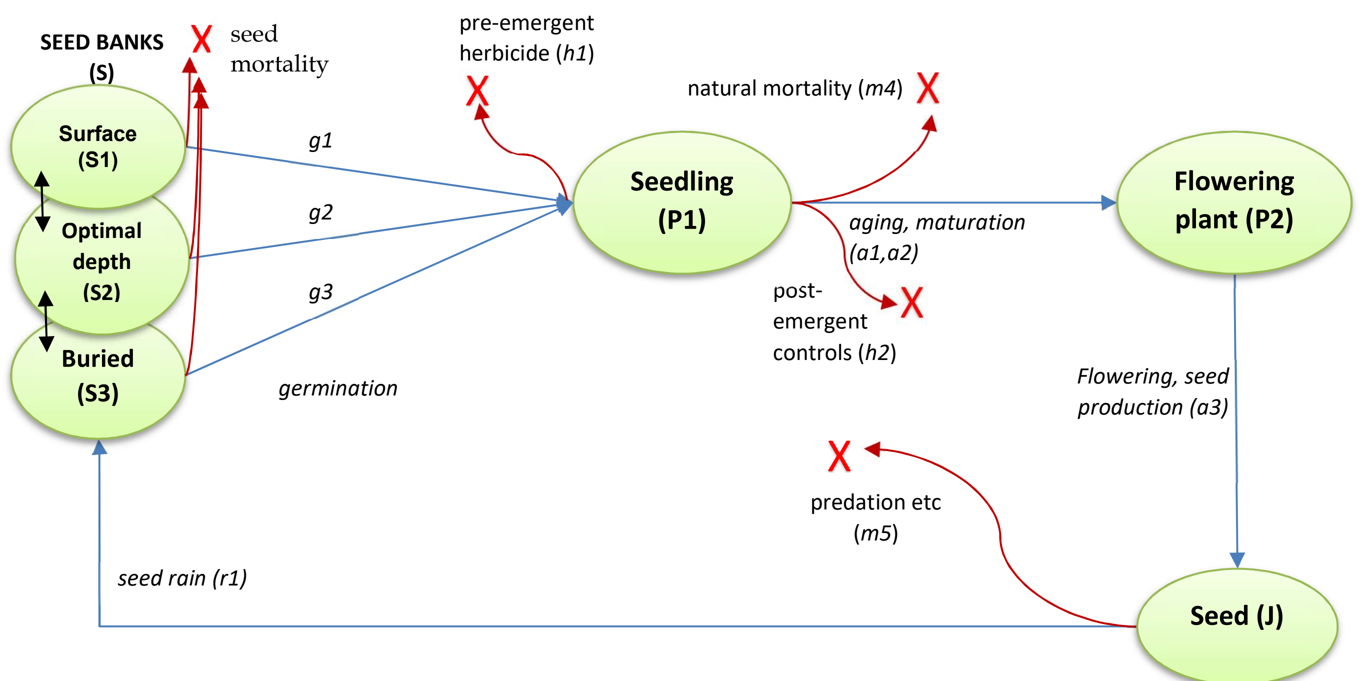


Figure 1.3.1. General structure of the Diversity model

Unlike most weed models, however, the structural focus of the Diversity model is on modelling discrete individuals proceeding through the possible fates of a real weed, rather than on pools or state levels, as are often used in mathematical models of ecology of various kinds. Individual-based modelling offers the capacity to deal with a great deal of system and biological variability, usually at the cost of longer simulation processing times. Simulation results thus are representative of the cumulative effect of many random events, as is arguably the case in real weed management. In the Diversity model, each weed is modelled as a list of variable values; for example: [3,1,117,3,0.01,1,2,2,0,0,0,3,0,0,0,0,3,0,0,0,0], and changing the values of these variables simulates growth, phenological development, seed production, and the likelihood of dying as a result of any of a large number of possible agronomic and ecological processes.

The model includes the capacity to model the evolution of resistance to three herbicides in up to four weed species with target-site and multiple non-target-site resistance (NTSR) mechanisms, as follows:

1. *EPSPS* gene amplification (# copies) for glyphosate resistance
2. ‘low resistance’ NTSR traits such as leaf waxiness/hairiness (# active traits)
3. Vacuolar sequestration (single semi-dominant gene)
4. Enhanced metabolism (single semi-dominant gene)
5. ‘other’ NTSR mechanisms (single semi-dominant gene)

The model also allows for the existence of multiple target site mutations that can be independent and in combination. Resistance types 3-5, as well as target site mutations, are tracked separately for each key herbicide. Type 1 applies only to glyphosate. A single value for type 2 genes provides low-level cross-resistance to all herbicides.

For the purposes of this project the three tracked herbicides are glyphosate, dicamba and glufosinate, but any combination of important herbicides could be modelled. Other herbicide applications are of course included, but resistance to these is not modelled in the simulations reported here. The Diversity model is designed to allow for relatively easy tracking of resistance to more than three herbicides, but each additional tracked herbicide increases the model's processing time, and would require some new data about resistance evolution and mechanisms.

Model scenarios are created by changing agronomic practices specified in simple text files. The user could include a theoretically unlimited number of different types of years (where a year 'type' includes particular rainfall, crop/fallow decision, and weekly weed management decisions) in arbitrarily long sequences. The model currently includes all the possible types of weed management decisions in Australian cotton, with many specified by herbicide type—such that a user could specify which weeks to apply trifluralin or paraquat or inter-row tillage, for example, as well as the three key herbicides.

The model tracks realistic application rates and includes functionality for automatically increasing rates as efficacy falls, modelling rate creep and the evolutionary effects of low and high herbicide rates. Each resistance mechanism acts to increase the 'fold' resistance value of each individual. The model determines an LD90 value for each individual whenever a herbicide is applied, based on the species' base LD90 for that herbicide multiplied by the fold value of resistance, and compares that with the actual dose applied to determine a probability of death. The model is stochastic, meaning that each run is different and multiple runs are used to investigate the success or failure of simulated strategies.

The sequence of events in DIVERSITY can be summarised as follows:

```
- Set initial variables, read functions, open data files
- Initialise yearly loop (for n years as specified by user)
    o Read weekly management data for the year
    o Do annual reporting on population sizes
    o Initialise weekly loop (for 52 weeks)
        ▪ Set up lists of individuals for the new week
        ▪ Turn on/off phenological switches for each species
          (flowering, end-of-season mortality)
        ▪ Process crop operations and predicted biomass as
          required
        ▪ Manage levels of pre-emergent herbicides present
        ▪ Process each individual:
            • For seeds:
                o Test for germination, death
            • For plants:
                o Increase biomass
                o Test for changes in phenological state
                o Test for natural mortality
                o Test for death by any weed control
                  methods present this week
                o Test for seed production
        ▪ Compile new weed lists and go to next week
    o Go to next year
- End simulation
```

In any given week, the user can choose to apply an arbitrary number of weed control methods. A user may simulate the application of single herbicides, tank mixes, or sequential (double knock) applications, at points they choose throughout the year. Some of the applications are of herbicides the model tracks for resistance evolution, and some are of other tactics. Resistance genes are used in the model to increase an individual plant's lethal threshold dose (LD90), by applying a 'fold' multiplier for each mechanism that is present in the individual. Depending on the user's specifications, two alleles of the vacuolar sequestration gene (gVAC) for a herbicide might confer an eight-fold resistance, and one allele of the metabolism gene (gMET) might confer three-fold resistance. Resistances are simplified to work additively, though more complex relationships might be present in real systems.

Plant death from herbicide applications (h1 and h2 in the model diagram) is determined randomly by comparing the individual plant's LD90 value with the applied rate of the associated herbicide. The actual applied rate of the herbicide is calculated as a labelled field rate for the species present, plus or minus an allowance for application variability. If the applied dose is equal to the plant's LD90 value, the probability of the plant dying from the herbicide application is, by definition, 0.9. If the LD90 is higher, the probability of death is estimated in equation 1:

$$pDeath = 1 - \sqrt{\frac{LD90 - Dose}{LD90}} \quad (1)$$

If the LD90 value is lower than the applied dose, the probability of death is estimated in equation 2:

$$pDeath = 1 - (e^{\frac{LD90 - Dose}{LD90}} * 0.1) \quad (2)$$

Susceptible individuals frequently have a probability of dying from a robust field rate of higher than 90 per cent. Nevertheless, some resistant individuals may still die from applications of the 'resisted' herbicide, and some susceptible plants may survive. These mechanisms are more flexible than simply assigning a single probability of death due to a herbicide application, taking into account a much larger range of genetic possibilities. Survivors of herbicide applications have their biomass reduced in proportion to *pDeath*. This simulates non-lethal tissue damage that may affect the total seed production of surviving plants, especially under competitive conditions.

The Diversity model uses a simplified, regionally-specific approach to modelling plant ecology. User modified files specify boundaries for possible parts of the year in which a species may germinate, and an optimum amount of stimulating rainfall that results in germinations. These boundaries are readily estimated for given biotypes and regional conditions. Effectively, this method splits an annual germination proportion, which is readily measured, over the number of wet weeks for the season, which is also easy to determine. This method reproduced realistic levels of germination over several seasons for *E. colona* using emergence and weather data for four years of our patch management experiment in Warwick, but more testing would be useful. Other aspects of plant phenology are modelled similarly, to make adjusting for different species and conditions easy.

1.3.4 Simulation plans and details: We have assessed a range of weed management strategies for use in cotton varieties with stacked herbicide resistance traits. In these scenarios we sought to determine a) the key risks for strategies that rely on the three over-the-top 'trait' herbicides; b) the most optimal of the practical strategies for relying on these three trait herbicides; and c) practical sets of effective, highly-diverse strategies using these and other weed management tactics. Throughout we were also interested to elucidate whether existing resistance recommendations (in particular our widely adopted 2+2+0 strategy) were still predicted to be effective with this more powerful modelling tool. Some key details:

- Throughout, we simulated three key species: flaxleaf fleabane, awnless barnyard grass, and common sowthistle. These species are of interest because they are widely distributed, economically important, and have a history of existing herbicide resistance in Australian cotton farming.
- Existing resistance (especially to glyphosate) is included in most simulations. The reality for new cotton varieties is that they will be planted in areas where resistance to glyphosate already exists. In some scenarios we also tested the potential for resistance evolution 'from scratch', which may be relevant in few situations but is useful for comparison with historical glyphosate use patterns.
- Tank mixes, stand-alone products, double knocks, and non-herbicide tactics are included, to simulate the expected real-world scenarios for cropping with these varieties.

We present below the results of the following groups of scenarios:

1. Effects of background resistance level, testing two weed management systems against three levels of existing resistance beyond known glyphosate resistance
2. Clean slate scenarios – wild-type levels of background resistance
3. Effectiveness of proposed US Xtendflex weed management strategies
4. Dryland rotations
5. Double knocks with glufosinate
6. Testing Group A herbicide resistance

Across the range of simulations in the plan, pre-testing scenarios, and repetitions, we have around 4000 sets of Diversity model results for analysis.

1.3.5 Scenarios: Effect of Background Resistance: In this set of scenarios, we tested the possible effects of introducing a triple stack herbicide resistance technology (like Xtendflex) in irrigated cotton. We assessed the effects of two management strategies on three prevalent species (awnless barnyard grass, fleabane, and sowthistle), under three possible levels of existing background resistance (from one in one million to one in one hundred plants containing a randomly-selected resistance trait, see below) to glyphosate, glufosinate, and dicamba. We assumed that the barnyard grass and fleabane populations already had glyphosate resistance (a weak target site mechanism in the case of barnyard grass, and a vacuolar sequestration mechanism for fleabane). We also assumed irrigated cotton was planted every year.

Our two management systems were the triple stack (TS) herbicides only, plus one double knock (glyphosate fb paraquat) prior to planting; and a BMP strategy in which we added several residual herbicides and some mechanical tactics to the TS strategy—see below for details. The BMP strategy was very similar to the BMP strategy used in our long-term barnyard grass patch management experiment at Warwick. (3.1)

Table 1.3.1 Scenario list for Background Resistance simulations

	In-crop weed management	
Starting resistance frequency†	*Triple-stack herbicides*	*Best Management Practice*
$1*10^{-6}$	TS-6	BMP-6
$1*10^{-4}$	TS-4	BMP-4
$1*10^{-2}$	TS-2	BMP-2

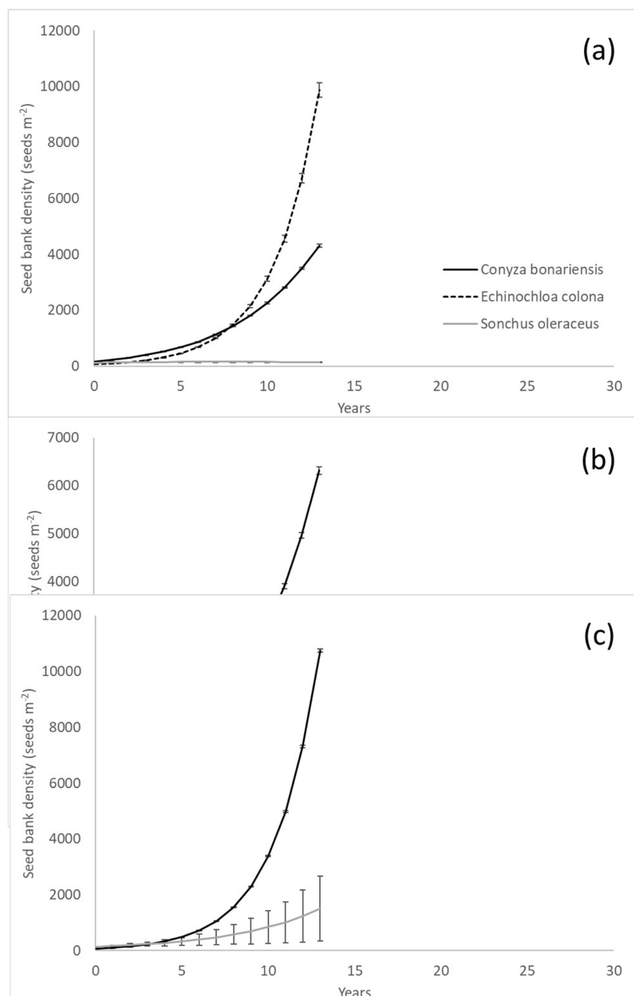
†Starting resistance frequency refers to the likelihood of one possible resistance trait being present as heterozygous resistant genes (rather than homozygous susceptible genes, as is the norm) in any seed in the initial population.

Weed management options applied in various phases of irrigated continuous cotton cropping systems simulated in the Diversity model

Point of application	Mangement strategy: *Triple-stack (TS)*	*Best Management Practice (BMP)*
Prior to planting	Glyphosate followed by† paraquat	Glyphosate followed by paraquat
At planting	-	Prometryn
Early in-crop	Glyphosate + dicamba‡; glufosinate§	Glyphosate+dicamba; Glyphosate+dicamba followed by glufosinate
Layby	-	Pendimethalin
Late in-crop	Glyphosate+dicamba	Glyphosate+dicamba followed by glufosinate; hand-hoeing
Winter fallow	Glyphosate+2,4-D; glyphosate	Cultivation; diuron; glyphosate+2,4-D if required

† The term 'followed by' indicates the use of a double knock, wherein the second herbicide is applied one week after the first, and has synergistic effects.
‡ Tactics separated by + are applied together, either in a single product or as a tank mix.
§ Tactics separated by semicolons can be applied sequentially in the same part of the season indicated, if and as weed germinations require additional control after the first tactic in the list.

Figure 1.3.2 Predicted end-of-year mean weed seed bank densities (over 10 runs of each scenario) for Diversity model scenarios TS-6 (a), TS-4 (b) and TS-2 (c). Values for Echinochloa colona are not present in scenarios TS-4 and TS-2 because runs of these scenarios that included E. colona failed too quickly



The TS management system (i.e. the three OTT herbicides glyphosate, glufosinate and dicamba, with minor additions of tactics outside the crop) was predicted to fail in controlling glyphosate-resistant awnless barnyard grass, even in the case of relatively low initial amounts of resistance to the other herbicides. The TS-6 scenario resulted in failure after 7-15 years in different runs, though plant numbers are problematically high after only a few years (Fig 1.3.2).

The loss of control of barnyard grass in this scenario was due to the inability of this system to control glyphosate resistant grasses, rather than selection of further resistance. Scenario BMP-6 shows that a more diverse management system provides adequate control of glyphosate-resistant barnyard grass over 30 years, (Fig 1.3.3), although there is a slow increase in population size over that time. On average, while the TS scenarios did result in somewhat higher levels of resistance in the last five years of each scenario than the BMP system, often in failed scenarios there was no time for significant increases in

resistance level (Table 1.3.3). Thus, the failures of these systems was most often due to poor control of existing glyphosate resistance.

Table 1.3.2 Seed bank densities of fleabane populations predicted by the Diversity model under two different management systems

	Triple-stack system		*BMP system*	
	TS-4	TS-2	BMP-4	BMP-2
Seed bank density after 5 years (seeds/m²)	770	481	223	404
Seed bank density after 13 years	6780	10778	814	1495
Seed bank density after 30 years	N/A	N/A	4611	8587

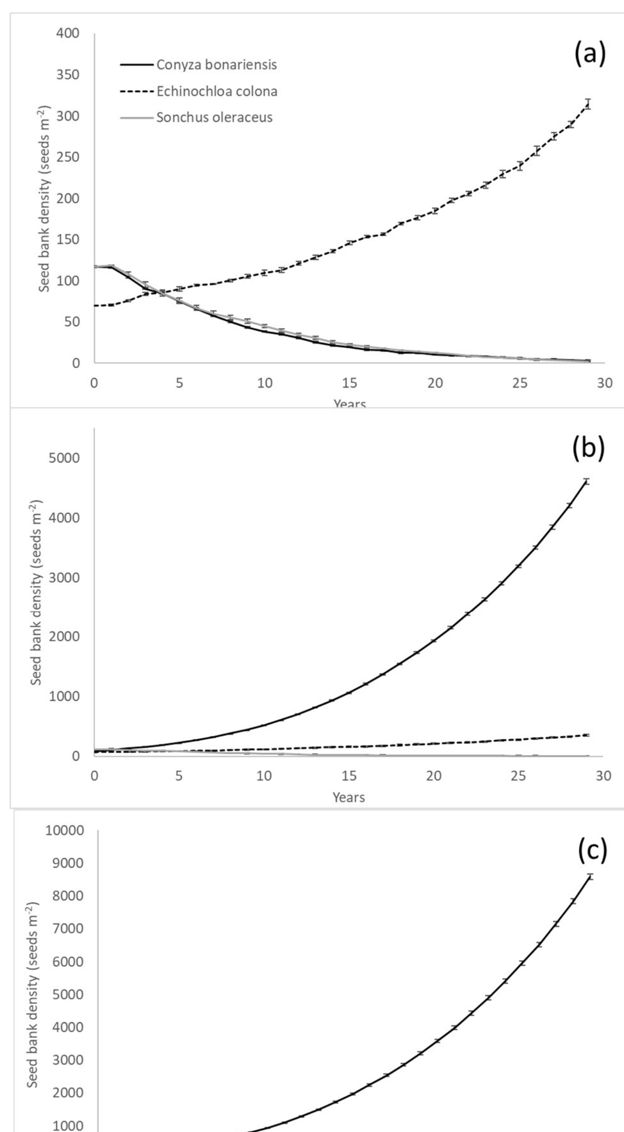


Figure 1.3.3 Predicted end-of-year mean weed seed bank densities (over 10 runs of each scenario) for Diversity model scenarios BMP-6 (a), BMP-4 (b) and BMP-2 (c)

High or very high levels of initial resistance gene frequency (other than glyphosate resistance in barnyard grass and fleabane, as noted) changed these results substantially. When we assumed each seed in the starting starting population has a 1 in 10,000 or 1 in 100 chance of having a random trait providing resistance to any of the three tracked herbicides, fleabane populations were consistently found to be uncontrolled in the medium to long term, under both the BMP and TS management systems.

No results are shown for TS-4 and TS-2 after 30 years, as these simulations failed after 13 years due to the high fleabane numbers. Interestingly, sowthistle populations were not predicted to be uncontrolled in the same scenarios—possibly due to being outcompeted by the other species, as well as more intrinsic factors. Glyphosate-resistant barnyard grass populations were well controlled in all of the BMP scenarios.

The key outcomes from this set of scenarios are:

1. Using the triple stack herbicides with one additional tactic is predicted to be insufficient for controlling glyphosate-resistant barnyard grass populations. The BMP system did control these populations well.
2. If there is substantial background of resistance to dicamba and/or glufosinate at the time of introducing the triple stack, using the TS strategy is predicted to result in gradual failure to control glyphosate-resistant fleabane. The BMP strategy is predicted to slow this process of failure, but still results in uncontrolled fleabane in the long term.

3. Both of these management systems are more diverse than glyphosate alone in a Roundup Ready/Flex system. Even in the TS system there may be time to notice gradual failure and make changes to management, except in the case where resistance is already present to dicamba and/or glufosinate in 1 per cent of plants, and for glyphosate resistant grasses. That said, the Diversity model suggests here that the advent of the triple stack will be an opportunity to promote the benefits of a more fully diverse BMP system, including residuals, chipping, tillage, and other knockdowns.

Table 1.3.3. Mean herbicide resistance level (-fold) in final five years of simulation, at three levels of background resistance to three herbicides under two management strategies

	Glyphosate			Glufosinate			Dicamba	
Scenario	ABYG	Fbane	Sthistle	ABYG	Fbane	Sthistle	Fbane	Sthistle
TS-6	7.5	9.01	1.1	1	1	1	1	1
TS-4	n/a	9.02	1.1	n/a	1.08	1	1	1
TS-2	n/a	9.1	1.1	n/a	1.11	4.4	1	5
BMP-6	4.8	5	1	1.01	1	1	1	1
BMP-4	5.4	6.8	1.15	1.03	1	1.15	1	1
BMP-2	5.6	6.85	1.1	6.02	1.04	1	2.5	1.1

Although over 10 runs of each scenario we did with the Diversity model, most of the results were qualitatively similar within scenarios, in some simulations there were a few results that were substantially different from the average. That is, resistance successfully took hold in the population and the system failed early. In BMP-6, there was one scenario out of 10 in which dicamba resistance reached approximately 6-fold in fleabane, and the seedbank of that species slowly climbed to 8000/m^2 over 30 years (data not shown). This suggests that it's important to investigate how often such statistically deviant results could occur, since the management requirements for the statistically different result would be quite different from the majority of paddocks under the same conditions (see below).

1.3.6. Scenarios: Clean Slate starting conditions Most of the simulations we have done with the Diversity model include the assumption that glyphosate resistance is already present in key weeds in simulated paddocks. We believe it is important that our recommendations take the current state of play into account. However, there is also value in testing proposed systems to see how resistance might evolve in weed communities with no existing resistance. These simulations allow us to compare systems developed for triple-stack varieties with previous modelling and historical data.

In Table 1.3.4, we show how often resistance greater than 2-fold to any herbicide evolved in 10 simulations each of four different scenarios. We tested two mutation rates—that is, how frequently a new resistance gene (randomly selected from all possible resistance genes) arose spontaneously in the population. High rate = 1 new resistant plant out of every 1 million seeds produced; low rate = 1 new resistant plant out of every 100 million seeds produced. Although these are large numbers, they are reached easily in large fields especially without good weed control. We tested irrigated scenarios using the TS and BMP strategies as detailed above.

In these scenarios, glyphosate (M) resistance was the most frequently evolved; all species were able to evolve glyphosate resistance at least some of the time. Fleabane usually evolved glyphosate resistance except in the low frequency, high-diversity system. Barnyard grass evolved resistance half the time or more under the TS system, and was still able to evolve resistance in some of the BMP simulations. Sowthistle, although poorly competitive with large populations of the other weeds, was nevertheless the only species predicted to (occasionally) evolve resistance to group I herbicides under these management conditions.

Table 1.3.4. Resistance evolution in three weeds simulated with the Diversity model

Mutation Rate	***Strategy***	***R frequency in Fleabane****	***R frequency in BYG****	***R frequency in sowthistle****
High	TS	1 (M)	0.6 (M, N)	0.4 (M, I)
Low	TS	1 (M)	0.5 (M)	0.3 (M)
High	BMP	0.5 (M)	0.2 (M)	0.3 (I)
Low	BMP	0.4 (M)	0.1 (M)	0

*Proportion of runs (out of 10) where resistance >2-fold was predicted to occur within 30 years, and which MOA/s the plants became resistant to.

1.3.7 Scenarios: High-diversity US Xtendflex systems In order to help assess Australian outcomes of the introduction of Bayer's Xtendflex system, we programmed simulations of several scenarios developed for Xtendflex cotton in southern USA by Larry Steckel at the University of Tennessee (Table 1.3.5). Note that these scenarios were devised for non-Australian weed environments; we tested them on our three key species with background glyphosate resistance in barnyard grass and fleabane, and three levels of background resistance to other herbicides in the same manner as the scenarios in 1.3.5 above.
On our species of interest, these strategies were mostly highly successful, with the stand-out failure of the Lubbock Reduced Tillage strategy notable for being the fastest to fail (within six years) of all non-test scenarios we developed. The remaining scenarios were highly diverse, with five to seven different herbicide modes of action applied each year, plus some use of cultivation.

Table 1.3.5 Four diverse weed management systems devised for Xtendflex in US cotton

Simulations	**Milan (Reduced Tillage)**	**Milan (Conventional Tillage)**	**Lubbock (Reduced Tillage)**	**Lubbock (Conventional Tillage)**
MOAs	*M, I, G, C, L, (K), Z*	*I, C, L, till, M, (K), Z*	*I, M, (K)/C,*	*D, I, M, (K), till, C*
Prior to planting	Glyphosate+ Dicamba+ Flumioxazin	Dicamba+ Fluometuron+ Paraquat	Dicamba+ Glyphosate	Trifluralin
	Possible tillage	Tillage		Tillage
At planting	Dicamba+ Fluometuron+ Paraquat	Dicamba+ Glyphosate+ Acetochlor	Acetochlor or Prometryn	
Early in-crop	Dicamba+ Glyphosate+ Acetochlor		Dicamba+ Glyphosate+ Acetochlor	Dicamba+ Glyphosate+ Acetochlor
	Or Glufosinate+ Acetochlor			
		Tillage at 3 and or 5 weeks		Tillage at 3 and or 5 weeks
	Dicamba+ Glyphosate	Dicamba+ Glyphosate	Dicamba+ Glyphosate	Dicamba+ Glyphosate
Layby	MSMA+ Diuron	MSMA+ Diuron	Glyphosate+ Diuron	Glyphosate+ Diuron
YEARS TO FAILURE*	**28**	**>30**	6	**>30**

* Average years to >20,000 seeds/m2 (if reached), for scenarios with background resistance set at 1×10^{-4}

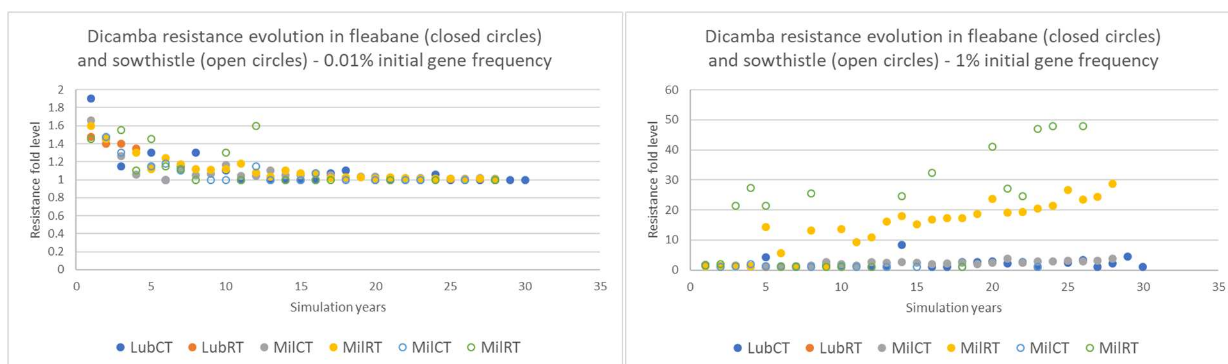


Figure 1.3.4. Dicamba resistance evolution in broadleaf weeds in several proposed Xtendflex weed management systems, assuming moderate (left) and high (right) levels of pre-existing resistance to dicamba

New resistance evolution—that is, resistance other than to glyphosate in barnyard grass and fleabane—was uncommon except in the case of high levels (1%) of pre-existing resistance (Fig 1.3.4). Dicamba resistance was observed commonly in these scenarios, and in some cases developed quickly. Resistance to dicamba above 20-fold (i.e. effectively immune to dicamba, and probably 2,4-D as well) was observed in both fleabane and sowthistle simulated populations, but only in the worst-performed scenario and the highest level of pre-existing resistance. In other scenarios, dicamba resistance was not predicted to increase. Meaningful increase in glufosinate resistance was never predicted to occur in these scenarios. This is likely for a few reasons. In the early-failing scenario, Lubbock Reduced Tillage, glyphosate resistant weeds out-compete the others and the system fails before new resistance has time to evolve. In the scenario where dicamba resistance did evolve, Milan Reduced Tillage, there is almost no glufosinate used, and the scenario succeeds long enough for dicamba resistance to evolve and cause issues. In the remaining scenarios, there is sufficient diversity—notably, with the inclusion of cultivation to clean up herbicide survivors at two points in the crop cycle—to slow resistance evolution. These are highly diverse systems, and especially in the two conventional tillage systems, no one mode of action is really the most important or dominant, especially with glyphosate already subject to resistance in two species.

In stage two of this subset of investigations, we adjusted these scenarios to fit Australian conditions, changing some of the chemistry applied and adjusting some application timings to optimise control. Having noted that these systems led to the application of 3, 5, 6 and 7 different modes of action, plus tillage, we decided to more directly investigate the relative value of increasing numbers of modes of action in our system.

The outcomes of these scenarios are summarised at the bottom of Table 1.3.6 in terms of years of predicted operation before >20,000 seeds/m2 are present in the soil, and in figures below.

Interestingly, the BMP program (6 MOAs plus tillage, here) is more effective than the 7 MOAs treatment. This suggests that at some high level of diversity there are diminishing returns to adding more modes of action because of the increasing need to add less effective herbicides in place of already used, but nominally more effective ones.

Systems from five MOAs (plus cultivation) upwards, in any case, are all quite effective and while there is an eventually large population of fleabane, numbers increase gradually enough for some extra interventions to be carried out after 15-20 years of operation.

Table 1.3.6. Five weed management systems of increasing diversity in irrigated cotton

Simulations	**3 MOAs**	**4 MOAs†**	**5 MOAs**	**6 MOAs‡**	**7 MOAs**
MOAs	*M, I, N*	*M, I, N, L*	*M, I, L, K, Z*	*M, L, C, I, N, D*	*M, I, L, K, Z, N, G*
Prior to planting	Glyphosate	Glyphosate fb paraquat	Glyphosate fb paraquat	Glyphosate fb paraquat	Dicamba+ Glyphosate fb paraquat
		Tillage	Tillage	Tillage	Tillage
At planting	Glyphosate+ dicamba	Glyphosate+ dicamba	Metolachlor	Prometryn	Metolachlor
Early in-crop	Glyphosate, glufosinate, dicamba	Glyphosate, glufosinate, dicamba	Dicamba+ glyphosate	Dicamba+ Glyphosate fb glufosinate	Dicamba+ Glyphosate fb glufosinate
			MSMA		MSMA
	Dicamba+ Glyphosate	Dicamba+ Glyphosate	Dicamba+ glyphosate	Dicamba+ Glyphosate	
Layby	Glyphosate, glufosinate, dicamba	Glyphosate fb glufosinate	Dicamba+ glyphosate	Pendimethalin + Diuron	Flumioxazin
		Inter-row cultiv'n	Inter-row cultiv'n	Inter-row cultiv'n	Inter-row cultiv'n
Late in crop	Glyphosate, glufosinate, dicamba	Glyphosate, glufosinate, dicamba	Glyphosate, dicamba	Glyphosate, glufosinate, dicamba	Glyphosate, glufosinate, dicamba
				Chipping	Chipping
YEARS TO FAILURE *	5	13	25	>30	27

* Average years to >20,000 seeds/m2 (if reached), for scenarios with background resistance set at $1x10^{-4}$
†This is effectively the TS strategy described above in this report
‡This is the BMP strategy described above in this report

As in the US systems above, new evolution of resistance did occur but was relatively uncommon, and for the same reasons—either the system failed quickly due to pre-existing glyphosate resistance, or there was sufficient diversity to slow resistance evolution markedly. While there is more use of (and pressure on) glufosinate compared to the US proposed systems, and dicamba use is quite frequent, these are by modern standards highly diverse weed management systems. We also see that because dicamba and glufosinate are only moderately effective against fleabane and sowthistle, the pressure for resistance to evolve seems to be lower under our set of assumptions. (It should be noted that in some other modelling projects, eg UWA's PERTH model, moderate effectiveness does not equate to lower rates of resistance evolution, but those projects focused on scenarios of much lower management diversity than we are dealing with here.)

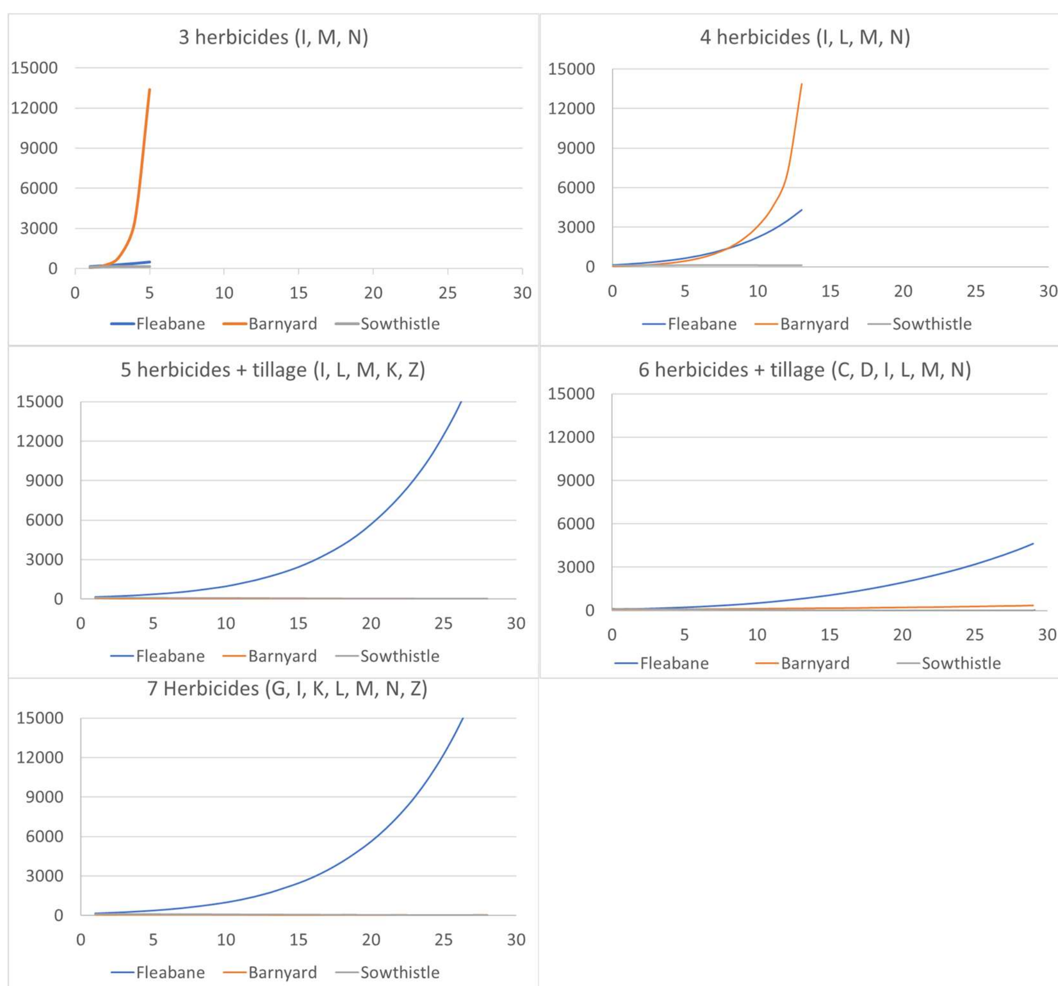


Figure 1.3.4. Seedbank density predicted over 30 year simulations of five weed management systems increasing in diversity

1.3.8 Dryland rotations and double knocks with glufosinate The 2+2+0 strategy was developed from our previous modelling work to extend a simple strategic rule for use especially in higher-risk dryland cotton cropping. Summer fallow weed management was found in that work to be critically influential over the speed of resistance evolution. We devised a range of dryland scenarios for the Diversity model to assess the same problem from a multi-herbicide, multi-species point of view.

We varied three factors in our dryland scenarios: cropping frequency (1 in 2 years vs 1 in 3 years), in-crop management (BMP vs TS scenarios as described in Table 1.3.1) and summer fallow management, as described below in Table 1.3.7.

The full list of combinations and a summary of model results for each is shown in Table 1.3.8. Each scenario was run five times and mean results are reported here.

In these simulations, failures (highlighted in Table 1.3.8) only occurred in scenarios with the TS strategy in crop, and the most rapid failures occurred when fallows were at the higher frequency (1 in 2). Of the summer fallow strategies, the DK and BMP strategies were the most successful at managing the populations; the M+I and Residuals strategies were less successful.

Table 1.3.7. Summer fallow weed management strategies used in dryland cotton scenarios with the Diversity model

Point of application	Fallow management strategy *Glyphosate + Group I (MI)*	*Double knocks (DK)*	*Residuals (R)*	*Best Management Practice (BMP)*
Post-harvest		Glyphosate fb glufosinate		Cultivation
Early fallow	Glyphosate +2,4-D	Glyphosate + 2,4-D	Glyphosate Pendimethalin*	Diuron Glyphosate + 2,4-D
Late fallow	Glyphosate	Glyphosate	Glyphosate	Glyphosate Cultivation Group A fb glufosinate
Preparation for planting	Glyphosate 2,4-D	Glyphosate fb paraquat	Metolachlor*	Glyphosate fb paraquat + metolachlor*

*Residuals used are representative of a type. Resistance and phytotoxicity are not tracked.

Table 1.3.8. Dryland scenario list and results summary from Diversity model simulations

In crop strategy	Fallow strategy	Cropping Frequency	Scenario ID	Years to system fail	Maximum dicamba resistance (x-fold)*	Maximum glufosinate resistance (x-fold)*
TS	**M+I**	1 in 2	TS-MI-2	9	1.9 (st)	1.5 (bg)
TS	**DKs**	1 in 2	TS-DK-2	>25	1.8 (fb)	1.4 (bg)
TS	**Residuals**	1 in 2	TS-R-2	9	1.6 (st)	1.2 (bg)
TS	**BMP**	1 in 2	TS-BMP-2	>25	1.6 (st)	1.6 (bg)
TS	**M+I**	1 in 3	TS-MI-3	12	1.4 (st)	1.3 (bg)
TS	**DKs**	1 in 3	TS-DK-3	>25	1.3 (fb)	1.4 (bg)
TS	**Residuals**	1 in 3	TS-R-3	12	1.6 (fb,st)	1.3 (bg)
TS	**BMP**	1 in 3	TS-BMP-3	>25	1.9 (fb)	1.8 (bg)
BMP	**M+I**	1 in 2	BMP-MI-2	>25	1.9 (st)	1.7 (fb)
BMP	**DKs**	1 in 2	BMP-DK-2	>25	1.6 (st)	1.6 (fb)
BMP	**Residuals**	1 in 2	BMP-R-2	>25	1.5 (st)	1.5 (st)
BMP	**BMP**	1 in 2	BMP-BMP-2	>25	1.6 (fb)	1.6 (fb)
BMP	**M+I**	1 in 3	BMP-MI-3	>25	1.6 (st)	1.4 (fb)
BMP	**DKs**	1 in 3	BMP-DK-3	>25	1.9 (fb)	1.9 (st)
BMP	**Residuals**	1 in 3	BMP-R-3	>25	1.9 (st)	1.5 (st)
BMP	**BMP**	1 in 3	BMP-BMP-3	>25	1.5 (st)	1.6 (st, fb)

There was in each case an accumulation of minor resistance genes in at least one species to dicamba and glufosinate (glyphosate resistance was already present and is not shown here), but no major resistance genes were found to have accumulated in a large proportion of the population. These relatively small amounts of resistance are reminiscent of rate creep, and would have some impact on the farming system as it progresses, without complete loss of control. The relatively low level of resistance predicted was not largely responsible for the loss of control in those simulations that failed; in these cases, poorly-managed existing glyphosate

resistance was the main cause of failure. We assumed a moderate level of background resistance to dicamba and glufosinate of 1×10^{-5}. Other simulations we've done here suggest that higher starting resistance frequencies would create somewhat different results. The influence of this variable supports the need for data on background resistance levels in real weed populations, preferably before commercial release of glufosinate and dicamba (or other) resistant cotton.

The DK fallow strategy is included as one method of implementing the 2+2+0 strategy across broadleaf and grass weeds. We included glyphosate fb paraquat, glyphosate fb glufosinate, and group A fb glufosinate as highly effective double knocks on relevant species (see section 2.2). Glufosinate's efficacy as a double knock partner is shown here, where it is part of two highly effective strategies. In real situations, glufosinate double knocks are unlikely to be used in fallow, if already used in crop, but our results emphasise their utility, and suggest that in this kind of rotation that includes mixtures, varied sequences, and double knocks, resistance evolution is not highly favoured. The BMP strategy is a modification of our previous findings from patch management work, which would be especially useful in fields where glyphosate resistance already exists in some species. Our results here show that both approaches are supported by the Diversity model's predictions.

1.3.9 Preliminary Group A resistance Grass selective group A herbicides appear to be an extremely useful tool against the glyphosate resistant summer grass weeds that are arguably the industry's most difficult resistance problem, except for one factor: they are well known for being prone to rapid selection for resistance themselves. Previous work by UA and UWA groups, and others around the world, has found that group A target site resistance genes are relatively common (estimated around 1 in 10,000) in unselected populations, which makes it easy to select them in the field. Added to this is the well-documented propensity in annual ryegrass to develop non-target site resistance to group A herbicides as well. In the field, group A resistance is often quoted as being likely to evolve after only a handful of annual applications, when there are no other herbicides targeting survivors. Thus, our industry has been extremely reluctant to recommend group A herbicides for glyphosate resistant summer grasses, even though it is safe and registered for use in crop.

Table 1.3.9. Modified weed management strategies incorporating the use of Group A herbicides for grass control

	Mangement strategy	
Point of application	***Triple-stack (TS)***	***Best Management Practice (BMP)***
Prior to planting	Glyphosate followed by† paraquat	Glyphosate followed by paraquat
At planting	-	Prometryn
Early in-crop	Glyphosate+dicamba; group A‡; glufosinate§	Glyphosate+dicamba; Group A followed by glufosinate
Layby	-	Pendimethalin
Late in-crop	Glyphosate; group A	Glyphosate; group A followed by glufosinate; hand-hoeing
Winter fallow	Glyphosate+2,4-D; glyphosate	Cultivation; diuron; glyphosate+2,4-D if required

† The term 'followed by' indicates the use of a double knock, wherein the second herbicide is applied one week after the first, and has synergistic effects.
‡ Tactics separated by + are applied together, either in a single product or as a tank mix.
§ Tactics separated by semicolons can be applied sequentially in the same part of the season indicated, if and as weed germinations require additional control after the first tactic in the list.

Our work on double knocks with glufosinate (2.2) suggest that strategies could be developed that protect group A susceptibility, while allowing their use on difficult to control glyphosate resistant populations of awnless barnyard grass and other species. Modelling group A use and resistance in cotton has been a minor priority for some years, and we elected to produce some preliminary simulations with the Diversity model.

We tested group A use in two strategies, being modified versions of the TS and BMP scenarios (designated TS-A and BMP-A). Two group A applications per year are added to each strategy, as shown below. In the modified TS-A strategy, these applications are without direct protection from a follow-up herbicide. In BMP-A, each group A application is part of a double knock with glufosinate.

We simulated irrigated continuous cotton with glyphosate-resistant barnyard grass and fleabane, and susceptible sowthistle, as in previous simulations described above.

Table 1.3.10. Resistance evolution to group A herbicides in two strategies simulated with the Diversity model

In crop strategy	Initial A resistance	Mean years to system fail	Minimum years to system fail	Maximum group A resistance (x-fold)*	Frequency of major resistance evolution
TS	$1x10^{-4}$	13	13	30.8	0.2
TS	$1x10^{-2}$	12	12	32.1	0.6
BMP	$1x10^{-4}$	>25	>25	1.3	0
BMP	$1x10^{-2}$	>25	>25	1.5	0

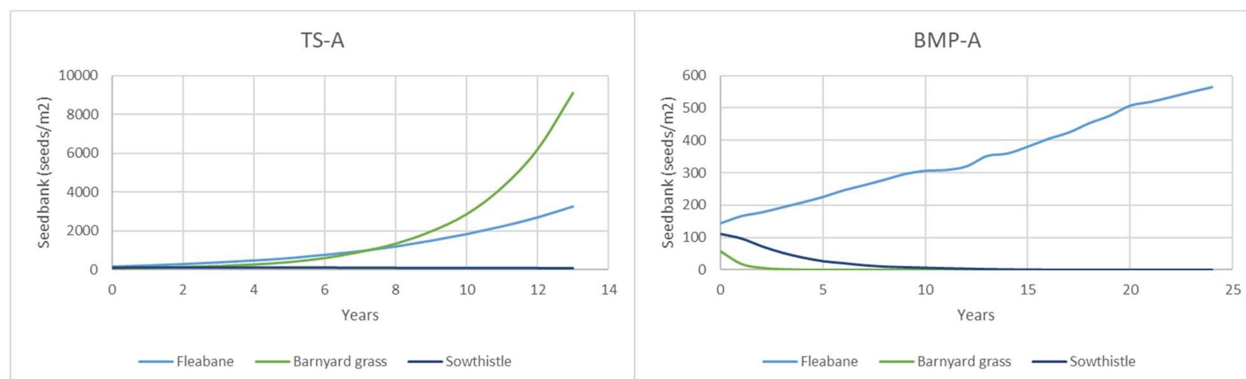


Figure 1.3.5. Example seedbank density in two scenarios with Group A for grass management with the Diversity model

Our simulations with the use of group A herbicides found substantially different results between the TS and BMP strategies. The BMP strategy was predicted to protect both weed management and group A susceptibility in the long term, while under the modified TS system both barnyard grass and fleabane were able to increase substantially after a decade. However, our results predicted that this loss of control occurred regardless of group A resistance evolution (Table 1.3.10), and that evolution was not observed in 100% of cases. These results are markedly different from studies on group A resistance in the field and in models, with other species in other systems, where resistance is reliably predicted within 5-10 years. Our simulations rely on a range of untested assumptions about theoretical group A resistance in barnyard grass, and so should be considered very preliminary. Nevertheless, the success of the BMP scenarios suggests that we need not ignore group A herbicides as a tool for managing summer grasses in crop. The glufosinate double knock has not been tested on group A resistant barnyard grass, and these results suggest this would be important data, but the potential utility

of glufosinate for protecting group A usefulness is starkly emphasised here. More work is clearly needed.

Future work

It's important to note that this implementation of the Diversity model only tracks resistance to three herbicides: glyphosate, glufosinate and dicamba. These are the three most frequently-used tactics in the in-crop strategies we tested, so there is greater evolutionary pressure on genes providing resistance to these three modes of action than to other tactics in the two management systems. However, in the real world evolution to other herbicides used in the system (notably paraquat, pendimenthalin, prometryn, diuron, grass selectives and 2,4-D) would certainly be possible, and may threaten the long-term viability of weed management in these systems. In previous, single-herbicide models of resistance, this possibility of non-key-herbicide resistance is ignored. While we have modelled resistance to three herbicides here, the Diversity model shows that it is not out of reach to model simultaneously all the substantial resistance risks for a given cropping system.

The Diversity model was presented for the first time at the Global Herbicide Resistance Challenge in Denver, May 2017. As a result of the presentation we were invited to produce a full paper on the model for a special issue of Pest Management Science.

1.4 - Develop a cotton-systems version of RIM to provide growers with a tool that allows them to assess the economics of their own current and future weed management

The cotton-systems version of RIM, known as BYGUM (Barnyard Grass Understanding and Management) was completed in late 2015. Since its release to industry, via workshops with ICAN and subsequent publication on the CottonInfo website, it has been downloaded by almost 300 unique users. Although we are unable to estimate the extent of use of the tool by and with growers, we are confident it has reached a substantial proportion of the Australian cotton industry.

BYGUM is a sophisticated Excel spreadsheet model, free to download, use, and transfer between users. It measures and estimates the economic impacts of weed management in cotton and mixed cropping systems, through realistic statistical modelling of weed and crop competition, user-defined prices and costs, and a range of other economic and accounting factors. Users are able to save and restore several scenarios in any one copy of BYGUM, and can also hold multiple copies of the model, allowing comparison between many different possible scenarios. The tool is designed to test current weed management systems and prospective ones, including those substantially different from the current system.

BYGUM is based on the University of Western Australia's Ryegrass Integrated Management (RIM) tool, a long-standing model of economic returns from weed management in western grains cropping. BYGUM retains much of the structure of RIM – that is, it is driven around the user defining a set of parameters for weed control tactics and crop types, and then devising a sequence of crops each with weed control options. The tool then estimates the size of the weed population in each year and reduces the expected maximum yield for each crop accordingly. The resulting yields and weed population trends are displayed on graphs and tables. The tool can save and load a set of different scenarios for easy comparison between options.

BYGUM differs from RIM in several important ways. In particular, it includes winter and summer cropping, as well as fallow weed management. These two changes have required a substantial reprogramming effort. We also cut the number of simulated years from ten winter crops (RIM) to five, in order to keep the tool to a single viewable page. Maintaining RIM's simplicity of use has been a major priority, but cutting the scenario to five years also reduces concerns about modelling economic future discounting and inflation.

The model underlying BYGUM has been altered from the original in RIM in several ways, including:

- Allowances for the effects of fallow and cover crops in winter and summer on subsequent crops. We used Australian rainfed cotton cropping data, APSIM simulation results, and expert-derived estimates of the value of one or two summer fallows between cotton crops. Little local data was available on the benefits of cover cropping during extended non-crop periods, so we relied here on APSIM and RIM simulations of cover crops and brown manuring. We derived estimates for the effect on weed seed banks and moisture retention for use in the next crop, and applied these in BYGUM as simple constants used in BYGUM's crop yield equations. Each of these model factors can be adjusted in the back end of BYGUM should new data or new situations require it.

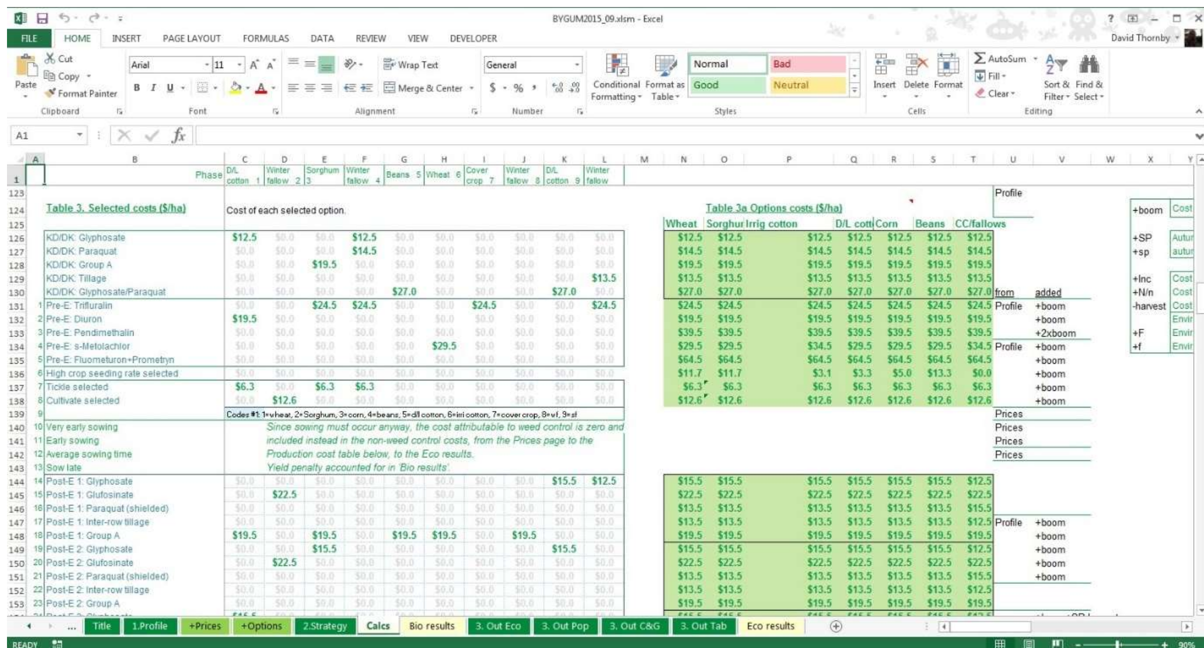


Figure 1.4.1. While BYGUM's back end is complicated, there are many user-definable model constants of interest here.

- The introduction of an irrigated crop. RIM has no functionality for irrigated cropping. In order to introduce it for BYGUM, we data-matched the mean observed effects of irrigation on weed emergence timing and abundance and added appropriate constants to BYGUM's weed biology models. These constants can be redefined by the user in the back end. Irrigation's advantages for crop yield are user-defined as part of the front end interface.
- Redefinition of herbicide types to suit subtropical farming systems. RIM's timing for in-crop operations was quite different from what is typically done in cotton and northern mixed cotton/grains operations. We devised a suitable setup of pre-crop, planting, in-crop, layby, harvest and post-harvest applications that can also be extended for sensible weed management in fallows. Management tactic applications can be chosen from a set of 19 different user-defined weed management methods (herbicides and tillage) across five different parts of the season. Users have three pre-planting and six post-planting weed management opportunities per phase, plus harvest weed seed

management in grains and the option to cultivate at planting, in which to choose from their defined options.

Figure 1.4.2 The 'Define' screen contains 19 different weed managment options that are user-defined, as well as a range of crop-related variables.

Figure 1.4.3. The user chooses up to eight weed control opportiunities, plus several slots for cultivation and harvest weed seed management, from the 19 user-defined tactics on the Define screen

- Addition of several crops. RIM's selection of crops is suitable only for western grains systems. We added several summer and winter cropping options suitable for a reasonable approximation (within time and space constraints) of northern region cotton and mixed cotton-grains farming systems. BYGUM allows users to construct rotations with dryland and irrigated cotton, corn, sorghum, beans (i.e. a single summer pulse crop, user-definable to mimic mungbeans (the default) or another species as desired), winter and summer fallows, a winter crop (which the user can parameterise as wheat

(the default) or another winter species of interest) and a cover crop. We used a range of data sources to provide parameters for competitiveness between the summer crops included and awnless barnyard grass, and APSIM to estimate weed effects on summer and winter fallow moisture retention efficacy. We elected to allow users full flexibility to create five-year rotations, which can lead to some nonsensical results if impossible combinations are chosen.

- Removal of mechanisms for long pasture phases.
- Functional and parameter changes to change the growth and population dynamics from annual ryegrass to awnless barnyard grass. We have a large database of barnyard grass data for a range of biological and ecological factors, and used this to estimate the required parameters. Detailed competition studies are still scarce, especially for the range of crops we included in BYGUM, so APSIM scenarios were created and analysed to fill in these information gaps.
- Cosmetic, background text, and tutorial changes were also required. A new colour scheme, logo, and visual elements were created to separate BYGUM from RIM. Each step in the model has an associated tutorial screen, and these were rewritten for BYGUM.

Figure 1.4.4. Branding changes were made to distinguish BYGUM from RIM

We have created a quick-start document for new users of BYGUM (see Appendices), and a range of background texts explaining the model for different audiences, for CottonInfo's website and other outlets.

BYGUM is useful for creating extension material with an economic message on summer weed management issues as they arise. We created and published a set of examples of these messages on aspects of residual herbicide choice, cover cropping, and the economic effects of different levels of glyphosate resistance, which were summarised in Spotlight and on the CottonInfo website. Full versions of these materials are included in the Appendices. Further development of extension message material would be a simple procedure, requiring only an extension specialist to become familiar with the operation of the tool. We anticipated CottonInfo and others being able to respond to within-season weed management issues and concerns with scenarios and extension packages built around them, in an ongoing way.

We held two sessions to gain industry feedback on top of presenting pre-launch at two CottonInfo/ICAN masterclass events, and made additions to the tool as a result, including conversion tools for bales/ha and changes to baseline cost estimates.

BYGUM is now an effective tool for simulating summer grass management (including the critical issue of glyphosate resistant awnless barnyard grass) with a focus on economics that is easy to use, requires only a recent copy of Microsoft Excel, and can be shared openly between users without risking data loss. Like RIM, it is a useful tool for teaching, workshops and for illustrating extension messages, as well as being useful for weed management planning between growers and their advisors. For detailed information on the programming and parameters used, the back end, accessible as a series of spreadsheet pages by selecting ‘Info’ and then ‘Lock/Unlock’ from the start page, contains the full set of data.

BYGUM is available for download from the CottonInfo website at https://www.cottoninfo.com.au/barnyard-grass-understanding-and-management-bygum

1.5 - Increase the ecological knowledge of key species relevant for improved control and defining risks for evolution of herbicide resistance

Introduction

Effective management of weeds in cropping systems can be achieved by gaining an understanding of the ecology and finding weak points in the lifecycle. Soil moisture is likely to have the greatest impact on emergence (Werth *et al.* 2017), and when moisture conditions are suitable, temperature significantly influences the time weeds emerge. Temperature also affects the rate at which plants grow and develop to stages such as tillering or stem elongation, and seed maturity. Understanding the timeframe to reach these stages is critical, as control tactics are more effective before grasses reach tillering and broadleaf weeds reach stem elongation. Long-term weed management is dependent upon driving down the soil seed bank. If weeds are not controlled in the early stages, or survive, then it becomes important to know how long before additional tactics need to be used before weeds reach maturity and replenish the soil seed bank.

The time of year species emerge can also have an effect on development in terms of day length or photoperiod (Huang *et al.* 2000). For species such as barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) the response to photoperiod in relation to growth is well known. Plants that emerge and grow with increasing day length are larger and produce more seed, whereas plants that grow with decreasing day length, mature faster but produce less biomass and seed (Keeley and Thullen 1989; Mitich *et al.* 1990). Redroot pigweed (*Amaranthus retroflexus* L.) has both a photoperiod sensitive phase for reproduction and a photoperiod insensitive phase after reproduction (Huang *et al.* 2000). Studies on Mitchell grass (*Astrebla* sp.) showed that photoperiod had little effect on the growth and development of tillers and initiation of anthesis, however did affect the numbers of seed heads that emerged (Jozwik 1970). Ngo *et al*. (2017) conducted growth and development studies on South Australian populations of feathertop Rhodes grass (*Chloris virgata* Sw.) and windmill grass (*Chloris truncata* R. Br.) and information is available on the biology of flaxleaf fleabane (*Conyza bonariensis* (L.) Cronquist) (Wu *et al*. 2007) and common sowthistle (*Sonchus oleraceus* L.) (Widderick *et al.* 2010).

Awnless barnyard grass, feathertop Rhodes grass, windmill grass, fleabane and sowthistle are among the most problematic weeds in cotton and grains systems. These are all small seeded species that have increased in prevalence due to an over-reliance on glyphosate, and a lack of cultivation and residual herbicides (Charles *et al*. 2004; Osten 2015; Werth *et al*. 2010, 2013). Glyphosate resistant populations of these species are now present throughout the cotton growing regions in Australia (Preston 2019). As the cases of resistance to glyphosate and other herbicides is likely to increase, understanding the life-cycle of these species and optimum timing of control tactics is crucial. This applies to both chemical and non-chemical tactics (Forcella 1993).

The purpose of this study was to determine how the time of emergence affected the growth and development of weed populations from the northern cropping region. The investigation of growth parameters for awnless barnyard grass, feathertop Rhodes grass, windmill grass,

fleabane and sowthistle in terms of biomass, seed production and thermal time as related to northern region populations may contribute to, and enable, more informed management decisions to be considered.

Methods

<u>Experimental procedure</u>

The growth and development of five key weed species was investigated in two consecutive glasshouse experiments established at the Leslie Research Facility, Toowoomba, Queensland. Both trials ran for 18 months with new plantings scheduled at four weekly intervals during the first 12 months of each trial, resulting in 13 plantings per year.

The experiment for year one commenced on the 07/10/14 and ran through to the final planting on the 15/09/15 with the last biomass harvest occurring on the 09/03/16. In the first year, the planting schedule was adhered to with the exception of the September planting being delayed by one week. In year two, the thirteen plantings occurred between the 13/10/15 and 13/09/16 with the final biomass harvest conducted on 28/02/17. In the second year, five scheduled plantings were delayed. The early March planting by two weeks and the late March, April and June plantings by one week. A delay of three weeks resulted in the August planting being removed from final analyses.

The glasshouse used for the experiment was fitted with evaporative coolers, activated to lower the internal temperature when it exceeded 25°C. When the evaporative coolers were not running, side vents were open, enabling air to circulate through the glasshouse. There was no heating facility. A Tinytag datalogger (Gemini Data Loggers Ltd, Chichester, UK) was suspended in the glasshouse in a semi-shaded position next to the thermostat sensor and data was downloaded to provide maximum/minimum temperatures.

The experiment was a randomised complete block design. Five replicates of each species were sown each planting date. Benches, numbered one to five, contained all the pots for the corresponding replicate.

Pots measuring 13 cm in diameter were filled with Searles Premium potting mix and topped with 2 - 3 cm of Scotts Osmocote professional seed raising and cutting mix. Approximately 50 seeds of each species were sown onto the surface of the mix, covered with a fine layer of seed raising mix and then misted with a fine water spray. The pots were placed onto benches according to the design layout and watered via an irrigation system until the trays under the pots were full. Seedlings were thinned to one per pot once established. Plants were fertilised on a monthly basis over the course of the experiment with Aquasol, prepared according to label specifications. An automated irrigation system was installed and pots were watered twice a day. If required, a supplemental watering into the trays was undertaken to ensure the pots did not dry out.

Prior to the establishment of the first trial, the evaporative coolers in the glasshouse developed faults. The decision was made to commence the experiment in the shadehouse and move into the glasshouse when the faults were rectified. This did not occur until the 30/10/14 and as the date for the second planting was the 04/11/14, the October pots were transferred into the glasshouse concurrent with the second planting.

Source of seeds

Standard lines of regional weed populations, originally collected in the field, are maintained via seed increases at the Leslie Research Facility. These include awnless barnyard grass (*QS - Bowenville*), feathertop Rhodes grass (*Dalby Agricultural College*), flaxleaf fleabane (*Tummaville*) and common sowthistle (*Dalby Agricultural College*). Mature windmill grass inflorescences were collected from along a fence-line and associated open ground at the Bowenville Reserve in 2014 and used in this experiment.

Growth and key development measurements

Plant development was monitored daily (Monday - Friday) and the dates plants reached each key stage (e.g. emergence, onset of tillering, inflorescence emergence, anthesis, onset of maturity) was recorded. While the time taken for the seedling to emerge was recorded, emergence date rather than planting date was used in all calculations.

Weekly measurements were taken for a number of growth parameters including plant height, number of tillers/inflorescences/buds and inflorescence lengths. An above ground biomass harvest was undertaken on the first four replicates at 24 weeks (6 months) from the date of planting. The fifth replicate was kept for a further 6 months to determine if the plants would continue to grow and potentially reach maturity if this stage had not been reached before the biomass harvest (data not presented in this report). During the growing period, dead leaves and inflorescences were removed from the plant and bagged to be included with the biomass. The plant matter was dried for 48 hours at 60°C to calculate the dry weight biomass.

Mature seeds were collected from the first five inflorescences to reach maturity on the grasses and the first five buds to open and retain seed on the broadleaves. Total seed production was then calculated by averaging out the seed produced by the five heads and multiplying that by the number of inflorescences (grasses) or the number of buds (broadleaves) produced. Due to the high seed production of fleabane, seed counts were not undertaken for every sample. Instead, the seed collected from each replicate was weighed and then the total number of seeds produced in replicate one for each planting was counted. The weight/1 000 seeds for each replicate one was calculated and then this value was used to estimate the number of seeds produced in replicates 2 - 5 for the same planting. Gaps in the data were due to key dates taking longer than 6 months to reach, or the stage was reached while staff were absent from the facility due to conferences, field work, etc.

Seed age and the effect on seed dormancy

In order to gain an understanding of the effect seed age has on dormancy, germination tests were conducted on the seed collected from each planting for awnless barnyard grass, feathertop Rhodes grass and windmill grass. The data obtained was used to calculate a time to germination for each monthly planting in addition to a total percentage germination.

Seed collected from the final planting in September 2016 matured between November and January depending on the species. Monthly collections of mature seed continued until the commencement of the germination tests. This allowed for an uninterrupted time line on seed age from fresh seed collected just before the germination tests through to the oldest seed that had matured from the first planting in October 2014. Awnless barnyard grass was the only species where seeds from all plantings were included in the germination tests. As the viability of feathertop Rhodes grass and windmill grass seeds diminishes with age, seeds from all the year two plantings were included for both species in addition to seeds from some of the year one plantings.

Seeds (50 seeds x 3 replicates) from each of the five replicates from each planting were placed into 8.5 cm Petri dishes lined with one filter paper (No. 1: Whatman International, Maidstone, UK) and 3 ml of deionised water was added to each dish. Up to nine petrie dishes were then placed into plastic zip-lock bags (27 x 35 cm) to retain moisture and for ease of checking before being placed into the incubators. Emergence counts were recorded on a daily basis for a period of 30 days with the emergence of the radicle being the determining factor in classifying the seed as having germinated. After 30 days, potassium nitrate (KNO_3) was added to feathertop Rhodes grass and gibberellic acid (GA_3) added to windmill grass to determine their viability, barnyard grass was subjected to the squeeze test. Potassium nitrate has been effective in improving germination in feathertop Rhodes grass (Hendrick and Taylorson 1974; Osten pers comm.) and GA_3 can promote embryo development and radical growth in some species (Naylor and Simpson 1961). Tetrazolium tests were not conducted on the feathertop Rhodes and windmill grass as results can be unreliable (Farley *et al*. 2013).

The germination experiments were conducted twice for each species using different Labtec refrigerated incubators (Model ICCBOD-300), each operating on a 12 hour day/night cycle with temperatures at 30°C/20°C.

Base temperature determination

The germination tests for base temperature determination commenced approximately 6 months after seed had been collected from year one plants to account for dormancy issues.

Seeds (50 seeds x 3 replicates) of each species were incubated at eight different constant temperature regimes (5, 10, 15, 20, 25, 30, 35 and 38°C), on a 12 hour day/night cycle. The highest temperature of 38°C was due to limitations in the incubator that prevented setting the desired maximum temperature at 40°C. Seeds were placed into 8.5 cm Petri dishes with two

layers of filter paper (No. 1; Whatman International, Maidstone, UK) and 10 ml of deionised water. The number of germinated seeds was recorded daily for at least 28 days, and recordings stopped when no further germinations were observed for 7 days. The tests were run twice in different incubators for each temperature regime.

Minimum temperature thresholds, and the reciprocal time to 50% germination were estimated using Steinmaus *et al*. (2000). Percentage germinations for each replicate were fitted to the logistic function:

$$Y = min+(max-min)/(1+(x/T_{50})^\wedge(-Hillslope)), \quad (1)$$

where Y is the percentage of cumulative germination, x is the time (d), the germination rate (T_{50}) is the time taken for half of the seeds to germinate, and Hillslope is the steepness of the curves. This was calculated using SigmaPlot 13.0 (Systat Software Inc., San Jose, California).

A linear regression was then performed by plotting the inverse of the germination rates against each incubation temperature. The base temperature (T_b) was then determined from where the regression intercepted the temperature axis.

Growing degree day calculation

The Tinytag datalogger, placed in the glasshouse at the beginning of the experiment, recorded the temperature at 15 minute intervals for the duration of the experiment. Maximum and minimum temperatures for each day were then obtained from the datalogger.

The estimated T_b value was then used to calculate the growing degree days (GDD) from emergence to tillering and emergence to seed set according to the following equation:

$$GDD = \sum[(T_{max} + T_{min})/2 - T_b], \quad (2)$$

where T_{max} is the daily maximum air temperature, T_{min} is the daily minimum air temperature, and T_b is the base temperature.

Statistical analysis

Growth and development parameters were analysed using ANOVA and Fisher's protected LSD multiple comparisons. When necessary, data was transformed using square root (sqrt) or log_{10} transformations. Base temperature data and growing day degree calculations were analysed by ANOVA, and Fisher's protected LSD multiple comparisons were used to differentiate between predicted means.

Due to the planting dates not being consistent for each year of the experiment and the increased glasshouse temperatures in year two, the data for both years were not combined for analyses.

Results and discussion

Growth measurements

In year one, the time of planting was significant in relation to many of the growth measurements recorded as well as the seed production of all five species. In year two, there were significant differences once again in growth measurements and seed production, with the exception of sowthistle seed production.

This report presents the results obtained for plant biomass and seed production of the five species.

Year one

- *Awnless barnyard grass*

The growth and development measurements significantly affected by the planting date ($p<0.001$) were dry weight biomass, plant height, tiller production, the length and number of panicles produced, and seed production.

Plants sown in October and November 2014, and September 2015, produced on average, significantly heavier biomasses during the 24-week growing period (13.9 - 14.4 g) (Figure 1). Similarly, the average biomass of plants from the early and late December plantings were not significantly different from each other but were from all other plantings at 9.24 and 8.4 g respectively. Seed production from these five plantings was also significantly higher than for the remainder of the year (Figure 2).

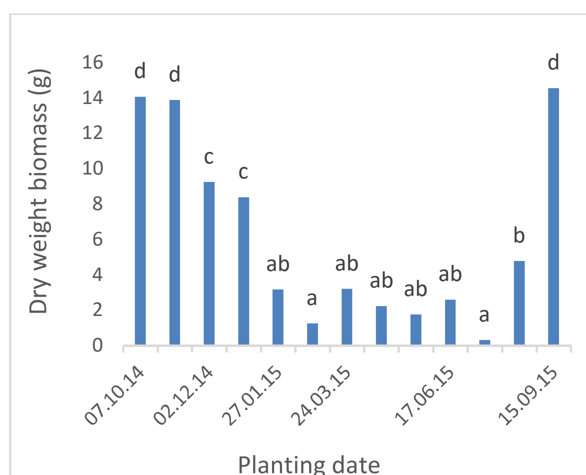


Figure 1: Average dry weight biomass (g) of awnless barnyard grass harvested at 24 weeks after planting (LSD = 3.21).

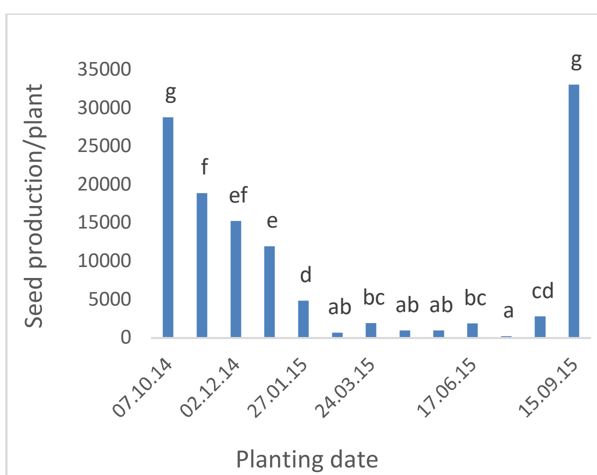


Figure 2: Back-transformed predictions of the average seed production/plant of awnless barnyard grass grown for 24 weeks from planting date (LSD on sqrt scale = 20.84).

The September 2015 planting generated the highest seed production with an average of 33 026 seeds/plant which was not significantly different from the October 2014 planting of 28 734 seeds. Seed production from plants sown between November and late December

averaged between 11 942 and 18 862 seeds/plant while plantings for the remainder of the year produced less than 5 000 seeds/plant over the 24 weeks growing period.

- *Feathertop Rhodes grass*

The growth and development measurements significantly affected by the planting date ($p<0.001$) were dry weight biomass, plant height, tiller production, the length and number of inflorescences produced, and seed production.

The average biomass of plants established in September 2015 (55.85 g) was significantly heavier than for the rest of the year, followed by the October and November 2014 plantings at 43.77 and 33.87 g respectively (Figure 3). With the exception of early December and August plantings (16 – 17 g), biomasses for the remainder of the year were, on average, under 10 g/plant. Seed production was significantly higher for plants sown in September 2015 (57 121 seeds/plant), followed by the October 2014 planting at 42 230 seeds/plant (Figure 4). While the November and early December plantings produced over 27 000 seeds/plant, seed production for the remainder of the year was less than 10 000 seeds/plant. Only two of the five February replicates had started producing mature heads before the biomass harvest and was removed from the analysis.

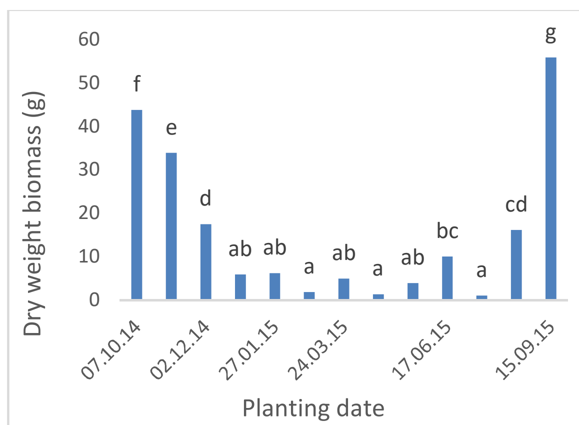


Figure 3: Average dry weight biomass (g) of feathertop Rhodes grass harvested at 24 weeks after planting (LSD = 7.00).

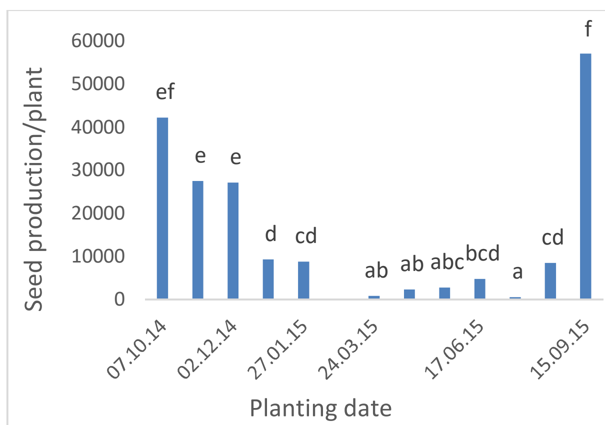
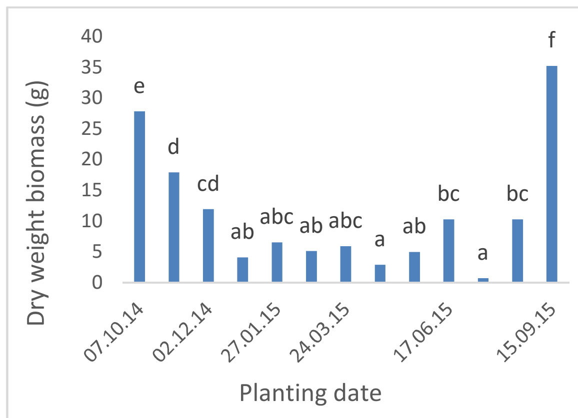


Figure 4: Back-transformed predictions of the average seed production/plant of feathertop Rhodes grass grown for 24 weeks from planting date (LSD on sqrt scale = 40.99).

- *Windmill grass*

The growth and development measurements significantly affected by the planting date ($p<0.001$) were dry weight biomass, plant height, tiller production, the length and number of inflorescences produced, and seed production.

Plants sown in October 2014 and September 2015 produced significantly heavier biomasses than plantings for the rest of the year at 27.84 g and 35.23 g respectively, followed by November (17.91 g) and early December (11.94 g). From late December through until August, biomasses were lower, ranging 10.26 g down to 0.68g (July planting) (Figure 5).



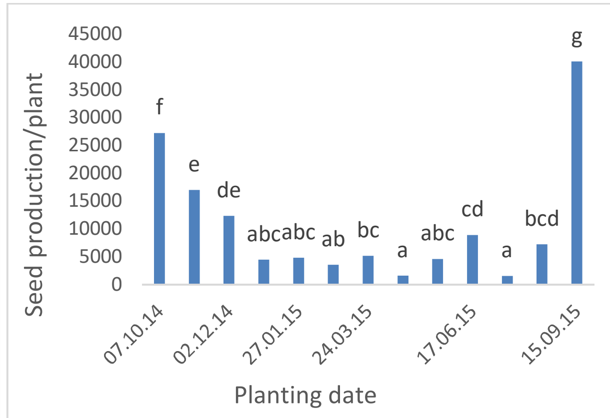


Figure 5: Average dry weight biomass (g) of windmill grass harvested at 24 weeks after planting (LSD = 6.23).

Figure 6: Back-transformed predictions of the average seed production/plant of windmill grass grown for 24 weeks from planting date (LSD on sqrt scale = 30.48).

Significantly more seed was produced from the September 2015 planting (40 076 seeds/plant) followed by the October 2014 planting (27 185 seeds/plant) (Figure 6). Between November and August, the plantings in November and early December still produced over 10 000 seeds/plant, with seed numbers decreasing to 1560 seeds/plant from the July planting.

In the glasshouse environment, the grass species were able to establish and produce seeds regardless of the time of emergence. When seedlings emerged during spring, larger plants with a higher seed production resulted. In particular, seed sown and emerging in September not only produced the largest biomasses for the three grass species but also a greater seed production across the species. There was a noticeable decrease in biomass and seed production in the summer and autumn emergences before starting to increase again with winter emergences.

- *Flaxleaf fleabane*

The growth and development measurements significantly affected by the planting date ($p<0.001$) were dry weight biomass, lateral and main stem heights, rosette diameter and seed production.

Plants sown in October 2014 produced, on average, heavier biomass (11.45 g) but were not significantly different from the biomasses of the November 2014 and January 2015 plantings (9.28 g and 9.03 g respectively) (Figure 7). The biomass produced from the July planting (0.001 – 0.013 g) was removed from the analysis as the residuals did not follow the distribution of other treatments and violated the assumptions of homogeneity required for analysis.

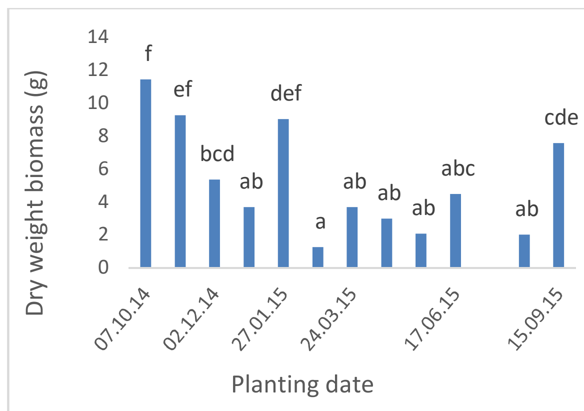


Figure 7: Average dry weight biomass (g) of flaxleaf fleabane harvested 24 weeks after planting (LSD = 3.79).

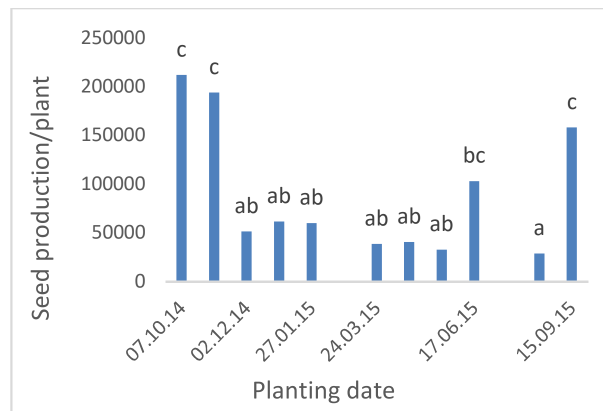


Figure 8: Back-transformed predictions of the average seed production/plant of flaxleaf fleabane grown for 24 weeks from planting date (LSD on sqrt scale = 143.4).

Average seed production was highest from plants sown in October 2014 (211 968 seeds/plant) but this was not significantly different from seed production of plants sown in November and June 2014, and September 2015 (range 102 913 – 193 864 seeds/plant) (Figure 8). In the February planting only 1 plant produced seed whereas in the July planting no seed was produced on any of the plants. Both plantings were removed from the analysis.

Flaxleaf fleabane was able to establish year round under glasshouse conditions. Seedlings emerging in spring and late summer produced heavier biomasses, with the spring emergences also producing the most seed. The seedlings that emerged in July did not progress beyond the rosette stage and did not have a growth spurt when the weather warmed up.

- *Sowthistle*

The growth and development measurements significantly affected by the planting date were dry weight biomass ($p<0.01$), height, rosette diameter and seed production ($p<0.001$).

Plants sown in June produced the heaviest biomass, averaging 28.30 g, but were not significantly different from the biomass produced by plants sown in the previous two months (Figure 9). For the remainder of the year, biomasses ranged from 13.17 g down to 3.83 g (July planting).

The June planting also had the highest seed production at 28 325 seeds/plant but this was not significantly different from the seed produced in the April and September plantings at 23 901 seeds/plant and 18 851 seeds/plant respectively (Figure 10). Significantly lower was the seed production for February at 2 725 seeds/plant followed by July at 3 226 seeds/per plant. For the remaining months seed production ranged from 6 416 to 14 400 seeds/plant.

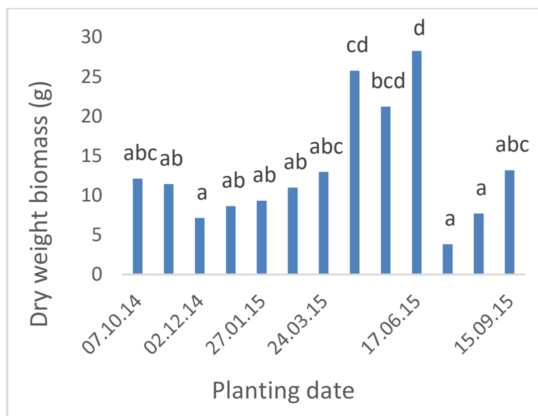


Figure 9: Back-transformed predictions of the average dry weight biomass (g) of sowthistle harvested 24 weeks after planting (LSD on sqrt scale = 1.67).

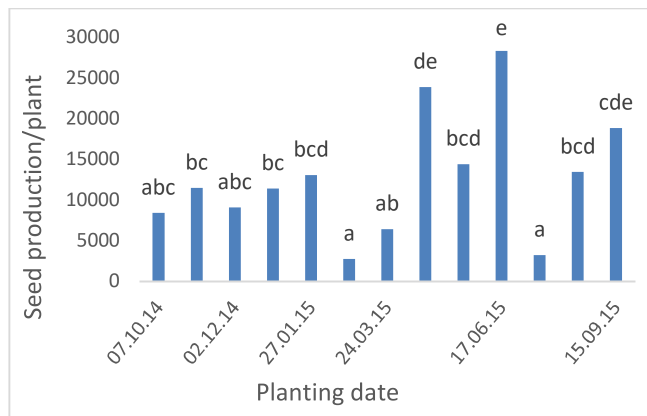


Figure 10: Back-transformed predictions of the average seed production/plant of sowthistle grown for 24 weeks from planting date (LSD on sqrt scale = 45.61).

Sowthistle was able to establish year round under glasshouse conditions. Seedlings emerging in autumn and early winter produced the greatest biomass and with the exception of May emergences, had the highest seed production.

Year two

In year two of the experiment, the daily maximum temperatures during spring and summer frequently exceeded the temperatures from the previous year (Appendix 1. Figure 1).

- *Awnless barnyard grass*

The growth and development measurements significantly affected by the planting date (p <0.001) were dry weight biomass, plant height, length and number of panicles produced, and seed production.

Plants sown in October 2015 and September 2016 produced, on average, significantly heavier biomass than other plantings throughout the year at 9.29 g and 11.43 g respectively (Figure 11). Biomasses for the remaining months ranged from 1.19 – 4.74 g. The late May planting was removed from the analysis as only one of the four plants to be sampled survived.

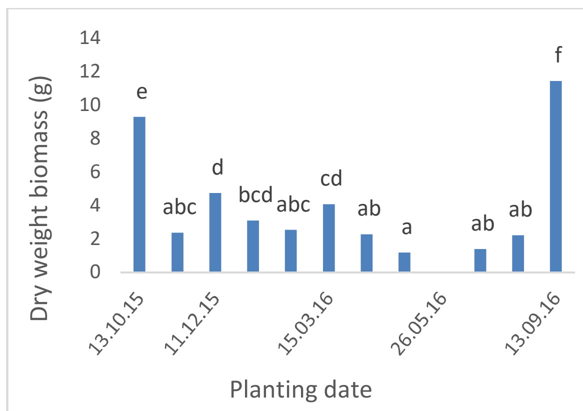


Figure 11: Average dry weight biomass (g) of awnless barnyard grass harvested at 24 weeks after planting (LSD = 1.78)

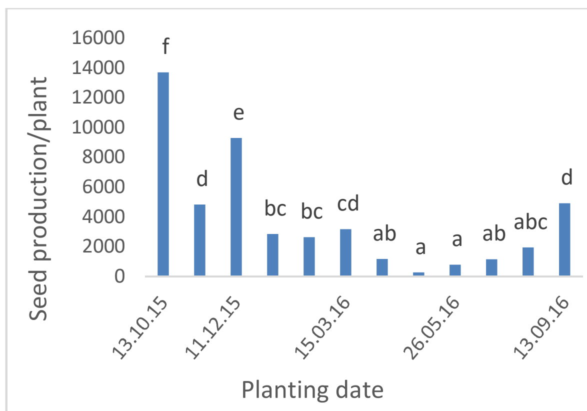


Figure 12: Average seed production/plant of awnless barnyard grass grown for 24 weeks from planting date (LSD = 1838.1).

The average seed production from the October and December 2015 plantings were significantly higher than for the remaining plantings at 13 710 and 9 304 seeds/plant respectively (Figure 12). For the remaining plantings, average seed production ranged from a low of 260 seeds/plant (early May) to 4 910 seeds/plant (September).

- *Feathertop Rhodes grass*

The growth and development measurements significantly affected by the planting date ($p<0.001$) were biomass, plant height, the length and number of inflorescences produced, and seed production.

Plants sown in September 2016 produced, on average, significantly heavier biomass compared to all other plantings (64.61 g), followed by October 2014 at 26.06 g (Figure 13).

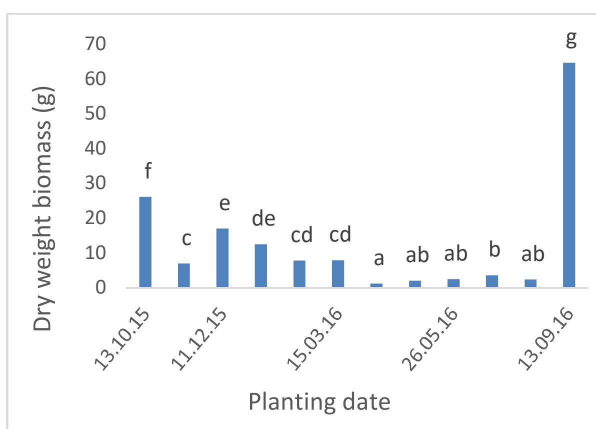


Figure 13: Back-transformed predictions of the average dry weight biomass (g) of feathertop Rhodes grass harvested at 24 weeks after planting (LSD on sqrt scale 0.75).

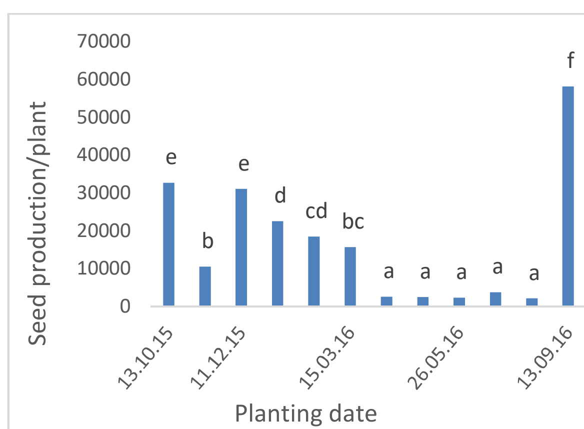


Figure 14: Average seed production/plant of feathertop Rhodes grass grown for 24 weeks from planting date (LSD = 6493.5).

Between November 2015 and March 2016 biomasses ranged between 6.99 g and 16.99 g compared to the significantly lower biomasses produced between April and late July (range 1.15 – 3.53 g).

Significantly more seed was produced by plants sown in September 2016 than all other months at an average of 58 150 seeds/plant (Figure 14). The lowest seed production occurred in the period between the April planting through until the late July planting (range 2 169 – 3 816 seeds). There was no significant difference between seed production during these months but they were significantly lower than for the rest of the year.

- *Windmill grass*

The growth and development measurements significantly affected by the planting date ($p<0.001$) were biomass, plant height, tiller production, the length and number of inflorescences produced, and seed production.

Plants sown in October 2015 and September 2016 produced, on average, significantly heavier biomass than all other plantings at 28.53 g and 23.92 g respectively (Figure 15). Biomasses from the remaining plantings ranged from 12.4 g (December) down to 3.22 g in early July.

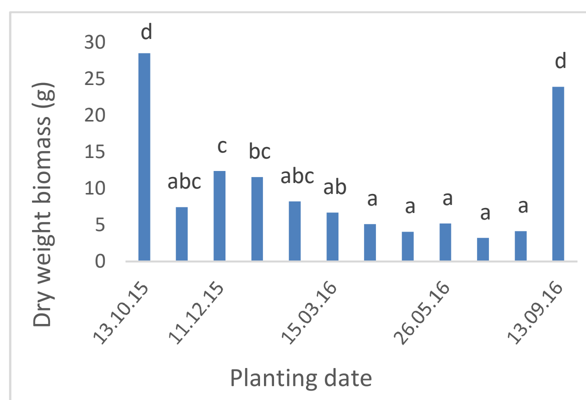


Figure 15: Average dry weight biomass (g) of windmill grass harvested at 24 weeks after planting (LSD = 4.99).

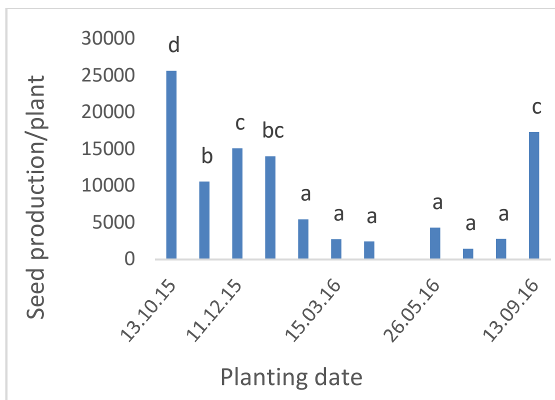


Figure 16: Average seed production/plant of windmill grass grown for 24 weeks from planting date (LSD = 4018.8).

Seed production was significantly higher from the October 2015 producing an average of 25 646 seeds/plant (Figure 16). There was no significant difference in seed production for plants sown between February 2016 and late July 2016 (range 1 426 – 5 433 seeds). The early May planting failed to produce any mature seed during the growing period. This was removed from the analysis.

In year two of the experiment, the grasses producing the largest biomasses and highest seed production emerged in spring, with a gradual decrease through the summer emergences.

- *Flaxleaf fleabane*

The growth and development measurements significantly affected by the planting date ($p<0.001$) were dry weight biomass, lateral and main stem heights, the rosette diameter and seed production.

Plants sown in September 2016 produced, on average, a significantly heavier biomass at 25.22 g than all remaining plantings (Figure 17). The lowest biomass produced was from plants in the April planting (2.35 g) while the remaining biomasses ranged from 4.44 g to 13.56 g.

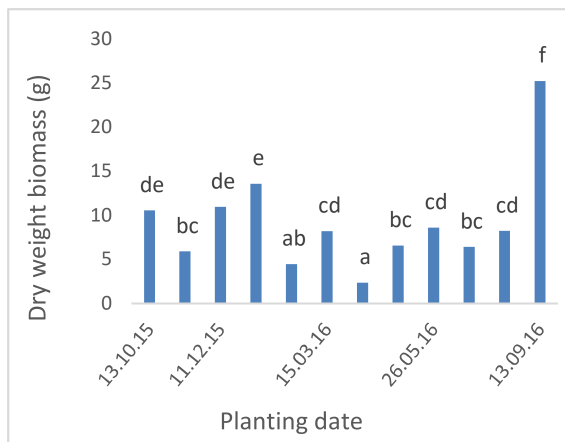


Figure 17: Back-transformed predictions of the average dry weight biomass (g) of flaxleaf fleabane harvested at 24 weeks after planting (LSD on sqrt scale = 0.67).

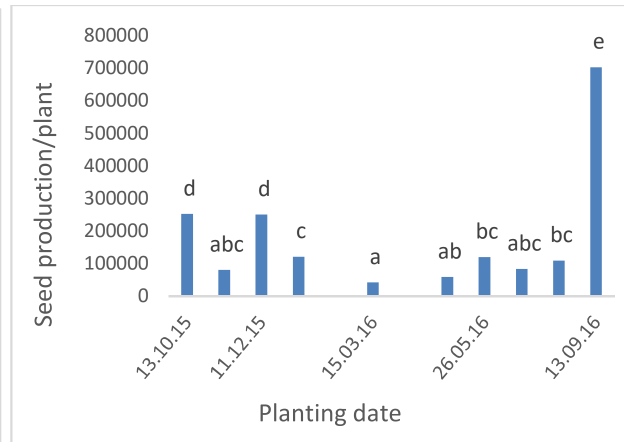


Figure 18: Back-transformed predictions of the average seed production/plant of flaxleaf fleabane grown for 24 weeks from the planting date (LSD on sqrt scale = 103.0).

Seed production was significantly higher in the September 2016 planting at 702 915 seeds/plant on average (Figure 18). Plants sown in February and April 2016 produced buds but failed to produce any mature heads and were removed from the analysis. The lowest number of seed produced was from plants sown in March at 42 684 seeds/plant.

Seedlings emerging in early spring produced heavier biomasses and had a higher seed production than the remainder of the year with seedlings emerging in February and April not progressing beyond producing buds during the 24-week growing period.

- *Sowthistle*

The growth and development measurements significantly affected by the planting date were dry weight biomass and rosette diameter ($p<0.001$), maximum height ($p=0.001$), but not seed production ($p=0.328$).

Plants sown in March produced the heaviest biomass at 20.37 g and were significantly different from all other plantings except April with a biomass of 16.73 g (Figure 19). Plant biomass increased from the lowest weight in October (3.53 g) up until the March planting and then gradually decreased.

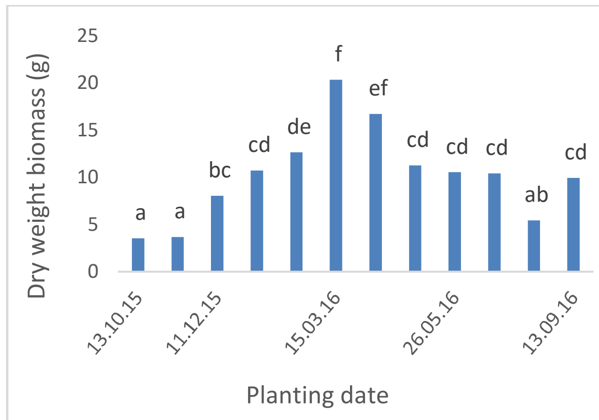


Figure 19: Average dry-weight biomass (g) of sowthistle taken at 24 weeks after planting (LSD = 4.37).

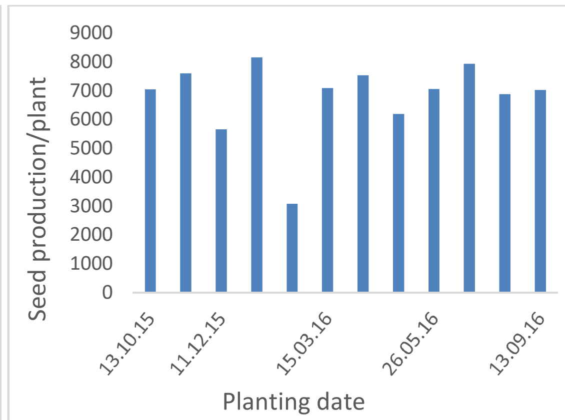


Figure 20: Average seed production/plant of sowthistle taken at 24 weeks after planting (no significant difference).

The time of planting was not significant in relation to seed production (p=0.328). Seed production averaged 6 777 seeds/plant and ranged from a high of 8 162 seeds produced in the January planting to a low of 3 082 from the following planting in February.

In year two of the experiment, seedlings emerging in early autumn produced plants with heavier biomasses, but unlike year one, there was no difference in seed production relating to time of emergence.

Seed age and the effect on seed dormancy

Dormancy did not break down in awnless barnyard grass until seed collected was approximately five months old. From that point germination in older seed collected gradually increased. The percent of total viable seed (germinated and dormant) remained relatively constant throughout the experiment. Holm *et al*. (1977) found that awnless barnyard grass had a dormancy period of approximately eight weeks. Windmill grass had a low number of seed germinate (1%) that was one month old through to four months old. Germination did not occur in larger numbers until seed was approximately nine months old. Other studies on windmill grass reported that it took 5 – 7 months after maturity for seed to reach maximum germination (Ngo *et al*. 2017; Borger *et al*. 2011; Maze *et al*. 1993). Feathertop Rhodes grass had virtually no germinations until the seed was approximately five months old. This took longer than studies by Osten (2012), where an after-ripening period of 6 to 10 weeks was required. Studies by Ngo *et al*. (2017) reported that germination did not occur until 2 months after maturity, and reached a maximum at 8 to 9 months. The addition of KNO_3 did little to improve germination of feathertop Rhodes grass. Gibberellic acid had a greater effect on improving the germination of windmill grass (Figure 21).

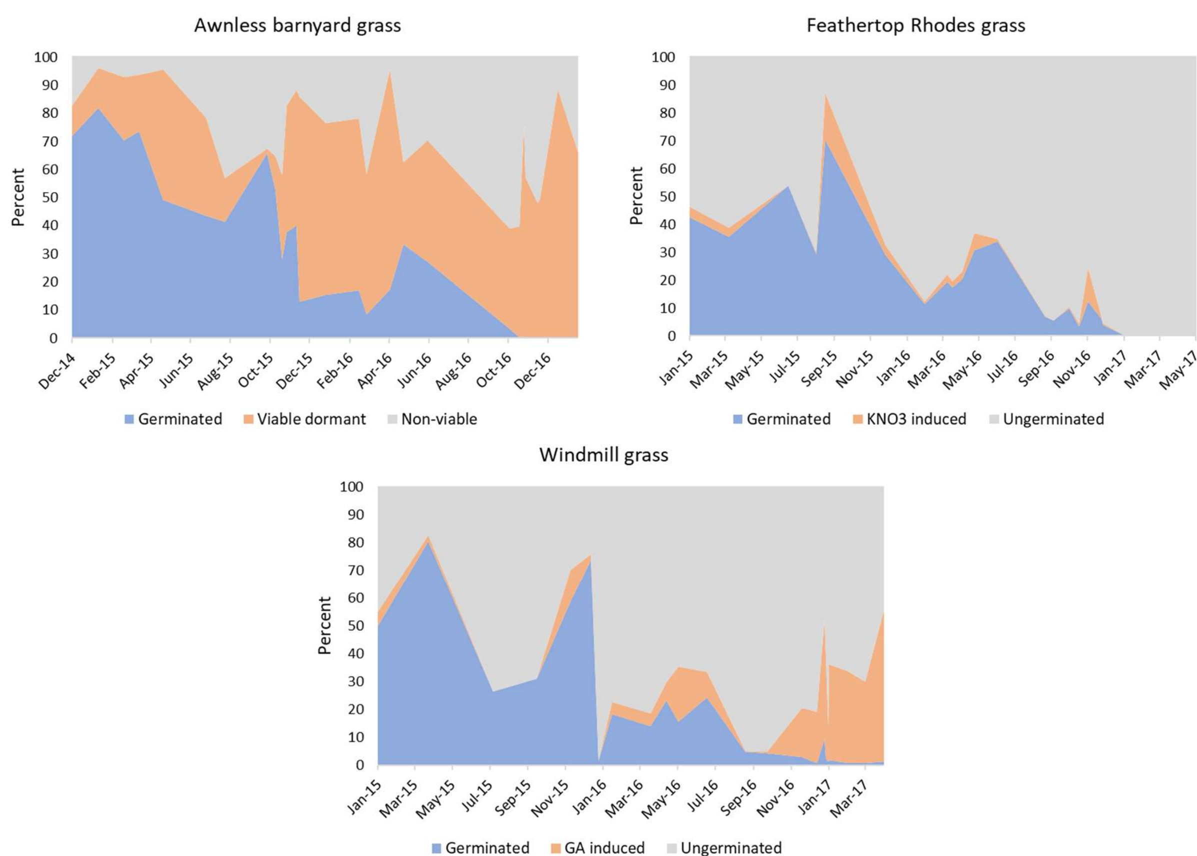


Figure 21: Germinability of seeds with respect to when mature seed was collected. Awnless barnyard grass were incubated in March 2017; feathertop Rhodes grass in June 2017, and windmill grass in April 2017.

Base temperature determination

Germination of each species occurred over a wide range of temperatures in the incubators (Table 1). An example of the curves used to determine time to 50% germination, and the 1/T50 estimates for each species are included in Appendix 1.

Table 1. Germination characteristics of each species in relation to temperature (°C), for base (T_b), range (T_r), and temperature at which maximum germination occurred (T_{max}); and the maximum germination (G_{max}). Detailed data is provided in Appendix 1.

Species	T_b	T_r	T_{max}	G_{max} (%)
Awnless barnyard grass	12.4	15-38	20	84
Feathertop Rhodes grass	6.5	10-38	20	29
Windmill grass	6.4	10-38	30	31
Fleabane	3.7	10-35	25	66
Sowthistle	0.8	5-38	15-30	100

Awnless barnyard grass had the highest predicted base temperature of all species tested at 12.4°C. Another study by Elahifard *et al*. (2014) predicted a lower base temperature of 9.7 -

9.9°C. However, in this study there were no germinations below 15°C. Awnless barnyard grass tends to commence emerging in late spring as is indicated by the temperature range in this experiment.

Both feathertop Rhodes and windmill grass that had base temperatures (T_b) of 6.5 and 6.4°C respectively can emerge at lower temperatures, which occur towards the start of spring. The estimated base temperature of windmill grass was lower than that estimated by Ngo *et al*. (2017) who reported T_b ranging from 9.2 - 11.2°C. The southern Australian populations of windmill grass that Ngo *et al*. (2017) tested had recorded no germinations at 10°C whereas this population had a 1% and a 5% germination in each run of the experiment. The predicted T_b for feathertop Rhodes grass was higher than Ngo *et al*. (2017) reported of 2 - 3°C, however was closer to the estimate of Zhang *et al*. (2015) who estimated a T_b value of 7.0°C.

The temperature range of germination for fleabane appears to have increased in comparison to studies by Wu *et al*. (2007), where no germinations occurred at 5 and 35°C. In this study, there was almost 30% germination at 35°C. This change supports anecdotal evidence that in the field, fleabane is emerging more in summer, as well as the main times of autumn and spring. The ability of fleabane to produce a large amount of seed has more than likely facilitated this adaptation to warmer conditions. The base temperature estimate for this study of 3.7°C was similar to Wu *et al*. (2007), who recorded 4.2°C.

Sowthistle germinations were consistently high over all the temperatures tested, with the lowest germination of 64% occurring at 38°C in the second run of the experiment. Sowthistle was previously considered a winter weed. However, as indicated by results from this experiment, it is now emerging in the field all year round. Widderick *et al*. (2010) also reported a wide range of temperatures at which sowthistle could germinate in populations collected in 1998 and 1999 with above 80% germination at both 5 and 35°C. The T_b estimate for sowthistle of 0.8°C in this experiment was lower than that predicted by Steinmaus *et al*. (2000) of 5.7°C. In this experiment, over 80% germination occurred at 5°C.

<u>Growing degree day calculations</u>

The time for each species to reach each developmental stage varied considerably throughout the two years of the experiment (Figure 22).

Awnless barnyard grass was the fastest of all the species to reach tillering and seed set. Its thermal time to reach tillering and seed set ranged from 80 – 387 GDD (11 – 35 days) and 398 – 767 GDD (42 – 74 days) respectively. In general, as the time to reach tillering increased or decreased, so did the time to reach seed set. Plants that emerged and grew with increasing day length took longer to reach each stage, than those that grew under decreasing day length. This is consistent with studies on barnyard grass (*Echinochloa crus-galli*) by Keeley and Thullen (1989) and Mitich *et al.* (1990) who reported that barnyard grass grown under long day length conditions took longer to reach maturity and were larger and produced more seed than plants grown under shorter day lengths. This also occurred in this study.

Awnless barnyard plants that emerged in late March and April, near the end of the preferred growing period, tended to take more days to reach each developmental stage. This is most likely due to temperatures declining below optimal. For example, plants that emerged on March 30th 2015 took 27 days to reach tillering and 74 days to reach seed set. Minimum temperatures in late April in that year were getting below 10C and maximums were often below 20C (Figure 4). As a result, plants were smaller and produced less seed.

The time for feathertop Rhodes grass to reach tillering and seed set ranged from 218 – 1180 GDD (16 – 56 days) and 1011 – 1995 GDD (76 – 129 days) respectively. This timeframe for development is comparable to that measured by Ngo *et al*. (2017), who found it took 260-330 GDD and 1145 – 1256 GDD for South Australian feathertop Rhodes populations to reach tillering and seed maturity respectively. There did not appear to be any relationship between day length and time to reach maturity. It is interesting to note that feathertop Rhodes generally had the lowest time to 50% germination values (T_{50}) of the species tested in the base temperature studies (Appendix 1); however was the slowest of the grasses to reach maturity. These results appear to conflict with anecdotal observations in the field, indicating that feathertop Rhodes reaches seed production much faster. It is thought that plants growing in low stress conditions with adequate moisture and nutrients in this study enabled them to grow and accumulate more biomass before setting seed.

Figure 22: Growing degree days and days for awnless barnyard grass, feathertop Rhodes grass, windmill grass, fleabane and sowthistle to reach tillering (grasses) or stem elongation (broadleaves) and seed set. Bars represent the standard error of the mean.

Thermal times for windmill grass were slightly shorter than for feathertop Rhodes grass. Time to reach tillering and seed maturity ranged from 200 – 660 GDD (15 – 36 days) and 1000 – 1710 GDD (61 – 150 days) respectively. These results were similar to those reported by Ngo *et al*. (2017) of 272 GDD and 747-786 GDD to reach tillering and the first mature seed. As with feathertop Rhodes grass, there appears to be little effect of day length on development.

Development of fleabane was slower than that of the grasses, taking 893 – 1739 GDD (44 – 124 days) to reach stem elongation, and 1729 – 2365 GDD (86 – 162 days) to reach seed

maturity. In the first year of the experiment, the cooling temperatures from March onwards resulted in an increase in the number of days for development, while there was not a noticeable increase in the thermal time to reach anthesis. Fleabane that was planted in February and April in the second year of the experiment did not reach seed set within six months of planting. This would have corresponded to 2950 and 2700 GDD respectively.

Sowthistle also varied considerably in its development throughout the experiment. Thermal times ranged from 576 – 1398 GDD (26 – 68 days) to reach stem elongation, and 1489 – 2249 GDD (65 – 132 days) to reach seed maturity. Development of sowthistle generally tended to be in between fleabane and the grasses. Analysis on GDD to reach stem elongation was mildly non-significant ($P = 0.067$) across the experiment, however GDD to reach set maturity was. The exact date that stem elongation occurred on plants that emerged in November and December was not recorded, so data is not shown in Figure 3.

Conclusion

These studies highlighted the variability in all species tested in terms of their growth and development. Awnless barnyard grass appeared to be the only species that responded to day length. In, terms of biomass and seed production of the summer grasses, plants that emerged at the start of summer grew larger and produced more seed than those emerging later. This is where the focus of control should be for the greatest impact. However, it is important to note that plants emerging later still produce seed and need to be controlled. Sowthistle now has the ability to emerge and grow well throughout the year. Fleabane also appears to be adapting to warmer temperatures, readily producing seed throughout spring, summer and autumn.

Appendix 1.

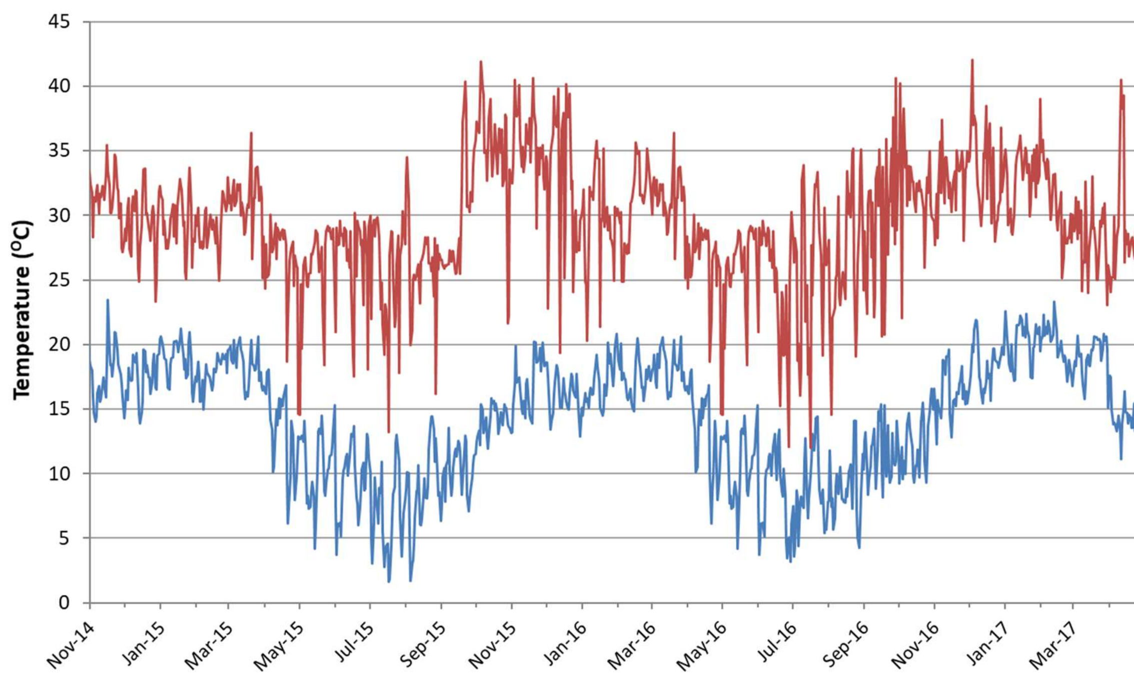


Figure 1. Maximum (red line) and minimum (blue line) temperatures recorded in the glasshouse throughout the duration of the experiment. Temperatures were recorded by a Tinytag datalogger every 15 minutes.

Table 1. Effect of temperature on germination rate (T_{50}, R^2 from Equation 1) and maximum germination (G_{max}) of awnless barnyard grass under 12 h alternating light/dark conditions.

	Experiment 1			Experiment 2		
Temp	T_{50}	R^2	G_{max}	T_{50}	R^2	G_{max}
C	(d)		(%)	(d)		(%)
5	-	-	-	-	-	-
10	-	-	-	-	-	-
15	-	-	0.0 ± 0.0 a	17.5 ± 1.6 d	0.97	9.1 ± 1.5 a
20	9.4 ± 0.9 c	0.97	40.1 ± 3.9 cd	6.6 ± 0.3 c	0.98	84.4 ± 1.5 c
25	5.3 ± 0.7 b	0.97	32.6 ± 4.3 bc	4.9 ± 0.4 bc	0.98	51.4 ± 5.3 b
30	3.8 ± 0.7 b	0.96	44.2 ± 2.6 d	2.7 ± 0.0 a	0.98	75.1 ± 2.8 c
35	3.3 ± 0.6 b	0.98	24.7 ± 3.1 b	2.8 ± 0.3 a	0.98	46.2 ± 8.9 b
38	3.7 ± 1.0 b	0.95	54.7 ± 1.3 e	3.0 ± 0.1 ab	0.99	79.8 ± 3.0 c

[a] Values (mean ± SE) within a column followed by different letters are significantly different (Fisher's protected LSD test: $P \leq 0.05$).

[b] The effect of each experiment was significant for T_{50} and G_{max}, so data is presented separately.

Table 2. Effect of temperature on germination rate (T_{50}, R^2 from Equation 1) and maximum germination (G_{max}) of feathertop Rhodes grass under 12 h alternating light/dark conditions.

Temp	T_{50}	R^2	G_{max}
C	(d)		(%)
5	-	-	-
10	11.5 ± 1.2 d	0.97	15.0 ± 2.2 a
15	6.3 ± 1.1 c	0.97	17.8 ± 3.2 a
20	3.5 ± 0.2 b	0.98	28.7 ± 3.8 c
25	2.1 ± 0.2 a	0.95	25.0 ± 1.7 bc
30	1.7 ± 0.1 a	0.98	26.6 ± 1.3 bc
35	1.5 ± 0.4 a	0.93	20.8 ± 2.2 ab
38	1.5 ± 0.2 a	0.96	17.8 ± 3.1 a

[a] Values (mean ± SE) within a column followed by different letters are significantly different (Fisher's protected LSD test: $P \leq 0.05$).

[b] The effect of each experiment was not significant for T_{50} and G_{max}, so data was pooled.

Table 3. Effect of temperature on germination rate (T_{50}, R^2 from Equation 1) and maximum germination (G_{max}) of windmill grass under 12 h alternating light/dark conditions.

	Experiment 1			Experiment 2		
Temp	T_{50}	R^2	G_{max}	T_{50}	R^2	G_{max}
C	(d)		(%)	(d)		(%)
5	-	-	-	-	-	-
10	-	-	1.3 ± 0.67 a	17.7 ± 0.9 e	0.96	5.6 ± 1.0 a
15	20.2 ± 0.1 e	0.97	4.9 ± 1.6 a	6.3 ± 0.2 d	0.98	16.2 ± 0.8 b
20	6.4 ± 0.2 d	0.98	23.5 ± 2.2 b	4.6 ± 0.4 c	0.98	27.0 ± 5.0 c
25	4.1 ± 0.2 c	0.98	23.2 ± 4.7 b	2.8 ± 0.2 ab	0.99	27.5 ± 4.7 c
30	3.2 ± 0.1 b	0.95	31 ± 7.5 b	2.3 ± 0.2 a	0.97	22.8 ± 1.4 bc
35	2.8 ± 0.1 a	0.95	25.2 ± 4.3 b	2.2 ± 0.3 a	0.98	26.4 ± 2.9 c
38	2.9 ± 0.2 ab	0.91	20.5 ± 1.5 b	3.9 ± 0.2 bc	0.97	27.7 ± 2.6 c

[a] Values (mean ± SE) within a column followed by different letters are significantly different (Fisher's protected LSD test: $P \leq 0.05$).

[b] The effect of each experiment was significant for T_{50} and G_{max}, so data is presented separately.

Table 4. Effect of temperature on germination rate (T_{50}, R^2 from Equation 1) and maximum germination (G_{max}) of fleabane under 12 h alternating light/dark conditions.

	Experiment 1			Experiment 2		
Temp	T_{50}	R^2	G_{max}	T_{50}	R^2	G_{max}
C	(d)		(%)	(d)		(%)
5	-	-	-	-	-	-
10	14.1 ± 0.4 e	0.98	35.7 ± 1.7 b	16.9 ± 0.3 d	0.99	32.3 ± 5.0 a
15	12.1 ± 0.0 d	0.97	20.1 ± 1.0 a	6.6 ± 0.1 c	0.97	52.9 ± 4.9 a
20	10.4 ± 0.2 c	0.98	48.5 ± 4.9 cd	6.9 ± 0.6 c	0.97	37.7 ± 2.6 a
25	7.6 ± 0.3 ab	0.99	65.5 ± 0.3 e	4.5 ± 0.1 ab	0.98	37.8 ± 9.3 a
30	7.0 ± 0.2 a	0.99	52.9 ± 0.7 d	3.1 ± 0.1 a	0.98	38.0 ± 8.9 a
35	8.2 ±0.2 b	0.95	42.3 ± 3.3 bc	4.9 ± 0.8 b	0.98	20.0 ± 3.0 a
38	-	-	-	-	-	-

[a] Values (mean ± SE) within a column followed by different letters are significantly different (Fisher's protected LSD test: $P \leq 0.05$).

[b] The effect of each experiment was significant for T_{50} and G_{max}, so data is presented separately.

Table 5. Effect of temperature on germination rate (T_{50}, R^2 from Equation 1) and maximum germination (G_{max}) of sowthistle under 12 h alternating light/dark conditions.

	Experiment 1			Experiment 2		
Temp	T_{50}	R^2	G_{max}	T_{50}	R^2	G_{max}
C	(d)		(%)	(d)		(%)
5	13.0 ± 0.6 f	0.99	80.2 ± 9.1 a	11.5 ± 0.4 e	0.99	94.0 ± 8.2 b
10	8.5 ± 0.3 e	0.99	91.5 ± 4.2 a	4.7 ± 0.1 c	0.99	96.7 ± 1.2 bc
15	5.7 ± 0.2 c	0.98	86.4 ± 0.0 a	3.4 ± 0.1 b	0.99	99.5 ± 0.1 bc
20	3.9 ± 0.2 b	0.99	95.6 ± 2.8 a	2.6 ± 0.2 ab	0.99	98.2 ± 2.0 bc
25	3.4 ± 0.2 ab	0.99	85.5 ± 2.1 a	1.7 ± 0.1 a	0.99	98.6 ± 1.2 bc
30	2.8 ± 0.1 a	0.99	96.6 ± 1.6 a	2.1 ± 0.1 a	0.99	100 ± 0.0 c
35	3.6 ± 0.5 ab	0.99	85.4 ± 5.9 a	1.9 ± 0.2 a	0.99	95.5 ± 2.4 b
38	7.2 ± 0.5 d	0.99	80.5 ± 2.9 a	8.3 ± 1.0 d	0.99	63.6 ± 3.0 a

[a] Values (mean ± SE) within a column followed by different letters are significantly different (Fisher's protected LSD test: $P \leq 0.05$).

[b] The effect of each experiment was significant for T_{50} and G_{max}, so data is presented separately.

[c] Gmax means for Experiment 2 are back transformed from arcsin(x)

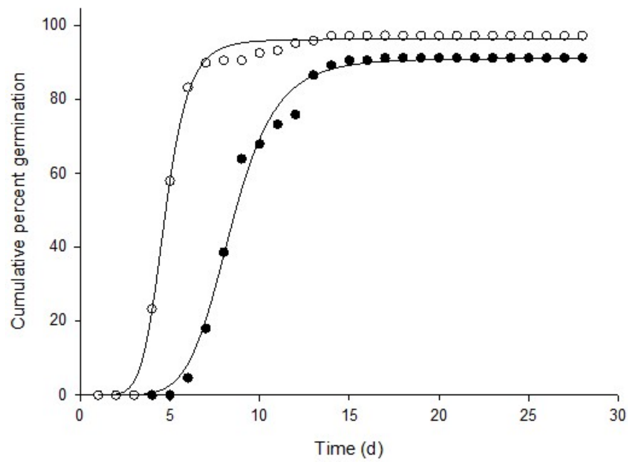


Figure 2: An example of the cumulative germination of Sowthistle at 10°C. The black circle represents run 1, the white circle represents run 2.

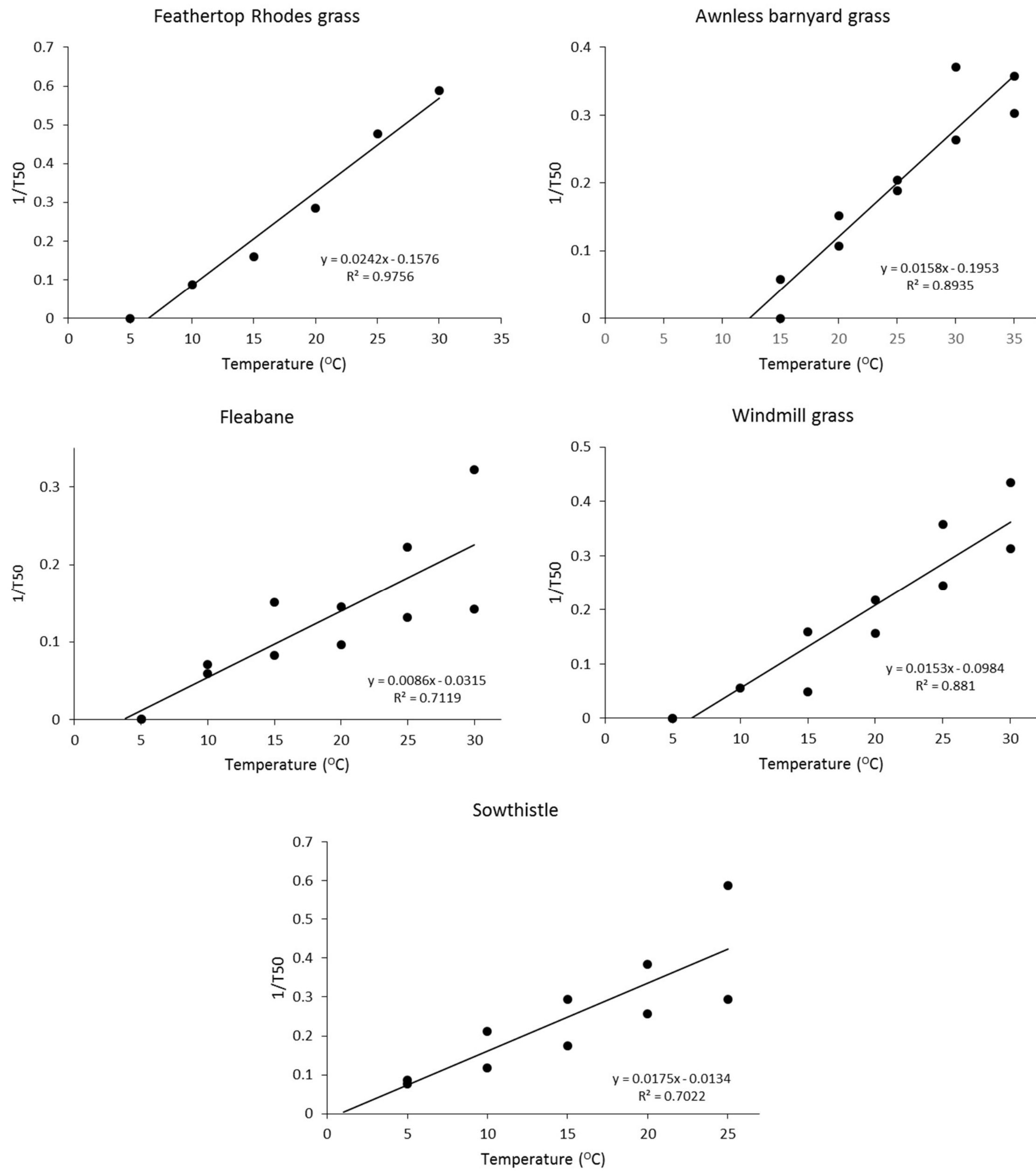


Figure 3: Base temperature estimates for awnless barnyard grass, feathertop Rhodes grass, windmill grass, fleabane and sowthistle. Each data point represents the average of each run, with the exception of feathertop Rhodes grass where data for each run were pooled.

1.6 - Investigate using molecular tools the spatial variation in resistance mechanisms and their role with gene-flow for glyphosate-resistant barnyard grass and fleabane

Key Findings:

Under this milestone we asked three questions, how connected are key weed populations across the cotton system? How many times has glyphosate resistance evolved? And how does resistance spread?

In Windmill grass, which is tetraploid, we found a genetically distinct population around Narrabri that has both a gene duplication and a target site mutation. The gene duplication resistance has evolved in one population from Walgett and has evolved twice in populations from SE Queensland, and one of these is already present in the southern NSW cotton-growing region. There are many populations in all regions, however, that do not have the gene duplication resistance mechanism. We showed that Windmill grass has very little evidence of outcrossing, and this helps to slow down multiple mode-of-action resistance. We still need to control the glyphosate resistant populations because every glyphosate resistant individual could possibly get a second mutation conferring resistance to another herbicide.

Fleabane is hexaploid, and our studies show that it has nine copies of the target site gene. This makes it hard for fleabane to get target site resistance, because many copies need to have the same mutation. We find that fleabane is genetically distinct between the regions of southern NSW, northern NSW and Queensland, although there is some evidence for long distance dispersal. Our data suggests that resistance may have evolved once and spread in Queensland, but we also find evidence for admixture and outcrossing in this species.

Sowthistle is an allotetraploid and our work shows that it has four copies of the *EPSPS* gene, this makes it hard for Sowthistle to get target site resistance and this explains why it took so long for Sowthistle to evolve resistance. Sowthistle has an NTSR mechanism, but it is unresolved. Our data show that Sowthistle populations are very mixed across geographic regions, which was predicted by their dispersal mechanism.

In Feathertop Rhodes Grass, we found more genetic diversity than expected, with evidence for separate colonisations in Queensland and NSW. This species is diploid, and this means that it is relatively easy for it to get resistance by target site mutations. Our data shows that this has happened over 10 times across the Australian cotton-growing region. One of these mutations is now widespread across Queensland. The sequence data and the response to glyphosate was well correlated, so there was no evidence of additional resistance mechanisms. We also show one property where the survivors in the field all have a resistance mutation, but the unsprayed population at the shed had no/low resistance mutations, illustrating how resistance evolves on farm and the importance of controlling survivors.

1.6.1 Population genetics of Windmill grass

Evolution of glyphosate resistance in Australian populations of Windmill Grass (*Chloris truncata*), target site amplification and target site mutation.

Abstract
Managing herbicide resistance requires knowledge of how many times resistance has evolved and how it spreads through the landscape. Windmill grass has evolved resistance to glyphosate in Australia and this was previously shown to be through increased *EPSPS* copy number. We combined population genetics and phylogenetic analysis with screening of resistance mechanisms to assess the species status of windmill grass and the evolution of glyphosate resistance in this species. Our population genetics and phylogenetic analyses indicate that although windmill grass is very closely related to *Chloris divaricata,* they are two separate species in population genetic terms. We also picked up several different species in our sampling, highlighting the importance of correct identification in weed and herbicide resistance research, especially in grass weeds. The qPCR results indicated four populations with high copies of *EPSPS* and four with a mid-level copy number. Mapping these results to the microsatellite data indicated that copy number resistance has evolved more than once. We found a *Pro-106-Leu* mutation in one population that also had increased copy number. The results demonstrate that windmill grass evolved herbicide resistance multiple times in different ways.

Introduction

Managing glyphosate resistance requires knowledge of the number of times that resistance has evolved, the way that it has evolved, and the way that it spreads across agricultural and natural environments. Although several glyphosate resistance mechanisms have been characterized in weeds, only one has been identified in windmill grass, the amplification of *EPSPS* (Ngo *et al.*, 2017). It is possible that different resistance mechanisms are present in this species. We still do not know how the increased copy number variation is inherited. It is also presently unclear whether the multiple EPSPS copies evolved once, or whether the same mechanism has evolved multiple times in windmill grass.

Windmill grass (*Chloris truncata*) is a short-lived (2-3 years) perennial grass and grows best when exposed to full sun (Brunharo *et al.*, 2016). Several modes of reproduction can be found in windmill grass populations, including sexual reproduction, but stolon reproduction predominates, and the species is widely believed to be self-pollinating (Whalley *et al.*, 2013). In suitable environments, windmill grass can grow up to 50 cm tall, with erect and tufted stems and narrow leaves (Chauhan *et al.*, 2016, Gardner, 2014, Storrie, 2014). The seed heads have 6-9 flower spikes with many tiny spikelets (Storrie, 2014), and are dispersed by wind. Windmill grass seed cannot remain viable for more than 18 months, and few seeds persist beyond 12-months regardless of burial depth (Chauhan *et al.*, 2016).

Chloris truncata is native to inland regions of Australia and generally grows in the arid tropical and temperate zones, but is not found in the Northern Territory (Werth *et al.*, 2013). The species is most prevalent in southern Australia, especially in the south-eastern region. A few records come from northern and inland Australia, but these may be identification errors as several species of *Chloris* (and even species in related genera) look similar to one another.

As a summer weed, windmill grass has become a threat to zero-till farming because it can reduce the production of winter crops by competing with them for nutrients and soil moisture (Borger *et al.*, 2010, Storrie, 2014, 2017). The eradication of windmill grass in autumn can take a long time, and this may delay sowing and cause financial loss (Borger *et al.*, 2010). In

addition, windmill grass is a common host for wheat streak mosaic virus (WSMV) and barley yellow dwarf virus (BYDV), so it provides indirect problems as well (Michael *et al.*, 2012, Hawkes and Jones, 2005).

Glyphosate is by far the most widely used herbicide worldwide and so is a key management tool against many weed species. Generally, glyphosate is considered to be a relatively environmentally friendly herbicide, based on its physio-chemical properties and biological mode of action. For example, glyphosate does not cause air pollution because it is non-volatile (Duke and Powles, 2008b). Glyphosate also has low toxicity to ground-water and is reportedly broken down rapidly in the soil (Baylis, 2000, Okada *et al.*, 2013, Powles and Preston, 2006). Glyphosate is non-selective (Duke and Powles, 2008a), and this allows simple, cheap, flexible and effective control of weeds under various situations (Baylis, 2000, Okada *et al.*, 2013, Pline-Srnic, 2006, Powles and Preston, 2006). Originally, the belief was widespread that resistance to glyphosate was unlikely to evolve in target organisms because of its particular mechanism of action (Baylis, 2000, Okada *et al.*, 2013, Pline-Srnic, 2006, Powles and Preston, 2006). However, glyphosate resistance has now evolved in many weed species (Duke and Powles, 2008a). The first weed to become resistant, *Lolium rigidum* (Poaceae), was reported in 1996 even though that particular population grew in a field without glyphosate resistant crops, so its exposure to the herbicide there must have been limited (Beckie, 2011, Pratley *et al.*, 1999, Preston *et al.*, 1996).

Glyphosate is a specific and potent inhibitor of the 5-enolypyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme, which is the penultimate enzyme in the shikimate pathway (Maeda and Dudareva, 2012). The shikimate pathway is crucial to the production of the aromatic amino acids (e.g. Tyr and Phe), which are all essential for protein biosynthesis in all plants (Maeda and Dudareva, 2012). Without these aromatic amino acids, plant dies. The reversible formation of 5-enolpyruvyl-shikimate-3-phosphate (EPSP) was catalyzed by the EPSPS enzyme catalyzes through the reaction of the first substrate shikimate-3-phosate (S3P) with the second substrate phosphoenolpyruvate (PEP) (Powles and Yu, 2010). The EPSPS enzyme is encoded in the nucleus but translocates into the chloroplast (Shaner, 2009). In terms of intrinsic glyphosate sensitivity of the enzyme, the EPSPS enzymes can be divided into two classes: Class I and Class II (Franz *et al.*, 1997). Class I enzymes exist in all plants and bacteria, whereas Class II enzymes are found only in bacteria (Franz *et al.*, 1997).

Target site mutations

The active site of the EPSPS gene is highly conserved (Powles and Yu, 2010). Typically, if the affinity to glyphosate in EPSPS mutants decreased, the affinity for the PEP will decrease as well (Funke *et al.*, 2009). This means that very few mutations can occur that provide resistance without causing the enzyme to become ineffective, which explains why resistance was considered unlikely. Pro106 mutation in the EPSPS gene causes glyphosate resistance in several species (Brunharo *et al.*, 2016). Mutations may cause proline at position 106 to be substituted with aminohydrophobic (e.g. Ala and Leu) or hydrophilic (e.g. Ser and Thr) amino acids through base substitutions at the first or second position of the amino acid and this usually causes 2-4 fold resistance (Sammons and Gaines, 2014).

Experiments have been conducted on *Escherichia coli* to determine the structural basis of glyphosate resistance in EPSPS (Funke *et al.*, 2009). More than one mechanism has been uncovered. The Thr102Ile/Pro106Ser double mutant EPSPS (TIPS EPSPS) confers high levels of glyphosate resistance (Funke *et al.*, 2009, Arnaud *et al.*, 1998). A single-site Thr102Ile mutation may also confer moderate glyphosate resistance on its own (Funke *et al.*, 2009). In the absence of the Pro106 mutation, the affinity for the substrate PEP is drastically decreased by the Thr102Ile mutation (Yu *et al.*, 2015, Funke *et al.*, 2009). This is because of

changes to the crystal structure change at the active site (Funke *et al.*, 2009). However, once the Pro106 mutations are present, the Thr102 mutation can evolve (Funke *et al.*, 2009). This TIPS mutation has also been detected in wild weed populations, i.e. *Eleusine indica* (Poaceae), where it results in very high levels of resistance (more than 180-fold) (Yu *et al.*, 2015).

Gene duplication is defined as heritable DNA segment replication (Innan and Kondrashov, 2010). Gene amplification is often used interchangeably with gene duplication when microorganisms are considered (Sammons and Gaines, 2014, Innan and Kondrashov, 2010), but is also sometimes used to refer to overexpression (De Prado *et al.*, 2012, Brooks and Roberts, 1999). The process of gene duplication and the subsequent divergence of the products in their functions has evolutionary significance in plants because novel gene functions may be acquired (Innan and Kondrashov, 2010). Gene duplication can be achieved through genome duplication, unequal crossing over events, or the insertion of reverse transcribed messenger RNA (Zhang, 2003). In several species, such as *Amaranthus palmeri* (Amaranthaceae), the glyphosate resistance level is correlated with EPSPS copy number, so the higher the EPSPS copy number, the higher the level of resistance (Sammons and Gaines, 2014). It has recently been shown that amplified EPSPS gene copies in glyphosate resistant *A. palmeri* are in the form of extrachromosomal circular DNA molecules (eccDNAs) and those are transmitted to their offspring with sexual reproduction (Koo *et al.*, 2018).

Herbicide resistance is an evolutionary phenomenon and may be influenced by several variables, including gene mutation, gene flow, fitness effects, mating patterns, herbicide selection pressure, and the initial frequency of resistance alleles, although gene mutation is considered the dominant factor (Jasieniuk *et al.*, 1996, Jasieniuk and Maxwell, 1994). Some herbicide-resistant weeds are highly self-fertilizing. Population genetic models suggest that in partially self-fertilizing populations, the probability of recessive mutations spreading and the rate of resistance evolution should be high, relative to that in outcrossing populations (Charlesworth, 1992, Jasieniuk *et al.*, 1996).

Recognizing species accurately is crucial in pest and weed management (Bickford *et al.*,2007, Hereward *et al.*, 2017, Paterson, 1991b). Although traditional taxonomic methods delimit species accurately in most cases, the common occurrence of morphologically indistinguishable cryptic species poses challenges for ecology and pest and weed management, and requires targeted research (Paterson, 1991a, Bickford *et al.*, 2007, Walter, 2003). Windmill grass is an Australian native grass, but there are many species morphologically close to it. There is also some morphological variation within what is defined as *C. truncata*. The species status of *C. truncata* must therefore be verified using population genetics approaches. Otherwise, we cannot be sure that it is windmill grass alone that is resistant to glyphosate, or if other morphologically indistinguishable or similar species are also resistant to glyphosate.

Materials and Methods

Sampling and DNA extraction

Twenty-nine populations of putative windmill grass were sampled in 2017 and 2018 across eastern Australia. For each population (Table 1.6.1.1), leaf tissue and seeds were collected from at least 30 plants randomly across each sampling area. The collected leaf tissue was immediately dried in sealed envelopes filled with silica gel. All collected samples were stored at room temperature until DNA was extracted. DNA was extracted by CTAB with a chloroform extraction and then 'DIY spin column purification' (Ridley *et al.*,

2016). DNA was also extracted from herbarium specimens of various *Chloris* samples (Table 1.6.1.2)

Table 1.6.1.1. The geographic details of sampled populations, presented in the sequence in which they were collected. The site from which each one was collected is also labelled according to whether it was from a farm (where glyphosate is likely to have been applied) or roadside (where potentially no glyphosate exposure).

Population Name	Population Code	Location	Date	Latitude	Longitude	Farm/ Roadside
Griffith1	WMG1	Griffith	Mar/16	-34.4405	146.0370	Roadside
Narrabri1	WMG2	Narrabri	Mar/16	-30.2113	149.6000	Farm
BaanBaa	WMG3	BaanBaa	Mar/16	-30.7325	150.0891	Farm
Dubbo	WMG4	Dubbo	Mar/16	-32.1868	148.4493	Roadside
Pampas	WMG5	Pampas	Mar/17	-27.7810	151.3084	Roadside
Jandowae1	WMG6	Jandowae	Mar/17	-26.7815	150.9459	Farm
Emerald	WMG8	Emerald	Mar/17	-23.4681	148.0834	Roadside
Dalby1	WMG7	Dalby	Mar/17	-27.2508	151.3473	Roadside
Dalby2	WMG9	Dalby	Mar/17	-27.1438	151.2588	Roadside
Jandowae2	WMG10	Jandowae2	Mar/17	-26.8832	151.0841	Roadside
Jondaryan1	WMG11	Jondaryan	Mar/17	-27.4003	151.6097	Roadside
Jondaryan2	WMG12	West prairie	Mar/17	-27.4282	151.3142	Roadside
Jondaryan3	WMG13	Jondaryan	Mar/17	-27.3993	151.5206	Roadside
Griffith2	WMG14	Griffith2	Feb/18	-34.4620	146.1958	Farm
Goolgowi	WMG15	Goolgowi	Feb/18	-34.0088	145.7351	Farm
DarlingtonPoint	WMG16	DarlingtonPoint	Feb/18	-34.6036	145.9358	Farm
Newell1	WMG17	Newell	Feb/18	-33.5559	147.6645	Farm
Warrawidgee	WMG18	Warrawidgee	Feb/18	-34.3943	146.7583	Farm
Kiacatoo	WMG19	Kiacatoo	Feb/18	-33.0587	146.7583	Farm
Griffith3	WMG20	Griffith	Feb/18	-34.3420	146.2081	Farm
Forbes	WMG21	Forbes	Feb/18	-33.4103	147.7993	Farm
RankinsSprings	WMG22	RankinsSprings	Feb/18	-33.8645	146.1976	Roadside
Griffith4	WMG23	Griffith4	Feb/18	-34.2585	145.9964	Roadside
Walgett	WMG24	Walgett	Feb/18	-30.0009	148.2086	Farm
WeeWaa	WMG25	WeeWaa	Feb/18	-30.1974	149.4884	Farm
Narrabri2	WMG26	Narrabri2	Feb/18	-30.2086	149.6001	Farm
Moree	WMG27	Moree	Feb/18	-29.2940	149.7772	Farm
Susceptible1	WS	Trangie		-31.9669	148.1534	Farm
Resistant1	WR	Trangie		-32.1459	147.5681	Farm

Table 1.6.1.2. Species details of the Queensland Herbarium *Chloris* samples.

Sample code	Species
CL 30	*C. divaricata var. cynodontoides*
AQ854141	*Indeterminate*
AQ780910	*C. pectinata*
AQ846170	*C. pectinata*
AQ814342	*C. divaricata var. cynodontoides*
AQ834034	*C. pectinata*
AQ280470	*C. divaricata var. cynodontoides*
AQ445929	*C. truncata*
AQ682362	*C. truncata*
AQ776015	*C. truncata*
AQ785308	*C. truncata*
AQ871736	*C. truncata*
AQ498201	*C. lobata*
AQ735764	*C. lobata*
AQ797075	*C. lobata*
AQ906240	*C. lobata*
AQ619758	*C. divaricata var. divaricata*

Gene sequencing, phylogenetics and EPSPS mutation detection

Following extraction, DNA was PCR amplified. Primers that were used for ITS, TrnL and EPSPS amplification are listed in Table 1.6.1.3. PCR conditions for ITS were: 3m initial denaturation (95℃) followed by 40 cycles of 95 ℃ for 20s, annealing at 52 ℃, and 72 ℃ for 30s and final extension at 72 ℃ for 7m. PCR conditions for TrnL and EPSPS were: 3m initial denaturation (95℃) followed by 40 cycles of 95 ℃ for 20s, annealing at 57 ℃, and 72 ℃ for 30s and final extension at 72 ℃ for 7m. PCR products were purified with one unit of Exonuclease 1 and one unit of Antarctic Phosphatase before sequencing. The EPSPS gene was included to test for any known resistance causing mutations. Sequences were corrected in the program CodonCode Aligner (CodonCode Corp, Centreville, MA USA). The optimal evolutionary model was determined with Jmodeltest (GTR+G for ITS and GTR+I for Trn L) (Darriba *et al.* 2012; Guidon and Gascuel 2003). Bayesian trees were constructed using MrBayes (Ronquist *et al.* 2012). Based on the ITS and TrnL phylogenetic trees, samples were divided into several groups, with these species respectively, *C. truncata*, *C. divaricata*, *C. virgata*, *Cynodon ambiguous* and *Enteropogon ramous*. Pie charts were constructed to present the relative frequencies of these species at each sampled site. To visualize haplotype relationships among individuals within *C. truncata* and *C. divaricata*, their sequence data (nuclear and chloroplast) were imported to PopArt to generate TCS networks as described by Clement *et al.* (2002). Nucleotide ambiguity was observed in the EPSPS sequence data and this made it hard to determine the haplotype relationship between *C. truncata* and *C. divaricata* because TCS ignores sites with ambiguities.

Table 1.6.1.3. Primer sequence for the amplification of ITS, Trn L and EPSPS.

Primer	Sequence
P1L	CTGTAGGTGAACCTGCGGAAGGATC
P2R	CTTTTCCTCCGCTTATTGATA
CtEPSPS Fwd	TGCGACCGTTGACAGCAG
CtEPSPS Rev	CACCACAGTTTGGCTACTAAGCAT
CtALS	GACTCCATCCCCATGGTC
CtALS	CGAGGTAGTTGTGCTTGGTG
CT_EPSPS_2820 Rev	TCCCTTGACACGAACTGGTG
CT_EPSPS_2430 Fwd	GACTCTCTGTGGAAGCGGAC
trnL-c_B49317	CGAAATCGGTAGACGCTACG
trnL-d-A49855	ATTTGAACTGGTGACACGAG

Microsatellite development and analysis

Twelve microsatellite markers were genotyped. These markers were identified from Illumina data (next generation sequencing, 'NGS') using QDD (Meglécz *et al.*, 2009) that designs primers with primer3 (Untergasser *et al.*, 2012). Their sequences are listed in Table 1.6.1.4. These primers were all synthesized by Integrated DNA Technologies (IDT) with an M13 tail added to the 5' end of the forward primer and a pigtail (GTTTCTT) added to the 5' end of the reverse primer to try to minimize stutter (Brownstein *et al.*, 1996). Fluorescent dyes (FAM, VIC, NED and PET) were added to the microsatellite PCR reactions following the M13 tailing method (Schuelke, 2000). Loci were amplified in 12 µl reactions, containing 1× reaction buffer, 0.1 µM of forward primer, 0.2 µM of reverse primer, 0.2 µM of microsatellite labelled primer and 0.5 Units of Taq polymerase. PCR conditions were as follows: 2-min initial denaturation (95°C) followed by 35 cycles of 95 °C for 15 s, annealing at 57 °C for 25 s and 72 °C for 30 s and 10 cycles of 95 °C for 15 s, annealing at 54 °C for 25 s and 72 °C for 30 s, and final extension at 72 °C for 7 mins. DNA labelled with FAM, VIC and NED were then pooled into the PET plate and cleaned with one unit of exonuclease I and one unit of Antarctic Phosphatase. Products (5 µl × 6 random replicates) were checked on a MultiNA (Shimadzu, Kyoto) microchip-based electrophoresis system. Purified PCR products were then sent to Macrogen (Soeul, South Korea) for microsatellite analysis.

Peaks were corrected, confirmed and binned using Geneious version 10 ((https://www.geneious.com)), and the allelic data were exported to Excel for genetic analysis and principle coordinate analysis (PCoA) using Genalex 6.5 (PE, 2012). PCoA is a multivariate statistical method that uses genetic distance to allow visualization of a distance matrix (Paliy and Shankar, 2016). To detect and remove any duplicated multi-locus genotypes in any population, clone correction was performed in popr in R (R Core Team 2015). Genetic relationships among individuals were visualised by constructing a minimum spanning network (MSN) in the popr package and population structure was explored by performing a Discriminant Analysis of Principal Components in adegenet (Jombart *et al.*, 2010). STRUCTURE was used to investigate population structure and assign individuals to potential populations (Porras-Hurtado *et al.*, 2013, Pritchard *et al.*, 2000). Expected and observed heterozygosity (HE and HO) were calculated in Genalex 6.5 to estimate genetic diversity within loci. FreeNA (Chapuis *et al.*, 2008) was used to detect null allele frequencies using the algorithm presented by Dempster *et al.* (1977). Because windmill grass is highly selfing, it was not possible to detect null alleles, because all populations were out of Hardy-Weinberg Equilibrium.

Gene expression analysis

To analyse EPSPS gene copy number variations, quantitative real-time PCR (qPCR) was used as per the methods of Ngo *et al.* (2017). Genomic DNA was extracted and used for glyphosate DNA copy number analysis. The fold change of EPSPS was measured relative to the housekeeping gene acetolactate synthase (ALS), which is considered to have consistent expression levels in windmill grass. Genomic DNA was standardised to 20 ng/µl. Serial dilutions were made (80, 40, 20, 10ng) to test the efficacy of the EPSPS and ALS primers. To assay the expression level of the EPSPS and ALS genes independently of one another in a qPCR reaction, each gene probe had a different fluorophore. Genomic DNA was amplified in 10 µl reactions, containing 1× Bioline Sensifast LO-ROX master mix, 0.2 µl of each of 10 µM EPSPS Forward/EPSPS Reverse and ALS F/ALS R primers, 0.1 µl of each 10 µM probe (EPSPS and ALS), 4 µl of 20ng/ µl genomic DNA. Amplification conditions were set as specified by Ngo *et al.* (2017). The EPSPS gene copy number was evaluated by calculating the ratio of EPSPS relative to ALS.

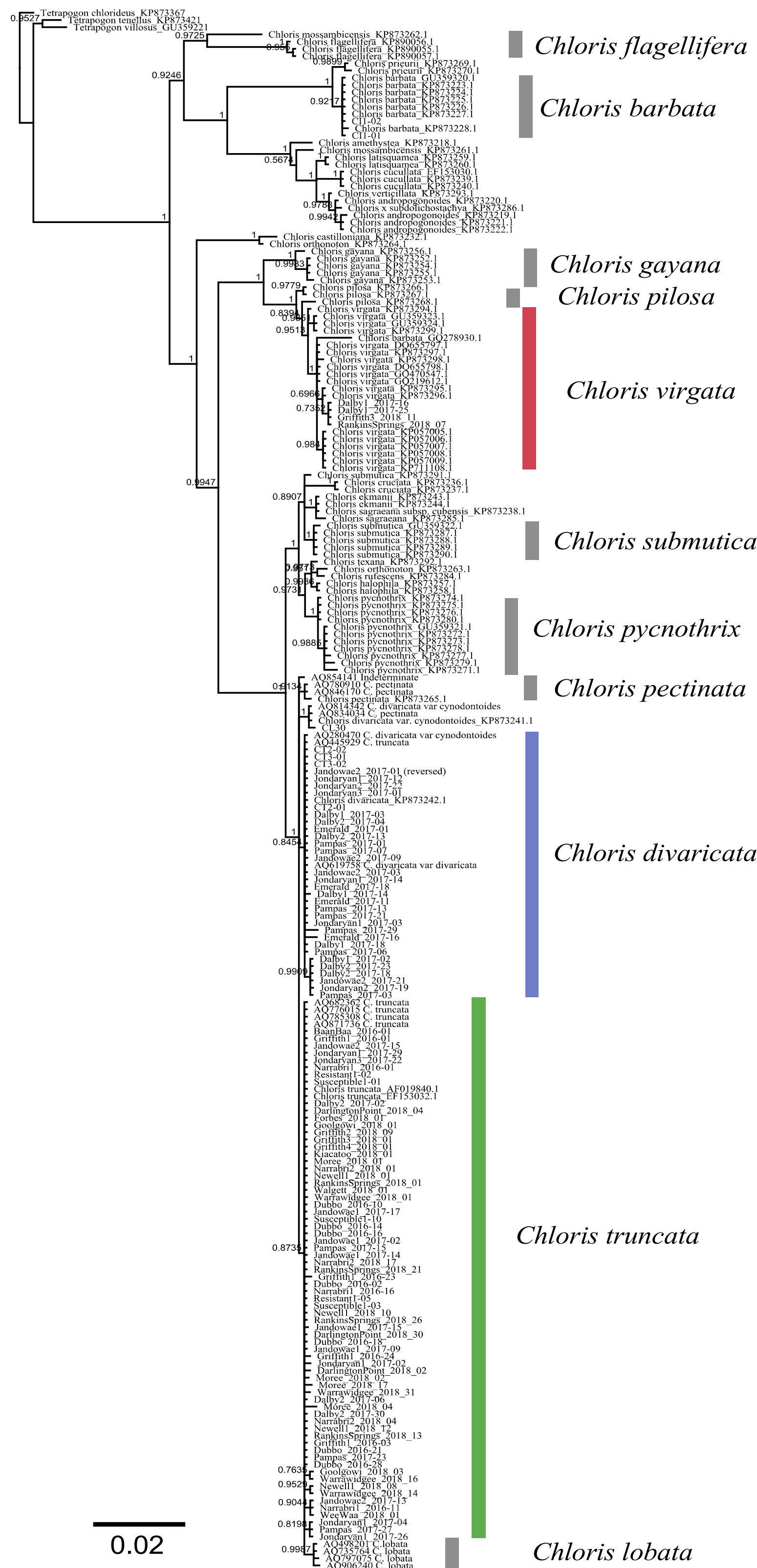
Results

Phylogenetic analysis and species status

The Bayesian phylogenetic tree placed *C. truncata* and *C. divaricata* relative to *C. virgata* (Fig. 1.6.1.1). The herbarium samples allowed the identification of the more closely related species to be established. They are consistent with those based on morphological identification (Table 1.6.1.2). Fig. 1.6.1.1 shows that *C. divaricata* and *C. truncata* are very closely related with only 2bp difference in the ITS sequence, as seen in the haplotype network (Fig. 1.6.1.3).

A phylogenetic tree based on the chloroplast DNA (Trn L) sequences was also constructed. Preliminary analysis with both genes (ITS and Trn L) separated *Enteropogon ramous* (Curly Windmill Grass) from the Chloris species, but these trees are not shown. However, the resolution of the chloroplast DNA tree was lower than ITS, and did not differentiate between *C. truncata* and *C. divaricata*. The species assignment of each population is listed in Figure 1.6.1.2. Most of the identified *C. truncata* samples were collected from New South Wales, while most of the *C. divaricata*, *E. ramosus*, *C. virgata* and *Cynodon ambiguous* samples were collected from Queensland, except for the Jandowae 1 sample being entirely *C. truncata*. Almost all the weed samples that were collected from cotton farms were *C. truncata*, but roadside populations were more mixed (Fig. 1.6.1.2).

Figure 1.6.1.1. Bayesian phylogenetic tree of a number of *Chloris* species. Samples within sites (Table 1) with identical sequences were removed. Numbers shown at nodes indicate the posterior probabilities. Species names are followed by Genbank accession numbers where appropriate. The *Chloris* species are marked in different colours. Unnamed and uncoloured branches reflect ambiguity about the species identity.



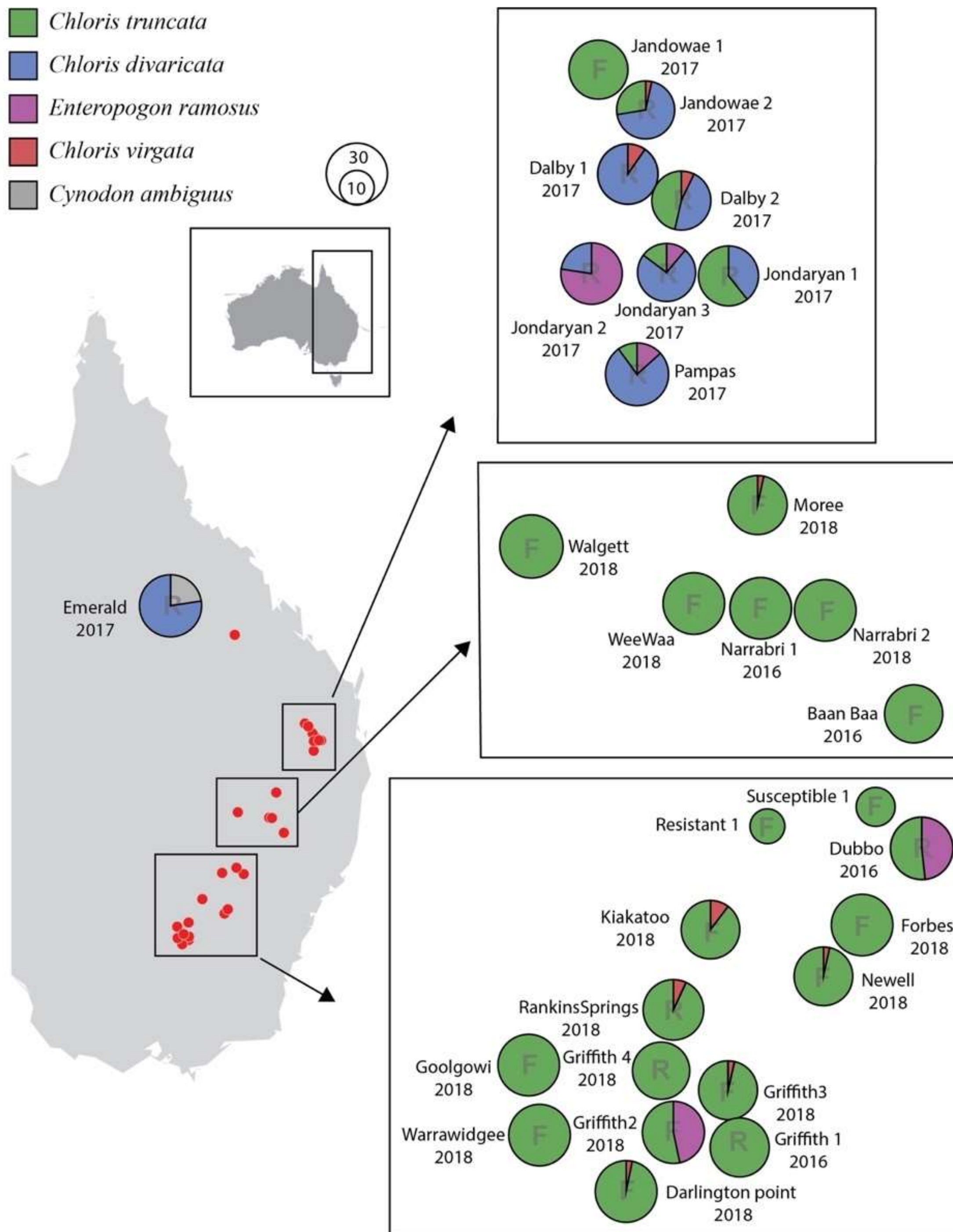


Figure 1.6.1.2. The species identity and geographical distribution of the putative windmill grass samples collected across southeast Australia for this study. Each sampling site on the map is expanded into one of the regional boxes on the right. Each pie chart has been scaled in proportion to the sample size at this site and the species are represented by colour (see top left) to indicate their proportional representation in the samples. Each sampling site is labelled by location and year (Table 1.6.1.1). Susceptible 1: previously identified as glyphosate susceptible samples (Table 1.6.1.1); Resistant 1: previously identified as glyphosate resistant samples (Table 1.6.1.1).

EPSPS sequence analysis

Target site mutations (Pro106Ser) were observed in three samples of *C. virgata* from one site (Dalby_1 Table 5), but this sample was *C. virgata*. We also detected mutations of proline to leucine at position 106, in eight samples of *C. truncata* from Narrabri 2 (Table 5).

Table 5. Target site mutations detected in sampled populations of *Chloris* species (Table 1.6.1.1, Fig. 1.6.1.2)

Name	Species	Proline 106	Threonine 102
Narrabri2_2018_01	*C. truncata*	Leucine	-
Narrabri2_2018_02	*C. truncata*	Leucine/Proline	-
Narrabri2_2018_04	*C. truncata*	Leucine	-
Narrabri2_2018_05	*C. truncata*	Leucine	-
Narrabri2_2018_06	*C. truncata*	Leucine	-
Narrabri2_2018_10	*C. truncata*	Leucine	-
Narrabri2_2018_20	*C. truncata*	Leucine	-
Narrabri2_2018_23	*C. truncata*	Leucine	-
Dalby1_2017-16	*C. virgata*	Serine	-
Dalby1_2017-25	*C. virgata*	Serine	-
Dalby1_2017-28	*C. virgata*	Serine	-

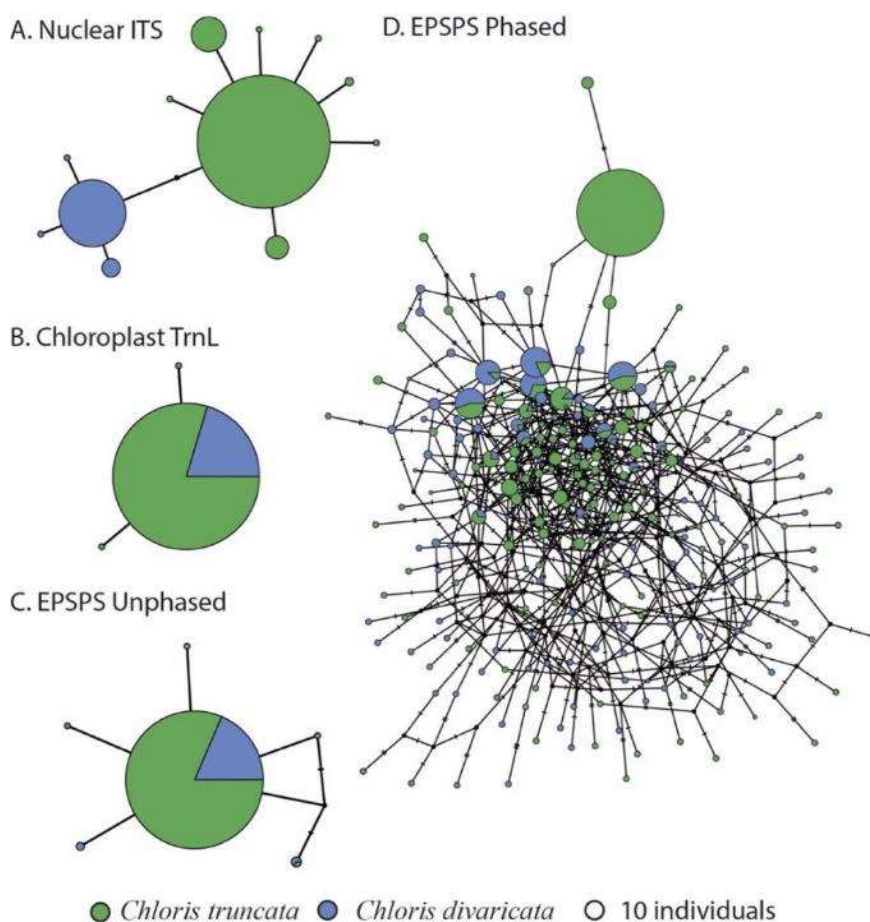


Figure 1.6.1.3. TCS haplotype networks inferred from nuclear sequences (ITS and *EPSPS*) and chloroplast (TrnL) sequences from two species, *C. truncata* (green) and *C. divaricata* (blue). The relative sizes of circles are proportional to the number of haploid individuals. Black dots indicate haplotypes that were not present. Mutational steps are indicated by dashes. EPSPS Unphased: nucleotide ambiguity was ignored.

Microsatellite analysis

Twelve new markers were developed, they amplified successfully in *C. truncata* but 2FAM did not work in almost all *C. divaricata* samples. According to the nuclear ITS sequence, samples were divided into groups. We exported individual names of every species from Geneious and then imported them to Excel. To test the relationship between *C. truncata* and *C. divaricata*, relevant individuals were than renamed their as ITS-designated species (Fig. 1.6.1.4 Structure plot). Genalex files were then created and PCoA analysis performed to see if the two species are distinguished by the microsatellite data. The PCoA showed three clusters, two for *C. truncata* (middle and bottom right) and one for *C. divaricata* (bottom left). There were two *C. divaricata* samples, from Pampas and Jandowae , that clustered with *C. truncata* (Fig. 1.6.1.4, structure plot). Also, some individuals were identified as *C. truncata* by ITS but clustered with *C. divaricata* in the structure plot (Fig. 1.6.1.4). These were collected from Trangie and Pampas. All structure plots were constructed based on clone corrected data. The duplicated multi-locus genotypes negatively affect the optimal assignments of individuals.

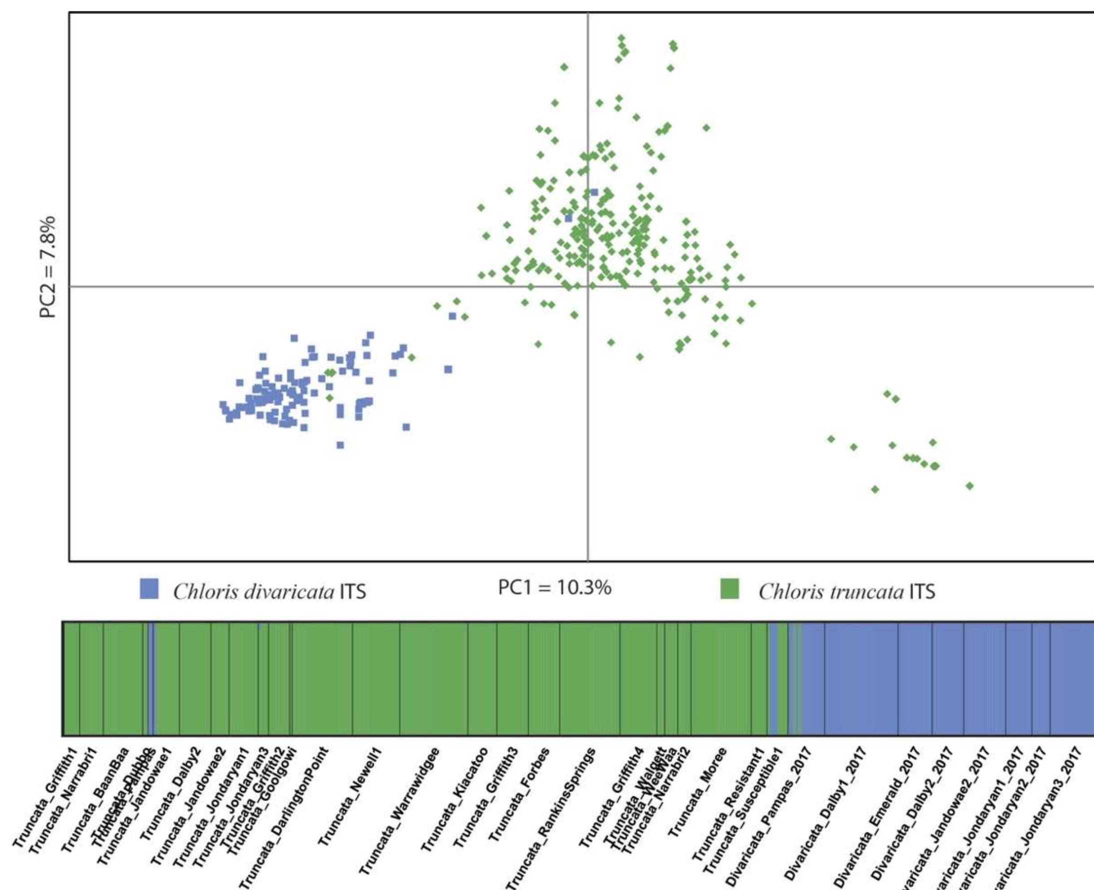


Figure 1.6.1.4. Principle coordinate analysis (PCoA) of genetic distance based on microsatellite data (above), and structure plot (bottom). The colour of the dots represents the categorization of individuals as either *C. divaricata* (139 individuals) or *C. truncata* (569 individuals) based on ITS sequences. The bar chart below shows the assignment of individuals to each of two clusters (K). In each bar, the height of each colour represents the posterior probability of assignment. The black lines separating the bar charts indicate each population, with the population name labelled below it.

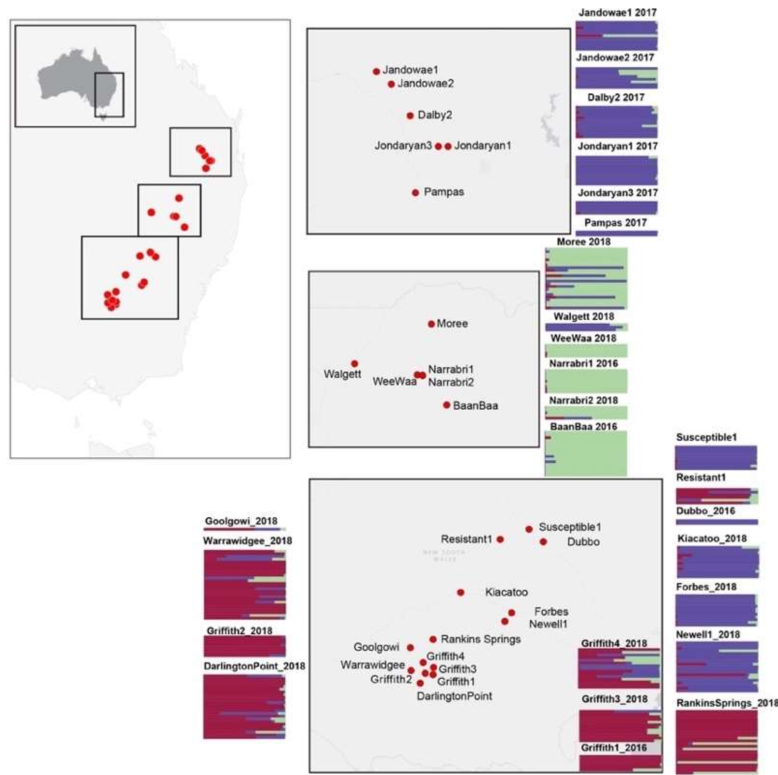


Figure 1.6.1.5. Structure plots of sites containing *Chloris truncata* showing the optimal assignment of inferred cluster to K=3. In each bar, the height of each color represents the posterior probability of assignment to each of these clusters.

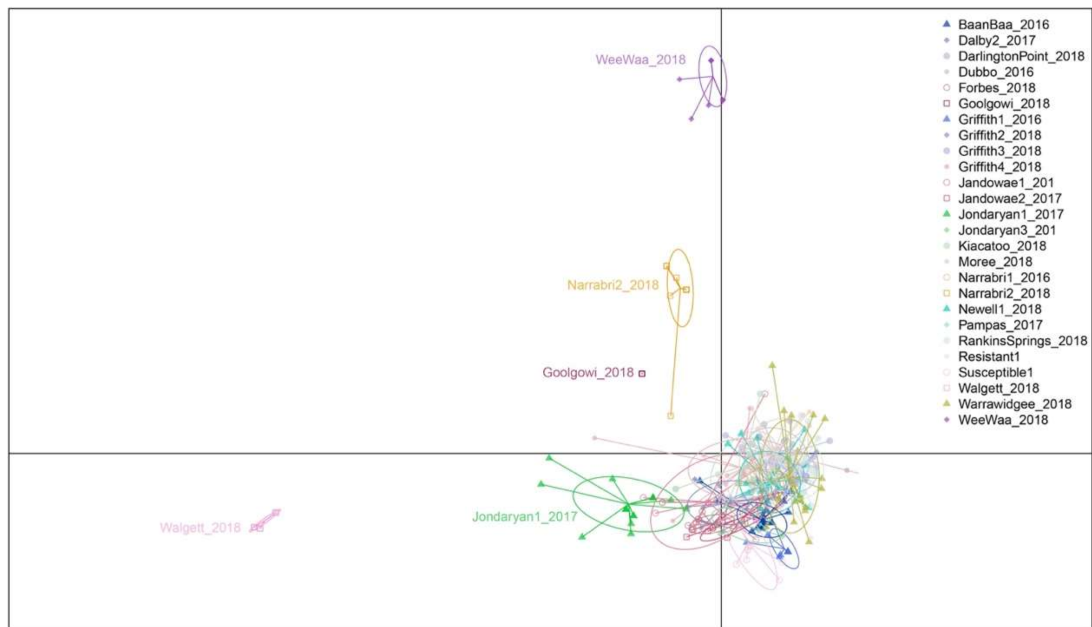


Figure 1.6.1.6. Scatter plot of Discriminant Analysis of Principal Components (DAPC) across 587 *Chloris truncata* individuals. Each population is represented by a different colour and dots represent individual genotypes.

Figure 1.6.1.7. Structure plots of sampled populations containing *Chloris divaricata* to show the optimal assignment of inferred cluster to K=2. In each bar, the height of each color represents the posterior probability of assignment to each of these clusters. The scatter plot below shows the Discriminant Analysis Principal Components (DAPC) across 138 *Chloris divaracata* individuals. Each population is represented by a different colour and dots represent individual genotypes.

The PCoA indicated two species clusters, *C. truncata* (upper middle and bottom right) and *C. divaricata* (bottom left) (Fig. 1.6.1.4). About 15 *C. truncata* individuals cluster together but far away from most of the other *C. truncata* samples. To confirm the population structure of *C. truncata*, we then ran DAPC in R. The 26 *C. truncata* populations formed 5 geographical clusters (Fig. 1.6.1.6) with clear separation of the Walgett_2018, Goolgowi_2018, Narrabri2_2018 and WeeWaa_2018 populations from all of the other populations, which grouped together at the bottom right of the plot. This result is consistent with the PCoA plots (Fig. 1.6.1.4). In Figure 1.6.1.7, Emerald_2017 and Pampas_2017 showed clear

differentiation from other *C. divaricata* population sites. And we can observe the clear differentiation between Pampas_2017, Emerald_2017 and other *C. divaricata* population sites (Fig. 1.6.1.7).

Table 1.6.1.6. (1) Genetic Diversity characteristics of *Chloris divaricata* in sampled populations. (2) Genetic Diversity characteristics of *C. truncata* in sampled populations N: the total numbers of *C. truncata* or *C. divaricata* in every population; Na: average number of alleles in every population; Ne: numbers of effective alleles; MLG: numbers of mutilocus genotypes (MLGs) observed; eMLG: expected MLGs; H: Shannon-Wiener Index of MLG diversity; Ho: overserved heterozygosity in every population; He: expected heterozygosity; uHe: unbiased expected heterozygosity; F: fixation index

(1) *C. divaricata*

Pop	N	Na	Ne	MLG	eMLG	H	Ho	He	uHe	F
Pampas	18	4	2.383	14	8.68	2.55	0.005	0.43	0.438	0.57
Dalby1	28	4.75	3.277	28	10	3.33	0.145	0.27	0.281	1
Emerald	22	2.333	1.489	13	7.42	2.35	0	0.44	0.461	1
Dalby2	12	3.583	2.855	12	10	2.48	0	0.4	0.411	0.96
Jandowae2	20	4.167	2.705	16	8.72	2.65	0.013	0.28	0.293	0.49
Jondaryan1	11	2.417	1.664	10	9.18	2.27	0.092	0.28	0.305	0.96
Jondaryan2	7	2.25	7	7	1.95	1.89	0.012	0.28	0.305	0.96
Jondaryan3	20	5.5	20	10	3	3.07	0.115	0.41	0.424	0.78

(2) *C. truncata*

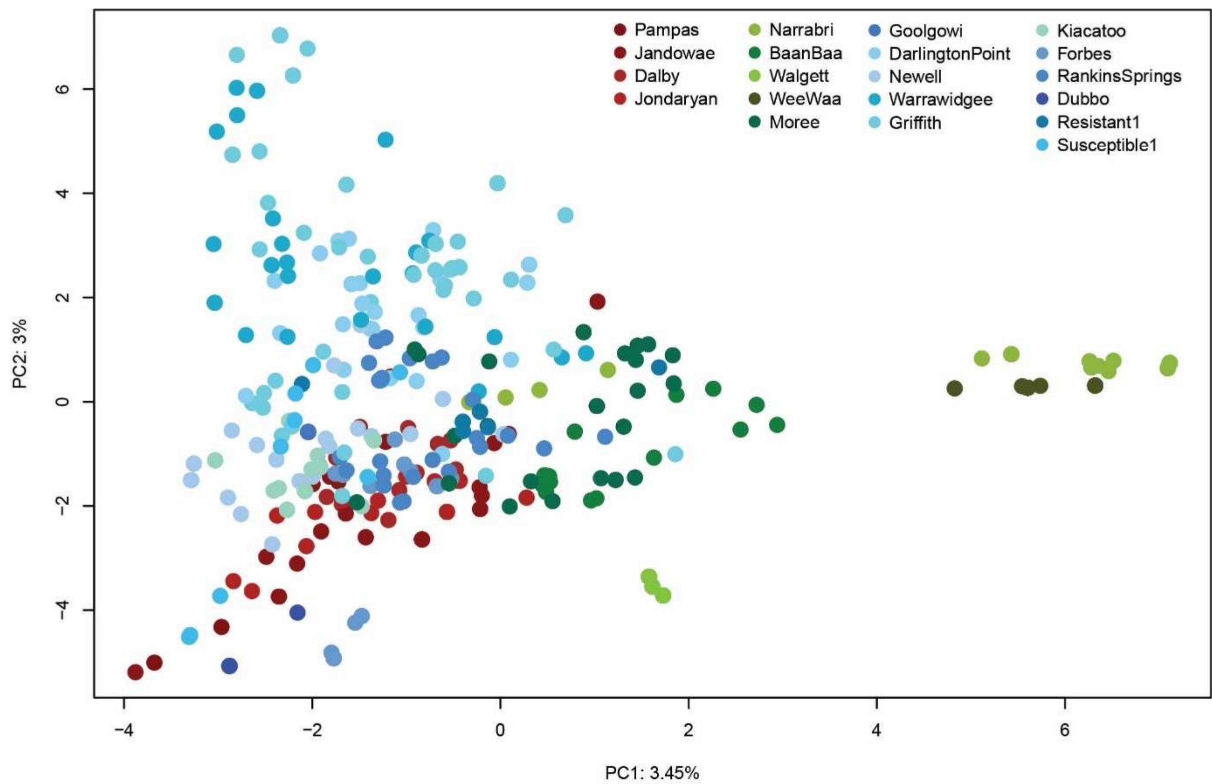
Pop	N	Na	Ne	MLG	eMLG	H	Ho	He	uHe	F
Griffith1	30	2.167	1.079	6	2.67	0.72	0.012	0.07	0.073	0.79
Narrabri1	30	1.917	1.324	9	4.55	1.58	0.009	0.24	0.241	0.96
BaanBaa	30	3.167	1.796	17	7.14	2.44	0.039	0.4	0.404	0.9
Dubbo	15	1	1	2	1.67	0.25	0	0	0	
Pampas	2	1.417	1.183	2	2	0.69	0.292	0.22	0.292	-0.3
Jandowae1	17	5.167	3.031	11	7.64	2.26	0.015	0.64	0.66	0.98
Dalby2	12	4.167	3.025	12	10	2.49	0.098	0.59	0.633	0.83
Jandowae2	9	4.333	3.174	8	8	2.04	0.021	0.65	0.689	0.96
Jondaryan1	18	3.5	1.956	11	7.19	2.18	0.054	0.43	0.44	0.79
Jondaryan3	5	3.833	2.738	5	5	1.61	0.392	0.61	0.675	0.37
Griffith2	16	3.167	1.977	8	5.62	1.65	0.042	0.47	0.489	0.91
Goolgowi	30	1	1	1	1	0	0	0	0	
DarlingtonPoint	31	6.5	3.363	24	9.18	3.09	0.045	0.65	0.663	0.93
Newell1	29	7.333	3.641	19	8.13	2.72	0.069	0.7	0.709	0.9
Warrawidgee	30	7.083	4.235	26	9.51	3.2	0.008	0.72	0.733	0.99
Kiacatoo	27	6.167	3.534	12	6.59	2.18	0.028	0.7	0.708	0.96
Griffith3	28	4	2.224	12	6	2.03	0.022	0.5	0.509	0.88
Forbes	30	3.75	2.648	12	6.07	2.05	0.017	0.6	0.614	0.97
RankinsSprings	29	3.75	2.213	24	9.16	3.06	0.026	0.49	0.493	0.94
Griffith4	29	5.5	2.76	15	7.21	2.41	0.027	0.62	0.627	0.96
Walgett	32	1.083	1.022	3	2.79	0.98	0.02	0.02	0.018	-0.1
WeeWaa	30	1.083	1.012	5	2.99	1.01	0	0.01	0.011	1
Narrabri	30	2	1.232	5	2.96	0.95	0.011	0.15	0.148	0.97
Moree	28	7	4.783	23	9.32	3.07	0.088	0.76	0.778	0.88
Resistant1	10	2.25	1.377	6	6	1.61	0.009	0.27	0.283	0.99
Susceptible1	10	3.417	2.776	9	9	2.16	0.033	0.6	0.637	0.94

To detect the genetic diversity within populations and see the relationship between genetic diversity and geographical grouping, we used clone corrected microsatellite data of both *C. truncata* and *C. divaricata* to perform distance analysis in Genalex and Poppr in R. Table 1.6.1.6 shows the fixation index which in *Chloris* species is very high (~0.9) in most of the sampled populations relative to the heterozygosity level. This is because windmill grass is a highly selfing weed. The numbers of MLGs also varied substantially.

Gene expression analysis

After passing quality control we classified three groups of EPSPS copy number, 'one' was the same as the reference standard and had no evidence of gene amplification, 'medium' corresponded to an estimate of around 40x copies based on the expression analysis, and 'high' corresponded to around 100x copies. We combined the expression data with the microsatellite data to determine how many times this resistance mechanism has evolved (Fig. 1.6.1.8).

We find evidence that the 'high' copy number evolved twice, and so did the 'medium', based on the clustering of the microsatellite genotypes and the highly selfing reproductive mode. We also find that the population with the Pro106Leu mutation also has the medium copy number and we confirm one individual that has both the mutation and the medium copy number.



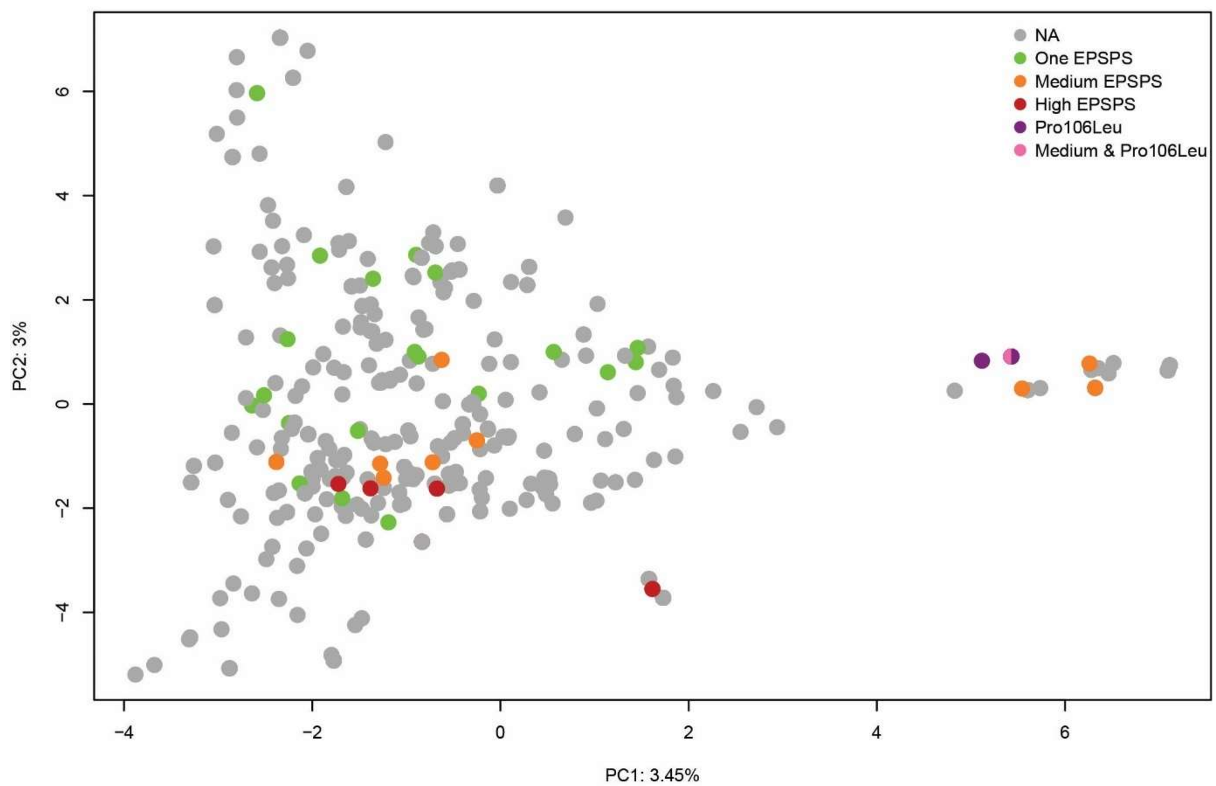


Figure 1.6.1.8 PCA plots of the microsatellite data with geography used to colour the points (top) and with the EPSPS copy number information used to colour the points (bottom).

Discussion

The results detailed above contribute to our understanding of the geographical connectivity of windmill grass populations, the species status of *C. truncata* and *C. divaricata*, and the evolution of glyphosate resistance in these weeds. We confirmed, with genetic data, that the two morphologically similar described species, *C. truncata* and *C. divaricata*, are indeed two distinct species that are closely related. They are identical at the chloroplast Trn L gene and only 2bp different at the nuclear ITS gene. However, both ITS sequence data (Fig. 1.6.1.1) and microsatellite data (Fig. 1.6.1.4) indicate that they are two distinct species. Issues related to the correct identification of these species are discussed below, as are aspects of their population genetics and ecology, and the implications for understanding and dealing with glyphosate resistance.

Species status of Chloris truncata and Chloris divaricata

Correct plant identification has proven to be crucial to understanding the resistance status and ecology of *C. truncata* and *C. divaricata*. Grass species identification can be difficult and challenging, and correct identification is important (Mangold and Parkinson, 2013). Incorrect identification can hinder the development of the most appropriate management strategy. Understanding glyphosate resistance and its evolution also requires accurate species identification.

Scientists have been seeking a universal plant barcode for use in species identification. The nuclear gene ITS has been proposed as such a standard. However, reports confirm that it can have fungal contamination, paralogy problems (common ancestry through gene duplication rather than speciation), amplification problems and, in any case, may not apply to all plants, especially those in large complex genera (Hollingsworth, 2011, Roy *et al.*, 2010, Group *et al.*, 2009). In species, like grasses, where identification is difficult, simply screening for glyphosate resistance across field-collected samples is not sufficient. We wanted to ensure that it was windmill grass, rather than morphologically indistinguishable species, that we were investigating. Several grass species in Australia are morphologically close to *C. truncata* and some of these were present in samples, including *C. divaricata* (Fig. 1.6.1.2) and *E. ramosus* (curly windmill grass). If grass species with glyphosate resistance are misidentified and then mistakenly included in samples for genetic resistance testing, then the number of times that resistance has evolved will be underestimated.

Although, *C. divaricata* and *C. truncata* have been shown to be two distinct species through the data presented, a number of individuals classified as *C. divaricata* by ITS but did cluster with *C. truncata* based on microsatellites. Hybridization could be one explanation for this, but it could also simply be a mistake in the assignment by ITS sequencing. If the individuals were hybrids, they would be expected to cluster between the two species in the PCoA plot (Fig. 1.6.1.4), but they do not. If hybridization does occur then glyphosate resistance could be passed from one to the other (Zelaya *et al.*, 2007). *Chloris divaricata* and *C. truncata* have different distributions, so if hybridization allowed glyphosate resistance to spread from *C. truncata* to *C. divaricata* then a much larger agricultural area could be affected.

Ecology and population genetics of C. truncata and C. divaricata

Knowledge about the genetic diversity of *C. truncata*, at both the population and species level, is vital for management of this weed and for understanding the evolution of resistance in this species (Mangolin *et al.*, 2012, Rao and Hodgkin, 2002). Compared to invasive species, native species tend to have higher genetic variability, and this may provide relatively more raw material for evolution (Sterling *et al.*, 2004, Mangolin *et al.*, 2012).

Heterozygosity is another aspect of genetic diversity, and can be strongly affected by the mating system (Enjalbert and David, 2000, Halkett *et al.*, 2005). Selfing species evidently have relatively lower genetic diversity, while outcrossing species generally have a higher level of genetic diversity (Godt and Hamrick, 1991, Aide, 1986). The fixation index (F) is the parameter used to specify the degree of heterozygosity. The higher the heterozygosity the smaller the value of F, and most *C. truncata* populations sampled had an F value close to 1 (Table 1.6.1.6), so the species is relatively homogeneous genetically and mostly asexual in reproduction.

Genetic diversity is expected to be lost following extreme selection, like that imposed by herbicide application, and this should be especially pronounced in a species that is an obligate self-pollinator (Vila-Aiub *et al.*, 2003). Apart from the level of heterozygosity within a population, the number and relative abundance of alleles within that population should also influence the genetic diversity available to selection. The BaanBaa population, for example, is resistant to glyphosate, but it still has 17 multilocus genotypes, whereas other populations (like that Goolgowi_2018) have only one multilocus genotype and they evidently do not have glyphosate resistance. Future work should check whether other populations with a lower number of such genotypes are glyphosate susceptible or resistant.

Glyphosate resistance in Chloris truncata

So far, target site amplification has been the only known glyphosate resistance mechanism in windmill grass (Ngo *et al.*, 2017). The evaluation of *EPSPS* expression levels and the sequence in the *EPSPS* region suggest these are two possible resistance mechanisms. The results presented in Table 1.6.1.5 show that target site mutations were present in some of the *Chloris* samples screened (Table 5). Three of these individuals had a Pro106Ser substitution and they were from the population "Dalby 1" (Queensland), and were identified as *C. virgata* based on ITS sequence data. Only four EPSPS target site mutations have been found in weeds generally (Pro106Ala, Pro106Leu, Pro106Ser and Pro106Thr) (Sammons and Gaines, 2014). Dual target site mutations confer more than 180-fold resistance, whereas single target site mutations can only confer moderate glyphosate resistance (less than 10 fold) (Sammons and Gaines, 2014, Yu *et al.*, 2015). In *C. virgata*, the target site mutations Pro106Ser and Pro106Leu on their own confer only 2.4-2.6 and 8.9-9.7 fold glyphosate resistance, respectively (Ngo *et al.*, 2018).

The Pro106Leu mutation was detected in one *C. truncata* population (Table 1.6.1.5). It is the first time that this mutation has been detected in Pro106 in windmill grass, but has not yet been verified as a glyphosate resistance mechanism. The probability is high, though, that it will confer resistance because leucine mutations have been associated with resistance in the related grass in *Lolium rigidum* (Kaundun *et al.*, 2011). Although, the farmer in BaanBaa, where samples were collected, said that the windmill grass there is resistant to glyphosate, target site mutations were not detected in samples from this population, so further investigation is needed. Our analysis of *EPSPS* expression levels confirmed that the resistant populations that we included in this study do have *EPSPS* gene amplification. Combined with the new target site mutation we discovered in windmill grass, this shows that glyphosate resistance has evolved at least twice in this species, and in different ways.

The increasing rate at which glyphosate resistance is reported in weeds generally, and the significant challenge to food production presented by glyphosate resistance (Vencill *et al.*, 2012), means that we need to understand how glyphosate resistance evolves and spreads geographically. The results presented here contribute to understanding these processes in windmill grass. The development of knowledge of glyphosate resistance is critical for the development of management strategies against weeds (Vencill *et al.*, 2012). If resistance evolution is a rare event then there is a possibility to focus resources on new cases of

resistance and thus eradicate all weeds in affected populations. If resistance evolves more readily, it could be a waste of resources to attempt eradication like this. These results show that resistance has evolved at least four times in windmill grass. So early eradication is likely not possible. The highly selfing nature of Windmill grass will make it more difficult for this species to get resistance to multiple modes-of-action, but every glyphosate resistant individual has the potential to develop resistance to another herbicide. We only tested a subset of our samples but over half of them only had a single copy of the *EPSPS* gene and these are all likely susceptible populations. This shows that there is still time to control the resistant populations and reduce the risk of multiple mode-of-action resistance.

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1.6.2 Population genetics of Fleabane

Regional genetic differentiation and the evolution and spread of glyphosate resistance in Fleabane, *Conyza bonariensis* (L.) Cronquist (Asteraceae).

Introduction

Conyza bonariensis (L.) Cronquist (Asteraceae), also known as flaxleaf fleabane, is a serious weed of north-eastern cropping systems in Australia and glyphosate resistant populations were first collected there in 2006 (Walker *et al.* 2011). More recent surveys show that practically all populations across Queensland are now glyphosate resistant (Jalaludin, pers comm). This species has evolved glyphosate resistance in at least nine different countries (Heap 2017) and several species in the genus have evolved glyphosate resistance. *Conyza bonariensis* is allohexaploid with a chromosome count of 2n = 6x = 54 (Paula & Pinto-Maglio 2015; Thebaud & Abbott 1995). Several species in this genus are polyploid but their ancestry and evolutionary relationships are not clear. Fleabane is regarded as an obligately selfing species and has wind dispersed seeds, so it is considered a species with good dispersal abilities. It is presently unclear whether resistance evolved once and spread, or if it evolved multiple times, and whether there is any outcrossing in this species.

In this study we surveyed fleabane from throughout the cotton growing region across three years. We were also able to access seed from the original resistant and susceptible lines kept at Queensland Department of Agriculture and Fisheries (QDAF) from the study of Walker *et al.* (2011). Fleabane seed has a short shelf-life and we were unable to germinate these lines, but we were able to extract DNA from the seeds and genotype them. We developed sixteen new microsatellite markers for this species. We genotyped all samples using these markers to assess population connectivity, the evolution of glyphosate resistance, and any evidence of outcrossing in fleabane.

Materials and methods

Samples and DNA extraction

We conducted three major sampling trips across the cotton growing regions of eastern Australia in 2016, 2017 and 2018. At each site leaf material was collected from 30 individuals and a bulk seed collection made. Leaf was collected directly into silica gel for subsequent DNA extraction. DNA was extracted using a CTAB/spin column protocol as outlined in 1.6.1 for windmill grass. We had seed collections available from the 2006-2008 surveys where glyphosate resistance was first reported in this species. We were unable to germinate these seeds because fleabane seeds have short shelf life, but we were able to extract DNA by pulverising the seed, adding 20% Chelex (Chelex 100 (Bio-Rad Corp) and heating at 98ºC for 10 minutes. We also grew several plants and tested them for herbicide resistance (Fig. 1.6.2.2), but they were all resistant and we did not continue testing. We also included 11 resistant populations screened by Adam Jalaludin from surveys in 2017/2018.

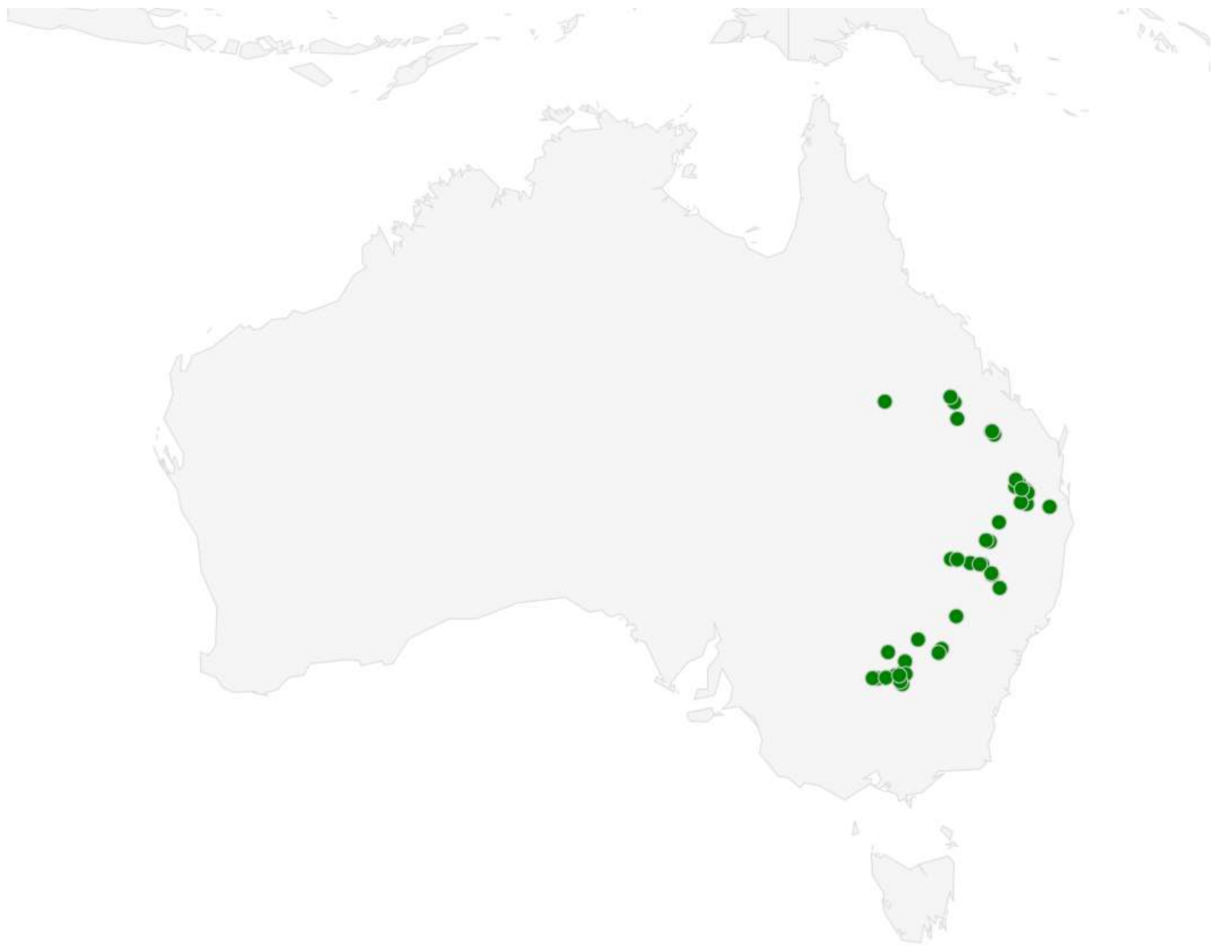


Figure 1.6.2.1 Map showing the sampling sites for populations used in this study.

Figure 1.6.2.2. Populations growing for resistance testing.

Genetic analysis

We identified microsatellite markers from next generation sequencing (NGS) sequence data using the QDD program (Version 3) (Meglécz *et al.* 2010, 2014), which uses Primer3 to design primers (Untergasser *et al.* 2012). Forty-eight primer pairs were screened across fleabane populations. From these, 16 variable markers were selected based on amplification success, size of PCR product (to enable them all to run on the same lane), and no stuttering pattern. An M13 tail (GTAAAACGACGGCCAG) was attached to each primer at the 5' end of the forward primer to allow PCR incorporation of fluorescent labels (Schuelke 2000). A PIG tail (GTTTCTT) was also added to the 5' end of the reverse primers to reduce stutter (Brownstein *et al.* 1996).

Markers were amplified in 12 µl PCR reactions composed of 2 µl DNA template, 0.5 U MyTaq polymerase (Bioline, Australia), 0.1 µM of forward primer, 0.2 µM of reverse primer, 0.2 µM M13 labelled primer with different fluorescent dyes (6-FAM, VIC, PET or NED) and 1x buffer. PCRs were performed under the following conditions: initial denaturation at 95 °C for 2 min, 35 cycles of 15 s at 95 °C, annealing at 57 °C for 25 s, extension at 72 °C for 30 s, 10 cycles of 15 s at 95 °C, annealing at 54 °C for 25 s, extension at 72 °C for 30 s, and a final extension of 10 min at 72 °C. Before genotyping (by Macrogen, South Korea), the quality of the PCR products was checked by gel electrophoresis, and then were cleaned using 1U each of Exonuclease I and Antarctic Phosphatase.

Microsatellite peaks were analysed using the microsatellite plugin in Geneious version 9.1.8 (http://www.geneious.com) (Kearse *et al.* 2012). The basic population genetic statistics, including Hardy-Weinberg probability test, were calculated using Hardy-Weinberg exact tests in Genepop version 4.6 (Rousset 2008) with 100 batches (10,000 iterations per batch). The locus-specific statistics (number of different alleles (N_a), the observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F)) were calculated using GenAlEx 6.5 (Peakall and Smouse 2006, 2012). The population assignment of MEAM1 was analysed using STRUCTURE version 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009). STRUCTURE runs were performed using the admixture model with a burn-in of 50,000 iterations followed by 500,000 iterations. Initially, K values were set from 1 to 10 with the same parameters as above. Five runs were then conducted, and these were permuted and plotted using CLUMPAK server (Kopelman *et al.* 2015). To estimate the most likely K value in the data set, we used Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) (Earl and vonHoldt 2012) and the "Evanno" method (Evanno *et al.* 2005).

A principal component analysis (PCA) was performed using the adegenet package (Jombart 2008; Jombart and Ahmed 2011) in R version 3.5.1 (R core team 2018). To test the correlation between genetic and geographical distance, isolation by distance (IBD) was tested using the adegenet package (Jombart 2008) in R version 3.5.1. Each test was permuted using a Mantel test based on 999 replicates.

Results

We amplified 14 microsatellites across 1,132 individuals, including ten herbicide resistant populations from the study of Walker *et al.* (2011) and two susceptible individuals. The markers amplified well, and missing data was 5% for the complete dataset after individuals were removed that had amplified at less than nine markers. None of the loci amplified more than two peaks, indicating that the primers were specific to one of the sub-genomes and the markers could be treated as diploid. A couple of populations did not amplify well, and this could be because they were a young *Conyza* of another species that was miss-identified. Overall, we genotyped 53 populations.

F_{IS} was 0.862 over all populations (obligate selfing usually returns a value close to one). When we plotted a PCA of the samples from the initial survey of 2006-2008 we found that the resistant and susceptible individuals clustered separately (Fig. 1.6.2.3).

Including all populations, the overall F_{ST} value was high, at 0.4, and there was geographic clustering evident in the data from the structure analysis (Fig. 1.6.2.4), and in the PCoA (Fig. 1.6.4.5). There was also an isolation by distance effect (simulated P = 0.002, Fig. 1.6.2.6) showing that genetic distance was correlated with geographic distance. The delta K analysis indicated that the most likely value for K, the number of populations was two, but there was some support for K=3. In the STRUCTURE plots, K=3 further separated the populations, but little resolution was gained at K=4 or K=5 (Figure 1.6.2.4).

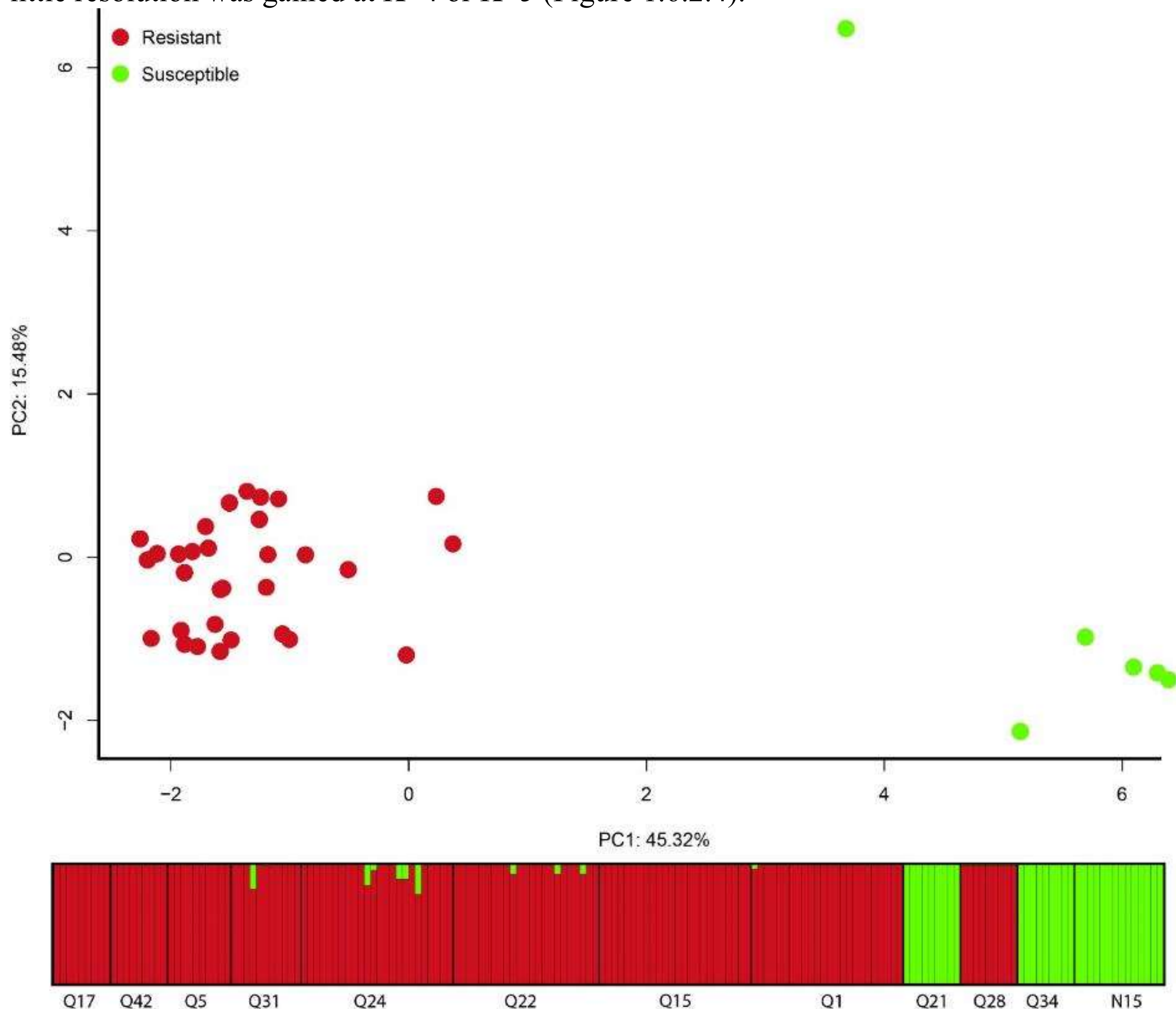
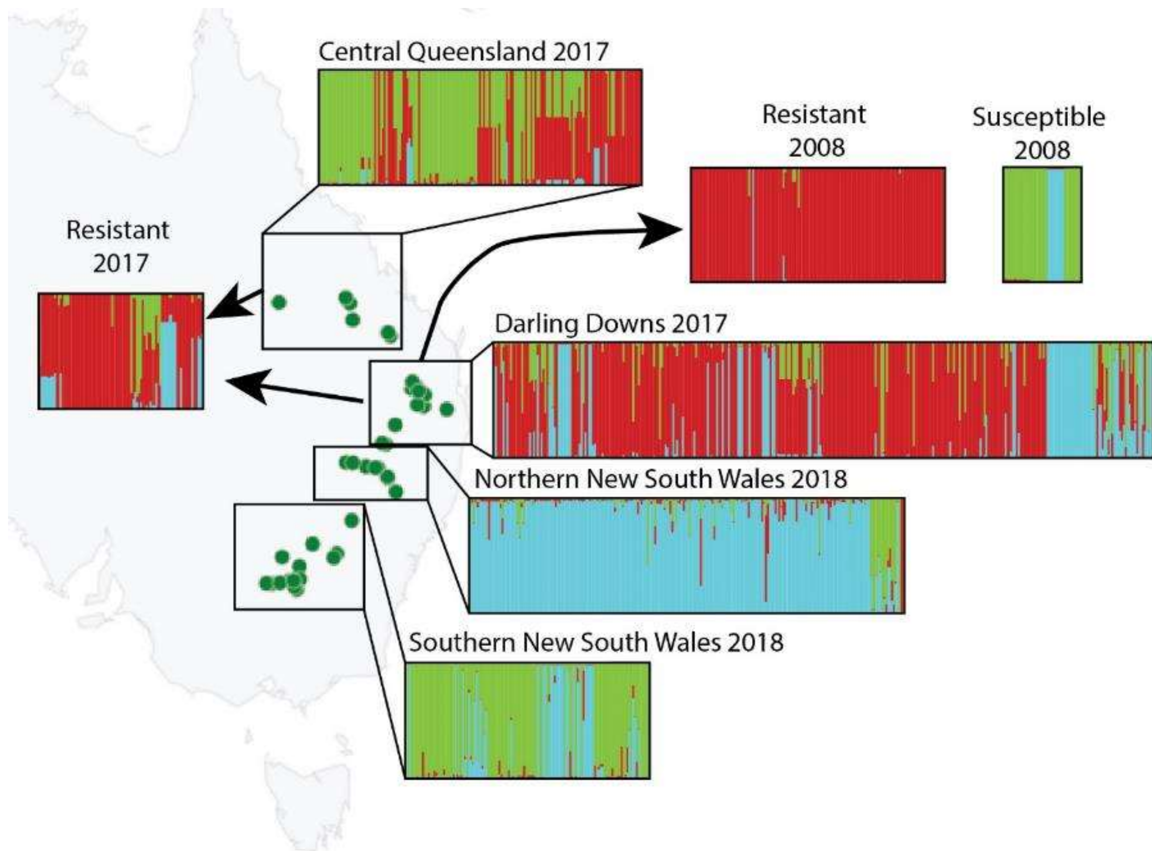


Figure 1.6.2.3 PCA of the microsatellite data of 12 populations from the survey of Walker *et al.* (2011), with resistant lines in red and susceptible lines in green (top), and structure plot of the same data (bottom).



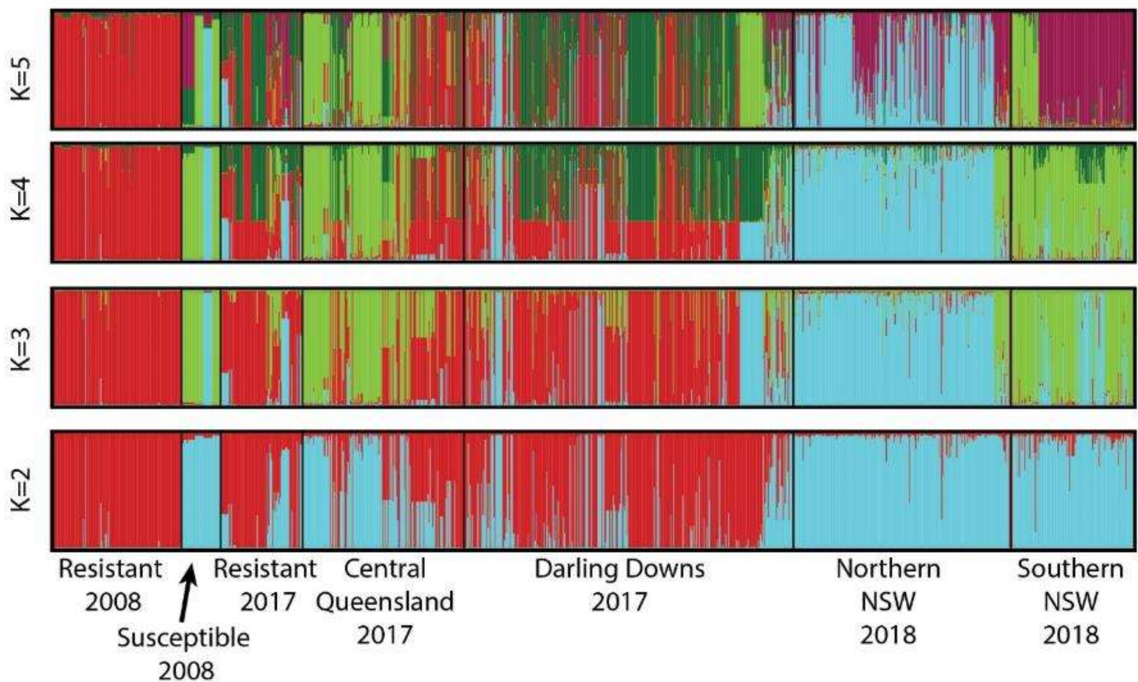


Figure 1.6.2.4 Structure analysis of the microsatellite data, each bar represents one individual and the colour represents that individual's posterior probability of belonging to each cluster, K. Resistant samples screened from 2017 were from central Queensland and the Darling Downs.

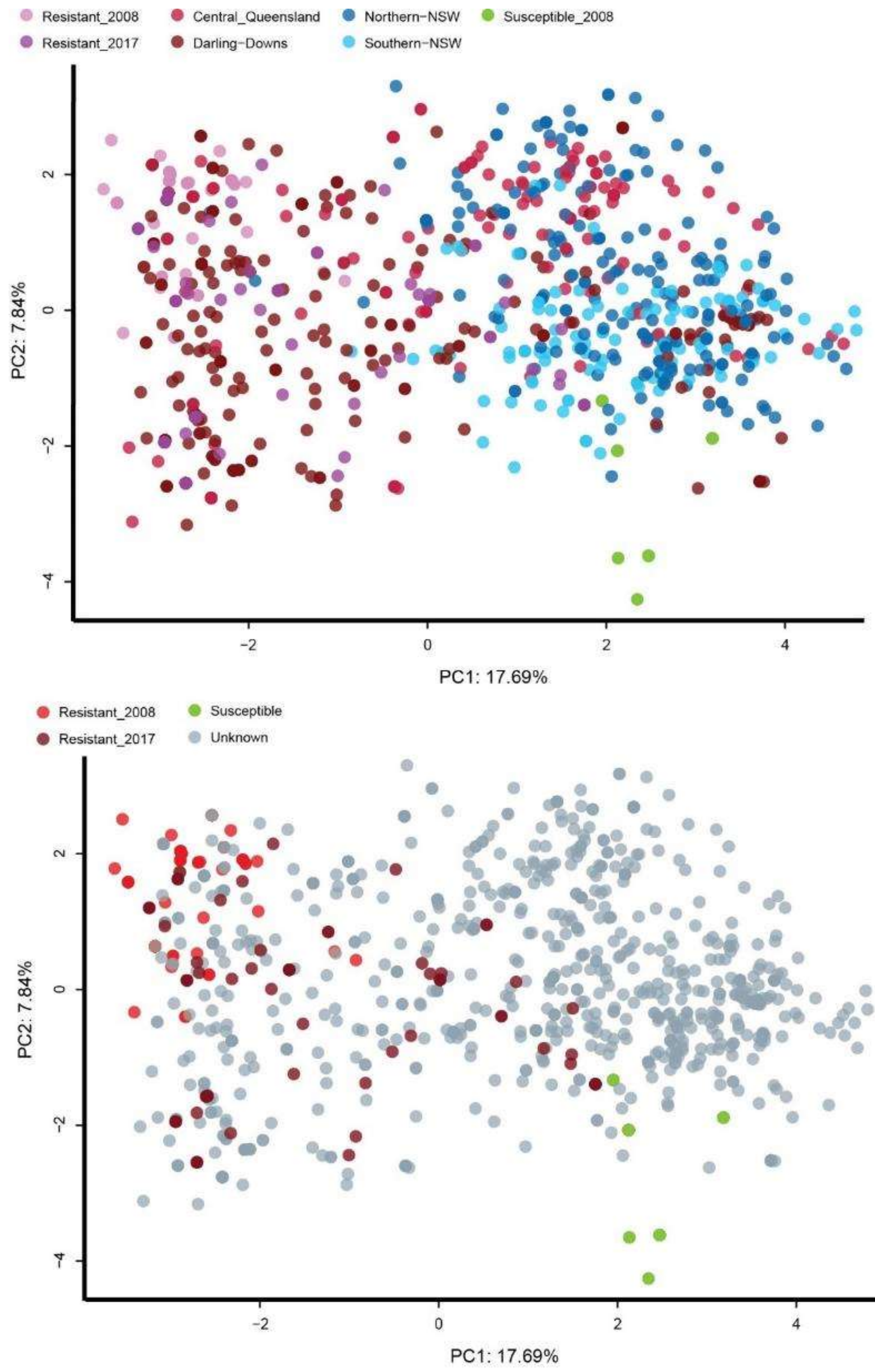


Figure 1.6.2.5 PCA plots of the genetic data showing the known resistant populations in relation to broad geographic regions (top) and with just the resistance data shown (bottom).

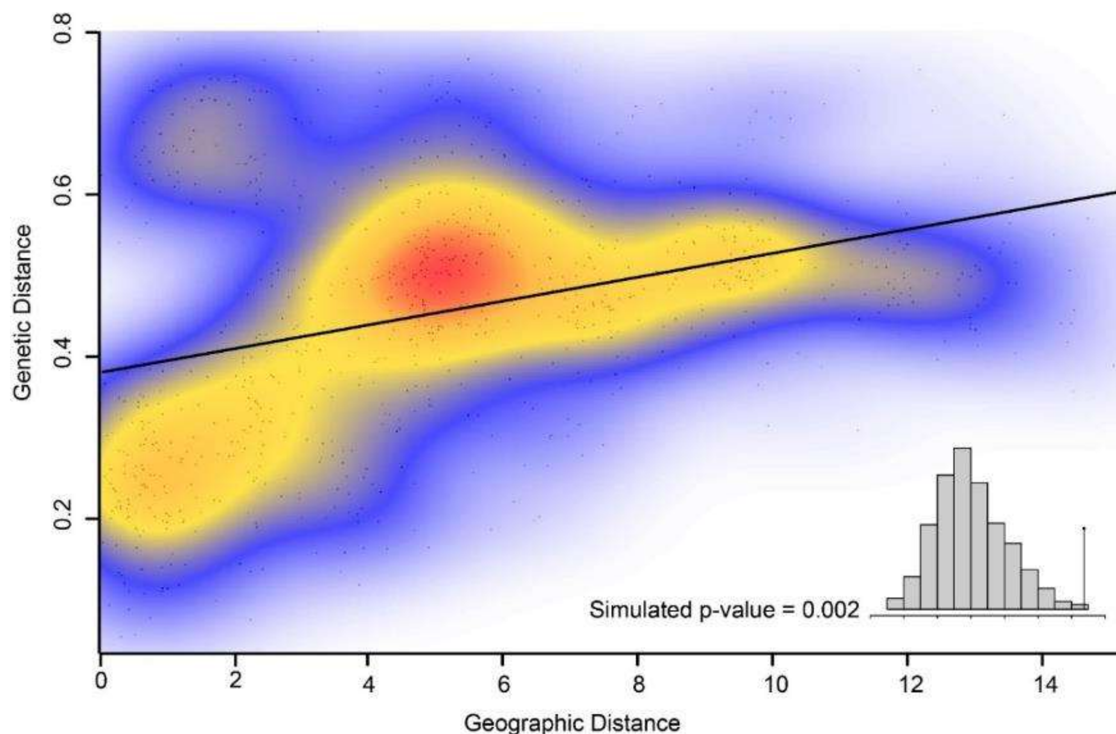


Figure 1.6.2.6 Isolation by distance plot showing the relationship between genetic distance and geographic distance.

Discussion

In this study we show that all the resistant populations from the study of Walker *et al.* (2011) were clustered together in the structure analysis and the PCA (Fig. 1.6.2.3), suggesting that this first case of resistance evolved once in southeast Queensland. The more recent resistant individuals, from surveys in central and southeast Queensland, also mostly cluster with the resistant samples from 2008 in the structure analysis (Fig. 1.6.3.4), although there appears to be some level of admixture with other populations.

There is strong geographic structure, with largely separate populations in southern New South Wales (NSW), northern NSW, and Queensland, although some populations in central Queensland cluster with individuals from southern NSW (Fig.1.6.3.4). Fleabane is considered to have very strong dispersal ability because the seed appears adapted for wind dispersal, but it has also been anecdotally claimed that the majority of the seeds fall to the ground and few enter the wind column. Fleabane is also present in very large populations in all of these regions, and perhaps the few seeds that do disperse across regions are not sufficient to affect the overall population structure.

There were suggestions of admixture in our data, and the genetic variation, combined with the F_{IS} of 0.862 indicate that fleabane is probably outcrossing under natural conditions, but perhaps only very low levels of outcrossing can result in the patterns we detect here. The resistant lines maintained in the glasshouse all have almost identical genotypes, but the field sampled plants had more genotypic diversity. This is important because it allows a weed to 'stack' different herbicide mode-of-action resistance mechanisms more easily.

When we combine the resistance data with the microsatellite genotyping data (Fig. 1.6.2.5) it appears that resistance appeared once in 2008. Our interpretation of what happened after that depends on how much outcrossing there is, if, as our results suggest, there is a non-negligible amount of outcrossing under natural conditions, then it is possible that resistance in glyphosate evolved once in Queensland and spread. Population Q28 comes out as different

when we analyse all populations in structure, and it came out differently in the PCA (Fig. 1.6.2.3). This population was originally recorded as resistant by Walker *et al.* (2011), but in the transcriptome experiment (Section 1.7.3) this line did not survive treatment, and its gene expression profile was quite different from other populations. This population could represent a second evolution of glyphosate resistance in fleabane, or it could have been miss-identified as resistant in the first place.

We consider all populations of fleabane on farms to be resistant to glyphosate in modern samples, and under this assumption, the populations in northern NSW and southern NSW have probably evolved resistance independently of the Queensland populations. We see evidence of long-distance migration in our data, with some individuals in northern and southern NSW being assigned to the Queensland cluster. This highlights possible routes for glyphosate resistance to spread from one region to another.

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1.6.3 Population genetics of Sowthistle

Population genetics of the wind-dispersed *Sonchus oleraceus* L. from Australia

Abstract

Sowthistle had been predicted to be a good candidate for the evolution of glyphosate resistance, but resistance was only first detected in 2008. Since then, resistance has spread rapidly, and this weed is considered to be well dispersed by wind. We assessed the population connectivity of sowthistle populations across the eastern Australian cropping system to see how connected by dispersal they are. We find that the populations are highly mixed, genetically, and this supports their reputation as strong dispersers.

Introduction

Sonchus oleraceus (L.) (Asteraceae), common sowthistle, originated in Europe but is now cosmopolitan and found in almost every ice-free part of the globe (Gleason & Cronquist 1991, CABI 2018). This plant is edible, with antioxidant properties (Xia *et al.* 2011), but is also an agricultural weed. Australian populations with glyphosate resistance were detected in 2014 (Cook *et al.* 2014).

The genus *Sonchus* is polyphyletic and polyploidy has played an important role in its evolution (Kim *et al.* 2007). Stebbins (1953) suggested that *S. oleraceus* is an amphidiploid (n=16, 2n=32) which received 18 chromosomes from *S. asper* and 14 from *S. tenerrimus*. More recent karyological analyses concur (Mejias & Andres 2004). Target site resistance to glyphosate is commonly found (Sammons & Gaines 2014), but being polyploid reduces a weeds ability to develop target site resistance (see section 1.7.3). We now think that it is polyploidy that caused glyphosate resistance to take a long time to evolve in sowthistle. Being polyploid also makes it harder to genotype individuals, because without a reference genome it is difficult to separate markers from the two genomes from each other. In this study we were able to use the draft reference genome developed in this study (See 1.7.1) to genotype sowthistle accurately.

The goal of this study was to assess how connected sowthistle populations are genetically.

Materials and Methods
Sample collection and DNA extraction.

Field surveys were conducted between 2016 and 2018 across the east coast of Australia, both roadside and on-farm populations were targeted (Fig. 1.6.3.1). At each site 30 individuals were sampled by taking leaf samples into silica gel, sampling only individuals separated by 1m. A representative bulk seed sample was also collected.

DNA was extracted using a combination of CTAB lysis and 'DIY spin-column purification' (Ridley *et al.* 2016). Around 1cm of dried leaf was used for each extraction. Details of the extraction protocol can be found online at www.jameshereward.org/CTABspincolumn.html. DNA was measured usingp PicoGreen™ (Molecular Probes, Eugene, OR) and normalised to 200ng in 40 ul.

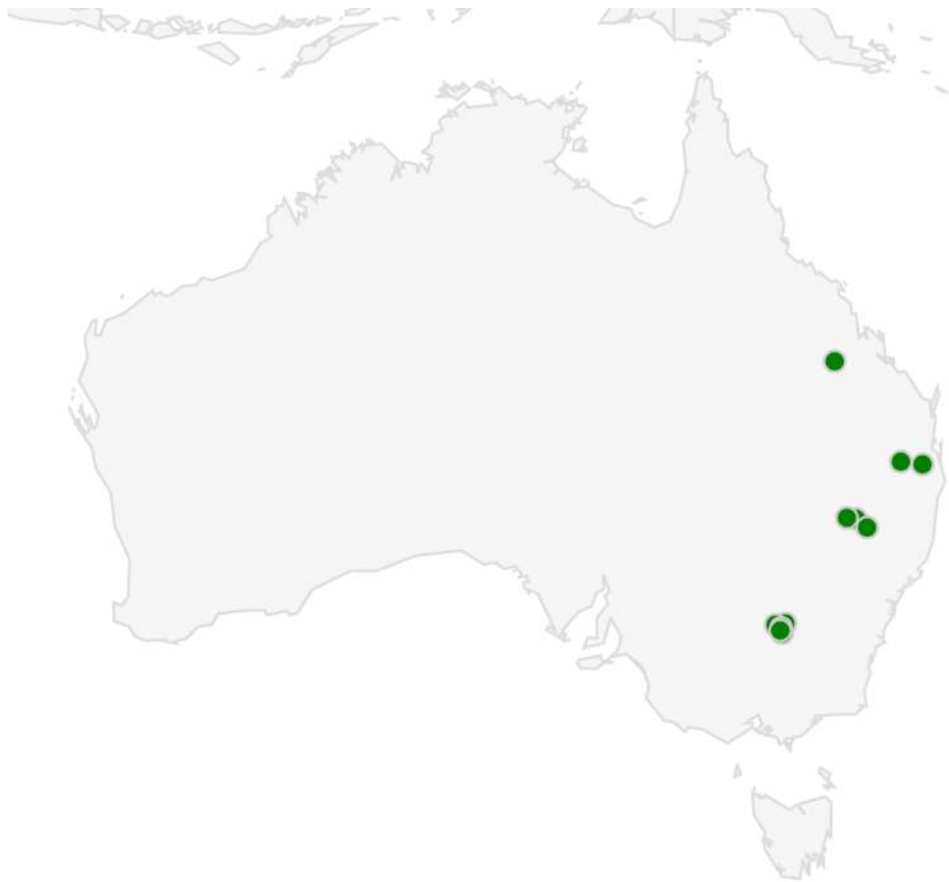

Figure 1.6.3.1 Map showing the sampling sites of sowthistle used in this study.

Genotyping by sequencing

We developed a *genotyping-by-sequencing* method based on Elshire *et al.* (2011), Poland *et al.* (2012), and Petersen *et al.* (2012), but using the Golay codes published by Caporaso *et al.* (2012). Briefly, we combined the forward adaptors from Poland *et al.* (2012) with the 12bp Golay barcodes of Caporaso *et al.* (2012) to add further stringency to the barcode recovery. We then used the reverse adaptors from Petersen *et al.* (2012), which allow for indexing using the standard Illumina index read (not possible using the reverse adaptors from Poland *et al.* (2012)), but we added the variable length barcodes to these adaptors to improve the quality of the reverse sequencing reads. We used PstI and MspI enzymes to produce the double digest, and then developed a modified protocol based on the three publications above that has minimal bead cleaning steps. The full protocol can be found on protocol exchange, and also at wwww.jameshereward.org/GBS.html. We designed 96 forward adaptors with Golay barcodes and 12 reverse adaptors with MTC barcodes, these 12 reverse barcodes are randomised against the forward barcodes to provide redundancy against the indexing (every individual in a lane has a unique combination of forward and reverse inline barcodes).

Data analysis

We demultiplexed the *genotyping-by-sequencing* (gbs) data using STACKS (Catchen *et al.* 2013). We removed adaptors, and quality-trimmed the end of the sequences of all samples to q10 using bbduk from the bbtools package (version 36) (Bushnell, 2018), with a kmer of 8. We mapped all reads to the sowthistle draft reference genome (see section 1.7.1) using BWA

mem v0.7 (Li & Durbin, 2009). We followed GATK best practices (Van der Auwera *et al.*, 2013) and called variants using HaplotypeCaller (GATK v 4.0.10.1) (McKenna *et al.* 2010).

We filtered single nucleotide polymorphisms (*snp*s) to quality Q30 using vcftools (Danecek *et al.* 2011), then to a minimum depth of 3 and maximum depth of 30. Following this we removed any marker that was missing more than 30% of the data, and then removed any locus with a minor allele count below three (e.g. one homozygote and one heterozygote). All indels and non-biallelic *snp*s were removed leaving only biallelic *snp*s.

We constructed a Principal Components Analysis (PCA) of the *snp* data using the adegenet package (Jombart 2008) in R version 3.5.1 (R core team 2018). To test the correlation between genetic and geographical distance, isolation by distance (IBD) was tested using the adegenet package (Jombart 2008) in R version 3.5.1. Each test was permuted using a Mantel test based on 999 replicates.

Results

We have genotyped 266 individuals and had 3,013 *snpsnp* loci remained after quality filtering. The PCA plot indicated that sowthistle populations are quite well mixed genetically (Fig. 1.6.3.2). The isolation by distance analysis (Fig. 1.6.3.3) revealed a significant relationship between genetic distance and geographic distance, indicating that there is some regional genetic structure. Overall F_{ST} was 0.118, which is lower than the other species, indicating less genetic structure than them. Overall F_{IS} was 0.438, with 0 indicating complete outcrossing and 1 representing full self-fertilisation.

We have collaborated with Chris Preston's group to further assess the population genetics of sowthistle, and his student has visited the lab to learn the *genotyping-by-sequencing* methods. We will add her results to our analysis to further understand how many times resistance has occurred in this species.

Discussion

Our results indicate that there is less genetic structure in sowthistle compared to the other species considered in this report. This is in line with expectations about the dispersal ability of this species. The high dispersal/connectivity of populations has allowed glyphosate resistance to spread rapidly from its initial detection in NSW in 2011 to now be widespread across Queensland. Our results do hint at some regional differences, with a positive isolation by distance slope, so the populations are not a random mix of genetic material.

We find that F_{IS} is lower for sowthistle (0.438) than for other species and this indicates that some level of outcrossing is occurring under natural conditions, although we were not able to detect this experimentally (see section 1.8). Outcrossing is important because it allows a weed species to stack different herbicide mode-of-action resistances more easily. This species is therefore the highest risk for developing resistance to other herbicide modes of action.

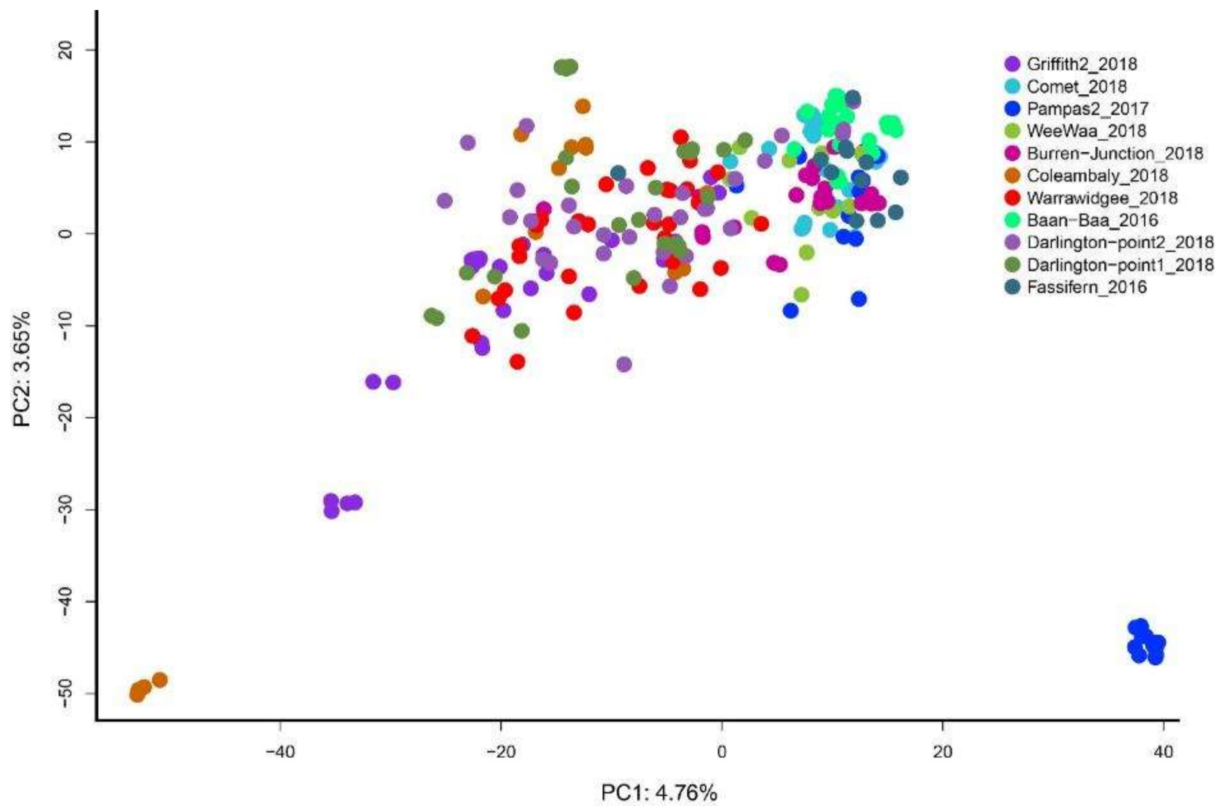

Figure 1.6.3.2 PCA plot of the sowthistle *snpsnp* data. Individuals are clustered by their genetic similarity.

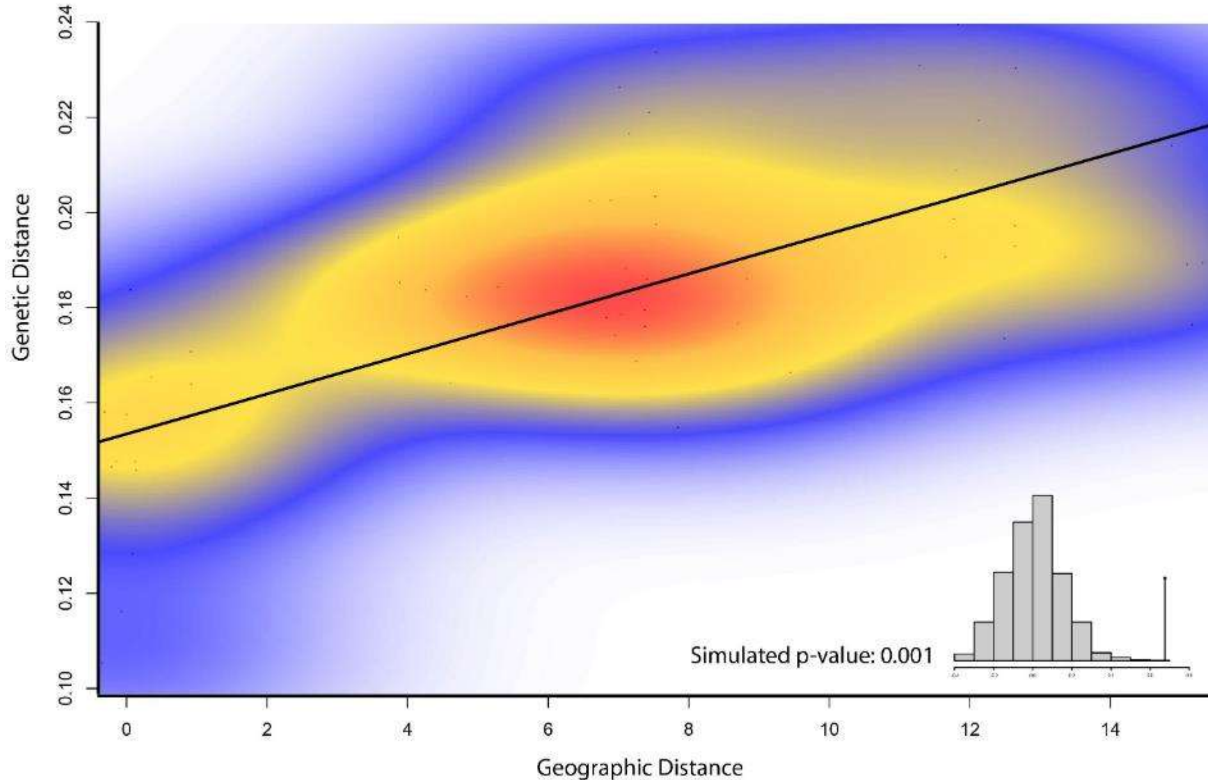

Figure 1.6.3.3 Isolation by distance plot showing a positive relationship between genetic distance and geographic distance.

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1.6.4 Population genetics of Feathertop Rhodes grass

Multiple evolution and spread of glyphosate resistance in Feathertop Rhodes grass *Chloris virgata*

Abstract

A key question for herbicide resistance, and evolution more broadly, is how many times resistance might evolve in one species by new mutations. The time lag between the widespread deployment of a given herbicide and the evolution of resistance would indicate that the evolution of resistance is a rare event. Conversely, the number of weed individuals in any species is very high, increasing the chances of rare mutation events. We tested this by investigating the number of times that glyphosate resistance has evolved in Feathertop Rhodes grass (*Chloris virgata*) by generating target-site gene sequence and genome-wide *snp* data. We find evidence that glyphosate resistance evolved at least 10 times in the samples included in this study through mutations to the target-site gene.

Introduction

The evolution of resistance to pesticides and herbicides provides a series of model systems to investigate the recent, repeated, and well replicated response of organisms to very strong selection. Although initially rare, resistance to the herbicide glyphosate is now widespread with 304 populations of 42 weedy species appearing to have evolved resistance to glyphosate around the world (https://www.abc.net.au/news/rural/2019-12-02/glyphosate-resistance-metabolism-based-found-in-barnyard-plant/11740574). Despite our good mechanistic understanding of the various ways that plants can evolve resistance to glyphosate, key questions about the number of times resistance evolves in each species and how it spreads, remain unanswered.

Glyphosate resistance is broadly defined as either "target-site resistance", which occurs through mutations in the *EPSPS* gene, and "non-target site resistance", which is every other way that resistance evolves. The *EPSPS* gene is the target for glyphosate and is part of the shikimate pathway that produces aromatic amino acids. There are very few mutations in the *EPSPS* gene that confer resistance to glyphosate without damaging the ability of the enzyme to process the substrate, polyvinylpyrrolidone ('PVP'). Laboratory enzyme evolution studies have shown how different mutations affect the enzyme kinetics. These lab studies have been broadly mirrored in field-evolved glyphosate resistance. The vast majority of species with evolved target-site glyphosate resistance have a mutation in DNA coding for the Pro106 amino acid (Sammons and Gaines 2014) which confers around 6-fold resistance to glyphosate. The double mutation Threonine101-Ileucine Proline106-Serine (TIPS) had also been detected in field-evolved populations of *Eleusine indica* (Yu *et al.* 2015). This double mutation confers around 180-fold resistance.

Our mechanistic understanding of the evolution of target site resistance to glyphosate might explain why it took a long time for resistance to evolve. Glyphosate has been widely used since the 1970's, but resistance has only become widespread more recently. The functional constraints on *EPSPS* binding mean that only a few, very specific, mutations can confer resistance without negatively affecting the survival of the plant. For a while, Monsanto claimed that it was not possible for weeds to evolve resistance to glyphosate. This claim was biologically implausible, but had some support from the situation in the field for several

decades. Eventually, through continued widespread use of this one herbicide, and sufficient numbers of weeds being produced and exposed to it, these rare mutations had a chance to occur and spread.

Does this mean that, within a species, target site resistance is likely to evolve only once and then spread? Or has enough time and exposure passed that multiple different target site mutations are likely to occur within a species across its distribution. This question is of direct relevance to the management of glyphosate resistance. If resistance is rare and only evolves once in a species and then spreads – there is a possibility for targeted management and containment when resistance first occurs. The question is also relevant to our understanding of evolution more broadly – the role of new mutations *vs* standing variation and the possibility of parallel evolution in response to a strong selection pressure.

Feathertop Rhodes (*Chloris virgata*) is a serious agricultural weed in Australian cropping systems because it is hard to control with many herbicides. It has always been considered "tolerant" of glyphosate, but could be previously controlled with higher rates. A risk assessment framework based on biological characteristics and farming practices identified *C. virgata* as a high risk weed for evolving glyphosate resistance (Werth *et al.* 2013). In 2015, evolved target-site resistance was detected (Ngo *et al.* 2015). Populations possessing a Pro-106-Ser mutation had 2.4-2.6-fold resistance to glyphosate, and populations possessing a Pro-106-Leu mutation had 8.7-9.7-fold resistance. The nucleotide substitutions responsible for these amino acid substitutions indicate that target-site resistance has evolved at least twice in Feathertop Rhodes (Ngo *et al.* 2015), but this was based on four resistant populations.

The goal of the present study was to combine population genetics, target-site (*EPSPS*) sequencing and resistance screening of multiple field-collected populations of Feathertop Rhodes to determine how many times glyphosate resistance has evolved, and how it spreads across agroecosystems.

Materials and Methods

Plant sampling and DNA extraction.

Field surveys were conducted between 2016 and 2018 across the east coast of Australia, both roadside and on-farm populations were targeted (Fig. 1.6.4.1). At each site 30 individuals were sampled by taking leaf samples into silica gel, taking care to make sure that plants were separate and not vegetatively propagated through tillers by sampling only individuals separated by 1m. A representative bulk seed sample was also collected.

DNA was extracted using a combination of CTAB lysis and 'DIY spin-column purification' (Ridley *et al.* 2016). Around 2-3cm of dried leaf was used for each extraction. Details of the extraction protocol can be found and online at www.jameshereward.org/CTABspincolumn.html. DNA was measured usingp PicoGreen™ (Molecular Probes, Eugene, OR) and normalised to 200ng in 40 ul.

Figure 1.6.4.1 Sampling sites for the Feathertop Rhodes populations used in this study.

EPSPS sequencing

We generated 10GB of Illumina 125bp paired-end sequence data for one individual and reassembled the complete *EPSPS* gene and ~ 6,000 bp of surrounding sequence by mapping to the *Eleusine indica* reference with extension by mapping in Geneious (https://www.geneious.com). This was annotated based on alignment to other full length *EPSPS* sequences and mRNA sequences from NCBI Genbank. We then designed primers, *EPSPS*_TS_Fwd and *EPSPS*_TS_Rev that target ~350bp around the known target site mutations and Pro106 and Thr XXX. We sequenced all 920 individuals with this primer pair. All primers were designed using primer3 (Untegrasser *et al.* 2012), implemented in Geneious (Kearse *et al.* xx, or (https://www.geneious.com)).

All sequencing reactions were conducted in 25ul reactions containing 2µl of DNA, 1X MyTaq buffer, 0.2µM of each primer and 0.5 units of MyTaq (Bioline, London, UK). Cycling conditions were: 40 cycles of 10s at 95°C, 30s at 52°C, and 30s at 72°C. PCR products were cleaned with Exo-AP, and sequenced in both directions by Macrogen (Seoul, South Korea), using the same primers as for PCR amplification. Sequences were edited in CodonCode aligner (CodonCode Corp, Centreville, MA USA). and aligned in Geneious. Haplotype networks were made using the TCS algorithm (Clement 2002) in PopART version 1.7 (http://popart.otago.ac.nz/).

Genotyping by sequencing

We developed a *genotyping-by-sequencing* method based on Elshire *et al.* (2011), Poland *et al.* (2012), and Petersen *et al.* (2012), but using the Golay codes published by Caporaso *et al.* (2012). Briefly, we combined the forward adaptors from Poland *et al.* (2012) with the 12bp Golay barcodes of Caporaso *et al.* (2012) to add further stringency to the barcode recovery. We then used the reverse adaptors from Petersen *et al.* (2012), which allow for indexing using the standard Illumina index read (not possible using the reverse adaptors from Poland *et al.* (2012)), but we added the variable length barcodes to these adaptors to improve the quality of the reverse sequencing reads. We used PstI and MspI enzymes to produce the double digest, and then developed a modified protocol based on the three publications above that has minimal bead cleaning steps. The full protocol can be found on protocol exchange, and also at www.jameshereward.org/gbs.html. The adaptor sequences are provided in supplementary XXX, and the plate layouts used are found in XXX. We designed 96 forward adaptors with Golay barcodes and 12 reverse adaptors with MTC barcodes, these 12 reverse barcodes are randomised against the forward barcodes to provide redundancy against the indexing (every individual in a lane has a unique combination of forward and reverse inline barcodes).

Resistance screening

Seeds of 39 *C. virgata* populations were germinated in 0.55L square pots (Masrac, Dry Creek, SA, Australia) containing standard potting mix (Boutsalis, Gill and Preston 2012). At the one-leaf stage, seedlings were transplanted into punnet pot trays (5x5x5 cm punnets, 20 punnets per tray). Each population was transplanted into one punnet tray, with two seedlings per punnet.

Plants were treated with 1080 g a.i h-1 Glyphosate (Glyphosate 540; Nufarm, Laverton North, Vic, Australia) at the 4–5-leaf stage. Nonionic surfactant (Wetter TX®, Nufarm) was added to the spray solution at 0.2% (v/v). Glyphosate was applied using a laboratory moving boom pesticide applicator and applied at an equivalent of 109 L ha−1 of water at a pressure of 250 kPa and a speed of 1 m s−1 using Tee-Jet 001 nozzles (Tee-Jet 8001E; Spraying Systems Co., Wheaton, IL). Survival was assessed 21 days after herbicide treatment.

Results

Evolution and spread of resistance

We capillary-sequenced 905 individuals for the *EPSPS* gene, with 320bp of sequence remaining after quality trimming. These sequences represented 12 different haplotypes (Fig. 1.6.4.2A), four of which were for the susceptible amino acid 106Pro, the other eight for known resistance mutations of this gene. We then generated genome-wide *snp* data and plotted a principal component analysis (PCA) using the specific *EPSPS* haplotypes to colour each individual (Fig. 1.6.4.2B), and a *tsne* plot, clustering the same *snp* data (Fig. 1.6.4.2C). These clustering analyses indicate that the Seriene5 *EPSPS* haplotype is present on three different genomic backgrounds and likely to have evolved three times. Together, these data indicate that glyphosate resistance evolved at least 10 times in the populations that we sampled.

Ploidy and reproduction

Our analyses of the whole-genome deep sequencing data indicate the *C. virgata* is a diploid, as was expected, has very low heterozygosity, and that the *EPSPS* gene is only present as a single copy. The *genotyping-by-sequencing* data indicate that F_{IS} is very high in this species

(0.905), and there is little evidence for outcrossing, although there is some admixture between the two distinct clusters in the PCA.

Geographic population structure

As Feathertop Rhodes grass is an invasive species in Australia (introduced for fodder), and self-fertilises, it was expected that there would be little geographic structure. Instead we found two genetically distinct clusters with a high degree of separation in the PCA plots (Fig 1.6.4.2). These loosely correlate to the geographic regions of Queensland and New South Wales. Overall F_{ST} was very high at 0.834, indicating strong genetic structure.

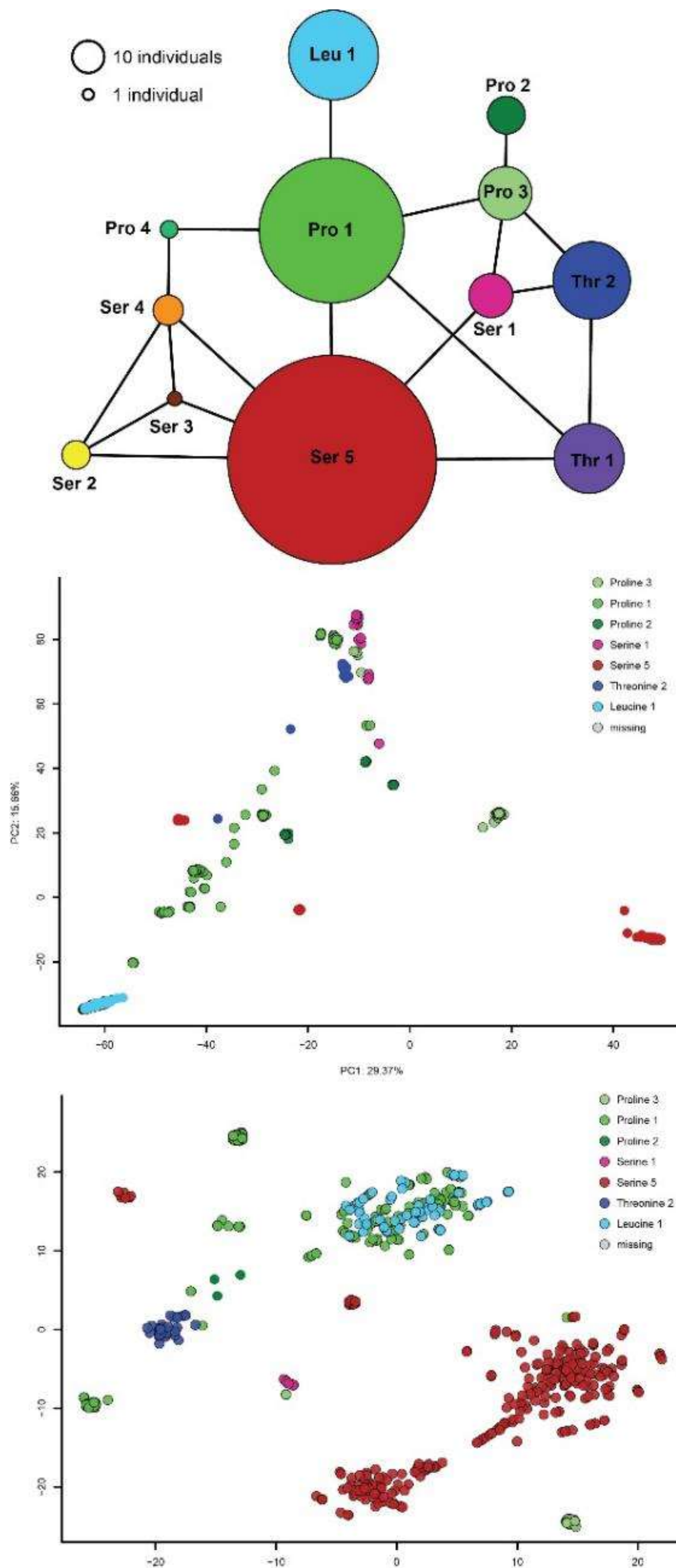

Figure 1.6.4.2 Haplotype network of the *EPSPS* sequence (top), a PCA of the *gbs* data with the *EPSPS* haplotype used to colour the points (middle) and a *tsne* plot coloured the same way (bottom).

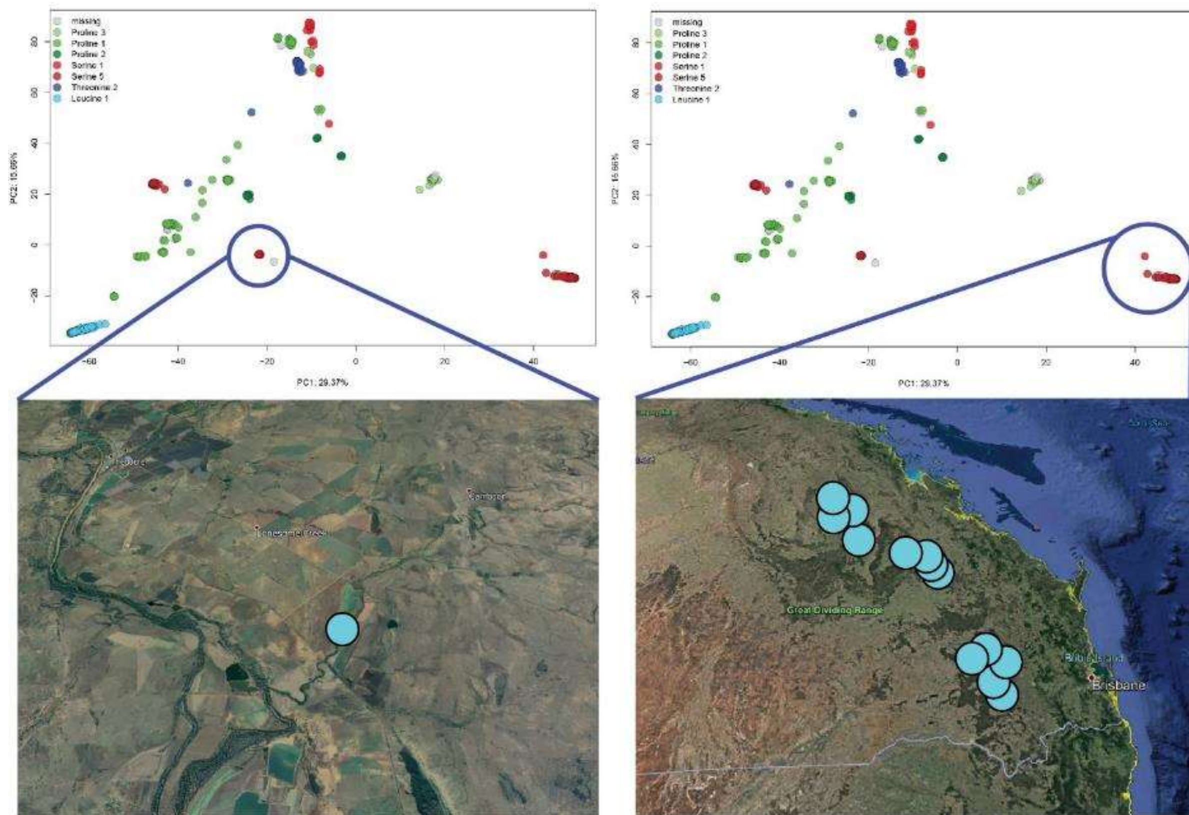

Figure 1.6.4.3 Spread of evolved glyphosate resistance, we show the location of one cluster of resistant individuals (left) that is restricted to one geographic site, and another that is present across a much larger area (left).

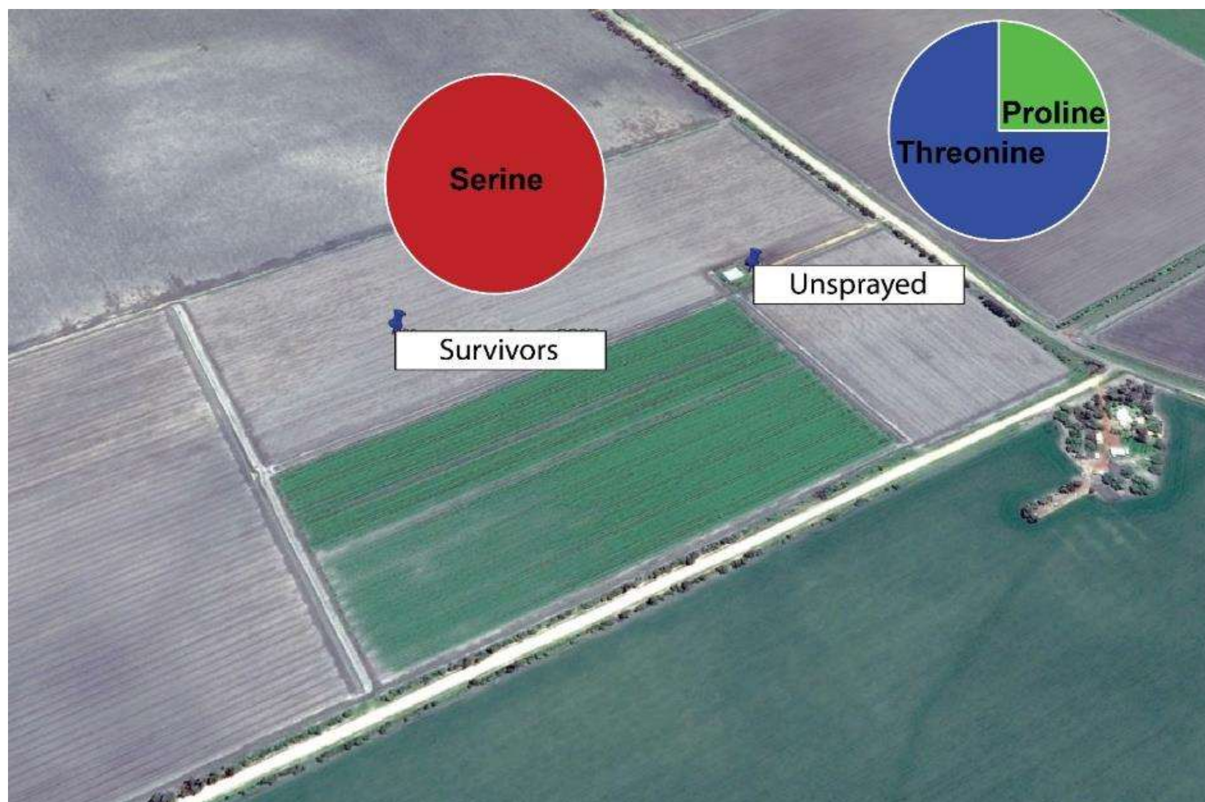

Figure 1.6.4.4 *EPSPS* sequence data for weeds that had survived in a sprayed field (left), where 24 individuals had the strong-resistance Serine mutation, and unsprayed individuals near the shed (right), 18 of these had the weakly resistant Threonine mutation and six of them had the susceptible amino acid, Proline.

Discussion

Feathertop Rhodes grass is diploid and this allows it to develop target site resistance more easily than species that are polyploid. Our study shows that target site glyphosate resistance has evolved over 10 times in this species. Feathertop Rhodes shows very little evidence of outcrossing, although there is some evidence for admixture between the two genetic clusters. These two genetic clusters likely represent two separate invasions of this species into Australia. This allows us to track the spread of resistance in this species and we can see some cases of evolved resistance that are restricted to one site, and some that have spread much further (Fig. 1.6.4.3).

We also find evidence of the active evolution of resistance on-farm in response to the strong selection pressure applied when herbicides are sprayed. One property still had susceptible populations in parts of the farm that were not sprayed with herbicide, but the survivors in the field had a strongly resistant gene mutation (Fig. 1.6.4.4). These individuals all represent an opportunity for glyphosate resistance to further spread throughout the landscape, and this highlights the importance of survivor control in preventing the evolution and spread of glyphosate resistance.

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1.7 - Determine the mechanisms of glyphosate resistance in key weed species

At the completion of this project we have established the glyphosate resistance mechanisms of three key weed species: fleabane, windmill grass, and Feathertop Rhodes. Sowthistle was initially investigated but work was continuing in Chris Prestons group in Adelaide, so this species was put on hold. We present results relevant to the resistance mechanism in Sowthistle from the genome sequencing project (1.7.1). We published the chloroplast genomes for all species studied as a resource for further work on herbicide resistance mechanisms in these species (1.7.4).

Key Findings:

Sowthistle was not investigated heavily as there was ongoing work in Chris Preston's group. We sequenced the genome of Sowthistle (1.7.1) and this revealed four copies of the *EPSPS* gene – two as a result of allotetraploidy, and two seem to be remnants of an ancient genome duplication that is shared with lettuce. Having four copies of the *EPSPS* gene will make it more difficult for this species to develop target site resistance and we believe that the mechanism will be an NTSR one. Work is ongoing in Chris Preston's group on the mechanism. His group has discovered that resistance is activated by temperature, and we included these lines in our experiment in milestone 1.9. The results of that experiment may shed further light on the resistance mechanism.

Fleabane naturally has nine copies of the *EPSPS* gene, three due to polyploidy and three due to ancient gene duplications (millions of years ago). This was established through genome sequencing (1.7.2) and a transcriptome experiment (1.7.3). This makes it hard for Fleabane to get target site resistance because several copies of the gene need to have a mutation at the same time. We found resistance-causing mutations in some copies of the gene, but these individuals had a susceptible phenotype confirming the dose-dependence of the *EPSPS* target site mutations. We conducted a transcriptome study (1.7.2) to assess this NTSR mechanism and find some support for a sequestration resistance mechanism.

Windmill grass resistance mechanisms were investigated through the work of Masters student Yu Shen. Details are provided in previous section 1.6.1. It had been established that *EPSPS* amplification was the resistance mechanism, so we screened populations for the copy number of the gene, and combined this with population genetics data to see how many times this evolved. There were two expression levels, medium and high, and each of these evolved twice. We also sequenced over 800 individuals for the *EPSPS* gene and five individuals from one population had a resistance-causing mutation. This population also had gene amplification, so there are two different resistance mechanisms in this population.

Feathertop Rhodes grass is diploid and had previously been shown to have a target site mutation. We investigated whether this is the only mechanism in this species and how many times it evolved (1.6.4). Populations were screened for the *EPSPS* gene, for their resistance to glyphosate, and they were genotyped at around 2000 *snp* markers. We found that all populations that survived glyphosate had the *EPSPS* mutation, and we find evidence that these mutations evolved at least 10 times across the cropping regions of Eastern Australia. We also found one property where the survivors in the field all had a resistance mutation, but the unsprayed population around the shed were all susceptible at the *EPSPS* sequence.

In conclusion, we learned through these experiments that glyphosate resistance mutations are dose-dependent, and this makes it much harder for polyploids to get target site resistance. The diploid Feathertop Rhodes evolved target site resistance over 10 times, but this has not occurred in Fleabane or Sowthistle once.

1.7.1 Sowthistle genome sequencing

In this project we developed a draft genome for sowthistle, we selected one resistant line and grew seeds. We extracted DNA using the CTAB/Spin column method and sequenced a full lane yielding 30Gb of 125bp paired-end sequence data. We also had mate-pair libraries constructed that sequence 125bp from each end of a 2,000bp and a 5,000bp fragment of DNA to help scaffolding.

Initial assembly was performed with Meraculous (Chapman *et al.* 2011) and this produced a good first draft of the genome, with 800mb represented in the 86,037 contigs. We used this assembly to assess the ploidy and the number of copies of *EPSPS*. We found evidence supporting the allotetraploid nature of Sowthistle, and we also found evidence for two more copies of the *EPSPS* gene. These two additional copies are very divergent and likely remain from the ancient genome duplication likely shared with the *lactuca* (lettuces, Reyes-ChinWo *et al.* 2017). These sequence data were provided to Chris Preston's group to further support their work on the mechanisms of resistance in sowthistle, and ALS sequences have also been provided.

The sowthistle genome has also been useful in the analysis of the sowthistle *genotyping-by-sequencing* data. It is hard to analyse this type of data in polyploid weeds, but the availability of the genome means that we can separate data from each of the two sub-genomes and then analyse it in the same way as a diploid organism.

The assembly is currently undergoing improvement, with Dovetail Hi-C and Chicago libraries being constructed (this was delayed due to trouble germinating the fleabane line). This scaffolding process will make the genome more complete (it will be contained in less fragments). The genome will then be annotated using the sowthistle transcriptome data we have.

The complete sowthistle genome will be made freely available on genbank to further research into resistance mechanisms in this species.

References

Chapman, JA, Ho, I, Sunkara, S, Luo, S, Schroth, GP & Dl S. Rokhsar. 2011. Meraculous: De Novo Genome Assembly with Short Paired-End Reads. PlosONE. 6(8) e23501.

Reyes-Chin-Wo, S, Wang, Z, Yang, X, Kozik, A, Arikit, S, Song, C, Xia, L, Froenicke, L, O. Lavelle, D, Truco, MJ, Xia, R, Zhu, S, Xu, C, Xu, H, Xu, X, Cox, K, Korf, I, Meyers, BC & RW Michelmore. 2017. Genome assembly with in vitro proximity ligation data and whole-genome triplication in lettuce. Nature Communications. 8, 14953.

1.7.2 Fleabane genome sequencing

In this project we developed a draft genome for fleabane. We conducted initial short-read sequencing on the highly resistant line Q17. We extracted DNA using the CTAB/Spin column method and sequenced a full lane yielding 24Gb of 125bp paired-end sequence data. We also had mate-pair libraries constructed that sequence 125bp from each end of a 5,000bp fragment of DNA to help scaffolding.

Initial assembly was performed with Meraculous (Chapman *et al.* 2011) and this produced a good first draft of the genome, with 1.14Gb represented in the 340,043 contigs. The assembly confirmed that fleabane is an allohexaploid, and one of the genomes is more divergent than the other two are. We find evidence in the genome assembly for three copies of the *EPSPS* gene in each of these three sub-genomes for a total of nine copies of the *EPSPS* gene. This makes it difficult for fleabane to develop target site resistance (see section 1.7.3).

The assembly is currently undergoing improvement, with Dovetail Hi-C and Chicago libraries being constructed. This was delayed due to trouble germinating the fleabane line Q17, despite repeated attempts. The transcriptome study (1.7.3) indicated that the genotype of Q42 was very close to that of Q17, so we germinated this line and have submitted it successfully. This scaffolding process will make the genome more complete (it will be contained in less fragments). The genome will then be annotated using the transcriptome data we have.

References

Chapman, JA, Ho, I, Sunkara, S, Luo, S, Schroth, GP & Dl S. Rokhsar. 2011. Meraculous: De Novo Genome Assembly with Short Paired-End Reads. PlosONE. 6(8) e23501.

1.7.3 Gene expression in response to glyphosate treatment in fleabane (*Conyza bonariensis*) – glyphosate death response and candidate resistance genes.

Abstract

BACKGROUND:

This study takes a whole-transcriptome approach to assess gene expression changes in response to glyphosate treatment in glyphosate-resistant fleabane. We assessed gene expression changes in both susceptible and resistant lines so that the glyphosate death response could be quantified, and constitutively expressed candidate resistance genes identified. There are three copies of the glyphosate target site (5-enolpyruvylshikimate-3-phosphate; *EPSPS*) gene in *Conyza* and as *Conyza bonariensis* is allohexaploid there is a baseline 18 copies of the gene in any individual.

RESULTS:

Many genes were differentially expressed in response to glyphosate treatment. Known resistance mutations are present in *EPSPS*2 but they are present in a glyphosate susceptible line as well as resistant lines and therefore not sufficient to confer resistance. *EPSPS*1 is expressed four times more than *EPSPS*2, further reducing the overall contribution of these mutations.

CONCLUSION:

We demonstrate that glyphosate resistance in *Conyza bonariensis* is not the result of *EPSPS* mutations or overexpression, but due to a non-target-site mechanism. A large number of genes are affected by glyphosate treatment. We present a list of candidate non-target-site-resistance (NTSR) genes in fleabane for future studies into these mechanisms.

Keywords: Fleabane, glyphosate, resistance, *EPSPS*, gene expression, transcriptome

1. Introduction

Conyza bonariensis (L.) Cronquist (Asteraceae), commonly known as flaxleaf fleabane, is a serious agricultural weed in Australia. Eastern Australian populations were detected with glyphosate resistance in 2006 (1), and resistance is now widespread (2). This weed has evolved glyphosate resistance in at least nine different countries (3), presumably representing at least nine different evolutionary origins of this trait. Glyphosate resistance is well studied (4, 5), and is generally categorised into target site or non-target site resistance mechanisms. The target site of glyphosate is 5-enolpyruvylshikimate-3-phosphate (*EPSPS*), which normally catalyses the reaction converting phosphoenolpyruvate (PEP) and 3-phospho-shikimate into phosphate and 5-enolpyruvylshikimate-3-phosphate. Few mutations that cause glyphosate resistance have been found in natural weed populations because most mutations that might confer resistance also reduce the affinity of the enzyme for its substrate (making them lethal). The most commonly observed resistance mutation occurs at position 106 (5), but a double mutation that provides much greater resistance has also been reported (6, 7). Target site amplification is another common glyphosate-resistance mechanism. Amplification is achieved through either the overexpression of a single copy of the gene or by means of genomic gene-copy increase, with the latter presumably through the action of transposable elements (although the actual mechanism has yet to be identified) (5, 8-12). All other resistance mechanisms are referred to as non-target-site-resistance (NTSR) mechanisms (13) and these include: reduced translocation of the chemical, subcellular compartmentalisation (vacuolar sequestration), rapid leaf necrosis, reduced absorption, glyphosate metabolism and potentially other unknown mechanisms (5).

Several species in the genus *Conyza* have evolved resistance to glyphosate, with *C. bonariensis* and *Conyza canadensis* the best studied of these. Based on the literature, different populations of *C. bonariensis* and *C. canadensis* appear to have repeatedly evolved a similar subcellular compartmentalisation resistance mechanism, but the molecular basis for this is somewhat contentious and unresolved.

Analysis of the sequence and expression of the *EPSPS* gene from populations of both species around the world has rarely found evidence for target site resistance. Early studies on resistant *C. canadensis* populations from the USA found no resistance mutations or expression differences in the three copies of the *EPSPS* gene (14). Glyphosate resistant *C. bonariensis* populations from Israel did not have any *EPSPS* mutations (15). Greek populations of glyphosate resistant *C. bonariensis* also had no resistance-causing mutations (16). Resistant populations of *C. canadensis* from Crete showed no expression differences for the *EPSPS* gene (17), whereas populations from mainland Greece showed 2-4 fold over-expression of the *EPSPS* gene following glyphosate treatment. Conversely, *EPSPS* expression was found to be stable in resistant and susceptible American populations of both *C. canadensis* and *C. bonariensis* (18). Basal *EPSPS* expression was, however, found to be higher in some resistant populations from Spain (19). Measuring the expression of *EPSPS* in these two species by quantitative PCR is somewhat complicated by the multiple copies of the gene (14). Most authors choose primer sequences that attempt to capture the expression of all three copies (to measure total *EPSPS* transcript production) (17, 18), but some studies only design primers based on one of the copies (19).

Experiments with nuclear magnetic resonance (NMR) in *C. canadensis* indicated that glyphosate resistance is due to vacuolar sequestration (20, 21). Radio-labelled (^{14}C) glyphosate and shikimate assay experiments indicate that resistant lines of *C. bonariensis* from Israel accumulate shikimate in the leaves and that the resistance mechanism is likely modified subcellular distribution of the herbicide (15) which could possibly be due to vacuolar or apoplast sequestration. Populations from Spain have been examined and reduced translocation of the herbicide out of the leaves was observed (19). These authors also

hypothesised that the reduced translocation observed is the result of a different, primary mechanism of cytoplasm exclusion (i.e. vacuolar or apoplast sequestration). Resistant lines of *C. bonariensis* from California show reduced translocation of the herbicide out of the leaf (18). There are also reports that Spanish populations of *C. canadensis* have reduced glyphosate mobility within the plant and can also metabolise glyphosate (22).

A de-novo transcriptome assembly has been produced for *C. canadensis* from 454 data to investigate the sequestration resistance mechanism (23). The expression levels of 17 ABC transporter genes in susceptible and resistant American lines were tested using real-time PCR (23). Two of these transporter genes (M10 and M11) were found to go from very low expression to quite high expression following treatment with glyphosate and the resistant lines expressed slightly more transcripts of these two transporters than the susceptible lines, implicating these two genes in the resistance mechanism in *C. canadensis*. Subsequent real-time PCR experiments on Greek populations seemed to support the role of these two transporter genes in resistance (17, 24, 25). Recently, the expression of M10 and M11 in American populations of both species has been tested; these transcripts were up-regulated in response to both glyphosate and paraquat, and a similar response was seen across lines, regardless of their sensitivity to the herbicide. These latter authors therefore conclude that these two ABC transporters are part of the stress response induced by the herbicide rather than a resistance mechanism.

Polyploidy has been posited to increase evolutionary potential by masking deleterious mutations, and by allowing duplicated gene copies to evolve new or slightly different functions (26, 27). In the case of herbicide resistance, however, polyploidy decreases the effect of each resistance mutation in a target site gene copy on the expressed protein phenotype (assuming all gene copies are expressed). This has been shown in glyphosate resistant populations of (hexaploid) *Echinochloa colona* in Australia, where a single mutation in the *EPSPS* gene is not sufficient to provide resistance at field rates of the herbicide (28). This gene dosage effect and dilution of target-site mutations in polyploids could favour non-target site mechanisms over target site mechanisms in the evolution of herbicide resistance in polyploid weeds. The genus *Conyza* is ploidy variable, with *C. canadensis* being a diploid with $2n = 2x = 18$, and *C. bonariensis* being allohexaploid with a chromosome count of $2n = 6x = 54$ (29-31). The copy number of the *EPSPS* gene is further complicated by there being three copies in the diploid *C. canadensis* (14, 17).

This experiment was established to explore the gene expression changes induced by glyphosate treatment in susceptible and resistant lines of Australian *C. bonariensis*. The aim of the experiment was to assess the expression levels and sequence of the multiple *EPSPS* copies, survey the transcriptomic response to glyphosate death induced stress, and potentially identify candidate NTSR resistance genes.

2. Materials and Methods

2.1 Transcriptome experiment

Four lines of fleabane from Queensland were selected for this experiment. These lines have been confirmed resistant / susceptible (1) and details of their resistance status and collection localities can be found in that paper. Since 2006 these lines have been maintained at the Leslie Research facility (Queensland Government, Toowoomba) by growing plants and collecting seed (as fleabane seeds have low viability after only a few years). Two resistant lines GR1 (previously Q17) GR2 (Q42), and two susceptible GS1 (Q21) and GS2 (Q34) were used in this experiment. Seeds were grown under controlled environmental conditions (25°C daily maximum) and artificial lighting. For each line, 9 plants were selected for the experiment (total 40 plants). At about the 8-leaf rosette stage total RNA was collected by

homogenising a single leaf into TriZol reagent (Life Technologies), plants were then immediately sprayed with glyphosate at a rate equivalent to 540 g acid equivalent/ha. Forty-eight hours after glyphosate treatment a second total RNA collection was made by homogenising a second leaf into TriZol. Plants were monitored for survival, and three replicates selected per line for transcriptome sequencing (both the pre-glyphosate and post-glyphosate treatments). Total RNA was subjected to DNAse treatment, and messenger RNA (mRNA) was isolated from the total RNA using a magnetic bead mRNA isolation kit (New England Biolabs). The 24 mRNA samples were then prepared for Illumina sequencing with the NEBNext Ultra Directional RNA kit (New England Biolabs), with each sample being given a unique index. All 24 sequence libraries were sequenced as a single pool by Novogene (China) across three lanes of a Hiseq2500 (Illumina) producing 125bp paired end reads.

Sequence quality was checked with FastQC (32), and screened for contaminants with Fastqscreen (33). Raw data were trimmed using Trimmomatic v0.35 (34) using aggressive adaptor clipping (option: ILLUMINACLIP 2:40:15) and relatively gentle quality trimming (option: LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15). For differential expression analysis it is important that all treatments are included in the transcriptome assembly (35, 36). Initial assemblies using all of the sequence data (all samples and all treatments) were fragmented. Analysis of this initial assembly (variant detection using Freebayes – see below) showed that the susceptible and resistant lines were quite different, but all resistant individuals were very similar as were all susceptible. The assembly was therefore conducted on individual GR1-1 and GS1-4 using data from both pre and post glyphosate treatment. Sequence data from these individuals was pooled, error correction was performed using tadpole from the BBmap suite (37), then *de novo* transcriptome assembly was constructed using Trinity v.2.4.0 (38, 39) with transcripts under 300bp discarded during assembly. Completeness of the assemblies was assessed by mapping to the three *EPSPS* genes (see next section), mapping to the complete annotated chloroplast genome of *C. bonariensis* (40), and assessment of contig lengths using the TinityStats script provided with Trinity (38, 39). The assembly was functionally annotated using trinotate (39).

Genetic variants were detected to assess the relationship of the samples and lines as revealed by their genotype. Quality trimmed reads were mapped to the isoform level reference assembly using BWA-mem (41). All sequence data for each individual (Pre and post treatment samples) were merged, readgroups were added to these merged SAM files, which were then sorted and compressed to BAM files with Samtools (42). The isoform level assmbly had mostly assembled the subgenomes independantly (see Results). Variants were therefore detected using Freebayes (43) with diploid settings. The resulting vcf files were filtered for quality (minq 40), with vcftools (44), indels were removed, as was any *snp* (Single Nucleotide Polymorphism) with a minor allele frequency below 0.3 (to reduce the number of *snp*S called in transcripts that represented more than one subgenome) and any variant that had missing data. This vcf file was then read into a Genind object in R (45) using a custom R script and PCA analysis performed using Adegenet (46).

2.2 EPSPS analysis

The *EPSPS* gene sequences for all three copies from *C. canadensis* (*EPSPS*1 = FR872820, *EPSPS*2 = FR872821, *EPSPS*3 = AY545668) and *C. bonariensis* (*EPSPS*1 = EF200070, *EPSPS*2 = EF200069, *EPSPS*3 = EF200074) were downloaded from GenBank. The Trinity assembly was mapped to each of these in Geneious (47), to identify *EPSPS* contigs, and also check the completeness of the assemblies. The final assembly had put each of the three *EPSPS* copies together independently, and each represented by at least one full-length transcript, but with some smaller isoforms within the trinity “gene” cluster. The trinity *EPSPS* contigs were removed from the trinity assembly, merged into a single full-length sequence for each gene, and then re-inserted into the reference transcriptome assembly.

Sequences from each sample (pre and post pooled) were then mapped to each of the three Trinity-assembled *C. bonariensis EPSPS* copies in Geneious using a custom sensitivity that only allowed three mismatches per read. Being allohexaploid there are six copies of each *EPSPS* gene in a *C. bonariensis* individual. We reconstructed the majority consensus for each of the three copies but were not able to resolve them further to separate genomes. Variants were detected using the Geneious variant caller using a minimum variant frequency of 0.02, and the Pro106 and Thr102 variants were tallied for each individual and each *EPSPS* copy. Expression values (RPKM = Reads Per Kilobase of transcript per Million mapped reads) values for each individual and each *EPSPS* copy were extracted from the BBmap outputs (see next section for details) and plotted using Graphpad Prism version 7.00, GraphPad Software, La Jolla, California, USA (www.graphpad.com).

The *C. bonariensis EPSPS*1 sequence generated in this study was used in a blast search and the top 100 hits were downloaded from GenBank. These sequences were aligned using MAFFT (48) and an initial neighbour joining tree produced to assess the phylogenetic position of the different *EPSPS* copies (Supplementary data S1). All *Conyza, Erigeron* and *Helianthus* sequences (including those from the current study) were then aligned using the Geneious aligner with *Daucus carota* (XM_017380930) as the root. The most appropriate substitution model was determined to be GTR+I+G using jmodeltest (49), and this model was used to produce a Bayesian phylogeny with mrBayes (50) deploying 1,000,000 iterations following a burn-in of 100,000 iterations.

2.3 Differential Expression analysis and candidate NTSR resistance genes

Quality-trimmed reads were mapped against the reference transcriptome using BBmap (37) with transcriptome specific settings (i.e. ambig=random, see supplementary data S2), and read counts were extracted for the gene-level (rather than isoform level) transcripts using a custom perl script.

Differential gene expression was tested using the DESeq2 (51) package in R (45), which uses empirical Bayes shrinkage for dispersion estimation. Differential expression (DE) was tested within each line in a post-glyphosate to pre-glyphosate comparison, the false discovery rate was set at 0.05. Lists of all DE genes (with an adjusted-p threshold of 0.05) were then exported for each comparison. MA plots were produced within DESeq2. The DE genelists were filtered to remove any genes that had an expression < logfold 2 and > logfold -2. The genes that are differentially expressed in the susceptible lines in response to glyphosate represent the glyphosate death response. A single non-redundant list was made of all the differentially expressed (DE) genes in lines GS1 and GS2. A non-redundant list of all genes DE in response to glyphosate treatment in GR1 and GR2 was also constructed. Any gene present in the glyphosate death response gene list was then subtracted from the resistant lines′ DE list to give a candidate NTSR resistance gene-list. These sequences were blastx searched to infer function. The PCA of count data indicated that there were significant differences between the susceptible and resistant lines prior to glyphosate treatment, we therefore pooled pre-glyphosate GS1 with GS2 to make treatment "susceptible pre", and pre-glyphosate GR1 with GR2 to make treatment "Resistant pre". Differential expression was tested in the comparison "resistant pre" to "susceptible pre" to assess the genes that were constitutively differentially expressed across these lines.

The sequences for the *C. canadensis* ABC transporter genes M10 and M11 were extracted from the literature ((53) supplementary data). The whole transcriptome assembly was mapped to each of these sequences and the RPKM values for each transcript that mapped

closely to M10 (three contigs from our assembly) and M11 (two contigs) were added for each gene, and then plotted using Graphpad.

3. Results & Discussion

3.1 Transcriptome experiment

All individuals from lines GS1 and GS2 died within two weeks following glyphosate treatment, and all from lines GR1 and GR2 survived. An average of 27,059,037 read pairs was returned after quality control and trimming. The assembly produced 279,586 contigs at the isoform level, and 81,545 at the "gene" level. The transcript N50 was 1444 and the mean transcript length was 1103. This assembly is provided as supplementary data (S3) as is the annotation report (S4). The number of genes is approximately that expected for one of the subgenomes; the recently sequenced (diploid) sunflower genome has 52,232 genes (54), and a transcriptome assembly of the polyploid artichoke (*Helianthus tuberosus*) returned 66,322 "gene" level transcripts (55). The isoform level assembly had over three times more transcripts than the number of "genes" reported by Trinity. Manual inspection of transcripts that mapped to *EPSPS* and a couple of other nuclear genes indicated that the isoform level assembly had separated transcripts from at least two of the three subgenomes, and in some cases three.

Initial data exploration and QC using a PCA of the count data indicated a problem with one of the samples; individual GR1-5 showed expression in the pre-glyphosate sample that was similar to the susceptible lines (despite there being considerable differences between the rest of the susceptible and resistant lines). This discrepancy was also evident in the genotype of that sample and assumed to be the result of a labelling error at some stage prior to the sequencing process. This individual was dropped from subsequent analyses.

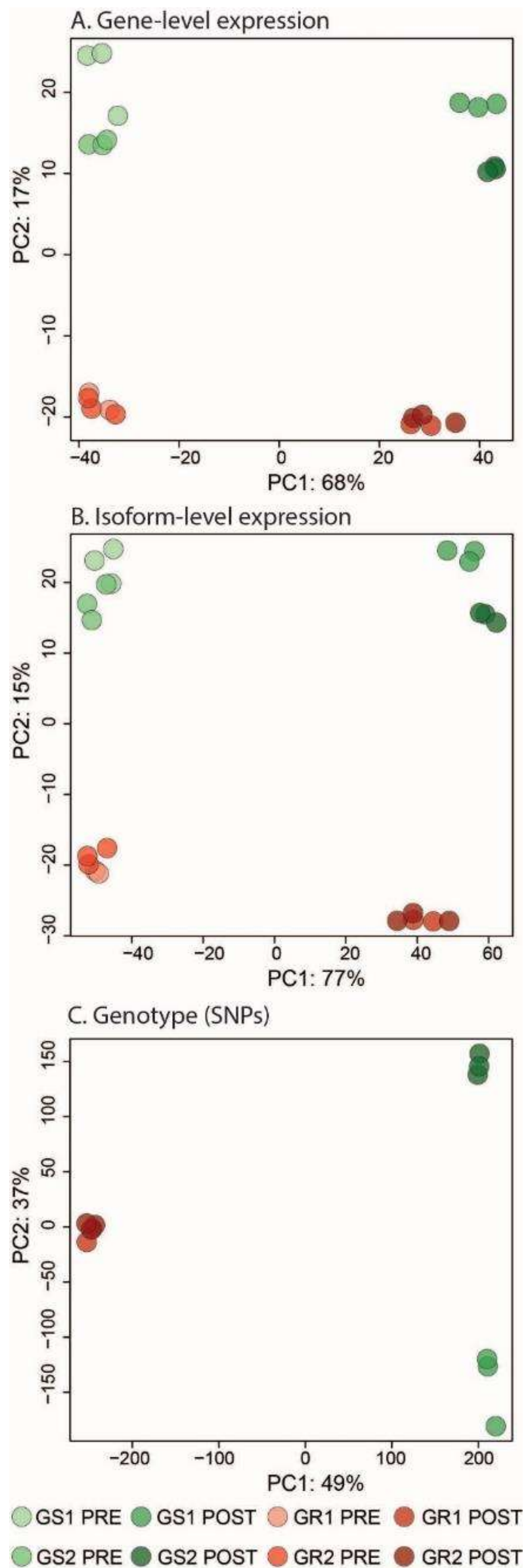

Figure 1.7.3.1. Principal coordinates analysis of the gene-level count data (A), the isoform-level count data (B) and the transcriptome genotype (*snp*s detected with Freebayes) (C). Red indicates glyphosate resistant (GR) lines and green indicates glyphosate susceptible (GS) lines.

Most of the variance (68%) in the PCA of the gene-level count data was explained by axis 1, and this axis separated the pre-glyphosate and post-glyphosate treatments (Fig. 1.7.3.1A). Axis 2 (17% of the variance) separated the resistant and susceptible lines in this analysis showing that there is an inherent gene expression difference across the resistant and susceptible lines even without glyphosate treatment. When the isoform-level counts were analysed (Fig. 1.7.3.1B) the clustering was very similar to the gene level counts, further confirming that the isoforms likely represented different subgenomes rather than line specific assemblies. Freebayes detected 2.1 mio variants but this was reduced to 239,055 *snp*s by the filtering process. This genotype data separated the susceptible and resistant lines across the first axis (49% of the variance), lines GR1 and GR2 have essentially identical genotypes, but susceptible lines GS1 and GS2 were separated by the second axis (37% of the variance, Fig. 1.7.3.1C).

3.2 EPSPS analysis

The initial *EPSPS* phylogeny indicated a duplication of the *EPSPS* gene into *EPSPS*1 and *EPSPS*2 sometime prior to the *Helianthus*/*Conyza* split, but that this duplication is not widespread across angiosperms (Supplementary data S1). The duplication of *EPSPS*2 is much more recent and appears restricted to *Conyza/Erigeron* (Fig. 1.7.3.2) (14). Discrepancy exists in the naming of *EPSPS*2 and *EPSPS*3 across the three *Conyza* species that have sequences available on GenBank (Fig. 1.7.3.2), so we followed the naming of *C. bonariensis* (EF200069-EF200074). Two sequences from *C. sumatrensis,* currently named as *EPSPS*2, are placed within the *C. bonariensis EPSPS*3 clade in our phylogenetic analysis and should probably be renamed as *EPSPS*3. The *C. canadensis* and *C. sumatrensis EPSPS*3 sequences form a clade that is separate to the *C. bonariensis EPSPS*2 and *EPSPS*3 clades (Fig. 1.7.3.2), so there are actually three clades on the *EPSPS*2/*EPSPS*3 branch of the tree. There have therefore been two gene duplications from the ancestral *EPSPS*2 into three different *EPSPS* copies within the *Conyza/Erigeron* group. The gene labelled *EPSPS*3 in *C. canadensis* (AY545668) is non-functional (14), having become a pseudogene, and this sequence is in this separate clade, which we refer to as *EPSPS*_NF (Fig. 1.7.3.2). The branch lengths are longer in this *EPSPS*_NF clade and our phylogeny indicates an earlier divergence compared to the other two, but this is likely a result of a higher mutation rate after functionality was lost rather than a true reflection of evolutionary history. Alignment of our *C. bonariensis EPSPS*2 and *EPSPS*3 sequences with the *C. canadensis* sequences (AY545668 and FR872821) showed that both *C. bonariensis* copies have very similar amino acid sequence to the functional *C. canadensis* copy. The *C. bonariensis* sequences also lack the Q181V mutation that disrupts catalytic activity (14) in *C. canadensis EPSPS*_NF. This indicates that *C. bonariensis* has functional copies of *EPSPS*2 and *EPSPS*3, in contrast to the non-functional clade *EPSPS*_NF.

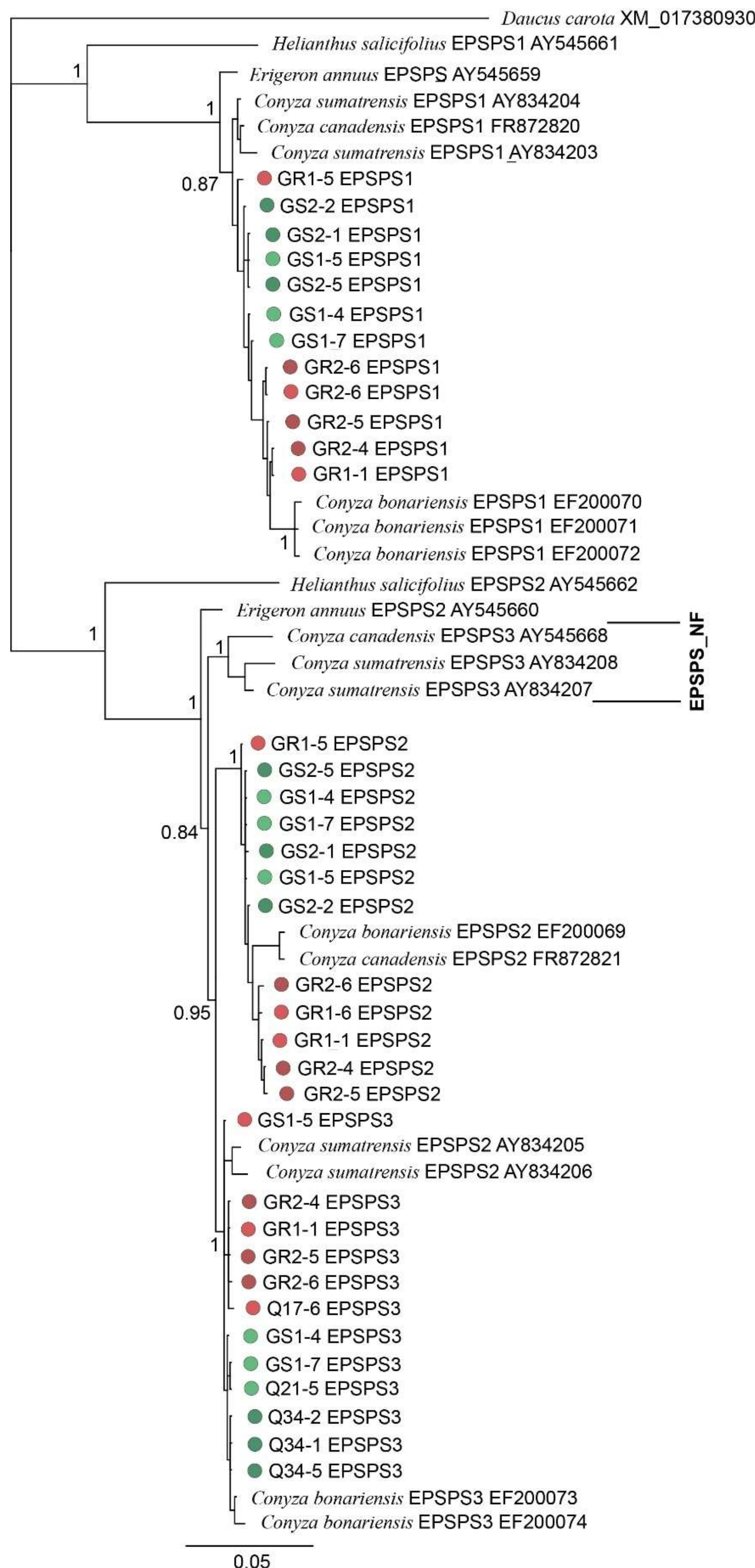

Figure 1.7.3.2. Bayesian phylogeny (1 mio iterations of mrBayes) showing the evolutionary relationships of the *Conyza*, *Erigeron* and *Helianthus EPSPS* gene copies, tree is rooted with carrot. Red indicates resistant lines and green indicates susceptible.

The evolutionary history of the different *EPSPS* copies means that *EPSPS*1 is highly divergent to the other two copies (with about 26% nucleotide difference across *EPSPS*1 and *EPSPS*2). *Conyza bonariensis EPSPS*2 and *EPSPS*3 are much more similar at the sequence level with 7% difference. Reads therefore reliably map to either *EPSPS*1 or *EPSPS*2, but mapping to *EPSPS*2 and *EPSPS*3 is somewhat ambiguous in some sections of the sequences.

Nevertheless, the Thr102 and Pro106 positions were sufficiently different for clear discrimination between them. Thirteen out of 15 individuals possessed the known resistance mutation Pro106Thr in *EPSPS*2 and two individuals have this mutation in *EPSPS*3 (Table 1.7.3.1). None of the *EPSPS* copies in any of the individuals had the Thr102Ile mutation (Table 1.7.3.1). The resistance mutations are present in both susceptible and resistant lines and therefore do not appear to contribute to the resistance phenotype. The frequency of these mutations (percentage of reads that mapped to that position) was lower than would be expected for a single gene copy in a hexaploid (16%). This could be explained by the presence of additional genomic copies (very recent gene duplications of *EPSPS*2 within *C. bonariensis*). We therefore confirmed that the three *EPSPS* genes are single copies within each of the component genomes by genomic DNA sequence mapping (Illumina PE125, Hereward unpublished data) and comparing the coverage to that of 10 random nuclear transcripts.

Table 1.7.3.1. Percentage of reads that map to each *EPSPS* copy that carry a resistance mutation

		*EPSPS*1		*EPSPS*2		*EPSPS*3	
Individual	Resistance	Thr102Ile	Pro106Thr	Thr102Ile	Pro106Thr	Thr102Ile	Pro106Thr
GS1-4	Susceptible	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
GS1-5	Susceptible	0.0%	0.0%	0.0%	0.0%	0.0%	2.7%
GS1-7	Susceptible	0.0%	0.0%	0.0%	3.5%	0.0%	3.5%
GS2-1	Susceptible	0.0%	0.0%	0.0%	10.7%	0.0%	0.0%
GS2-2	Susceptible	0.0%	0.0%	0.0%	9.9%	0.0%	0.0%
GS2-5	Susceptible	0.0%	0.0%	0.0%	5.6%	0.0%	0.0%
GR2-4	3.5 fold	0.0%	0.0%	0.0%	7.5%	0.0%	0.0%
GR2-5	3.5 fold	0.0%	0.0%	0.0%	7.4%	0.0%	0.0%
GR2-6	3.5 fold	0.0%	0.0%	0.0%	11.5%	0.0%	0.0%
GR1-1	6 fold	0.0%	0.0%	0.0%	7.4%	0.0%	0.0%
GR1-6	6 fold	0.0%	0.0%	0.0%	5.6%	0.0%	0.0%

The relative expression of *EPSPS*1 (mean RPKM = 23.5) prior to glyphosate treatment was about four times higher than *EPSPS*2 (mean RPKM = 4.1) and twice as high as the *EPSPS*3 gene (mean RPKM = 9.1, Fig. 3). The relative expression of *EPSPS*1 decreased substantially following glyphosate treatment (mean RPKM post = 5.6). The relative expression of *EPSPS*2 and *EPSPS*3 did not appear to be strongly affected by glyphosate treatment (Fig. 1.7.3.3B & 1.7.3.3C). However, the expression data for these two copies are susceptible to read mismapping across sections of them. Previous qPCR expression studies on *C. bonariensis* and *C. canadensis* have tended to deploy primers that amplify all copies of *EPSPS* (17, 18, 56). We therefore calculated the sum of RPKM values for all *EPSPS* copies (Fig. 1.7.3.3D). This revealed that *EPSPS* expression (of all three genes) was reduced following glyphosate treatment, but not as dramatically as the effect on *EPSPS*1 alone. A slight (often not significant) decrease in *EPSPS* expression following glyphosate treatment has been detected in other studies (17, 18). The reduced expression of *EPSPS*1 but not *EPSPS*2 and *EPSPS*3 indicates that the transcription of the different genomic copies is controlled differently, the transcription of *EPSPS*1 may be affected by some aspect of a stress-response pathway, for example. Some authors report higher expression of *EPSPS* in resistant lines of *C. bonariensis* (19), but we observed no difference across the resistant and susceptible lines in terms of *EPSPS* expression (Fig. 1.7.3.3).

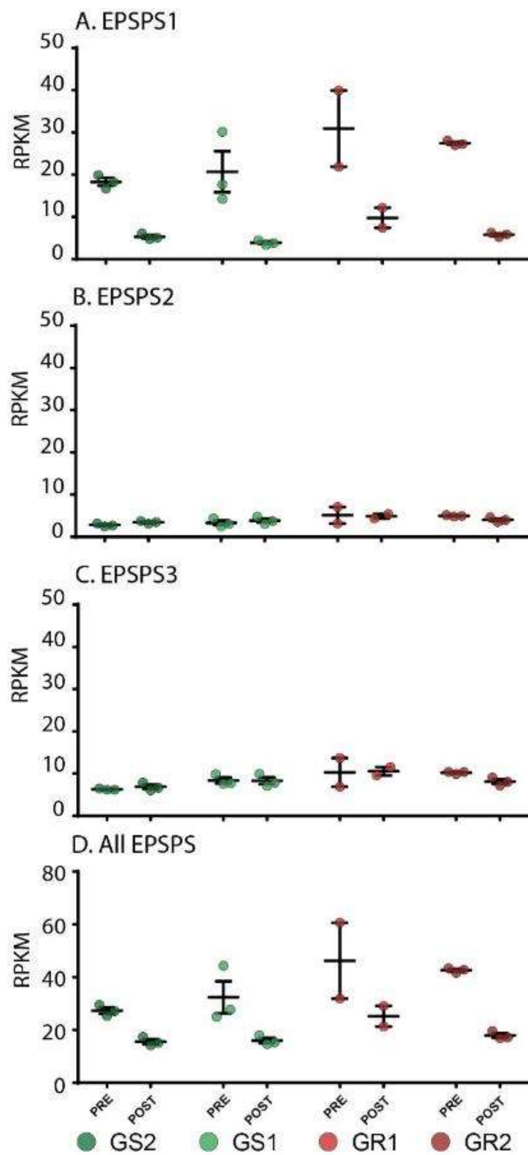

Figure 1.7.3.3. Expression (Reads Per Kilobase of transcript per Million mapped reads) of the three different *EPSPS* gene copies in *Conyza bonariensis* before and 48hrs after glyphosate treatment. *EPSPS*2 and *EPSPS*3 have similar sequence and experience some read mapping ambiguity in sections. Red indicates resistant lines and green indicates susceptible.

Although there is a resistance mutation in at least one of 18 copies of the *EPSPS* genes present in *C. bonariensis* its effect is diluted not only by the other copies (within and across genomes), but also by expression differences. Transcriptional bias appears to be widespread in polyploids, and, for example, was detected in 40% of homeologous genes in polyploid cotton (57). The resistance percentages of the *EPSPS*2 gene seem to provide evidence of this; one out of six copies (three diploid genomes) would produce 16% of transcripts (if all were expressed equally), but we always detected less than this. The expression of *EPSPS*2 is also lower than *EPSPS*3 and *EPSPS*1, representing only 10% of expressed *EPSPS*. The resistance mutations therefore account for 1% or less of total expressed *EPSPS* and it is not surprising that they do not contribute to the resistance phenotype. Mutations in *EPSPS*1 would have a greater effect, but there are still six copies of this gene in allohexaploid *C. bonariensis*, and it is not yet clear how many would need to be mutant to affect the phenotype.

Populations of tetraploid *E. colona* from California with a Pro106Ser mutation in one of the two homeologous copies of *EPSPS* show two to four-fold resistance to Glyphosate (58). Populations of (likely hexaploid) *E. colona* from Australia, with one or two resistance alleles, also show about two-fold resistance, which is not sufficient for resistance at field rates in cool

conditions, but does provide resistance when glyphosate is applied in hot conditions (28). These studies indicate that maybe 50% of homeologous *EPSPS* copies bearing mutations in a polyploid can cause a resistance phenotype, but our data highlight the importance of assessing the relative expression of each genomic copy of the *EPSPS* gene.

Together, these results indicate that neither target site mutations or target site overexpression are responsible for glyphosate resistance in Australian populations of *C. bonariensis*. Resistance must therefore be the result of a Non-target-site-resistance (NTSR) mechanism.

3.3 Differential Expression analysis and candidate NTSR resistance genes

Many transcripts were differentially expressed following glyphosate treatment in both susceptible and resistant lines of *C. bonariensis* (Fig. 1.7.3.4). When genes with expression < logfold 2 and > logfold -2 were excluded a mean of 4,140 transcripts (5% of 81,539) were up-regulated in the susceptible lines GS1 and GS2 and 6,336 (8%) down-regulated. In the resistant lines (GR1 and GR2) 2,356 (3%) were up-regulated and 2,932 (4%) down-regulated (Supplementary data, S5). We define the glyphosate death response as the sum of all genes that were differentially expressed in the susceptible lines at a fold change greater than 2 following glyphosate treatment. This consisted of 16,350 (20% of 81,539) genes indicating widespread transcriptional changes caused by the herbicide induced death. Few other studies report whole transcriptome responses to glyphosate treatment, but they generally detect lower numbers of DE genes than recorded here on *C. bonariensis* (59-61). These other studies generally run the experiment for less than ours (48hrs) and it would be useful for future studies to assess a time series of the transcriptional glyphosate response. The number of DE genes in the resistant lines in response to glyphosate treatment was lower, but still high (Fig. 1.7.3.4). This shows that glyphosate has a large effect on the gene expression of the resistant lines. This is to be expected because the resistant individuals, despite surviving the glyphosate treatment, still lost a lot of leaves before re-growing.

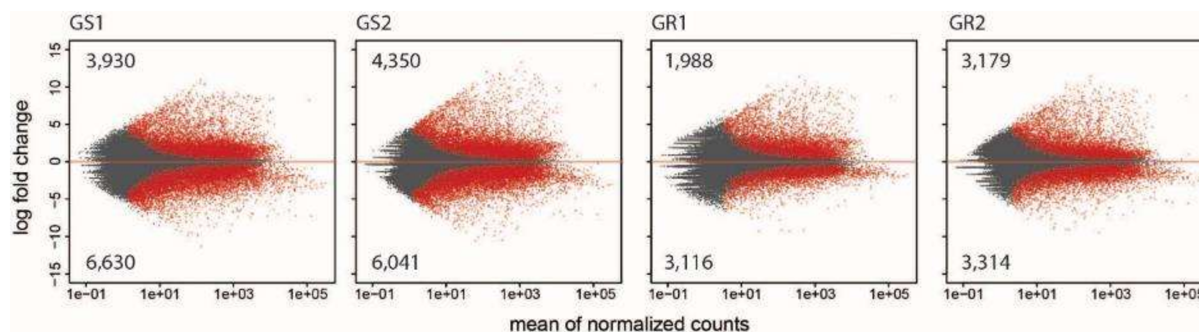

Figure 1.7.3.4. MA plots showing the differential expression of genes in each line before and 48 hrs after glyphosate treatment. Dots above the line are up-regulated and dots below are down-regulated, red dots indicate significant expression at an adjusted-p threshold of 0.05. The plots show all differentially expressed genes, and the numbers above and below the red line indicate the number of upregulated and downregulated genes after a logfold cut-off of 2 was applied.

The expression of candidate resistance genes M10 and M11 was similar to that observed in the study that initially characterised these ABC transporters (53), going from low expression to high expression following glyphosate treatment (Fig. 1.7.3.5). In our *C. bonariensis* lines, however, expression was slightly higher in the susceptible lines than in the resistant lines, and we categorise these genes as part of the glyphosate death response. Our interpretation is in line with that of the authors of a recent study who observed a similar pattern of expression in both *C. canadensis* and *C. bonariensis* in susceptible and resistant lines and in response to both glyphosate and paraquat stress (18). Even when working from mechanistic principles the

identification of NTSR genes can be hampered by stress related genes that are activated by the herbicide. In this instance, having a whole-transcriptome assay of gene expression changes helps to identify the extent of the stress response, and remove it from the candidate resistance gene list.

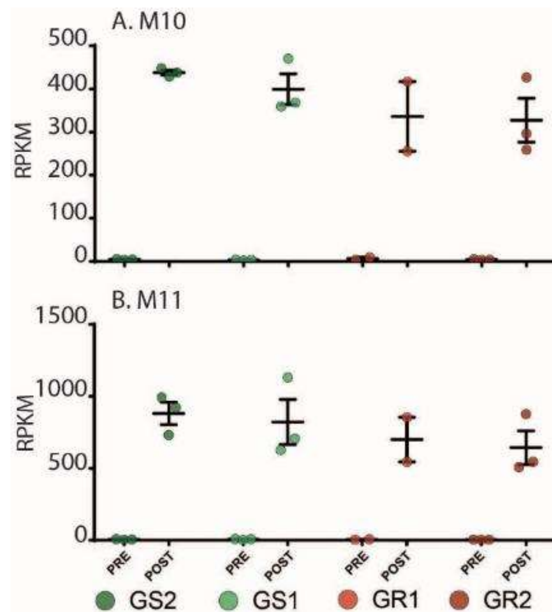

Figure 1.7.3.5. Expression (Reads Per Kilobase of transcript per Million mapped reads) of the *C. bonariensis* version of the M10 and M11 genes that are reported to be involved in glyphosate resistance in *C. canadensis*. Red indicates resistant lines and green indicates susceptible.

Comparison of the pooled resistant lines (pre-glyphosate) with the pooled susceptible lines revealed that 2,807 genes (3%) were up-regulated and 2,327 (3%) down-regulated. This is the inherent gene expression difference between the resistant and susceptible lines in this study. There is considerable constitutive difference in gene expression between these lines, and also considerable genotypic difference (*snp*s) between the susceptible and resistant lines (Fig. 1.7.3.1C). The resistant lines do show a different phenotype, growing slower and being more compact (M. Keenan, pers. obs). We do not consider this list to be a candidate resistance gene list. Although some of the constitutive DE genes may be part of the resistance mechanism (or activated as a by-product of the resistance mechanism) it is also likely that many of these DE genes simply represent biological differences between the resistant and susceptible lines.

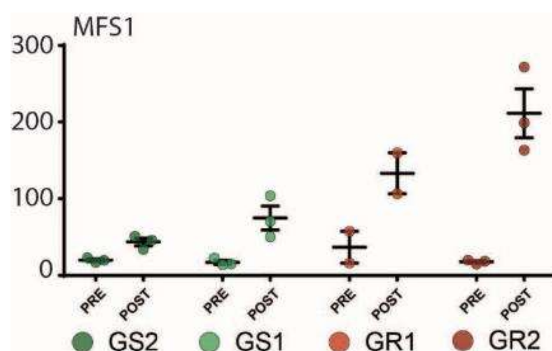

Figure 1.7.3.6. Expression (Reads Per Kilobase of transcript per Million mapped reads) of the *C. bonariensis* Major Facilitator Superfamily gene, a candidate Non-target-site-resistance gene (DN24711_c1_g1 in the assembly). Red indicates resistant lines and green indicates susceptible.

The full list of induced candidate non-target-site-resistance genes (anything differentially expressed in either of the resistant lines but not in susceptible lines after treatment) contained 834 transcripts. Line GR1 (6-fold resistant) had 357 candidate NTSR transcripts, and Q42 (3.5-fold resistant) had 557 with only 78 being common across the two resistant lines. One gene in particular is potentially relevant to glyphosate resistance, being identified as part of the "Major facilitator superfamily" of genes. This is the second largest family of transporter genes after the ABC transporters and they are known to have functions that include the extrusion of deleterious compounds (62). This locus in *C. bonariensis* was up-regulated in response to glyphosate in resistant lines GR1 and GR2 (Fig. 1.7.3.6). The expression of this locus is higher in GR2 than in GR1, even though GR2 displays a lower resistance phenotype. This could indicate that there is more than one resistance mechanism in these two lines, despite their having essentially identical genotypes. Future work will further validate and characterise the candidate NTSR resistance genes identified here.

4. Conclusions

Glyphosate resistant lines of *C. bonariensis* in Australia have low level target site mutations but they only represent about 1% of transcribed *EPSPS* due to historical gene duplications, polyploidy, and differences in the expression of homeologs. Target site mutations and *EPSPS* expression do not correlate with the resistance phenotype in these lines and the resistance mechanism must be non-target-site.

Many genes are differentially expressed in response to glyphosate application, and the previously identified candidate resistance genes M10 and M11 seem to be part of this glyphosate death response. Our results highlight the inherent difficulty in disentangling NTSR mechanisms from stress responses. We present a list of 834 candidate NTSR resistance genes that warrant further investigation of their potential role in the glyphosate resistance mechanism of *C. bonariensis*. The transporter gene MFS1 appears to be a good candidate as part of the resistance mechanism.
In general, the results indicate that a whole-transcriptome approach is useful for dissecting expression levels of target site genes with multiple copies in polyploid species, assessing the death response to herbicides, and identifying target NTSR mechanisms.

Acknowledgements

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Data availability

EPSPS sequences have been deposited at GenBank (Accessions TBA), sequencing reads have been deposited at the short-read archive (Acessions TBA). Assembled contigs, annotations, and the scripts required to reproduce these results are provided as supplementary data as below:

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1.7.4 Chloroplast genomes

We published chloroplast genomes for all species under investigation, these resources were then used in the population genetics and mechanism work presented in this report and are made available for future researchers. References provided:

JP Hereward, JA Werth, DF Thornby, *et al.*, 2016, 'Complete chloroplast genome sequences of six lines of *Echinochloa colona* (L.) link', *Mitochondrial DNA Part B*, 1 (1) 945-946 doi:10.1080/23802359.2016.1261612

JP Hereward, JA Werth, DF Thornby, *et al.*, 2016, 'Complete chloroplast genome sequences of two species of Chloris grass, *Chloris truncata* Sw. and *Chloris virgata* R.Br', *Mitochondrial DNA Part B*, 1 (1) 960-961 doi:10.1080/23802359.2016.1266705

JP Hereward, JA Werth, DF Thornby, *et al.*, 2017, 'Complete chloroplast genome of glyphosate resistant *Conyza bonariensis* (L.) Cronquist from Australia', *Mitochondrial DNA Part B*, 2 (2) 444-445 doi:10.1080/23802359.2017.1357441

JP Hereward, JA Werth, DF Thornby, *et al.*, 2018, 'Complete chloroplast genome of glyphosate resistant *Sonchus oleraceus* L. from Australia, with notes on the small single copy (SSC) region orientation', *Mitochondrial DNA Part B*, 3 (1) 363-364 doi:10.1080/23802359.2018.1450682

1.8 - Establish whether the same non-target-site resistance (NTSR) mechanisms have evolved in windmill grass and feathertop Rhodes grass

Feathertop Rhodes grass has a target-site mutation and this milestone was closed early, but we added an outcrossing experiment to replace it and this is reported here.

Outcrossing experiment - Fleabane and Sowthistle.

Introduction

The rate of selfing *vs* outcrossing has a big impact on the evolution of herbicide resistance, in particular the evolution of multiple mode of action resistance. This is because weeds that only self cannot recombine resistance traits with one another to create natural 'stacks' of resistance. In species that only self, resistance to multiple herbicides can only evolve by multiple different mutations in the same lineage, whereas in outcrossing species, two different individuals can evolve resistance and the mutations can be exchanged.

Windmill grass and feathertop Rhodes have very high rates of selfing because they exhibit both very high fixation indices (i.e. fixation indices close to one), and high rates of apparent clonality in the genetic data. The population genetic data for sowthistle and fleabane was less clear, so we undertook an experiment to grow the plants in the presence of natural pollinators and assess the rate of outcrossing using parentage analysis.

Methods

Thirty plants from each population were established in a glasshouse at the end of September 2017. For each species, we placed ten pairs in the open with access to pollinators. Each pair was comprised of one glyphosate-susceptible and one glyphosate-resistant line, these lines had already been shown to be genetically distinct from each other. A successful cross would have been used to establish a mapping population for the resistance trait. Plants were placed together (less than 10cm distance) when the first flower buds appeared, and left together for two months. Seed heads were bulked for each plant and stored. Sowthistle seed was collected from December 2017 to February 2018.Fleabane seed was collected from January 2018 to February 2018.

We grew 200 seedlings from the susceptible and the resistant plant of one cross from each species, and collected leaf material from the F1 plants as well as the parents. We extracted DNA and then conducted *genotyping by sequencing* as outlined in section 1.6.4. The genotype data was filtered in the same manner as in 1.6.4, and we then constructed a principal components analysis (PCA) for each species using adegenet in R (Jombart *et al.*, 2008, R Core team 2018).

Results

Hoverflies were seen regularly on the sowthistle, and the plants were observed to be regularly frequented by a range of insects. Following genotyping, the PCA analysis indicated that none of the fleabane (Fig. 1.8.1) and none of the Sowthistle (Fig. 1.8.2) had crossed. All the offspring clustered with their parents and there were no individuals in between the two parental clusters.

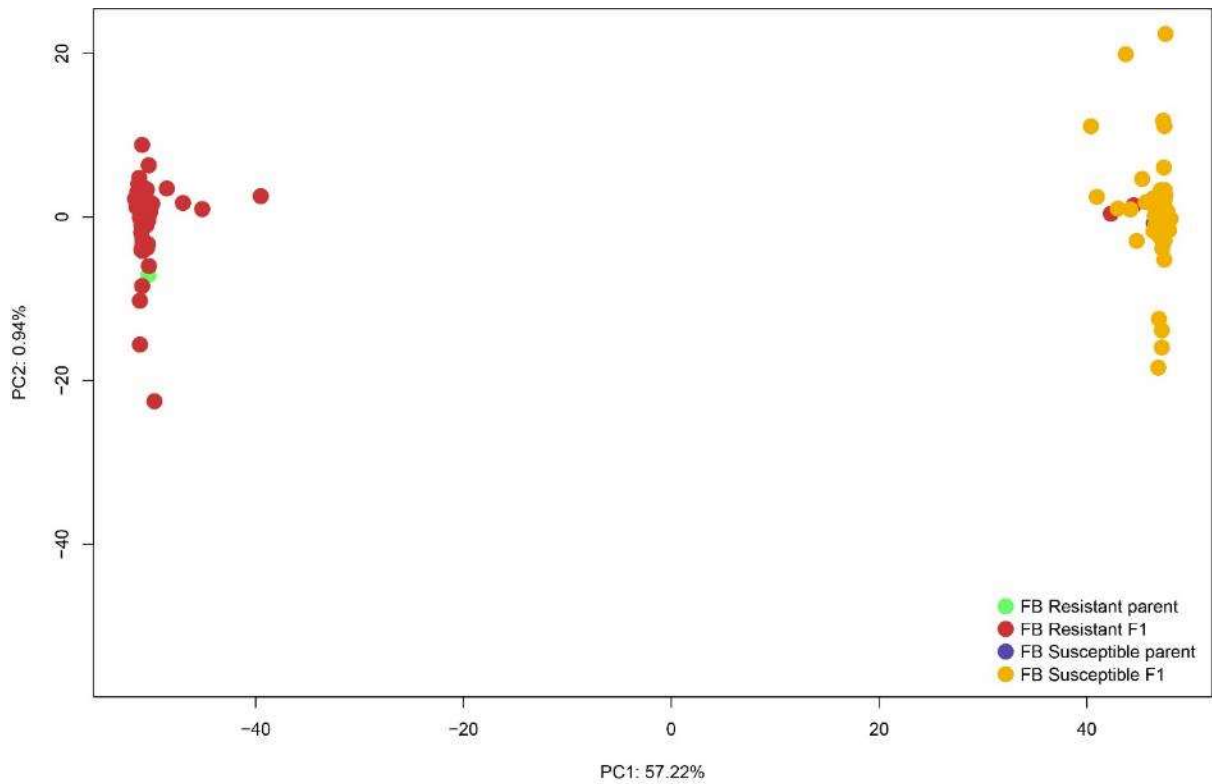

Figure 1.8.1 PCA of the fleabane outcrossing genotype data, there are two clusters representing the parental genotype and no individuals are found in the centre where outcrossed individuals would be expected.

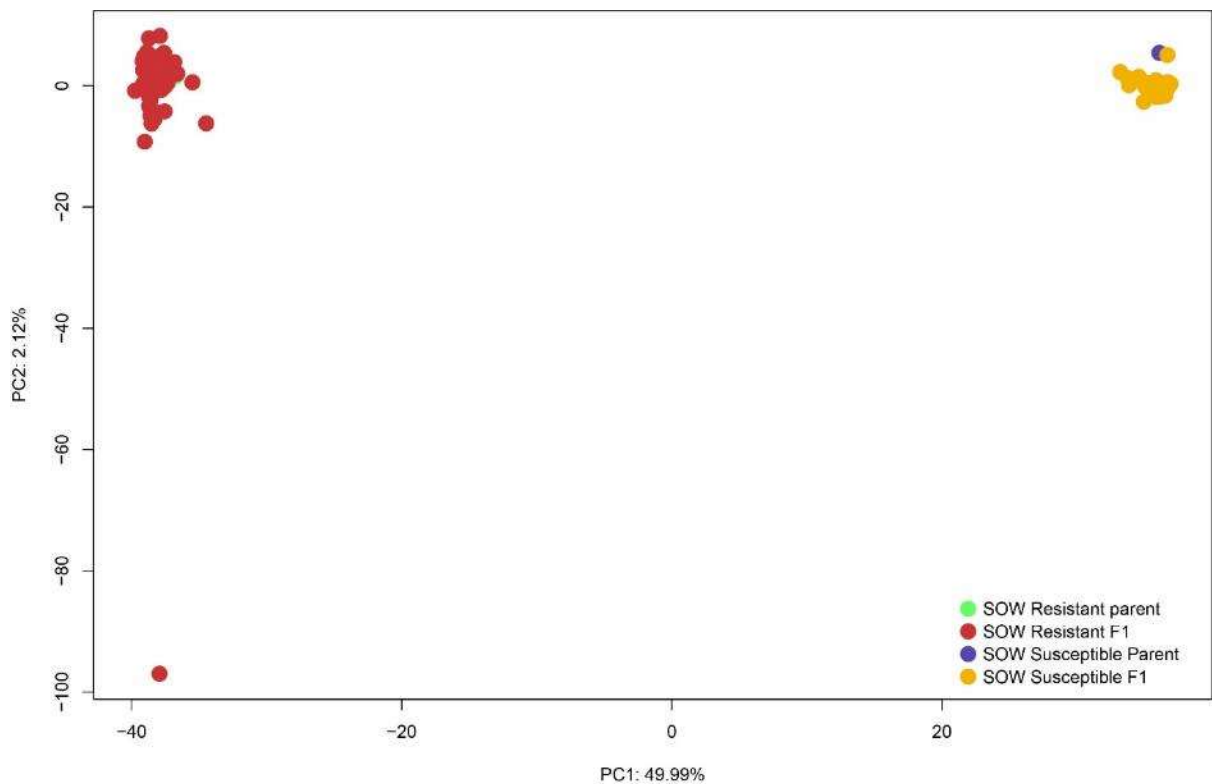

Figure 1.8.2 PCA of the sowthistle outcrossing genotype data, there are two clusters representing the parental genotype and no individuals are found in the centre where outcrossed individuals would be expected.

Discussion

This experiment demonstrates that outcrossing was below one in 200 (i.e. undetectable), which is under the experimental conditions. In contrast, the population genetics data appear to indicate some level of outcrossing in sowthistle, at least, but perhaps this suggests that outcrossing occurs at a very low rate under field conditions.

Taken together with the population genetics data, we can say that feathertop Rhodes and windmill grass have extremely low rates of outcrossing (in the field), and are less likely to 'stack' different mode-of-action resistance mechanisms. In fleabane and sowthistle it appears that there may be low rates of outcrossing, even if this occurs rarely, there are numerous weed individuals per generation and this creates an opportunity, even if limited, to exchange resistance mechanisms. In practice, even for weed species that only self-fertilise, if we allow the glyphosate resistant population numbers to get too high, then there will be a lot of individuals within which a second mode-of-action mutation could evolve. We can think of self-fertilisation as a tool to help combat multiple mode-of-action herbicide resistance, but only if we keep our glyphosate resistant populations small.

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1.9 - Investigate interaction between stress and herbicide applications with potential implications to activate non-target site resistance (NTSR) mechanisms

Introduction

Non-target site resistance (NTSR) has been hypothesised to be a co-option of natural stress response genetic systems in weeds. Under this model, herbicides induce stress in a weed. The plant cell receives herbicide stress signals via sensors. The herbicide stress signal is transducted to regulators and triggers regulation cascade(s) that modulate the expression of 'protectors' at the DNA, mRNA and/or protein level, and can generate herbicide 'stress imprint' by epigenetic modifications. The herbicide signal also triggers the modulation of regulators via feedback, enabling the adjustment of NTSR over time (Delye 2013). But how similar is herbicide-induced stress to abiotic stress?

Glyphosate is reported to be more effective at controlling ragweed at 29 ºC compared to 20ºC due to increased translocation of the herbicide (Ganie *et al.* 2017). Conversely, in Australian populations of glyphosate resistant barnyard grass, resistance increases at 30 ºC compared to 20 ºC, and it is suspected that it has a NTSR mechanism that becomes more active at higher temperatures (Nguyen *et al.* 2015). Sowthistle (*Sonchus oleraceus*) also seems to have an NTSR mechanism that is expressed in response to temperature, with plants that are susceptible at 20 ºC becoming resistant at 30 ºC.

It is also generally not recommended to apply herbicide when plants are under extreme stress because they may have shut down internal systems for translocation or absorption.

Sowthistle is an allotetraploid with one genome coming from *S. asper* and one from another species (related to *S. tenerrimus*). This species, as with lettuce and other members of this group also had an ancient genome duplication (Reyes-Chin-Wo *et al.* 2017). This means that there are now four copies of the *EPSPS* gene based on whole-genome sequencing. Copies A and B come from the ancient duplication event, and B1 and B2 (and A1 and A2) are the result of polyploidy. One of these genes has a resistance mutation, but we do not know whether all four copies are expressed or not.

The aim of this study was to assess the gene-expression changes in glyphosate resistant and susceptible populations of Sowthistle at different temperatures, to further understand the interactions between herbicide resistance, temperature, and stress. Assessing the genes that are differentially expressed between resistant and susceptible lines at 20 ºC *vs* 30 ºC (A1 *vs* B1) should reveal clues to any constitutively expressed NTSR mechanisms. Assessing differential expression before *vs* after glyphosate treatment (A2 *vs* A1, B2 *vs* B1) will reveal any induced NTSR mechanisms (after subtracting the glyphosate death response genes). Comparing the glyphosate stress – induced differential expression with the heat stress induced differential expression, will allow us to assess how similar these stress responses are (C1 *vs* A2 *vs* B2). Comparing the heat stress with the 30 ºC and 20 ºC will show how the temperature activated resistance potentially interacts with heat stress (C2 *vs* C1 compared to B2 *vs* B1 compared to A2 *vs* A1).

Questions

1. Do NTSR mechanisms share pathways with general stress responses?
2. Can NTSR mechanisms be activated by stress?
3. What are the expression levels of the four *EPSPS* copies in Sowthistle?
4. What genes may be involved in the temperature – activated glyphosate resistance in Sowthistle?

Materials and Methods

For each treatment, five replicates of each population were established. Seeds from each population were germinated on 0.6% (w/v) agar and transplanted after six days into forestry pots (6.5 x 6.5 x 16 cm) filled with Searles Premium Potting Mix topped with 2 cm of seed raising mix. The seedlings were kept in a growth room at 25/15 for eight days after transplanting before being relocated into growth chambers. Plants were assigned to replicates according to size (1-smallest to 5-largest), with 20 pots (4 populations) per tray. Trays, containing the plants were moved around within the chambers on a daily basis as a way to ensure randomisation.

The following treatments were applied to each of the populations:

1) Control: 20°C/15 ^{O}C no glyphosate
2) Low growth temperature: 20°C/15 ^{O}C w/ glyphosate
3) High growth temperature: 30ºC/20 ^{O}C w/ glyphosate
4) Heat stress: 20°C/15 ^{O}C + 35ºC (four hours before and four hours after glyphosate application); return to 20°C/15 ^{O}C

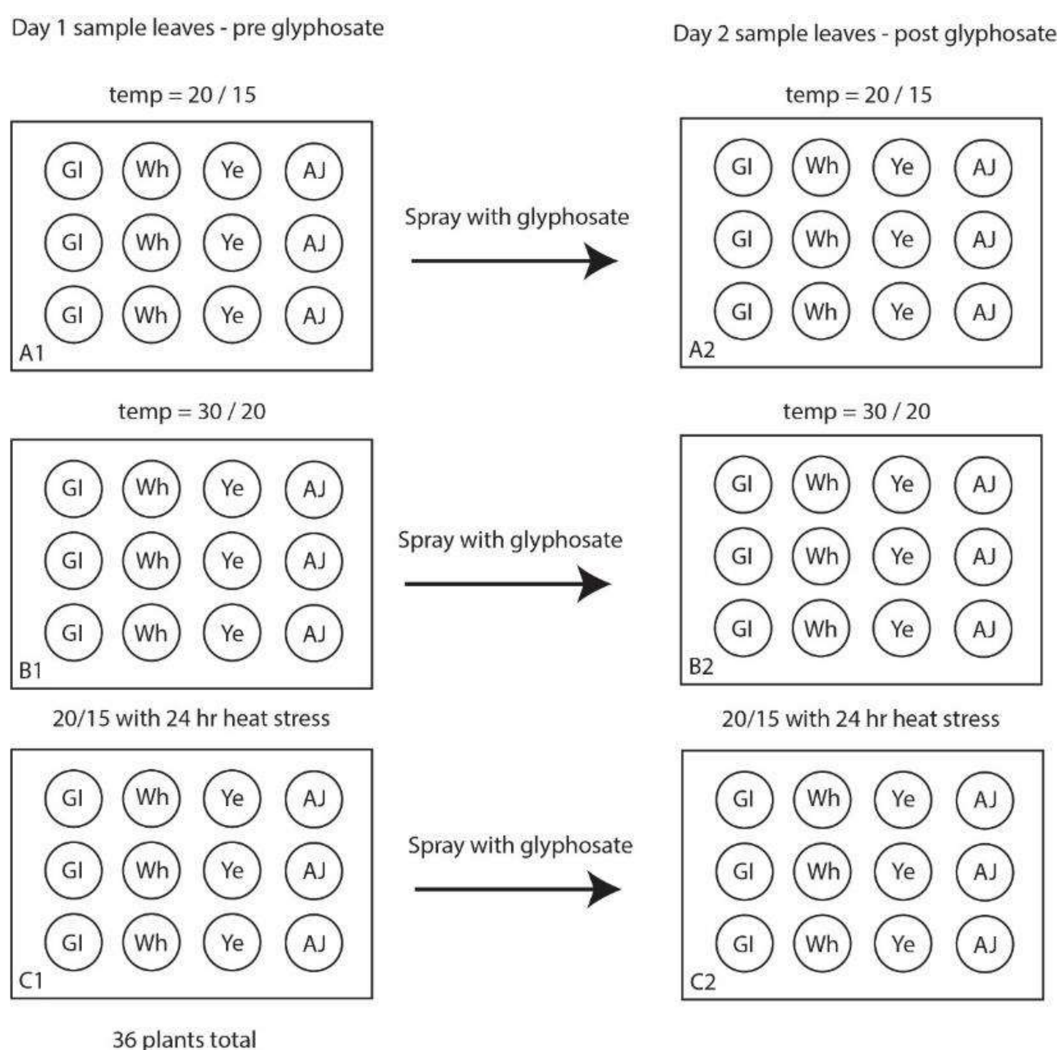

Figure 1.9.1. Schematic of the treatments and populations in this study.

The plants were grown for a total of 23 days before being sprayed with glyphosate at 540 g ae/ha (1L Weedmaster ARGO) with 114 L/ha water, in the spray cabinet. At the time of spraying, most plants had between 4-6 leaves. The afternoon before the plants were due to be

sprayed, those being subjected to the 35ºC heat stress were moved into an incubator set at the night time temp of 15 ºC and programmed for 35 ºC day time temperature from 6:00 am - 10:00 am the following day.

With the exception of the control treatments, all plants had leaf material sampled before being sprayed. After spraying, all pots were returned to the same pre-spraying conditions. The pots that had been in the incubators at 35 ºC were returned to the incubators for a further four hours of heat stress, and then moved into the 20 ºC /15 ºC growth chambers.

An above-ground dry weight biomass was taken at 21 days. The plants were harvested at the soil surface and dried in an oven for 24 hours at 60 ºC.

Leaf material was taken directly into TRIzol™ Reagent (Invitrogen) and homogenised. RNA was extracted from the leaf material using a standard Trizol extraction protocol. The RNA samples were shipped to Novogene Corporation (Beijing, China) for stranded RNA sequencing, targeting 40 million 150bp paired-end reads per individual.

Results

Following several unsuccessful initial attempts at seed germination, a moderately successful first run of the experiment was conducted in March and April 2019. However, the root systems of some of the plants were weak, and we decided that the quality of the plants was not sufficient given the expense of the transcriptome sequencing. As part of the extension to project UQ1501 we were able to re-run the experiment in October 2019. This experiment was successful, and RNA has been extracted and shipped for sequencing, however, the results have not been returned in time for this final report.

Further results will be reported to the Cotton Research and Development Corporation (CRDC), as they become available.

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1.10 – Overseas travel to "The 2nd Global Herbicide Resistance Challenge"

David Thornby and James Hereward joined several Australian delegates to the Global Herbicide Resistance Challenge in Denver, Colorado, in May 2017. There were approximately 350 delegates overall. The US, Australia, South America and the UK were well represented. As in the previous iteration of this meeting, in Fremantle, there was a spread of focus on the genetics of resistance, resistance management, and policy and regulation as it applies to resistance.

The sociological approach to the resistance problem was one particularly new theme. Sociology classifies resistance as a 'wicked' problem—one without definite solutions, since susceptibility cannot really be recovered, with strong human (emotional) dimensions and lots of actors involved. Sociological theory says that traditional problem-solving applies poorly to wicked problems, and in particular we shouldn't expect to create change simply by finding the 'right' information and communicating it to individual stakeholders. The sociological talks suggested that encouraging communal/community action is a better way to generate change.

There was also discussion of the regulatory environment for herbicides and traits in a sociological context, with the key suggestion being that an optimal approach (at least for the US) is for industry to organise who is responsible for what in setting agreements between and among companies and users, and regulation only used to make those industry-led agreements enforceable.

There was a notable absence of new information on RNAi or similar novel technology; presumably the delivery problem is still intractable, at a table discussion of the application of weed genomics to management a Monsanto representative indicated that the technology has not been abandoned. He also indicated that there is ongoing work on the topical application of RNAi to honey bees. Technical herbicide work seemed to be focused mainly on less frequently-used chemistries (in the US) such as on stretching the sustainability of HPPD inhibitors. There also seemed to be somewhat less new information on resistance mechanisms than in previous meetings. The story of gene amplification in palmer amaranth continues to develop, however, but gets more confusing as more research is conducted. The mobile element has been sequenced and is over 300kb, it is suggested that this mobile DNA is present as a single circular copy at some stage but the mechanism of its jumping through the genome is still unresolved. Doug Sammons presented data that showed lines of palmer amaranth with high copy number but no resistance, and suggested that this was due to a small RNA silencing mechanism that has somehow been activated naturally within the plant as part of the gene jumping process.

There is a push to develop an "International Weed Genomics Consortium" which will seek to attract some funding from chemical companies and seeks to develop high quality reference genomes for key weed species. One of the species in the list of targets is *C. bonariensis*. James Hereward is in discussion with Karl Ravet so that if this happens we can use our genomic data and line Q17 as part of this reference.

Scientists and extension professionals in the US appear to have caught up with Australian and Canadian messages on resistance management, and US growers were reported as largely being confident that they can control their glyphosate-resistant populations at present. The Western Australian message about harvest weed seed management in grains cropping received a substantial amount of attention, including a dedicated workshop. It appears there is some movement towards adopting these techniques in North and South America. (See Appendix 2 for David's presentation)

1.11 Investigate the extent of glyphosate and 2,4-D resistance in sowthistle populations

Between May and July 2019, field surveys were undertaken to sample weed populations, including sowthistle. A total of 20 sowthistle populations were collected from the Darling Downs region but seed numbers were low and of variable quality. Germination trials to establish the quality of the seed and provide enough emergences to transplant ran from July through to September. Seeds were germinated on 0.6% agar preparation and were transplanted into trays contained a sand/peat moss mixture at around 9 days which has been found to provide the best survival rate for sowthistle. However, germination rates and transplant survival rates were low with twelve of the populations either not germinating with enough numbers to transplant, or not surviving the transplant procedure. The eight remaining populations were transplanted but had low survivors by the time of spraying (< 20 in most cases). Only 3 populations had both glyphosate and 2,4-D treatments applied, a further 3 populations only 2,4-D and the remaining 2 were glyphosate only treatments.

The rates used were 1350 ml/ha of Glyphosate 540 (540g/L) and 1500 ml/ha of 2,4-D (700g/L). The treatments were applied to plants approximately 3 cm in diameter/height with survivor counts taken at 21 days after treatment (DAT).

There were no survivors at 21 DAT for either herbicide.

2.1 - Define role of dicamba and glufosinate with current and potential cotton weeds in a gaps analysis

The role of dicamba and glufosinate was determined as part of the TIMS herbicide tech panel discussion paper on future transgenic technology. The section on impacts for weed control in the Australian Cotton System is contained below. A copy of the whole report may be obtained from CRDC/TIMS

XtendFlex® system use case for weed control in the Australian Cotton Farming System

Proposed Weed Species Responses for Extendflex

The likelihood of improved control with Extendflex was assessed using other hericide labels as a guide i.e Cadence for dicamba, Liberty and Basta for Glufosinate. This is summarised in Table 2. It is important to note, Basta (glufosinate) was included for consideration. This formulation has control of a wider range of weeds than liberty, mostly likely due to the higher herbicide and water rates allowed for on the Basta label.

Key points for noting:

- Glufosinate has not been commonly used in cotton rotations to date, it provides better control of a number of broadleaf weeds than glyphosate including the *Ipomea* and *Convolulus* species
- Recent trials indicate that glufosinate may be a handy double knock partner to glyphosate±group A as an alternative to paraquat on the summer grasses. If this carries through to the field a glyphosate+glufosinate stack will allow double knock practices to be carried out in-crop.
- There have been few post-emergent broadleaf control options in-crop, the ability to apply dicamba OTT should create an effective option for controlling a number of broadleaf weeds, including some poorly controlled by glyphosate.
- Overall control of broadleaf weeds is likely to be improved (Sowthistle, Dwarf Amaranth, Caltrop and Bell vine), with two additional MOA's able to be used in-crop
- Control of fleabane may be improved by the ability to use glyphosate+dicamba in-crop
- Control of feathertop Rhodes grass, and caustic weed is not likely to be improved by the addition of either herbicide, so control options for these species remains limited
- Peachvine, is already on the Liberty label, not on dicamba (Cadence) label, however proposed for Bayer dicamba (Extendimax) label
- Fleabane is not present on either the Glufosinate or dicamba label, however also proposed for the Extendimax label
- Summer grass control may be slightly improved, however this depends on the following factors
    - Effectiveness of double knock tactics involving glufosinate as the second knock herbicide
    - The effectiveness of glyphosate on summer grasses could be considerably reduced
- For glufosinate to be effective, weed growth stage is critical. As a result control levels achieved are likely to be more variable and the incidence of escapes may increase

A series of double knock trials were conducted in the glasshouse using glyphosate±dicamba and dicamba fb glufosinate as a double knock partner in the place of paraquat. These were conducted on awnless barnyard grass, feathertop Rhodes grass, windmill grass, fleabane and Sowthistle. Clethodim was used on the summer grasses in place of dicamba. These trials found that:

- Control of awnless barnyard grass was improved with either glyphosate fb glufosinate, clethodim fb glufosinate and glyphosate+clethodim by glufosinate on both glyphosate resistant and susceptible populations
- Control of Sowthistle was improved with either glyphosate fb glufosinate, dicamba fb glufosinate and glyphosate+dicamba fb glufosinate on both glyphosate resistant and susceptible populations
- Control of fleabane was improved with dicamba fb glufosinate and glyphosate+dicamba fb glufosinate on both resistant and susceptible populations
- Control of feathertop Rhodes grass was improved with either glyphosate fb glufosinate, clethodim by glufosinate and glyphosate+clethodim fb glufosinate, however timing of follow-up application needed to be around 7-10 days for consistent results on both glyphosate resistant and susceptible populations
- Windmill grass was harder to control with the most consistent control achieved using clethodim fb glufosinate or glyphosate+clethodim by glufosinate 7-10 days later on both glyphosate resistant and susceptible populations

Table 2 Weed species that control is likely to improve (0 = no improvement 4 = strongly improved) on in a glyphosate+glufosinate+dicamba stack. Indications on control achieved were assessed due to presence on relevant herbicide labels.

Common name	RR Herbicide	Basta	Liberty	Cadence/Dicamba	Extenflex rating
Sowthistle	Yes	Yes	Yes	Yes	4
Bell vine			Yes	Yes	3
Caltrop	Yes	Yes	Yes	Yes plus glyphosate	3
Dwarf amaranth		Yes	Yes	Yes	3
Pigweed (Red)	Yes	Yes	Yes		3
Bathurst burr	Yes			Yes	2
Bladder ketmia	Yes		Yes		2
Buckwheat	Yes	Yes		Yes plus glyphosate	2
Cobbler's pegs		Yes		Yes	2
Deadnettle	Yes	Yes			2
Fat hen		Yes		Yes	2
Green Amaranth		Yes		Yes	2
Mintweed	Yes	Yes		Yes plus glyphosate	2
Native Amaranth		Yes		plus glyphosate	2
Noogoora burr	Yes			Yes plus glyphosate	2
NZ spinach	Yes	Yes		Yes	2
Prickly Lettuce		Yes		plus glyphosate	2
Thornapple	Yes			Yes plus glyphosate	2
Wild gooseberry		Yes		Yes (annual gooseberry)	2
Wireweed	Yes	Yes		Yes plus glyphosate	2
Annual polymeria			Yes		1
Annual ryegrass	Yes	Yes			1
Awnless barnyard grass	Yes	Yes			1
Crowsfoot grass		Yes			1

Johnsongrass	Yes	Yes			1
Liverseed grass	Yes	Yes			1
Native sensitive plant				Yes?	1
Nightshades				Yes (Blackberry)	1
Peachvine			Yes		1
Redshank				Yes	1
Rhyncosia			Yes		1
Sesbania			Yes		1
Spear thistle	Yes			Yes	1
Spiny emex				Yes	1
Summer grass		Yes			1
Tarvine				Yes	1
Turnip weed	Yes			plus glyphosate	1
Variegated thistle	Yes			Yes	1
Wild turnip				Yes (cereals)	1
African turnip	Yes				0
Annual ground cherry	Yes				0
Anoda weed					0
Australian Bindweed					0
Barley grass					0
Black pigweed	Yes				0
Boggabri weed	Yes				0
Button grass	Yes				0
Camel melon	Yes				0
Caustic weed	Yes				0
David's spurge					0
Devil's claw					0
Feathertop rhodes grass					0
Flaxleaf fleabane					0
Indian hedge mustard					0
Maloga bean					0
Malvestrum					0
Marshmallow					0
Mexican poppy	Yes				0
Nutgrass	Yes				0
Paradoxa grass	Yes				0
Stinkgrass	Yes				0
Sweet summer grass	Yes				0
Take-all					0
Velvetleaf					0
Wild oats	Yes				0
Wild radish					0
Windmill grass					0

Current weed practice and weed issues

The use case for Extendflex should be considered in the Australian context. A 2016 survey conducted by Monsanto identified the top 5 weeds targeted for control in cotton were fleabane, barnyard grass, milk/sowthistle, feathertop Rhodes grass and peachvine. (http://cdn-au.mailsnd.com/53263/3jiL4Mw99_KEcxkddAvhTMUY7URu4koAsDPm2aOCfcE/2655195.pdf)

The industry survey of consultants has found that fleabane is particularly costly and wide spread, with only 2% of growers indicating no financial impact and 9% estimating >$50 impact from fleabane (Figure 1). Reliance on glyphosate has seen resistant weeds become widespread across the industry (Figure 2), and the emergence of other hard to control weeds (figure 3).

Figure3: Estimated financial impact of weeds (2017/18 CCA qualitative survey)

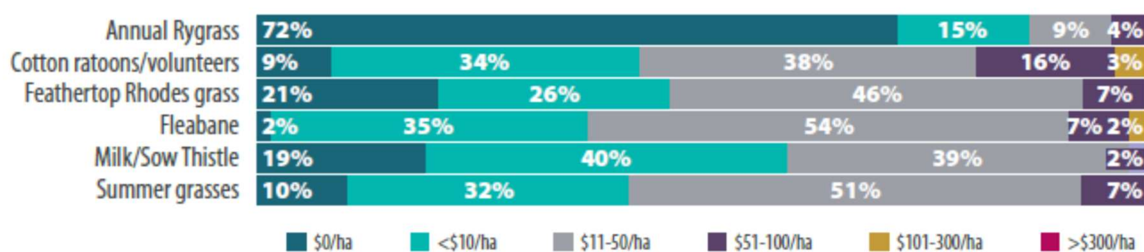


Figure-2. Estimate total areas believed to contain populations of glyphosate resistant weeds (2017/18 CCA qualitative survey)

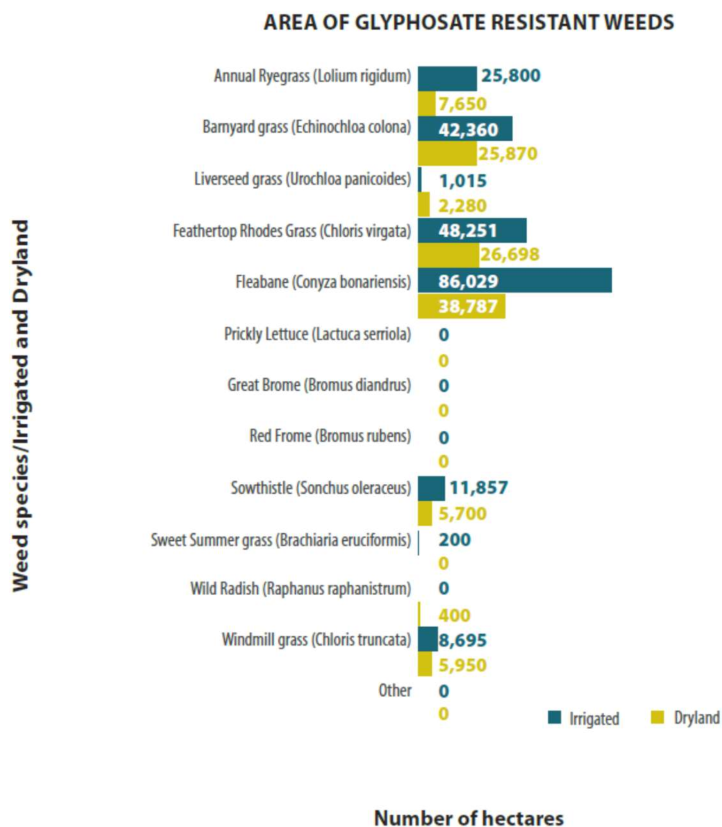
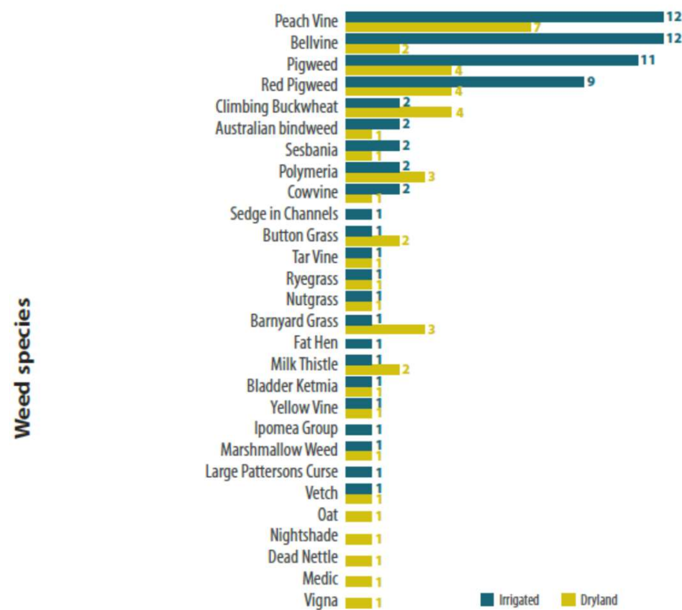


Figure-3. Aside from weed species known to have glyphosate resistance (as per previous question), other weed species that are becoming more challenging to control in the irrigated and dryland systems (2017/18 CCA qualitative survey)

OTHER WEED SPECIES CHALLENGING TO CONTROL IN IRRIGATED AND DRYLAND SYSTEMS

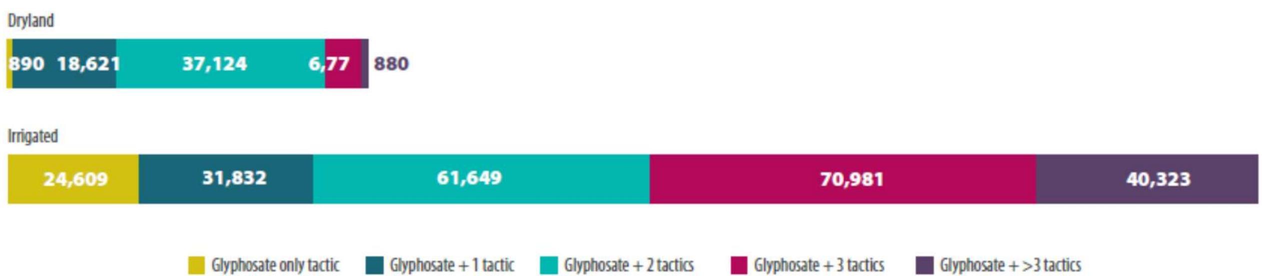


Number of responses

There is some evidence of reduced reliance on glyphosate for weed control in cotton. Figure 4 shows there is reasonable adoption of the HRMS recommended strategy of 2 or more tactics and figure 5 shows residual usage has lifted slightly from its 2012 low of 8% of total herbicides. However the industry is still largely reliant on glyphosate, making up 80% of herbicide use in cotton.

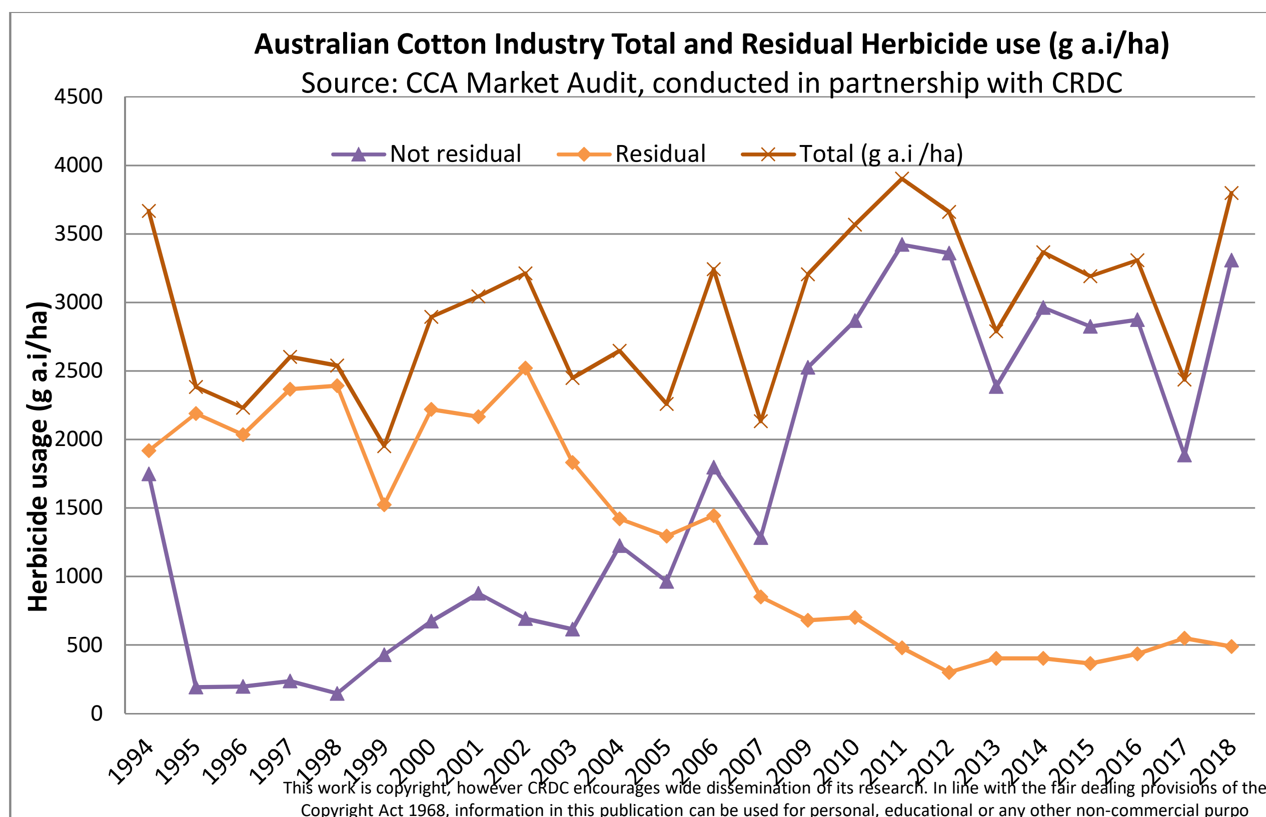
Figure-4. Estimated number of tactics used over irrigated and dryland cotton hectares in 2017/18. A tactic is considered a weed control operation such as cultivation, herbicide, chipping (2017/18 CCA qualitative survey)

WEED CONTROL TACTICS



Number of hectares

Figure-5. Total herbicide use compared with residual and non residual herbicices



Consultants Expected Use Patterns of Xtend Flex Herbicide Technology & Current Use Patterns of Dicamba & Glufosinate:

Emerald

- My thinking is that it will usually be a single use pattern for us, unless there is a reason that develops for us to double knock. At this stage I would say it would be a glyphosate-dicamba 1st application then on the second flush a glyphosate only (probably by air hence leaving Dicamba out)
- We have used a very small percentage of glufosinate in fallows through Weed-Its, but otherwise virtually none and no dicamba

MacIntyre

- Dicamba would be handy in-crop for some of our difficult broadleaves, particularly fleabane (dryland), peach vine, bell vine and sesbania. Whether this is on its own or with Roundup I don't know.
- Currently we often use a Group A early in the crop – often with the first in-crop Roundup. I'd assume, mixing this with Dicamba may have the potential to impact the grass control so would need to give this some thought.
- We use Dicamba occasionally in fallows – mainly specific cases with vetch or chickpea volunteers. Not general use by any means
- I've never used Glufosinate, so not having much experience with it, I'm a bit in the dark but if it offers some double-knock for the bigger weeds above, this would be useful. The higher water rate and ground-only restrictions of this product may give some resistance to its use
- These two products add minimal value for summer grass weed control which is our biggest weed challenge generally.

Darling Downs

- At present we don't use any Dicamba or Glufosinate. However, anything that will help with any of the vines would be of benefit as they are certainly becoming more of an issue on the downs. If the price is right then I don't see why there would be an issue with using it in the future.

Liverpool Plains

- Dicamba could be used pre-plant. If we haven't been able to get diuron or terbyne in the system in July-Sept, we will often end up with late fleabane or milk thistle to take out close to planting. Low rate of Starane followed by paraquat PSPE is common in this dryland scenario. Instead of paraquat PSPE could use Glufosinate early in-crop to finish any survivors.
- Xtend Flex would enable Dicamba to be used instead of Starane and thus reducing risk exposure to herbicide carryover. Also makes management of volunteer legumes, especially faba beans a bit easier too if dicamba was an option.
- Could also use Glufosinate as a double knock if going RR + Dicamba + Verdict for grass rather than paraquat in the cotton crop so should get better control of FTR, Windmill Grass, Glyphosate resistant BYG, Liverseed Grass, Button grass etc
- Potential weeds include peach vine, fleabane, milkthistle, volunteer legumes. Also bindweed and native bindweed if effective at higher rates.
- Using very little glufosinate due to inconsistency and price. Used in WeedIT it does go well. Dicamba use limited given cost but do use for faba control at times where lontrel isn't an option. Also limited given its drift risk to sensitive crops through the summer such as current BG3 and its volatilisation risk pending formulation quality.

Gwydir

- Expect Dicamba will help on peach vine & bell vine in crop where glyphosate is becoming less effective, also opens up the potential to use Dicamba in fallows leading up to planting cotton,
- Some of the earlier work on Glyphosate + Dicamba followed by Glufosinate looks promising, will certainly be a great help in dryland where we don't want to cultivate Fleabane, could see growers preferring this to relying on residuals
- Could also use dicamba (or glufosinate) as a directed spray during the final cultivation before row closure, as after that coverage of any OTT herbicide is an issue
- Main concerns are Dicamba drift issues onto sensitive crops/vegetation and the high water rates required for efficacy of Glufosinate, and also pricing of all products
- Currently use little Dicamba, mainly for control of volunteer Chickpeas, little bit of Glufosinate through optical sprayers

Macquarie

- Problem weeds are in order fleabane, plains grass (very unscientific, it just grows quick and glyphosate can struggle when it's bigger). Red pig weed too is becoming more prominent as well. Fleabane is a bigger problem than I had in the north.
- Interested in looking at dicamba on fleabane when it's young and to a lesser extent red pigweed. Also, double knock of all weeds with glufosinate where required.
- Glufosinate may take a bit to get effective control, so I think it would be field specific. Maybe dicamba would be a first pass by ground rig.
- I never use glufosinate, so a lot to learn broad acre. Have used it spot spraying and it worked well with coverage. Don't use dicamba other than in some corn years ago. Also, realise it has limitations.
- No silver bullets just options to attack specific problems.

2.2 - Investigate effectiveness of glufosinate and dicamba in cotton with range of herbicide-tolerance traits

Introduction:

Transgenic cotton varieties with tolerance to glyphosate have been in use in the Australian cotton industry since the year 2000. Its introduction initially made weed control in-crop more flexible as it allowed for glyphosate applications over-the-top of the crop in addition to other weed management tactics that were available. However, the over-reliance of glyphosate in-crop as well as in fallow has resulted in the evolution and proliferation of glyphosate resistance in five key weeds in cotton growing systems. These weeds are Awnless barnyard grass, feathertop Rhodes grass, Windmill grass, Fleabane and Sowthistle. As a result, growers have had to reduce their reliance on glyphosate at its effectiveness has somewhat diminished, and bring back tactics such as residual herbicides and cultivation that were dropped out of their weed management programs.

Xtendflex® cotton with tolerance to glyphosate, glufosinate and dicamba is now commercially available in the United States and will soon become available in Australia. It is proposed that the addition of these herbicides into weed management in cotton will provide growers with more flexibility in weed management and aid in the control of glyphosate resistant species.

Glufosinate-tolerant cotton has also been available since 2006. However after some initial adoption, this trait has been underutilised for a number of reasons including limited activity of glufosinate on some of the key species such as fleabane, and the summer grasses, tighter requirements for application and timing than glyphosate, and variability of control in non-ideal conditions.

Dicamba has been in use in winter cereals for a number of years, and apart from some summer fallow use, weeds that grow in the cotton season have generally had little exposure to this chemical previously. However, past dicamba use in winter cereals means that Sowthistle and Fleabane have possibly had considerable previous exposure to this herbicide (although the Fleabane is more likely to have had more exposure to 2,4-D).

Double knock combinations with paraquat or paraquat+diquat as the follow-up herbicide have proven successful in the control of a number of glyphosate resistant populations of these key. While glufosinate alone is expected to contribute little to the control of the summer grasses, it is proposed that its most effect use is likely to be in a double knock situation as the follow-up herbicide. A major component of this experiment was to determine the effectiveness of glufosinate as a double knock partner in place of paraquat, as Extendflex cotton would allow this type of application in-crop. In addition, it is important determine the most effective timeframe for the follow-up glufosinate applications to occur in order to achieve maximum control.

The introduction of glufosinate and dicamba should prove beneficial to weed management in Xtendflex® cotton, particularly for broadleaf weeds in general. This study was designed to investigate how the introduction of these herbicides is likely to affect control of the five key weeds in cotton growing regions which already have existing glyphosate resistant populations.

Methods:

Research into the effectiveness of glufosinate and dicamba was conducted with shade house experiments on fleabane, featherthop Rhodes and Windmill grass from 2015 to 2017. For both grass species, clethodim (Sequence) was used in place of dicamba.

For each species, control on glyphosate resistant/tolerant populations was compared to glyphosate susceptible populations to determine if the combinations of herbicide were effective on both. The fleabane populations were sourced from the Queensland Department of Agriculture and Fisheries (QDAF). The susceptible (Q21) and resistant (Q42) populations were previously used in an experiment conducted by (Werth, Walker, Boucher and Robinson 2010). Glyphosate resistant Sowthistle was sourced from Tony Cook at the New South Wales Department of Primary Industries (NSW DPI), and the susceptible population from QDAF. Both resistant (QBG4) and susceptible Awnless barnyard grass populations were sourced from QDAF. QBG4 was found to have a 3-4 fold resistance to glyphosate (Werth, Thornby, Walker, Charles and McDonald 2010). Both feathertop Rhodes grass populations were sourced from QDAF and had some tolerance of glyphosate. The "susceptible" population previously showed a 78% reduction in biomass compared to the untreated control, while the "resistant" population only had a 6% reduction in biomass (Walker *et al.* unpublished data). Both Windmill grass populations were sourced from Tony Cook.

Plants were sown onto the surface pots containing potting mix in a shade house. After emergence they were then thinned to four plants per pot. Plants were allowed to grow to large rosettes and initiation of stem elongation for Fleabane and Sowthistle, and mid-tillering for the grasses before being sprayed. Plants were sprayed in a research track sprayer at 93 L/ha water with DG95015EVS nozzles at 2 bar. Assessments were made by determining dry weights of plants harvested 28 days after the last spray.

Treatments were allocated (Tables 2-6) investigating combinations of glyphosate ± dicamba/clethodim with glufosinate applied as a double knock partner at set intervals after the first spray. Glufosinate timings were investigated to determine if there is an optimal timing for the second knock as has been found with paraquat in previous research. This use pattern was viewed as an appropriate long-term use pattern in Extendflex cotton systems to achieve high levels of control while mitigating against resistance evolution in the dicamba/clethodim and glufosinate:

Herbicide rates were allocated according to label rates for the respective herbicides in cotton (Table 1). Due to the high herbicide rates, plants were sprayed at mid-tillering in order to determine optimum intervals for double knock timings with glufosinate.

Table 1. Herbicides and rates used in all experiments

Herbicide	Label rate	Trial rate
Glyphosate	1.5 kg RR (1035 g ae/ha)	1.5 kg RR (1035 g ae/ha)
Dicamba	1.1 L/ha (480 g ai/ha) Clarity	1.1 L/ha Clarity
Clethodim	0.25-0.375L/ha (240g/L ai)	375 ml/ha Sequence + 20ml bonza
Glufosinate	3.75 L/ha Liberty (750 g ai/ha)	3.75 L/ha Basta

Statistical Analysis

The experiment was designed as a randomised complete block with four replicates. For Fleabane and Windmill grass in both experiments and Sowthistle and feathertop Rhodes grass in experiment 2, treatments that had 100% control in both glyphosate and resistant

populations were removed from the analysis. Data was then angular transformed, and an analysis of variance was conducted.

Results:

Fleabane

Control of fleabane was quite effective in both experiments, particularly where Dicamba or Glyphosate+Dicamba were followed by glufosinate in a double knock (Table 2). In the first experiment Dicamba alone had significantly less control on the glyphosate susceptible (80%) compared to the glyphosate resistant population (91%). Control with dicamba alone improved in both populations in the second experiment. Control was further improved with the addition of glyphosate or glufosinate as a tank-mix partner.

Each herbicide when used individually gave variable control, however when used in combinations control was improved. The glyphosate fb glufosinate double knock treatments also had variable control, with significantly less control to the glyphosate resistant population in the second experiment at all double knock timings. This result indicates the importance of not relying on glyphosate alone for fleabane control, even when following up with glufosinate. The timing of the follow-up glufosinate application also did not affect control in either experiment when either dicamba or dicamba+glyphosate was used as the initial herbicide.

Table 2. Percent of untreated control of glyphosate resistant and susceptible populations of fleabane. Numbers in brackets are arcsin transformed with the means being back transformed. Means without transformed numbers in brackets were removed from analysis as they did not fit the normality assumption required for analysis of variance. Analysis compared resistant to susceptible population for each year independently.

Initial Herbicide	Follow-up Herbicide	Interval (Days)	Experiment 1 Resistant	Experiment 1 Susceptible	Experiment 2 Resistant	Experiment 2 Susceptible
Glyphosate			67.0 (54.9)*	100.0 (90.0)	61.6 (51.7)*	88.6 (70.3)
Dicamba			91.0 (72.6)	80.1 (63.9)*	98.3 (82.5)	100.0 (90.0)
Glufosinate			99.4 (85.6)	95.5 (77.7)	79.2 (62.9)	79.3 (62.9)
Glyphosate + Dicamba			100.0	100.0	100	100
Glufosinate + Dicamba			-	-	99.7 (87.0)	100.0 (90.0)
Glyphosate	Glufosinate	1	99.5 (86.1)	100.0 (90.0)	88.0 (69.8)*	98.8 (83.7)
Glyphosate	Glufosinate	3	99.9 (88.4)	100.0 (90.0)	79.5 (63.1)*	98.9 (83.8)
Glyphosate	Glufosinate	7	100.0	100.0	94.8 (76.8)*	100.0 (90.0)
Glyphosate	Glufosinate	10	99.7 (86.7)	100.0 (90.0)	82.6 (65.3)*	97.0 (80.0)
Dicamba	Glufosinate	1	100.0	100.0	100.0	100.0
Dicamba	Glufosinate	3	100.0	100.0	100.0	100.0
Dicamba	Glufosinate	7	100.0	100.0	100.0	100.0
Dicamba	Glufosinate	10	100.0	100.0	100.0	100.0
Glyphosate + Dicamba	Glufosinate	1	100.0	100.0	100.0	100.0
Glyphosate + Dicamba	Glufosinate	3	100.0	100.0	100.0	100.0
Glyphosate + Dicamba	Glufosinate	7	100.0	100.0	100.0	100.0
Glyphosate + Dicamba	Glufosinate	10	100.0	100.0	100.0	100.0
LSD Population x Treatment (P = 0.05)			(8.4)		(8.2)	

* Indicates control of the resistant population significantly differed from the susceptible population.

Feathertop Rhodes grass

Consistent control of feathertop was only achieved in when the herbicides were used in a double knock combination (Table 3). Timing of the follow-up glufosinate had a slight effect on the control achieved in both experiments, with 99-100% control only reached with 7 and 10 day intervals between initial herbicides and glufosinate in both experiments. This was independent of what the initial herbicides used were.

Clethodim alone provided above 90% control of both glyphosate resistant and susceptible populations, which was slightly better than when clethodim and glyphosate were combined in a tank-mix. The effect of clethodim alone being more effective and clethodim+glyphosate was less noticeable in the double knock treatments intervals of 1 and 3 days for the follow-up glufosinate.

Control with Glufosinate alone was lower in the 2nd experiment with a significant difference between the glyphosate susceptible (57%) compared to the resistant population (78%). There was also significantly reduced control of the glyphosate resistant compared to the susceptible populations at the one and three day timings between glyphosate and glufosinate double knocks.

Table 3. Percent of untreated control of glyphosate resistant and susceptible populations of feathertop Rhodes grass. Numbers in brackets are arcsin transformed with the means being back transformed. Means without transformed numbers in brackets were removed from analysis as they did not fit the normality assumption required for analysis of variance. Analysis compared resistant to susceptible population for each year independently.

			Experiment 1		Experiment 2	
Initial Herbicide	Follow-up Herbicide	Interval (Days)	Resistant	Susceptible	Resistant	Susceptible
Glyphosate			56.2	93.5	2.2 (8.5)*	98.3 (82.5)
Clethodim			97.0	98.6	91.7 (73.3)	90.8 (72.4)
Glufosinate			99.1	99.9	77.9 (62.0)	57.0 (49.0)*
Glyphosate + Clethodim			89.6	89.7	99.4 (85.6)	90.7 (72.3)*
Glufosinate + Clethodim			-	-	89.3 (70.9)	84.0 (66.5)
Glyphosate	Glufosinate	1	100.0	100.0	38.2 (38.2)*	84.3 (66.7)
Glyphosate	Glufosinate	3	99.2	100.0	48.9 (44.3)*	86.8 (68.7)
Glyphosate	Glufosinate	7	100.0	100.0	100.0	100.0
Glyphosate	Glufosinate	10	99.5	100.0	100.0	100.0
Clethodim	Glufosinate	1	100.0	99.8	96.2 (78.8)	98.3 (82.6)
Clethodim	Glufosinate	3	100.0	100.0	99.3 (85.2)	99.5 (86.1)
Clethodim	Glufosinate	7	99.7	100.0	100.0	100.0
Clethodim	Glufosinate	10	100.0	100.0	100.0	100.0
Glyphosate + Clethodim	Glufosinate	1	97.5	99.3	97.7 (81.2)	90.5 (72.1)*
Glyphosate + Clethodim	Glufosinate	3	93.5	100.0	99.6 (86.5)	96.3 (78.9)
Glyphosate + Clethodim	Glufosinate	7	99.4	100.0	100.0	100.0
Glyphosate + Clethodim	Glufosinate	10	99.6	100.0	100.0	100.0
LSD Population x Treatment (P = 0.05)			-		(8.9)	

* Indicates control of the resistant population significantly differed from the susceptible population.

Windmill grass

Windmill grass generally proved to be more a difficult species to achieve total control even in glasshouse circumstances as can be seen by the results in Table 4. No treatment provided total control of glyphosate resistant windmill in both years, although the clethodim fb glufosinate 10 days later and the glyphosate + clethodim fb glufosinate 7 and 10 days later gave 95% control or higher in both years. The treatments with a glufosinate application 10 days after the initial herbicides appear to be generally most effective across both glyphosate resistant and susceptible populations.

It is interesting to note the in the susceptible population in Experiment 2 the control achieved by glyphosate alone was higher than glyphosate fb glufosinate 1, 3 and 7 days later, where control was not only lower but variable. This may indicate that in some circumstances there could be some antagonism if glufosinate is applied too soon after glyphosate, and may impede the action of the glyphosate. This effect was not observed for the other species tested.

Table 4. Percent of untreated control of glyphosate resistant and susceptible populations of Windmill grass. Numbers in brackets are arcsin transformed with the means being back transformed. Means without transformed numbers in brackets were removed from analysis as they did not fit the normality assumption required for analysis of variance. Analysis compared resistant to susceptible population for each year independently.

			Experiment 1		Experiment 2	
Initial Herbicide	Follow-up Herbicide	Interval (Days)	Resistant	Susceptible	Resistant	Susceptible
Glyphosate			65.6 (54.1)*	97.6 (81.1)	69.6 (56.6)*	98.4 (82.7)
Clethodim			96.5 (79.2)	98.6 (83.2)	95.9 (78.3)	96.6 (79.3)
Glufosinate			94.1 (75.9)	97.3 (80.6)	98.2 (82.3)	86.1 (68.1)*
Glyphosate + Clethodim			92.4 (74.0)	85.8 (67.8)	95.1 (77.2)	97.0 (80.1)
Glufosinate + Clethodim			93.8 (75.7)	93.3 (75.0)	91.1 (72.7)	93.6 (75.3)
Glyphosate	Glufosinate	1	82.5 (65.2)	92.4 (74.0)	75.0 (60.0)	77.1 (61.4)
Glyphosate	Glufosinate	3	100.0	100.0	77.2 (61.5)*	95.2 (77.3)
Glyphosate	Glufosinate	7	100.0	100.0	79.3 (63.0)	74.1 (59.4)
Glyphosate	Glufosinate	10	96.5 (79.3)*	100.0 (90.0)	100.0	100.0
Clethodim	Glufosinate	1	99.4 (85.6)	95.1 (77.2)	87.1 (68.9)*	97.5 (81.0)
Clethodim	Glufosinate	3	100.0	100.0	95.9 (78.3)*	100.0 (90.0)
Clethodim	Glufosinate	7	100.0	100.0	97.7 (81.3)	99.9 (89.3)
Clethodim	Glufosinate	10	99.7 (87.1)	100.0 (90.0)	100.0	100.0
Glyphosate + Clethodim	Glufosinate	1	96.8 (79.8)	97.7 (81.3)	81.5 (64.6)	93.2 (74.9)
Glyphosate + Clethodim	Glufosinate	3	100.0	100.0	95.8 (78.2)	84.5 (66.8)
Glyphosate + Clethodim	Glufosinate	7	100.0	100.0	99.6 (86.2)*	91.1 (72.7)
Glyphosate + Clethodim	Glufosinate	10	99.8 (87.5)	100.0 (90.0)	100.0	100.0
LSD Population x Treatment (P = 0.05)			(9.8)		(11.6)	

* Indicates control of the resistant population significantly differed from the susceptible population.

Awnless barnyard grass

Control of awnless barnyard grass was very successful with these combinations of herbicides on both glyphosate and resistant populations in both experiment (Table 5). The only exception was the reduced control from glyphosate on the glyphosate resistant population.

Table 5. Percent of untreated control of glyphosate resistant and susceptible populations of Awnless barnyard grass. Statistical analysis was not performed on this species as data did not fit the normality assumption required for analysis of variance.

			Experiment 1		Experiment 2	
Initial Herbicide	Follow-up Herbicide	Interval (Days)	Resistant	Susceptible	Resistant	Susceptible
Glyphosate			46.9	96.9	59.8	99.9
Clethodim			100.0	100.0	100.0	100.0
Glufosinate			100.0	100.0	100.0	100.0
Glyphosate + Clethodim			99.4	99.8	95.8	99.8
Glufosinate + Clethodim						
Glyphosate	Glufosinate	1	100.0	100.0	99.4	98.2
Glyphosate	Glufosinate	3	93.0	100.0	100.0	100.0
Glyphosate	Glufosinate	7	100.0	100.0	100.0	100.0
Glyphosate	Glufosinate	10	96.3	100.0	100.0	99.9
Clethodim	Glufosinate	1	100.0	97.3	100.0	100.0
Clethodim	Glufosinate	3	100.0	100.0	100.0	100.0
Clethodim	Glufosinate	7	100.0	100.0	100.0	100.0
Clethodim	Glufosinate	10	100.0	100.0	100.0	100.0
Glyphosate + Clethodim	Glufosinate	1	100.0	100.0	100.0	99.9
Glyphosate + Clethodim	Glufosinate	3	100.0	100.0	100.0	100.0
Glyphosate + Clethodim	Glufosinate	7	100.0	99.7	100.0	100.0
Glyphosate + Clethodim	Glufosinate	10	100.0	100.0	100.0	100.0

Sowthistle

For sowthistle, the double knock treatments with glufosinate provided effective control regardless of resistance to glyphosate (Table 6). The timing of the second glufosinate knock was not significant in the experiment, with the exception of glyphosate fb glufosinate 2 day later in Experiment 2 (although above 90% control was still observed). However, the best control of both populations was achieved with glufosinate applications 7 days after initial treatment, irrespective of what the initial treatment consisted of.

No differences in efficacy between glyphosate resistant and susceptible populations were measured in the first experiment. However, the reduced control from glyphosate alone was significant in the 2nd experiment. Also, in the 2nd experiment Dicamba alone was significantly less effective on the glyphosate resistant population species (76% control of susceptible, 53% control of resistant). There was also some reduced control of the glyphosate resistant population, which was significant when glyphosate was followed by glufosinate one day later (99% control of susceptible, 92% control of resistant).

There were no differences noticed in the effectiveness of, dicamba + glyphosate or glufosinate between glyphosate resistant and susceptible populations in either experiment.

Table 6. Percent of untreated control of glyphosate resistant and susceptible populations of Sowthistle. Numbers in brackets are arcsin transformed with the means being back transformed. Means without transformed numbers in brackets were removed from analysis as they did not fit the normality assumption required for analysis of variance. Analysis compared resistant to susceptible population for each year independently.

			Experiment 1		Experiment 2	
Initial Herbicide	Follow-up Herbicide	Interval (Days)	Resistant	Susceptible	Resistant	Susceptible
Glyphosate			97.6	100.0	64.8 (53.6)*	99.9 (89.2)
Dicamba			100.0	94.1	52.6 (46.5)*	75.6 (60.4)
Glufosinate			100.0	100.0	99.7 (86.7)	99.2 (85.0)
Glyphosate + Dicamba			100.0	100.0	93.0 (74.7)	97.0 (80.1)
Glufosinate + Dicamba			-	-	100.0	100.0
Glyphosate	Glufosinate	1	100.0	100.0	92.4 (74.0)*	99.7 (86.9)
Glyphosate	Glufosinate	3	100.0	100.0	98.0 (82.0)	100.0 (90.0)
Glyphosate	Glufosinate	7	100.0	100.0	99.9 (88.6)	100.0 (90.0)
Glyphosate	Glufosinate	10	100.0	100.0	98.8 (83.7)	100.0 (90.0)
Dicamba	Glufosinate	1	100.0	100.0	99.9 (88.6)	99.3 (85.2)
Dicamba	Glufosinate	3	100.0	100.0	99.9 (88.0)	100.0 (90.0)
Dicamba	Glufosinate	7	100.0	100.0	100.0	100.0
Dicamba	Glufosinate	10	100.0	100.0	99.9 (88.9)	100.0 (90.0)
Glyphosate + Dicamba	Glufosinate	1	100.0	100.0	100.0	100.0
Glyphosate + Dicamba	Glufosinate	3	100.0	100.0	99.9 (88.0)	100.0 (90.0)
Glyphosate + Dicamba	Glufosinate	7	100.0	100.0	100.0	100.0
Glyphosate + Dicamba	Glufosinate	10	100.0	100.0	99.9 (88.6)	100.0 (90.0)
LSD Population x Treatment (P = 0.05)			-		(9.5)	

* Indicates control of the resistant population significantly differed from the susceptible population.

Conclusion

The combination of these herbicides has generally resulted in effective control of both glyphosate resistant and susceptible populations of the weeds tested in this experiment. The most consistent results have come from using glufosinate as a double knock partner, particularly where the timing of the follow-up application is 7 and 10 days. There was also no consistent results to determine that control of either dicamba, glufosinate or clethodim alone was reduced on glyphosate resistant compared to susceptible populations. This shows there is no indication that resistant to glyphosate in these species is linked to any of the other herbicides.

The introduction of Xtendflex® cotton should be beneficial weed management in-crop. However it is critical to state that these herbicides should be used in addition to existing tactics such as pre-emergent herbicides and non-chemical tactics to ensure the seed bank is kept low and to minimise the chances of resistance evolution to these herbicides.

References:

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2.3 - Define potential impact of non-chemical tactics, such as pupae busting and crop rotations/cover crops, on seed-bank dynamics

Introduction

The increase in herbicide resistance has started to change the focus of weed control towards non-chemical and cultural tactics. These tactics include tillage, crop competition, harvest weed seed control and cover crops. Tillage has been commonly used in cotton systems, particularly before the introduction of glyphosate-resistant technology. Since the technology was introduced, the amount of tillage before and within the crop reduced. Pupae busting is essentially a tillage operation after the end on the season that can bury seed and possibly reduce subsequent weed emergences, particularly for small seeded species. Crop competition in cotton crops is hard to achieve unless the crop is kept weed free for at least eight weeks in a 1 m row spaced irrigated crop. Cotton is initially an uncompetitive plant, and row spacing, particularly in non-irrigated crops is generally greater than 1 m.

Cover crops are used by some growers to help with water infiltration, reduce evaporation and runoff, and improve soil structure, carbon and nitrogen. The impacts of these cover crops on weed populations in cotton systems in Australia have not been studied in detail. Research in North America has shown that cover crops can provide some suppression of early season weeds in a number of crops including cotton (White and Worsham 1990; Norsworthy 2011). Research by Teasdale (1996) showed that residues from cover crops could reduce the available light and moisture for weeds when they germinate.

Winter annual cover crops investigated in the US have included cereal rye and wheat. Cereal rye in particular has been shown to have a high biomass potential and some allelopathic properties (Reeves *et al.* 2005). These cover crops are typically terminated 3 weeks prior to cotton planting. This late termination of the cover crop is possible in the US due to higher rainfall resulting in less soil moisture constraints. We propose that a winter cover crop in Australia would need to be terminated earlier to minimise soil moisture loss and allow more time for rainfall between cover crop termination and cotton planting.

There are some growers on the Darling downs who utilise a summer crop of millet in a 1 in 3 year dryland cotton rotation. The millet is planted the following summer after the cotton crop and terminated before setting seed. The stubble then remains for the next summer fallow and is broken down enough to plant cotton in the following season. This study aimed to test this scenario for the effect on weed germination. In order to test the winter cover crop prior to cotton planting, we chose to use barley as it is considered more competitive against weeds than wheat, and little cereal rye is grown in Australia. We decided to leave pupae busting out of this experiment so we could focus on the cover crop, as well as equipment limitations and another CRDC project that was focusing on pupae busting in more detail.

Materials and methods

Trial design

A dryland experiment was established at the Queensland Department of Agriculture and Fisheries research farm at Wellcamp. The experiment consisted of eight treatments (Table 1). The first treatment was a 1 in 3 year cotton rotation. The second treatment started with a millet cover crop followed by cotton the next season, and then a summer fallow then cotton. The third and fourth treatments had a summer fallow between the millet and the cotton crop. The third treatment started with the millet crop and the fourth started with cotton. This was done so that more information could be gathered on the effect on weed germinations from the

millet. The fifth treatment was a standard cotton cereal rotation, the cereal is often used to break down the soil and provide some cover after the previous cotton crop. The sixth and seventh treatments were barely planted prior to the cotton crop with two different termination times. The first termination time in treatment 6 was planned at mid-tillering (approximately 8 weeks prior to cotton being planted). The second termination time in treatment 7 was planned at booting (approximately 5 weeks prior to planting cotton). Treatment 8 was a standard cotton-sorghum rotation.

Table 1. Cover crop trial rotations for the experiment at Wellcamp Research Station.

	Year 1		**Year 2**		**Year 3**		**Year 4**	
Trt No.	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**
1	Cotton	Fallow	Fallow	Fallow	Cotton	Fallow	Fallow	Fallow
2	Millet LS	Fallow	Cotton	Fallow	Fallow	Fallow	Millet LS	Fallow
3	Cotton	Fallow	Millet LS	Fallow	Fallow	Fallow	Cotton	Fallow
4	Millet LS	Fallow	Fallow	Fallow	Cotton	Fallow	Millet LS	Fallow
5	Cotton	Barley LS	Fallow	Fallow	Cotton	Barley LS	Fallow	Fallow
6	Fallow	Barley ES	Cotton	Fallow	Fallow	Barley ES	Cotton	Fallow
7	Fallow	Barley LS	Cotton	Fallow	Fallow	Barley LS	Cotton	Fallow
8	Sorghum	Fallow	Fallow	Fallow	Cotton	Fallow	Sorghum	Fallow

The trial was a randomised complete block design with four replications. Individual plots were 3 m x 20 m. Cotton was planted at 2 rows per plot 1m apart in the middle of the row. When adjacent plots of cotton were planted this formed a single skip configuration. Cotton was planted at a rate of 10 seeds/m to achieve 8 plants/m. In the first year Sicot 74 BRF was used, and Sicot 746 B3F in subsequent years. Sorghum (MR Blazer) was planted in 3 rows, 75 cm apart to achieve a plant population of 75 000 plants/ha. White French Millet was planted at 8 kg/ha in 25cm rows. Barley was planted at 50 kg/ha in 25 cm rows.

Trial Procedure

Before the trial commenced the area was sprayed with glyphosate to control winter weeds. Seeds of awnless barnyard grass, feathertop Rhodes grass, sowthistle and fleabane were spread by hand across the trial area. Each year, the areas to be planted to cotton were fertilised with Urea at a rate of 180 kg/ha, placed adjacent to the rows with the “Kinze” double disc opener planter. Cotton, sorghum and millet were then planted into their respective plots in November. Cotton received an additional 40 kg/ha of MAP Zn at planting. The sorghum received 120 and 60 kg/ha Urea respectively at planting, plus 40 kg/ha MAP Zn. Soil tests were taken in each plot in treatment 2 each year to monitor nutrient levels.

Weed emergence counts were taken throughout the season after rainfall events using 6 x 0.5 m^2 quadrats up the centre of the rows. The millet and sorghum were both sprayed out with glyphosate just prior to anthesis. Samples to measure the biomass from the barley and millet stubble were taken from treatments 2, 3, 6 and 7 prior to planting in Year 2. This was done taking samples from 3 x 0.5 m^2 quadrats. This was also planned prior to Year 4, however dry conditions meant the cover crops did not produce enough biomass to be worth sampling. Cotton was defoliated using Dropp Liquid at 100 ml/ha plus DC Tron at 1 l/ha. Cotton was picked and weighed with a plot picker. After picking, the cotton plots were then slashed and disced to prevent volunteers.

Results

Weed emergence

Of all the weed species planted, awnless barnyard grass was the only one that consistently emerged in large enough numbers to count (Table 2). Results from this experiment did not clearly show that the cover crops suppressed weeds in the absence of herbicides. Initial emergences in Year 1 resulted in fewer plants emerging in the treatments that began in fallow, compared to treatments that had a crop planted. This was most likely due to soil disturbance at planting and subsequent irrigation to get the trial started stimulating awnless barnyard grass emergence. The highest initial emergences did occur in all the treatments that started with cotton. Cotton is a poor competitor initially, and photos from Figure 1 show the increased competitiveness of millet at the same age. As a result, it appears that there could be a combination of soil disturbance from the planter, and initial competitiveness of the crop that led to the initial emergence counts, however these results are not conclusive. Throughout the remainder of the season, the treatments with fallow had slightly higher emergences, however these were only low numbers overall, and were not significantly different from the other treatments.

Table 2. Total awnless barnyard grass emergence at the end of each season with respect to each crop and previous cover crop.

	Year 1		Year 2		Year 3		Year 4		
No	Current Crop	Plants/m^2	Previous /Current crop	Plants/m^2	Previous /Current crop	Plants/m^2	Previous /Current crop	Plants/m^2	Total
1	Cotton	86.3 e	Fal/Fal	6.9	Fal/Cot	4.6 a	Fal/Fal	3.3 d	72.5
2	Millet	50.8 bc	Fal/Cot	16	Fal/Fal	7.3 a	Fal/Mil	0.6 ab	69.2
3	Cotton	81.6 e	Fal/Mil	6.5	Fal/Fal	6.3 a	Fal/Cot	3.0 d	66.1
4	Millet	56.5 cd	Fal/Fal	12.1	Fal/Cot	23.7 c	Fal/Mil	1.2 abc	69.5
5	Cotton	73.1 de	Bar LS/Fal	10.1	Fal/Cot	12.5 ab	Bar LS/Fal	0.2 a	88.8
6	Fallow	19.8 a	Bar ES/Cot	18.3	Fal/Fal	8.6 ab	Bar ES/Cot	2.3 bcd	79.6
7	Fallow	33.3 ab	Bar LS/Cot	11.1	Fal/Fal	12.3 ab	Bar ES/Cot	2.6 cd	104.9
8	Sorghum	30.1 ab	Fal/Fal	16.1	Fal/Cot	18.9 bc	Fal/Sor	2.4 cd	90.2
LSD (P = 0.05)		*21.1*		*13.0*		*10.9*		*1.7*	*43.7*

The second year of the experiment saw no differences in weed emergence between the treatments. The highest number of emergences of 18 plants/m^2 occurred in the treatment 6, where cotton followed on from the barely ES sprayed out at mid-tillering. The residue from the cover crops did not appear to reduce weed emergence, however it appeared that the treatments that had some stubble remaining may have created an environment favourable for weed emergence. This effect was more noticeable when the stubble remaining was from the millet, barley and sorghum (treatments 2, 6, and 8 respectively). However, plant numbers overall were low due to dry conditions and the effect was not significant.

The stubble loads from the cover crops were measured just after cotton planting (Table 3). The barley LS spayed out at booting had significantly more stubble at the time of cotton planting than the other crops (Figure 2). The heavier stubble load in the barley LS treatments (5 and 8) had lower emergences than most other treatments towards the start of the season. However, as the season progressed and the stubble broke down, the number of barnyard grass emergences increased. This extra stubble provided a challenge for planting cotton as the planter was not equipped with trash-workers to cut through the stubble, and stubble in the planting row had to be removed manually. The winter of 2016 had sufficient rainfall to establish the cotton crop 5-8 weeks after the barley was sprayed out. The dry conditions following planting also resulted in reduced cotton yields compared to the previous season.

The average yield across the majority of cotton plots as 4 bales/ha. However, the cotton planted into the Barley LS treatments yielded an average of 8.5 bales/ha (Table 4). This was most likely due to the higher stubble load reducing moisture loss from the soil.

Table 3. Amount of stubble remaining in October 2016 after planting cotton.

Treatment No.	Crop	t/ha
2	Millet	2.0 a
3	Cotton	1.8 a
6	Barley (ES)	2.1 a
7	Barley (LS)	5.3 b

Emergences at the start of year 3 were highest in Treatment 4 that had millet in the first year and long fallow (21 plants/m^2), this was followed by Treatment 8 that had sorghum in the first year than then fallow (16 plants/m^2). This effect was significant; however, Treatment 1 had the lowest emergences (4 plants/m^2) with cotton in the first year and then a long fallow. Significant effects at this point appeared to be coincidental with field variability. There may have been some effect of the small amount of residual stubble from previous crops in other treatments, however this is unlikely.

The remainder of the trial suffered from lack of sufficient soil moisture. The trial was irrigated prior to planting the barley cover crop, however despite this initial irrigation, the lack of rainfall and inability to irrigate in a timely manner resulted in the barley suffering from severe drought stress. Cotton was able to be planted with some rainfall in November, however emergence of the crop was very patchy. Due to this patchy emergence and lack of rainfall, the cotton was not picked in the final year. Weed emergences were also very low and showed no real response to the previous cover crop.

Table 4. Cotton yields with respect to each treatment and year. Cotton was not picked in year 4 due to patchy emergence and dry conditions.

	Year 1		Year 2		Year 3	
No	Current Crop	Bales/ha	Previous /Current crop	Bales/ha	Previous /Current crop	Bales/ha
1	Cotton	14.4 ± 0.8	Fal/Fal		Fal/Cot	7.0 ± 0.2
2	Millet		Fal/Cot	4.6 ± 0.9	Fal/Fal	
3	Cotton	14.9 ± 1.2	Fal/Mil		Fal/Fal	
4	Millet		Fal/Fal		Fal/Cot	7.0 ± 0.7
5	Cotton	11.9 ± 0.4	Bar LS/Fal		Fal/Cot	5.7 ± 1.2
6	Fallow		Bar ES/Cot	3.6 ± 0.7	Fal/Fal	
7	Fallow		Bar LS/Cot	8.6 ± 2.3	Fal/Fal	
8	Sorghum		Fal/Fal		Fal/Cot	6.7 ± 0.2

Figure 1. Awnless barnyard grass emerging amongst cotton (top), millet (middle) and sorghum (bottom) as initial weed counts were taken in December 2015.

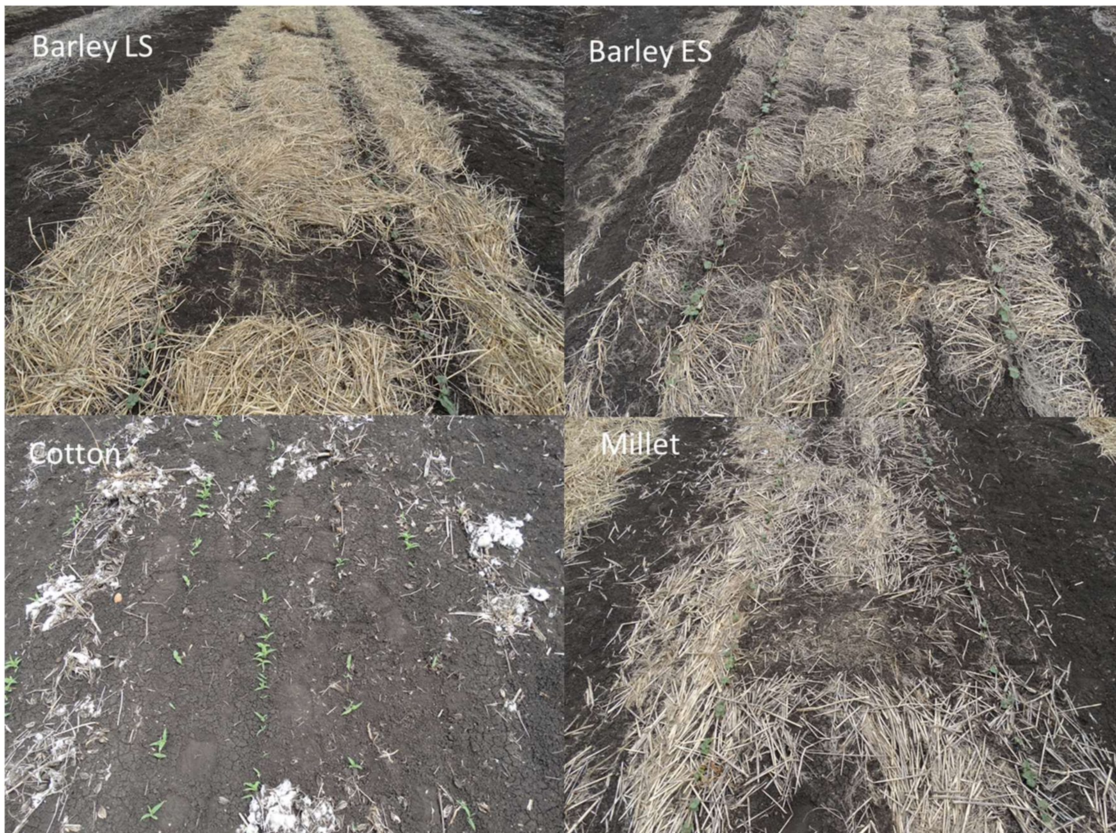

Figure 2. Stubble remaining in October 2016 with respect to the previous crop. Cotton and millet were planted in November 2015. Barley was planted in July 2016.

Potential for genomics and new genetic control methods for weeds of the Australian cotton industry

Introduction

Recent years have seen dramatic advances in our ability to turn DNA into digital data. As the field of genomics enters its golden years, opportunities arise for the cotton industry in relation to weed research and potentially new methods of weed control further into the future.

Opportunities in Genomics

Genomics has fundamentally changed genetic research. Without a genome, genetics is like looking for a needle in a haystack. A genome can be thought of as a map that we can use to find what we are looking for. Genome sequencing can be thought of as an infrastructure project, one that creates a resource from which many future discoveries will be made. Sometimes it is hard to predict what these future discoveries might be, but we will focus here on the areas where genomics can help weed research now.

An international weed genomics consortium has been developed (https://www.weedgenomics.com/about/) to foster collaboration and data sharing for weed genomes, and a review has been published outlining some of the opportunities for genomics in weed research (Ravet et al 2018). This highlights the growing momentum around weed genomics, which is largely due to technological advances that make genomics much more affordable.

Understanding the evolution of resistance
Other parts of this report demonstrate how genomics helps us to understand resistance and make predictions about how different weed species will evolve herbicide resistance in the future. The number of copies of a target site gene is a crucial factor in the way that resistance evolves, species with a single copy of the gene are more likely to evolve target site resistance and species with many copies are more likely to evolve non-target site resistance. A weed species might have multiple copies of a target site gene because of polyploidy, but even diploid species can have several copies of a gene through ancient gene duplications and this is something that we have learned thanks largely to genomics.

Having a genome available also contributes to our ability to conduct population genetic studies in polyploid weeds as demonstrated in this project with the sequencing of the sowthistle genome. Genotyping is difficult in a polyploid species because it is hard to figure out which markers belong to which subgenome, the availability of a reference genome allows us to establish this, and then conduct population genetics in the same way as for a diploid organism.

Establishing the mechanisms of Non-Target-Site-Resistance
The mechanisms underpinning non-target-site-resistance have been difficult to establish using traditional genetic approaches. The availability of a genome provides a reference to guide these studies. In this project we developed a genome for sowthistle, and this is currently being used to further investigate NTSR mechanisms in this species by Chris Preston's group in Adelaide. We also used genomic approaches to characterise the mechanism of NTSR in fleabane, which has yielded a promising candidate gene that may be involved in a vacuole-sequestration resistance mechanism. The fleabane genome will enable

further investigation of this candidate, other parts of this sequestration system, and specific mutations associated with resistant and susceptible lines.

Genetic tests for resistance
Fully characterising NTSR mechanisms will allow genetic tests for resistance to be designed. In this project, only one of the four species had a target-site mutation (feathertop Rhodes) – designing a genetic test for resistance in this species is straightforward. It is not so simple in species with NTSR resistance mechanisms, because we generally do not know the molecular basis of NTSR resistance. Once these mechanisms are fully characterised, and it has been established that only one mechanism has evolved in that species, then genetic tests can be designed that would speed up resistance testing and provide information to growers much more rapidly.

Precision herbicide development
Chemists are now able to use protein predictions from genomes to identify new targets for herbicide development. Genomics therefore opens up new ways to guide herbicide development. Key to this process is establishing how many copies of a particular target gene there are, and how many different versions of that protein might have to be targeted.

Potential for genetic control of weeds

Genetics may provide ways to control weeds in the future, two technologies are currently proposed as promising candidates, gene drives and RNA interference. Both technologies are targeted at specific species, and this will rely on the availability of a genome sequence for that species. This should be taken into consideration when prioritising genome sequencing projects in weeds.

Gene drives
Gene drives are not a new idea, but the invention of CRISPR/Cas9 genome editing has given new impetus to the consideration of synthetic gene drives. These gene drives push a variant through a population faster than mendelian inheritance would allow. This is achieved by the insertion of a genome-editing mechanism so that a single inherited copy of the allele is copied across chromosome pairs within an individual. It has been proposed that this kind of synthetic gene drive could be used to eradicate pest populations (Webber et al 2015) or perhaps even reverse herbicide resistance (Hoffmann et al 2017).

Sterility-inducing gene drives have been modelled as a means to eradicate invasive mouse populations (Prowse et al. 2017). Two strategies (homozygotic embryonic non-viability and homozygotic female sterility) worked well, especially if multiple guideRNA′s were introduced to counteract the evolution of resistance alleles. Many important agricultural weeds are self-compatible, and the possible effect of high rates of selfing on gene-drive interventions have not been explored. Previous modelling of cytoplasmic incompatibility as a mechanism of weed control indicated that it would only be effective in species with a low effective selfing rate (Hodgkins et al).

In agricultural weeds, there may be options for pushing weed-killing mutations through a population, or simply reversing the evolution of herbicide resistance so that herbicides become an effective control method again. Many authors have called for more modelling of the potential of gene drives in agricultural weeds (Neve 2018). This is clearly required before further research on the development of gene drive technology in weeds.

Many aspects of weed population biology might cause issues for gene drives, for example, annual generations, the seed bank, and very large population sizes. Sexual reproduction is key to the success of any gene drive, and high rates of selfing are a considerable barrier to the

success of any potential gene drive program. Some authors have suggested that it may be possible to reverse self-compatibility in weeds as part of the gene drive process, but this will be difficult and may only work for some species.

Herbicide resistance-reversing gene drives are likely to be more palatable to the public and regulators than methods that aim to eliminate an entire species. However, if we consider the nine key glyphosate resistant weeds in cotton, only two of these are obligate outcrossing (table 1). Work in this project has shown that windmill grass and feathertop Rhodes have very low rates of outcrossing. Although there is some evidence for outcrossing in fleabane and sowthistle from the population genetics data, our experiment did not detect any outcrossing in 200 individuals of each cross, suggesting that the rates are very low. More modelling is required of the effect of different outcrossing rates on gene drive systems, but it seems likely that gene drives will have limited utility in major glyphosate resistant weeds of the cotton growing system in Australia. Annual Ryegrass is the most obvious candidate for exploring this technology.

Table 1. Nine key glyphosate resistant weeds of the cotton growing region and their mating system.

Common name	Scientific name	Mating system
Annual Ryegrass	*Lolium rigidum*	Self-incompatible outcrossing
Flaxleaf Fleabane	*Conyza bonariensis*	Selfing - rare outcrossing
Feathertop Rhodes	*Chloris virgata*	Selfing - extremely rare outcrossing
Awnless Barnyard Grass	*Echinochloa colona*	Selfing - rare outcrossing
Sowthistle	*Sonchus oleraceus*	Selfing - rare outcrossing
Windmill Grass	*Chloris truncata*	Selfing - extremely rare outcrossing
Wild / Black Oats	*Avena fatua*	Selfing - ~0.5% outcrossing
Liverseed Grass	*Urochloa panicoides*	Selfing – with potential outcrossing
Silverleaf Nightshade	*Solanum elaeagnifolium*	Self-incompatible outcrossing & vegetative reproduction

RNA interference

RNA interference is a technology where a small RNA molecule is used to disrupt the expression of a gene. The genetic machinery to make RNAi molecules can be inserted into an organism (making it a GMO) as in the corn-rootworm killing RNAi inserted into corn (Wu et al 2018). RNAi can also be applied directly to an organism (Dalakouras et al. 2019), but the instability of the RNAi molecule outside of an organism has created a roadblock to the development of this kind of product.

RNAi has several attractive qualities for weed control. It can be designed in a way that is highly species specific (so the crop is not affected and the product can be applied in-crop). There are many potential targets for RNAi in any given weed species (many genes are essential for the survival of the weed and these are all potential targets). Lastly, if and when a weed species evolves resistance to the RNAi product, the molecule can be redesigned to incorporate resistance mutations, hit a slightly different area of the gene, or a different gene target altogether.

In 2012, Australia hosted the inaugural herbicide resistance challenge conference in Perth. At this conference Monsanto announced the development of RNAi technology that was capable of reversing glyphosate resistance by blocking the expression of the *EPSPS* gene. The RNAi was also effective in killing susceptible plants by blocking the expression of *EPSPS.* However, following this announcement no products have yet eventuated due to the issues of

not being able to deliver the RNAi to a crop in a field because of the instability of the molecule.

Recently, and with funding support from CRDC, Neena Mitter and her team at UQ have developed a method of incorporating RNA molecules into clay particles which protect the RNA molecule and release it slowly. This provides a mechanism of delivering RNA to crops in the field. Currently this technology is in the research phase and only being tested against pathogens and insect pests. There may be opportunities in the future, however, for this technology to be tested against weeds.

RNAi is simpler to develop for diploid species, but technically it would also be possible to develop solutions for polyploid species as RNA molecules can be designed for each different copy of a target gene. Thinking about which species might be good candidates for testing in the Australian cotton system, the best candidates would be hard to control with herbicides, and not a polyploid. Feathertop Rhodes would probably be the best species to test initially, and this species should therefore be a priority for genome sequencing.

Regulatory environment

The Australian regulatory body overseeing all of this is the Office of the Gene Technology Regulator (OGTR). The rules have recently changed regarding gene editing and other technologies, James Hereward provided comment for a news article in nature on this topic (Mallapaty 2019).

Essentially these rule changes exempt genome editing from regulation provided no new genetic material is inserted into an organism. At the same time, regulations on gene drives have been tightened, with more oversight required for any experiments in this area. RNAi regulations have also been adjusted so that RNA sprayed onto plants is not regulated provided that it does not cause changes to genomic sequence, allow translation of a novel protein, or cause the formation of an infectious agent. This means that applying RNAi directly to a plant is no longer regulated, but the regulation and future prospects for gene drives are less certain.

Conclusions

Genomics is already having a big impact on weed research, but we are still at the start of the genomics era in weed research, due to the recent affordability of genomic technology. Gene drives might have limited utility against major weeds of the cotton system in Australia, but RNAi approaches do hold a lot of promise. Genome sequencing is a pre-requisite for genetic control methods, and this report should help to guide genome sequencing efforts in weeds of the Australian cotton system.

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2.5 - Evaluate potential cover crops for suitability to northern region farming systems

As part of the project extension, the project team collaborated with the GRDC project "US00084 – Innovative weed solutions for northern region cropping systems" in the component that is examining multi-species cover cropping systems for weed suppression in the northern grains region. The winter trial conducted at Kingaroy Research Facility included monocultures and mixtures of purple vetch, field pea, tillage radish and grazing oats. Annual ryegrass and mustard were used as mimic weeds. Results from the experiment are in the process of being analysed, however initially there did appear to be some effect particularly with the tillage radish that was competitive and produced considerable biomass. Summer trials are also planned at Kingaroy with monocultures and mixes of white French millet, Japanese millet, buckwheat, sorghum, lablab and cowpea to be investigated. These experiments are also being conducted at Wagga Wagga in conjunction with the team at Charles Sturt University.

Case studies on growers using and planning to use cover crops

Two case studies with growers using or planning to use cover crops with a focus on weed management were also conducted. These are currently in the process of being compiled into an article for CRDC's Spotlight magazine. A draft of the article is below:

Cover crop grower case studies

The interest in including cover crops in cotton and broadacre systems is growing.
Cover crops provide the potential to preserve soil moisture, improve soil health and compete with weeds.

As part of the Cotton Research and Development Corporation (CRDC) -funded project, "Staying ahead of weed evolution in changing cotton systems," the Department of Agriculture and Fisheries (DAF) Weed Science team investigated the impact of cover crops on weed suppression. This included conducting two grower case studies; Jamie Grant has had over a decade of experience with growing cover crops, and James Traill is about to include them in his rotation.

We gathered information on:

- why they include or plan to include cover crops
- what were their cover crops of choice, the effect on weeds
- key learnings and further research they would like to see undertaken.

Case study #1: Jamie Grant

Jamie is a dryland cotton grower near Jimbour, Darling Downs.

Jamie's current crop rotation is cotton every second year and a millet cover crop every other year. He has included French white millet as a cover crop in his rotation for nearly a decade and as a result, he has been able to change from cotton every third year to every second.

Jamie said his main reason for including the cover crop is to preserve soil moisture.

"The cover crop increases infiltration from rainfall, prevents the majority of run-off in larger events, and also prevents evaporation of moisture from the soil," he said.

“Weed management was not a major focus for the inclusion of the cover crop, however the cover from the millet does give an additional benefit in terms of weed control.”

Jamie also highlights the importance of a dedicated cover crop, as compared to a cash crop that is harvested for grain.

“The main purpose of the cover crop is to preserve moisture and cover,” he said.

“When a crop is allowed to reach harvest maturity, it has taken extra moisture from the soil profile contrary to the objectives of a cover crop.”

Jamie has settled on French white millet as his cover crop, planted in 15” (38 cm) rows. As the focus is to preserve soil moisture, millet is a short duration crop and can be grown to near maturity in six weeks from planting in October to December. In this time, the millet provides maximum cellulose to give the maximum length of cover from the stubble.

“While growing, the millet only uses approximately one foot or 30 cm of stored soil moisture,” Jamie said.

“The gains in soil moisture has improved fallow efficiency from 30 per cent in fallow to 70 per cent with the cover crop.”

Before the inclusion of the cover crop, the soil profile required approximately 600 mm of rainfall to refill. Now the profile is refilled after 300 mm.

The millet creates enough cellulose that the cover remains adequate until cotton is planted the following season.

Jamie’s research has shown that legumes tend to break down too quickly to provide the length of cover required, and French white millet has the right characteristics.

“I find that if I plant in October, I generally have 40 per cent cover the following November, when I’m ready to plant cotton,” Jamie said.

“I don’t use sorghum as a cover crop, as the wider row spacing does not provide the cover needed, and the gaps in the stubble create a suitable microenvironment for weed germination and growth.

“I also noticed that in lighter rainfall events in sorghum and wheat stubble, the rainfall runs down the stalks of the standing stubble and creates a wet patch at the base. This is where the weeds grow and creates weedy patches across the field.

“A good millet cover crop is more even and allows the rain to penetrate the stubble evenly, and the stubble cover reduces weed emergence and the need to spray.”

Cover crops must reach maturity to create the maximum amount of cellulose for longevity. Other crops such as sorghum, wheat and barley take too long to reach maturity and as a result use too much moisture.
The main weeds on Jamie’s farm include sowthistle, feathertop Rhodes grass, fleabane and others. Jamie places a high importance on weed control, however stated that - “If you can grow good weeds, you can grow good crops.”

Jamie's focus on weed management in the cover crop is to ensure adequate cover across the whole field, as gaps in cover create a haven for weeds.

"I do this by ensuring good germination, with quality seed, and I put as much effort into growing a good cover crop as I do growing cotton," he said.

"Double knocks are still an important part of the herbicide program, and controlling weeds prior to crop emergence (both for millet and cotton) ensure the crop can get a head start to out compete the weeds.

"An in-crop spray of MCPA and Starane is always done in the millet to control volunteer cotton. However, if a heavy cover crop is grown, a spray to control volunteer cotton is not always needed."

Jamie also uses controlled traffic system (CTS), as he considers minimising the impacts of soil compaction to be very important, has been using Weed Seeker technology on a large boom for a number of years, and is now using a "Swarm Farm" robot mounted with a WEED-IT sprayer across his fields.

"The big boom is generally used for broadacre spraying, with the relevant herbicide mixture for the weeds present," he said.

"The Weed Seeker, and now the Swarm Farm robot with the WEED-IT, will be mainly used to control weeds in fallows between rain events, and broadacre sprays on mass germinations.

"The spray rig is also rotated across the tramlines in the CTS, so that it does not constantly run up and down the same wheel tracks. This allows subsequent sprays to control weeds that were run over by the rig in the previous spray."

Jamie's key learnings and advice to growers considering growing cover crops is to "work it backwards."

"Grow the cover crop that can accumulate the most moisture, and then grow the cash crop that will take the best advantage of the moisture. It is important to work out your moisture availability and your crop frequency. The moisture holding capacity of the soil will be better with a cover crop independent of soil type. The lower the capacity of the soil to hold moisture, the greater the effect evaporation has. This increases the importance of having a cover crop."

Jamie has spent a couple of years determining how to germinate and grow a good cover crop. He also stressed the importance of purchasing quality seed.

"Patience is the key," he said.

"It is important to do a good job with proper seedbed preparation at planting. An example of this when planting millet, is that it does not like to break through a crust while emerging."
If Jamie gets enough rainfall for planting millet, he checks the forecast to ensure a further heavy rainfall event is not likely to occur within the next 7-10 days. If rainfall is forecast, he holds off planting until the event occurs. This ensures the millet has the right conditions for germination and a good start. Jamie finds that putting the effort into the millet crop means he reaps the benefit in the following cotton crop.

"A new tactic I'm considering is intercropping, by planting millet in-between the cotton rows that are 60" (152 cm), and then spraying the millet out after 3-4 weeks," he said.

“This will increase ground cover in the cotton crop, with the benefits of increased weed competition, better rainfall infiltration and reduced moisture evaporation in-crop, for the sacrifice of some surface moisture that will evaporate in summer anyway.

“It is also of key importance to let neighbours know what cover crops you have, in to minimise the risk of spray drift, which will reduce their effectiveness by either killing areas or impeding growth and creating areas of less than adequate cover. Using “SataCrop” is an important tool to do this.”

Case study #2: James Traill and Ashley Tunks

James and Ashley manage irrigated and dryland farms for One Tree Agriculture, between Warra and Jandowae.

The main crop rotation used is cotton once every four years, with a cereal, sorghum double cropped to chickpeas, cereal and then back to cotton.

At this stage, James has not included cover crops into the rotation. The reason for this has been the dry conditions resulting in a lack of soil moisture to plant most crops.

“I conducted a fair amount of research into cover crops, by talking to other farmers including Jamie Grant and Hugh and James Pursehouse in northern New South Wales, and reading Nuffield reports undertaken by Alex Nixon and Rob Blatchford,” James said.

“Initially, I was hesitant about including cover crops in the rotation as no grain would be harvested from them. However, when the longer-term benefits of moisture preservation and potential yield benefits from subsequent crops were raised, the case for including cover crops became more convincing”.

The main reason James is keen to include cover crops is for moisture preservation; in the current conditions, moisture preservation in the highest concern, and weed management tends to be a lower priority.

“I can see potential benefits that the cover crops can provide in terms of weed suppression and competition,” he said.

“In years with higher rainfall, where the growth of weeds will become a larger problem, I’d like to see some savings in the cost of chemicals used.”

White French millet will be the starting point as James undertakes the process of determining what cover crops will fit into the system.

“I’m aware of the potential benefits of a range of crops, including tillage radish that may provide benefits in areas of compaction, particularly in wheel tracks,” he said.

“I may also look into include some legumes in the mix to provide benefits in terms of nitrogen. This approach was taken by Hugh and James Pursehouse, who started looking at straight millet and are now exploring multi-species cover crops.“

The main characteristic James has identified for a cover crop, is a short season crop that can provide adequate cover while allowing enough time for subsequent moisture infiltration and

preservation before the main crop is planted. As a result, James plans to start with millet and is also considering Lablab, among others, to include in the mix.
"The main weed problem across the farms that I manage is feathertop Rhodes grass, particularly on lighter country. Fleabane is also present, however management of it is under control. I use a residual program including metolachlor, terbuthylazine/diuron and paraquat – I know of the impact of glyphosate resistance - I'm keen to preserve glyphosate, and don't want to rely on it and 2,4-D."

James employs a contractor with a WEED-IT sprayer to manage escapes, and takes additional time to physically remove any escapes, particularly for feathertop Rhodes grass.

He understands that the inclusion of millet and other cover crops will have an impact on the residual program currently used. However, he is confident on advice from his consultants, Paul Castor and Tony Taylor, who have both had experience with herbicide programs for cover crops and recognise their importance in the rotation.

"I'd like to see information from research on how the growth stages of the different cover crops line up in terms of reaching the appropriate termination time to maximise cover and limit seed production.

"Also, I'm interested in effective measures for volunteer control if any of the cover crop species set seed, and the impact on weed suppression and competition, although moisture is the key concern presently.

'Finally, I want to know more about the most appropriate planter setups and planting depth when planting multi-species, and the time it takes for cover crop residues to breakdown."

Research on cover crops in the CRDC project "Staying ahead of weed management in changing cotton systems" has shown that cover crops can provide a benefit in terms of weed control. However, in order for them to be effective, it is important to start with a clean crop and ensure that the cover provided is adequate and evenly spread. Similar to findings from Jamie Grant, research showed that when the cover was not adequate that lower amounts of cover provided a haven for weeds to germinate. It was also shown to be important to ensure the cover crop was able to get a head start on the weeds in order to out compete them. Therefore ensuring a clean start and a healthy crop was critical.

The project also examined the effectiveness of the 2+2 and 0 strategy (2 non-glyphosate tactics in crop, plus 2 non-glyphosate tactics in fallow and zero survivors or incursions). This strategy was found to be effective, and the use of tools such as the WEED-IT can provide an effective way to incorporate other herbicides, and particularly follow-up for effective survivor control.

Jamie Grant discussing the impact of the millet cover crop preserving soil moisture and reducing weed emergence to Jeff Werth and Annie Ruttledge. Jamie also talked about utilising the WEED-IT on the Swarm Farm robot to manage smaller patches of weeds (Annabel Twine and Jamie in the field)

2.4 - Evaluate the potential for pre-harvest weed seed control in cotton crops

Introduction

Harvest weed seed control (HWSC) has become an important component of weed management in grains systems, particularly in southern and western cropping regions, where growers are managing weeds with resistance to a number of herbicides. HWSC provides a non-chemical option aimed at collecting and destroying weed seed through attachments/modifications to a combine harvester in the forms of chaff carts, Harrington Seed Destructor, bale direct, and placing weed seeds and chaff into windrows and destroying.

At this stage, HWSC is not possible with a cotton picker as it only picks the lint off a cotton plant and leaves everything else in the field. Therefore the focus of this milestone was to determine how weed seed viability can be reduced before picking has commenced. Experiments in the previous project "UQ1203 – IWM in transgenic farming landscapes" showed that herbicides such as glufosinate applied at flowering to barnyard and feathertop Rhodes grass were effective at reducing viability. Fleabane was also trialled in previous experiments, however its potential to regrow and produce viable seed meant that the only effective herbicide was 2,4-D+picloram, which is not an option in a cotton crop.

This experiment was designed to examine the effectiveness of a range of herbicides and a defoliant (Dropp Liquid) on barnyard grass, feathertop Rhodes grass and sowthistle. Paraquat has a registration for Spraytopping to reduce seed set of annual ryegrass in pulse crops. Liberty Link cotton provided the ability to use glufosinate in crop, and glufosinate will be an option when Extendflex cotton is introduced. These herbicides used in the previous experiment were tested again, as their effects on sowthistle were not tested before. We also included Flamprop-methyl (known as Mataven, now sold as Oatmaster) as this herbicide has a registration to reduce seed viability on Wild Oats in wheat. Dicamba was also included to examine its effects on sowthistle as this herbicide will also become an option when Extendflex traits are introduced into the industry.

Materials and Methods

Seedlings of the three species were initially established in forestry tubes in December 2016 to be later transplanted into the field. However due to a record heat wave when the plants were ready to be transplanted it was decided that the experiment should remain in the shadehouse where the plants could be properly cared for. Plants were then transplanted into individual 14 cm pots. The experiment was a randomized complete block design with five replicates.

Plants were sprayed at flowering with their respective herbicides (Table 1) in a spray cabinet using Tee jet DG95015EVS even flat fan nozzle at 2 bar delivering 93 l/ha of total spray solution. Heads/buds that had commenced flowering prior to spraying were marked so that they could be separated from those that flowered after herbicide application.

Table 1. Herbicides and rates used in the experiment in December 2016. Verdict was only used on the grasses, and Clarity (dicamba) was only used on Sowthistle

Herbicide	Active	Herbicide rate (mL or g/ha)
Gramoxone	Paraquat	2500
Clarity	Dicamba	1100
Basta	Glufosinate	3750
Dropp* + Canopy	Thidiazuron	100 + 500
Oatmaster + Uptake	Flamprop-methyl	1875 + 40
Verdict + Uptake	Haloxyfop-methyl	150 + 40

*The rate of Dropp was increased to 150 ml/ha in the repeat experiment.

After spraying, plants were placed back in the shadehouse and seed was collected, counted and stored for later germination tests.

Germination tests

Germination tests were conducted on sowthistle immediately as the seed does not have dormancy. For the grasses, seed was stored at room temperature for 6 months to allow dormancy to break down before testing.

Seeds (50 seeds x 3 replicates) of each species were incubated at 25/15 for sowthistle and 30/20 for the grasses, and a 12 hr day/night regime. Seeds were placed in 9 cm Petri dishes with two layers of filter paper (No. 1; Whatman International, Maidstone, UK) and 10 ml of deionised water. The number of germinated seeds was recorded daily for at least 28 days, and recordings stopped when no further germinations were observed for 7 days.

The experiment was repeated the following season.

Statistical analysis

The percent viable seeds was multiplied by the total seed production to determine the numbers of viable seed produced for each treatment and species. Results for each species were analysed by ANOVA for treatment and year. For awnless barnyard grass, the interaction between year and treatment was non-significant so data from both years was combined. For the remaining species, the interaction between year and treatment was significant, so results from each year were kept separate.

Results

Basta was the most effective herbicide in reducing the numbers of viable seed produced across all species. Verdict and dicamba also reduced viable seed production significantly in both years. For awnless barnyard grass (Figure1) Verdict significantly reduced the total seed production to 430 seeds/plant compared to the nil treatment that produced 7000 seeds/plant. The majority of the seed produced was unviable, with only 5% germinating in one replication. Glufosinate and paraquat reduced the amount of viable seed by 93% and 88% respectively. The application of Oatmaster and Dropp had no effect on awnless barnyard grass. In the first run of the experiment, Dropp was applied at 100 ml/ha which was the lowest rate for ideal conditions at the time of defoliation. This was increased to the higher rate of 150 ml/ha in the second year, however increasing the rate did not improve the control.

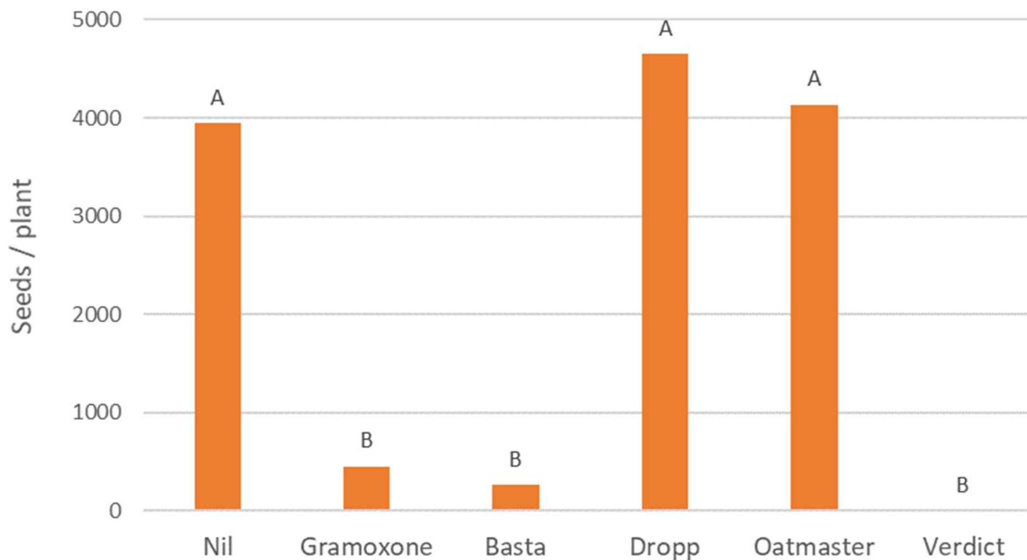

Figure 1. Viable seed production of awnless barnyard grass with respect to each treatment applied at flowering. Treatments with different letters are significantly different.

Basta was the most effective herbicide to reduce the seed production on feathertop Rhodes grass (Figure 2). Plants did produce seed in both years (245 seeds/plant in year 1; 1960 in year 2), however none of them were viable. Results overall for feathertop Rhodes grass were similar to that of awnless barnyard grass; however, paraquat was slightly less effective. Gramoxone reduced viable seed production by 82% in year 1 and 62% in year 2. Dropp had a significant reduction of 39% in year 2. , possibly due to the increased rate. Oatmaster reduced viable seed production by approximately 50% in both years. Verdict was also effective at reducing viable seed production by over 99% in both years

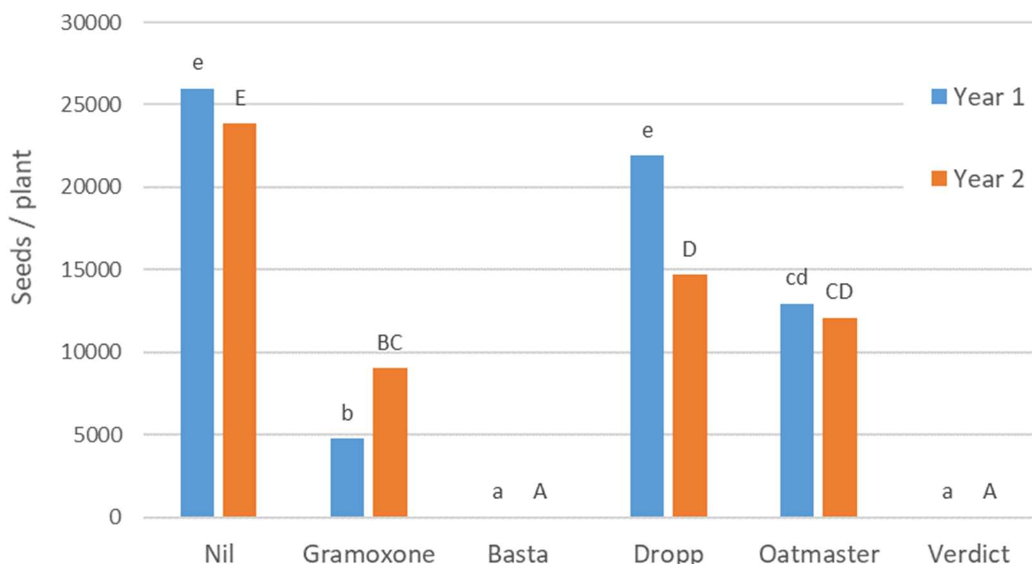

Figure 2. Viable seed production of feathertop Rhodes grass with respect to each treatment applied at flowering. Years 1 and 2 were analysed separately. Treatments with different letters are significantly different.

Basta and Clarity were the most successful in reducing seed viable seed production in sowthistle (Figure 3). Although in the 2nd year of the experiment, the effect of Basta was not significant, viable seed production was reduced by nearly 75%. Dicamba killed the plants in both years, with the exception of one replication in the first year where the plant was able to produce approximately 200 seeds that were non-viable. The application of paraquat on

sowthistle resulted in plants dying and therefore producing no seed in the first year. In the second year however, plants did survive and produced more seed than untreated plants. Dropp and Oatmaster both had no effect on seed production, and plants actually produced significantly more seed than untreated ones.

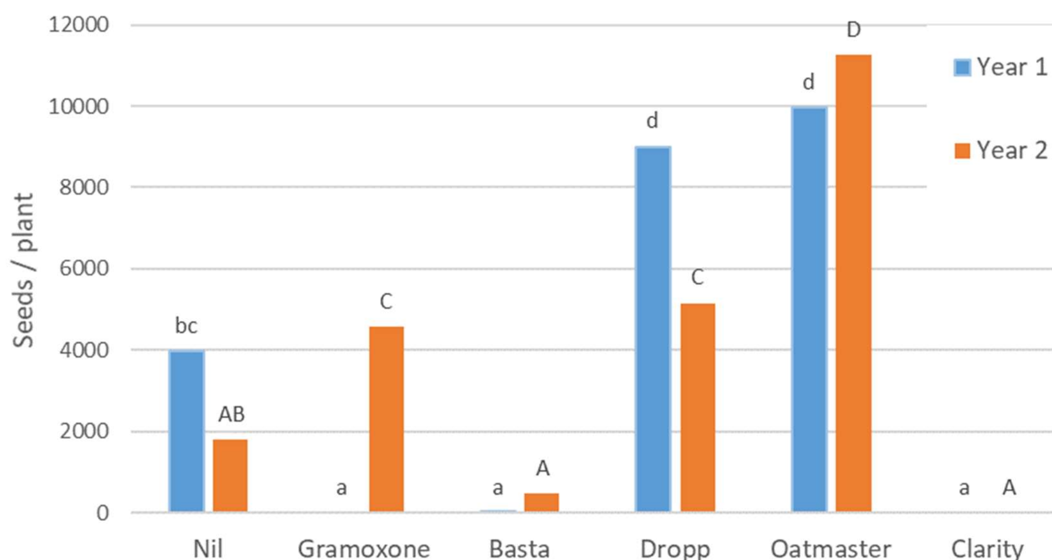

Figure 3. Viable seed production of Sowthistle with respect to each treatment applied at flowering. Years 1 and 2 were analysed separately. Treatments with different letters are significantly different.

Conclusion

The optimum time to control weeds is when they are small. This is when herbicides and other control measures are more effective. However, this experiment has shown that there is some potential to reduce the amount of seed returning to the seed bank on plants that escape control measures for whatever reason. Glufosinate was the most consistent herbicide across all species, and is an option in fallow and Extendflex crops when they are available. Glufosinate has been underutilised in cotton and broadacre cropping, so at this point the risk of resistance developing to it is low. Verdict was also very successful on the grasses, however due to the high resistance risk of the group A's, this option if used should be considered carefully. Dicamba was also successful on sowthistle, which is also an option in fallow or Extendflex cotton. However, resistance to group I herbicides is increasing, and research in southern Australia has shown that a sowthistle population found resistant to 2,4-D was also resistant to dicamba and clorpyralid. There appears to be no additional benefit of reduced seed production when defoliating cotton, other than perhaps Spray.Seed which contains paraquat and diquat.

3.1 - Determine long-term impacts of weed management approaches on weed seed-bank in the current patch eradication research

Introduction

Awnless barnyard grass (barnyard grass) is an important weed in Queensland and New South Wales and is commonly found in grains and both irrigated and non-irrigated cotton systems (Walker *et al.*, 2005; Werth *et al.*, 2013). There has been a heavy reliance on glyphosate since the introduction of glyphosate-resistant cotton to Australia in 2000. This has resulted in reductions in the use of pre-emergent herbicides, tillage and hand hoeing in-crop (Werth *et al.*, 2011), in addition to an existing heavy reliance on glyphosate in fallow (Walker *et al.*, 2005). As a result of the increased reliance on glyphosate, resistant populations of barnyard grass are now present in these areas (Preston, 2017).

Barnyard grass has a dormancy period of approximately two months (Holm *et al.*, 1977). Seed bank persistence studies by Walker *et al.* (2010) found that less than 5% of seeds remained viable when left on the soil surface after 24 months. Seed viability increased to 24% after two years when buried to a depth of 10 cm (Walker *et al.*, 2010). Overall seed bank life of is estimated at around 6-8 years (Burnside *et al.*, 1996; Martinkova *et al.*, 2006), so we would expect *E. colona* to be similar. It is a predominately self-pollinating species (Madsen *et al.*, 2002; Thornby and Walker, 2009) with its main dispersal methods being machinery and water from flood irrigation and overland flows.

Simulations on glyphosate resistance management of barnyard grass have predicted that using two non-glyphosate tactics in crop and in fallow, and preventing any survivors from setting seed is an effective strategy (Thornby *et al.*, 2013). This approach of reducing reliance on glyphosate has become critical in the management of palmer amaranth in the US. Where palmer amaranth is present, growers are increasing their use of non-glyphosate tactics such as other herbicides, tillage and hand weeding to ensure control (Sosnoskie and Culpepper, 2014). Research into the ability of palmer amaranth to dramatically spread from a small patch has also placed critical importance on not allowing survivors to set seed (Norsworthy *et al.*, 2014). The seed production of barnyard grass is smaller than palmer amaranth (42 000 cv. 1.8 million seeds plant^{-1}) (Mercado and Talata, 1977; Norsworthy *et al.*, 2014). However, taking measures to prevent population increases and stopping resistant plants setting seed is a critical factor in resistance management of any species (Thornby *et al.*, 2013). The approach of adding two non-glyphosate tactics in crop and fallow and preventing survivors (known as the "2+2 & 0" approach) has been promoted by the industry as a Best Management Practice (BMP) in the form of an industry wide Herbicide Resistance Management Strategy (Maas, 2017).

In the field, resistance generally appears first as small patches that spread if not correctly managed. Allowing resistant populations to proliferate is not a long term option. When populations of barnyard grass are detected, what is the appropriate strategy to deplete the seed bank? To answer this question, a systems experiment was established in order to understand the effect of these approaches to manage patches of glyphosate-resistant barnyard grass in the field. This experiment examines the effect of the "2+2" approach on barnyard grass patches, and what additional tactics need to be applied in order to be effective.

Materials and methods

The trial was located at the Queensland Department of Agriculture and Fisheries' Hermitage Research Facility near Warwick, Queensland (28.21S, 152.10E). The site was rain-fed (dryland) and had an existing population of glyphosate susceptible barnyard grass. These

plants were allowed to remain and set seed for a year in order to build up the population before treatments were applied in September 2012.

The experiment consists of nine treatments (Table 1) with three replications in a randomized complete block design. Plot sizes were 2.5 m x 15 m. Glyphosate was always applied at a low dose (170 g a.e. ha^{-1}), aiming for 30 – 40% survivors from each application. The registered rate for barnyard grass control on the Roundup Ready Herbicide label ranges from 360 g a.e. ha^{-1} to 1035 g a.e. ha^{-1}. This level was designed to imitate when it is likely that glyphosate resistance could be identified in the field and control measures would then be taken. Control tactics are designed on the basis of a rain-fed cotton-fallow system with minimal soil disturbance, and subsequent reductions in the amount of stored soil moisture. Tillage was not included in this experiment, although it is considered an important tactic for resistance management. All herbicides were applied with AM110015 nozzles at 200 kPa.

Table 1. Treatment details on the Hermitage patch management site. Treatments 3-9 contained an additional (Add) tactic in each respective phase.*

Treatment No.	Treatment
1	Glyphosate only (sub-lethal)
2	BMP*
3	BMP + Add (phase 1)
4	BMP + Add (phase 2)
5	BMP + Add (phase 3)
6	BMP + Add (phase 1 and 2)
7	BMP + Add (phase 1 and 3)
8	BMP + Add (phase 2 and 3)
9	BMP + Add (phase 1,2 and 3)

*Best Management Practice (BMP) treatments contained glyphosate plus two non-glyphosate tactics in both crop and fallow. BMP +Add treatments contained a further non-glyphosate tactic in each phase or combination of phases listed. Phase 1 went from October to mid-December; Phase 2 went from mid-December to mid-February; and Phase 3 went from mid-February to the end of March each year.

In Southern Queensland, cotton is planted around October/November and picked in March/April. The experiment is broken up into three main phases each season: 1 - Early season (October – mid-December); 2 – Mid-season (mid-December – mid-February); and 3 - Late season (mid-February - March). Treatments 3-9 consisted of an additional tactic applied in one or more of each phase as per Table 3.

In the 2012/13 season (cotton), all treatments received four applications of glyphosate (one pre-plant, three early-mid season). Treatments 2-9 all received a paraquat (625 a.i ha^{-1}) plus pendimethalin (1502 a.i ha^{-1}) application post-plant-pre-emergence (PPPE) and a mid-season layby application of diuron (1800 g a.i. ha^{-1}). Treatments 3, 6, 7 and 9 received a metolachlor (1440 a.i. ha^{-1}) application pre-plant (PRE) in phase 1. Treatments 4, 6, 7 and 9 received a shielded paraquat in phase 2. Treatments 5, 7, 8 and 9 received and a hand hoeing in phase. In 2013/14 (fallow), all treatments received two glyphosate applications (phase 1 and 3). The treatments 2-9 received two double knocks (glyphosate fb paraquat seven days later) (phase 1 and 3). There was an additional metolachlor application in treatments 3, 6, 7 and 9 in phase 1. This season a dry spell between December and February (Figure 1) severely affected the experiment, so the only other additional treatment required was a hand hoeing in phase 2 in treatments 4, 6, 8 and 9.

In 2014/15 (fallow), glyphosate was applied once in each phase to all treatments. The treatments 2-9 received paraquat and haloxyfop-methyl (78 g a.i. ha^{-1}) in phases 1 and 3 respectively. An additional metolachlor was applied in phase 1 to treatments 3, 6, 7 and 9. An additional paraquat application occurred in phase 2 to treatments 4, 6, 8 and 9. Paraquat was also applied in phase 3 to treatments 3, 6 7 and 9).

In 2015/16 (cotton) glyphosate was applied pre-plant and over-the-top in phase 1, and over-the-top again in phase 2 to all treatments. Pendimethalin was applied to treatments 2-9 at planting, and diuron applied as a layby in phase 2. Metolachlor was applied pre-plant to treatments 3, 6, 7 and 9). Paraquat was applied in phase 2 to treatments 4, 6 8 and 9. Dry conditions towards the end of the season negated the need for further control tactics in phase 3.

Measurements and Statistical analyses.

Plant counts were taken approximately one to two weeks after rainfall to measure emergence, and two-three weeks after post-emergent herbicide applications to measure survival rates (data not shown). Counts of plants remaining at the end of each season were recorded at the end of the season in March. Counts were taken using quadrats (0.5 m x 1 m) in the center of the plot with five quadrats per plot.

Soil cores were taken in August 2012 before treatments were applied, and then after every season in June to determine seed bank changes with respect to treatment. Cores were taken with a hand corer 10 cm in diameter and 10 cm deep, with nine cores taken per plot and soil cores washed and the number of barnyard grass seeds per core counted (Werth *et al.*, 2008; Dawson and Bruns, 1962).

Data collected in relation to barnyard grass emergence and plants remaining at the end of the season was analysed by analysis of variance (ANOVA) using GenStat (16th edition, VSN International, Hemel Hampstead, HP1 1ES, UK). Where data did not meet requirements for ANOVA, Log(x+1) transformations were used. Plants remaining at the end of 2013/14 were not analysed as data did not meet the requirements of ANOVA even when transformations were applied. The barnyard grass seeds counted in the soil cores were pooled for each plot and then analysed by ANOVA in the same manner.

Table 2. Breakdown of barnyard grass control tactics used in each season and phase for respective treatments listed in Table 1*†

Phase	Glyphosate only	Best Management Practices (BMP)	Additional tactics (Add)
	<u>*2012/13 – Cotton*</u>		
1. Early season	Glyphosate (PRE) Glyphosate (OTT)	Paraquat + Pendimethalin (PPPE) *(Treatments 2-9)*	Metolachlor (PRE) *(Treatments 3, 6, 7 and 9)*
2. Mid-season	Glyphosate (OTT) Glyphosate (OTT)	Diuron (Layby) *(Treatments 2-9)*	Paraquat (Shielded) *(Treatments 4, 6, 8 and 9)*
3. Late season	Nil	Nil	Hand-hoeing *(Treatments 5, 7, 8 and 9)*
	<u>*2013/14 - Fallow*</u>		
1. Early season	Glyphosate	Paraquat (DK) *(Treatments 2-9)*	Metolachlor *(Treatments 3, 6, 7 and 9)*
2. Mid-season	No action needed	No action needed	Hand-hoeing *(Treatments 4, 6, 8 and 9)*
3. Late season	Glyphosate	Paraquat (DK) *(Treatments 2-9)*	No action needed
	<u>*2014/15 - Fallow*</u>		
1. Early season	Glyphosate	Paraquat (DK) *(Treatments 2-9)*	Metolachlor *(Treatments 3, 6, 7 and 9)*
2. Mid-season	Glyphosate	Nil	Paraquat *(Treatments 4, 6, 8 and 9)*
3. Late season	Glyphosate	Haloxyfop *(Treatments 2-9)*	Paraquat *(Treatments 5, 7, 8 and 9)*
	<u>*2015/16 – Cotton*</u>		
1. Early season	Glyphosate (PRE) Glyphosate (OTT)	Pendimethalin (PPPE) *(Treatments 2-9)*	Metolachlor (PRE) *(Treatments 3, 6, 7 and 9)*
2. Mid-season	Glyphosate (OTT)	Diuron (Layby) *(Treatments 2-9)*	Paraquat (Shielded) *(Treatments 4, 6, 8 and 9)*
3. Late season	Nil	Nil	No action needed

*Herbicide rates used are as follows: glyphosate at 170 g a.e. ha^{-1}, metolachlor at 1440 a.i. ha^{-1}, paraquat at 625 a.i. ha^{-1}, pendimethalin at 1502 a.i. ha^{-1}, diuron at 1800 g a.i. ha^{-1}, haloxyfop-methyl at 78 g a.i. ha^{-1}. Glypohsate, paraquat and haloxyfop were applied with 100 L ha^{-1} water. Pendimethalin and Diuron were applied with 120 L ha^{-1} water, and metolachlor was applied with 150 L ha^{-1} water.
†Best Management Practice (BMP) treatments contained glyphosate plus two non-glyphosate tactics in both crop and fallow. BMP +Add treatments contained a further non-glyphosate tactic in each phase or combination of phases listed. Phase 1 went from October to mid-December; Phase 2 went from mid-December to mid-February; and Phase 3 went from mid-February to the end of March each year.

Results and discussion

Barnyard grass emergence

The total barnyard grass emergence for all treatments throughout the 2012/13 season was quite high, although this was expected due to the very high starting seed bank (Table 3). The highest emergence of 1118 plants m^{-2} occurred in the glyphosate only treatment where no pre-emergent herbicides were used. This was significantly higher than all of the other treatments. The pre-plant metolachlor reduced emergence by 65% in the treatments that included this as part of the additional tactic in phase 1 (Treatments 3, 6, 7 and 9). The remaining treatments that received a pendimethalin at planting (Treatments 2, 4 and 8) missed the first major cohort, and as a result had slightly higher emergence. Treatments 3 and 4 that only received additional tactics in phases 2 and 3, respectively, had significantly higher emergence throughout the season than those that either had the additional tactic in phase 1 alone, and in phases 1 and 2, 1 and 3, or all phases.

Table 3. Total *Echinochloa colona* emergence in each year of the experiment. Means with the same letters are not significantly different.

Treatment	2012/13 Cotton* (plants m^{-2})	2013/14 Fallow*	2014/15 Fallow	2015/16 Cotton*
1. Glyphosate only	1118.4 d	316.7 b	87.4 b	118.1 d
2. BMP	176.0 bc	45.0 a	39.1 a	88.5 cd
3. BMP + Add (phase 1)	116.8 ab	56.7 a	18.9 a	23.4 ab
4. BMP + Add (phase 2)	257.8 c	27.3 a	34.3 a	26.8 ab
5. BMP + Add (phase 3)	324.1 c	44.8 a	40.5 a	60.9 bcd
6. BMP + Add (phase 1 and 2)	81.6 a	42.1 a	9.2 a	29.1 ab
7. BMP + Add (phase 1 and 3)	129.6 ab	54.9 a	6.5 a	32.3 abc
8. BMP + Add (phase 2 and 3)	191.8 bc	26.4 a	17.5 a	16.8 a
9. BMP + Add (phase 1,2 and 3)	108.4 ab	43.4 a	8.1 a	11.9 a
P-value	<0.001	0.014	0.005	0.004

*Means back-transformed from Log(x+1)

Barnyard grass emergence in 2013/14 and 2014/15 generally declined in all treatments. The glyphosate only treatment still had significantly higher emergence than the remainder of the treatments. This did not appear to be due to the use of early season metolachlor in phase 1 of both years, as treatments 2-9 were all statistically the same. When metolachlor was applied in both seasons there was adequate rainfall for incorporation within 1-2 days (19 mm in October 2012, and 12 mm in November 2015). Barnyard grass emergence did not occur until approximately 4 weeks later in each case. This should have resulted in some control from metolachlor, however did not. As a result the higher emergences in the glyphosate only treatment appears to be most likely due to seed carry-over from more surviving plants the previous season. In 2015/16, metolachlor reduced emergences by 83% compared to the glyphosate only treatment, and 9% more than the BMP treatments which had pendimethalin at planting. The reduced additional benefit in 2015/16 is most likely attributed to the reduced seed bank.

Plants remaining at the end of the season

The glyphosate only treatment had the highest number of plants remaining at the end of 2012/13 (24.4 plants m^{-2}) compared to the next highest (7.5 plants m^{-2}) in the BMP + Add (phase 1) treatment (Table 4). Paraquat in the form of a "double knock" as the additional

tactic in phase 2 (Treatments 4, 6, 8 and 9), provided 95 – 100% control compared to 60-90% control where glyphosate was applied alone. The phase 3 additional treatment of hand-hoeing (Treatments 5, 7, 8 and 9) generally resulted in fewer plants remaining than the other treatments, but did not perform as well as expected, with 77% control achieved. At the time of hand-hoeing, many of the barnyard grass plants were quite large, with large root systems, making them difficult to control effectively.

The hot dry spell in 2013/14 negated the need for the planned additional control measures in phase 3. There was therefore no difference between the BMP and BMP + Add treatments. The slight difference in plants remaining at the end of the season was only due to slight variability in the phase 2 double knock application.

The double knock tactics applied in the 2014/15 of the experiment, particularly in phases 2 and 3 were effective at controlling barnyard grass with approximately 97% control. This, combined with the overall lower emergence, led to no survivors in a number of treatments (4, 5, 6, and 9). The initial double knock achieved approximately 72% control, which resulted in a slight but significant increase in the number of plants remaining in the BMP treatment compared to the BMP + Add treatments. The glyphosate-only treatment had significantly more plants remaining than the other treatments; however, as stated earlier, emergence and plants remaining were generally lower for this season across treatments.

There was again a low number of emergence in 2015/16. This, combined with the reduction in emergence from the residual herbicide applications, and relatively low rainfall, resulted in low numbers of plants remaining. The glyphosate only treatment had significantly more plants remaining than the other treatments. However even with effective double knock applications in treatments 4, 6, 8 and 9 (89% control), the BMP treatment finished with slightly but not significantly more plants remaining than the treatments that included the additional measures.

Table 4. Density of *Echinochloa colona* plants remaining at the end of each season after respective treatments have been applied. Means with the same letters are not significantly different.

Treatment	2012/13 Cotton* (plants m^{-2})	2013/14 Fallow†	2014/15 Fallow	2015/16 Cotton
1. Glyphosate only	24.4 d	27.6	1.9 c	10.8 b
2. BMP	3.5 abc	0	0.8 b	2.0 a
3. BMP + Add (phase 1)	7.5 c	0	0.3 ab	1.7 a
4. BMP + Add (phase 2)	6.3 bc	0.3	0.0 a	1.2 a
5. BMP + Add (phase 3)	1.8 a	0.3	0.0 a	1.9 a
6. BMP + Add (phase 1 and 2)	4.4 abc	0	0.0 a	1.2 a
7. BMP + Add (phase 1 and 3)	2.9 ab	0.3	0.1 ab	0.9 a
8. BMP + Add (phase 2 and 3)	2.2 a	0.1	0.4 ab	0.5 a
9. BMP + Add (phase 1,2 and 3)	2.0 a	0	0.0 a	0.5 a
P-value	<0.001	-	<0.001	<0.001

*Means back-transformed from Log(x+1)

†Not analysed due to not meeting requirements of ANOVA.

Effects on the seed bank

Differences in the seed bank were not significant between treatments in the first two years of the experiment (Table 5). In June 2015, the glyphosate only and BMP treatments were similar, whereas treatments that contained additional measures, particularly in two or more phases, had significantly higher reductions in the seed bank. These differences between the BMP and the BMP + Add treatments did carry over to June 2016, and all of these were significantly better than the glyphosate-only treatment.

One of the difficulties of this type of experiment is "simulating" a glyphosate-resistant population. In the field, we would expect the glyphosate-resistant population to increase under the continual use of glyphosate alone, but this was not the case with this experiment. Glyphosate still achieved some control in the glyphosate only treatment (ranging from 54% to 95% control). Initially, there was a decrease in the seed bank and the corresponding number of emergence, but towards the end of the experiment the barnyard grass population appeared to stabilize in the glyphosate only treatment while it continued to decrease in the other treatments.

Table 5. *Echinochloa colona* seed bank measurements at the beginning of the experiment and the end of each season, with respect to each treatment. Means with the same letters are not significantly different.

Treatment	Aug 2012	June 2013 Cotton	June 2014 Fallow	June 2015 Fallow*	June 2016 Cotton*
	(Seeds m^{-2})				
1. Glyphosate only	84454	12963	1154	4538 cd	5648 b
2. BMP	53933	14595	1000	5345 d	124 a
3. BMP + Add (phase 1)	110734	19976	1665	1058 abc	81 a
4. BMP + Add (phase 2)	66727	10077	439	627 ab	22 a
5. BMP + Add (phase 3)	82936	13515	736	1827 bcd	262 a
6. BMP + Add (phase 1 and 2)	102137	12468	481	293 a	25 a
7. BMP + Add (phase 1 and 3)	97261	13501	976	958 ab	272 a
8. BMP + Add (phase 2 and 3)	49076	9172	429	643 ab	77 a
9. BMP + Add (phase 1,2 and 3)	94012	17542	990	368 a	68 a
P-value	0.348	0.767	0.627	0.010	0.022

*Means back-transformed from Log(x+1)

Conclusions

In this experiment, the BMP treatment containing two non-glyphosate tactics in crop and fallow considerably reduced the barnyard grass seed bank. The additional tactics in the other treatments were slightly more effective, though not significantly. However, in this experiment, year 3 had a relatively dry start (September – November 2014) which coincided with when barnyard grass tends to emerge. There was also a hot dry spell in Year 2 between December 2013 and February 2014 that resulted in the death of survivors (Figure 1). The higher than expected contribution of glyphosate across the treatments may also have made the BMP treatments more effective than they would be in the field. The obvious result of the experiment is that a glyphosate only approach will not be sustainable, even if some level of control is achieved (Price *et al.*, 2011). Diversification of weed management tactics that do not include glyphosate will result in more sustainable long-term management of resistant populations (Bagavathiannan *et al.*, 2013; Thornby *et al.*, 2013). The BMP (2+2 approach), a

successful strategy in this experiment, should also be able to be applied to drive the seed bank down in a glyphosate-resistant population. The reduction in emergence that was achieved when at least two non-glyphosate tactics were included each year are should result in further seed bank reductions in subsequent years. It is likely that additional tactics may be needed in seasons with more rainfall events, and cohorts emerging. However, emphasis should be placed on monitoring fields to determine control measures required as well as their success. Even on larger farms, the ability to monitor for survivors is achievable though somewhat time consuming. Monitoring is of critical importance to containment, particularly to prevent seed production and continuation of the population (Norsworthy *et al.*, 2014). It is also important to note that the "double knock" applications did not consistently achieve 100% control, therefore allowing some seed bank replenishment. This once again highlights the importance of monitoring, even when the "double knock" tactic is used. This experiment has shown that BMP (2+2) strategy should be used as a baseline for resistance management, and the additional measures employed when required to prevent the proliferation of resistant populations.

3.2 - Devise new strategies using new information from above activities

Management strategies from these activities have centred around the 2+2 and 0 (2 non-glyphosate options in crop and in fallow with no survivors of new incursions) approach. This has formed the basis of the HRMS (Herbicide resistance management strategy) which is published in the Crop Pest Management Guide each year. Results from the Diversity model both support the continued use of the 2+2+0 strategy and suggest how it can be implemented and refined for multi-species, multi-resistance thinking (see 1.3). These results could be used directly in reformulating and refining the HRMS tables.

The double knock utilising glufosinate as the second chemical will also be useful in replacing paraquat to minimise paraquats resistance risk and enhance user safety. Glufosinate should also become an important chemical in Extendflex cotton when it is introduced into Austalia.

Research on resistance mechanisms has also been utilised to form the Herbicide Tech Panel advice to TIMS on the introduction of Extendflex.

The BYGUM model, while not directly a strategy development tool, has been useful in producing predictions around timely problems and issues in cotton, such as the economics of residual use. BYGUM outputs make good extension material, adding economic considerations to other strategic discussions. HRMS and other strategies could be modelled in BYGUM to test and extend their economics.

3.3 - Develop weed identification app with QBIT team using Lucid

The Weeds of Australian Cotton ID key was released as an app for iOS and Android (available from the Apple and Google Play stores) and for internet browsers (freely available via website) in July 2018, following limited release for testing in 2017. It allows users to identify 50 key weeds of cotton, by querying a database of key information and viewing photos, and is free for use and downloading.

Figure 3.3.1. Weeds of Australian Cotton splash screen

The app uses the Lucid key software system, which allows for the construction of a key using all the typical features and factors that are used in traditional dichotomous keys, from characteristics including leaf, cotyledon, stem, flower and fruit appearance. Lucid allows users to proceed through the key in a non-linear fashion, unlike dichotomous keys: they can narrow down their choices using whichever characteristics are easy for them to gather first, and then use the remaining characteristics and/or photos to make a final identification. Each weed also has a fact sheet associated with it in the app that allows the user to understand the ecology and problematic nature of each species as it relates to cotton production.

The Lucid system is a property of Identic, a UQ-owned company that was developed to perform the Lucid business functions of the former QBIT Lucid key team. We contracted with Identic to supply the key framework and two pieces of software used for building Lucid keys. Identic also supplied the app's graphical elements (buttons, etc) and approximately a third of the key information and photos for the species of interest, which they had in their existing database.

We created key information for the remaining species, checked and updated a range of the supplied key data, and updated the text for each species as well as a large number of photos. Most of the new photos for the app were supplied by Graham Charles; most of Identic's existing database of photos were originally taken by Sheldon Navie. While a range of keys including these species exist, in print and digitally, one of the important additions in the new app is the ability to identify species based on cotyledon shape. We collected a new set of photos and data for each of the species concerned regarding cotyledon and early seedling morphology, and emphasise throughout that the early identification the tool provides is essential for best management of weeds. The capacity to apply an effective control method while the weed is still small is critical.

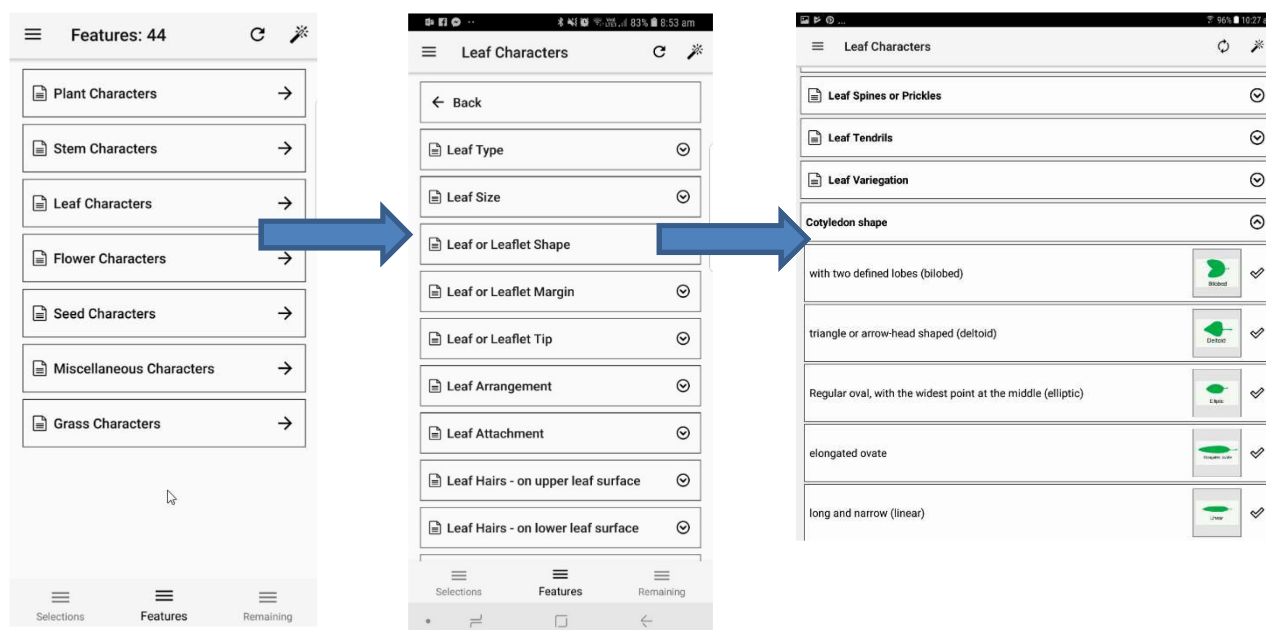

Figure 3.3.2. ID App workflow: selecting groups of characteristics, individual characteristics, and type

We identified 50 important species for inclusion in the app, in consultation with Graham Charles and Ian Taylor. It is to be expected that species of greatest interest will shift over time, and the list of species would benefit from periodic review. The current species are shown in the list below.

Table 3. List of species included in the Weeds of Australian Cotton ID app and key

Abelmoschus ficulneus
Amaranthus macrocarpus
Argemone ochroleuca
Boerhavia dominii
Bromus catharticus
Carthamus lanatus
Chamaesyce drummondii
Chenopodum album
Chloris gayana
Chloris truncata
Chloris virgata
Convolvulus erubescens

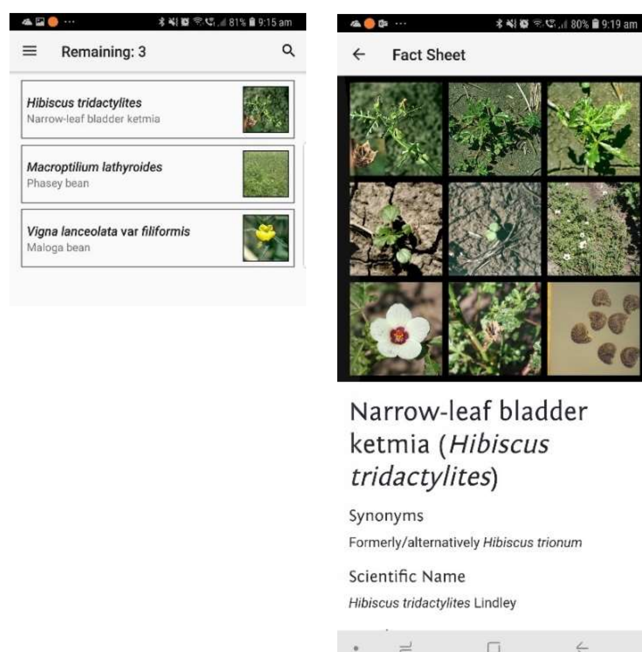

Figure 3.3.3. ID app users select from final key candidates with reference photos, and each species has an attached fact sheet with multiple photos and detailed information on species biology, ecology, problematic status, and other data

Cullen tenax
Cyperus bifax
Cyperus eragrostis
Cyperus rotundus
Datura ferox
Echinochloa crus-galli
Echinocloa colona
Fallopia convolvulus
Hibiscus tridactylites
Hibiscus verdcourtii
Lamium amplexicaule
Leptochloa fusca
Lolium rigidum
Macroptilium lathyroides
Malva parviflora
Phalaris paradoxa
Physalis minima
Polygonum aviculare
Polymeria longifolia
Polymeria pusilla
Portulaca oleracea
Rapistrum rugosum
Salvia reflexa
Sesbania cannabina
Sisymbrium thellungii
Solanum nodiflorum
Sonchus oleraceus
Sorghum halepense
Trianthema portulacastrum
Tribulus micrococcus
Tribulus terrestris
Urochloa panicoides
Vigna lanceolata var. filiformis
Xanthium italicum
Xanthium occidentale
Xanthium orientale
Xanthium spinosum

We produced a quick start guide for new users, which is available on the CottonInfo website at https://www.cottoninfo.com.au/weeds-australian-cotton-app. A copy is also attached in the Appendices.

Updating and future work

Updates to the app versions of the key are handled by Identic. While maintaining the app's presence on the two app stores and assuring some basic compatibility between Lucid Mobile and the operating systems (iOS and Android) are the responsibility of Identic, by and large any future incompatibilities (which, given the history of app and OS development, is probable some time in the next few years) will come with a cost for updating. Updates that add new species, photos, key data or other information will also be at some cost—and as noted earlier, species importance is always likely to shift over time, providing an incentive to keep the tool up to date

in terms of species included. A modest commitment to maintaining the app, should it prove useful to industry, could be achieved for around $2000 every two or three years, at current prices, and depending on the number of new species added. The browser-based version, which is held on Identic's Lucid Key Server, can be updated at no charge whenever desired, providing no new Identic IP (existing key data, photos or text) is required. The app and browser versions each have some advantages and disadvantages: the browser version can be updated without cost or extra processing by Identic, and if desired could be hosted on the CottonInfo site or another similar server, which would make the tool fully independent of Identic. The app version requires cost and processing by Identic at each update. However, the app version is fully downloaded to the user's device, allowing offline use, while the browser version requires a constant internet connection.

3.4 - Update WeedPak with recommendations for weed control in cotton systems

At this stage, updates to WeedPak have been on hold as the project team was under the impression that the format of WeedPak had been changed to smaller factsheets on key species. Results from work conducted in the project should be ready to convert into a format for WeedPak when required.

Experiments on the glufosinate double knock, and results from the SHeRA model will be more applicable when Extendflex technology has been introduced to the industry.

3.5 - Contribute to development of HRMS for glyphosate-resistant weeds in cotton with herbicide-tolerance traits

The project team in conjunction with the TIMS Herbicide Tolerance Tech Panel has contributed to the development and updating of the HRMS for glyphosate resistant weeds. The team has also contributed to updating the HRMS annually as part of its inclusion in the Cotton Pest Management Guide. The team will also endeavour to continue to update the HRMS to ensure its relevance when Extendflex cotton is introduced.

As noted in 1.3 above, data from the Diversity model simulations are suitable to feed directly into devising updates to and new versions of the HRMS. Specific strategic information from Diversity simulations includes the need to base management systems on providing control of existing glyphosate resistant weeds, especially grasses, under new triple-stack transgenic varieties; and that the 2+2+0 guideline remains a workable basis for HRMS strategies, provided each '2' is approached separately for grass and broadleaf weeds.

Extension Opportunities

1. **Detail a plan for the activities or other steps that may be taken:**
   (a) to further develop or to exploit the project technology.
   (b) for the future presentation and dissemination of the project outcomes.
   (c) for future research.

Information gained from the project will continue to be used to update the HRMS and the Cotton Pest Management Guide. There should also be the ability to update the HRMS, CPMG and potentially WeedPak with information around stewardship of Extendflex Technology when it is commercially introduced into Australia.

The cover crop case study article will be published in Spotlight.

The team will aim to present findings at the various Cotton and Weed conferences if funding permits.

There are also plans to publish the Glufosinate Double knock, Growth and Development and Genetic work in the relevant journals.

9. A. List the publications arising from the research project and/or a publication plan. (NB: Where possible, please provide a copy of any publication/s)

Journal Papers

Werth J., Keenan M., Thornby D., Bell K. and Walker S. 2017. Emergence of four weed species in response to rainfall and temperature. Weed Biology and Management 17, 29-35.

Hereward JP, Werth JA, Thornby DF, Keenan MD, Chauhan BS and Walter GH. 2016. Complete chloroplast genome sequences of six lines of Echinochloa colona (L.) link. Mitochondrial DNA part B: Resources 1(1).

Hereward JP, Werth JA, Thornby DF, Keenan MD, Chauhan BS and Walter GH. 2016. Complete chloroplast genome sequences of two species of Chloris grass, Chloris truncata Sw. and Chloris virgata R.Br. Mitochondrial DNA part B: Resources 1(1).

Hereward J, Werth J, Thornby D, Keenan M, Chauhan BS, Walter G. (2017) Complete chloroplast genome of glyphosate resistant *Conyza bonariensis* (L.) Cronquist from Australia. *Mitochondrial DNA*. 2(2) 444-445.

Thornby D, Werth J, Hereward J. Keenan M. and Chauhan BS. 2018. Herbicide resistance evolution can be tamed by diversity in irrigated Australian cotton: a multi-species, multi-herbicide modelling approach. *Pest Management Science* 74, 2363-2375.

Thornby D, Werth J, Hereward J. Keenan M. Chauhan BS and Walter GH. 2018. Gene expression in response to glyphosate treatment in fleabane (*Conyza bonariensis*) – glyphosate death response and candidate resistance genes. *Pest Management Science* 74, 2346-2375.

Conferences

Werth J, Thornby D, Keenan M and Walker S. (2014) Managing patches of glyphosate resistant *Echinochloa colona*: can they be eradicated? In *Proceedings of the 19th Australasian Weeds Conference – Science, Community and Food Security: the Weed Challenge*, 1–4 September, Hobart, Tasmania, ed. M. Baker. (Tasmanian Weed Society, Hobart).

Thornby D, Werth J, and Walker S. (2014) Patch management solves early infestations of glyphosate resistant awnless barnyard grass. In *Proceedings of the 19th Australasian Weeds Conference – Science, Community and Food Security: the Weed Challenge*, 1–4 September, Hobart, Tasmania, ed. M. Baker. (Tasmanian Weed Society, Hobart).

Thornby D. (2015) BYGUM – a new tool for BarnYard Grass Understanding and Management. 2nd Australian Cotton Research Conference, Toowoomba, September 2015

Keenan M. (2015) Growth and development of three key summer grasses in Australian cotton systems. 2nd Australian Cotton Research Conference, Toowoomba, September 2015

Hereward J. (2015) Genetics of glyphosate resistance. 2nd Australian Cotton Research Conference, Towoomba, September 2015

Werth J (2015) Can patches of glyphosate-resistant *Echinochloa colona* be eradicated?. 2nd Australian Cotton Research Conference, Toowoomba, September 2015

Werth J (2016). Managing herbicide resistance in herbicide-tolerant cotton in Australia: The situation now and planning for future traits. 7th International Weed Science Congress. Praque, Czech Republic. June 19-25, 2016.

Thornby, D. (2017). The Diversity Model. 3rd Australian Cotton Research Conference, Canberra.

Hereward J. (2017). Population genetics and transcriptomics of glyphosate resistant *Conyza bonariensis* populations in Australia. 3rd Australian Cotton Research Conference, Canberra.

Keenan, M. (2017). Growth and development of feathertop Rhodes grass (*Choris virgata*). 3rd Australian Cotton Research Conference, Canberra.

Werth, J. (2017). Eradication or mitigation? How to manage patches of glyphosate resistant *Echinochloa colona*. 3rd Australian Cotton Research Conference, Canberra.

Thornby D. How many herbicides does it take to screw in a lightbulb? Australian Cotton Conference. Broadbeach. August 2018

Hereward J. Genome sequencing of glyphosate resistant fleabane (*Conyza bonariensis*) and sowthistle (*Sonchus oleraceus*). 21st Australasian Weeds Conference. Manly. September 2018.

Werth J. Eradication or mitigation? How to manage patches of glyphosate resistant *Echinochloa colona*. 21st Australasian Weeds Conference. Manly. September 2018.

Articles and Workshops

Jenson M, Thornby D, and Werth J (2015) "Sowthistle: A diligent approach required" Spotlight Magazine, Winter 2015

Hereward J (2017). Genetics of glyphosate resistance in key weeds of the northern region. Australian Cottongrower. Oct-Nov 2017.

Werth J. (2017) "Using the 2+2&0 to "Slam" resistant patches and keep them subdued" Australian Cottongrower, December 2017-January 2018, page 18.

"Weed Management Case Study: Managing Resistant Barnyard grass." http://www.cottoninfo.com.au/sites/default/files/documents/Weed%20management%20case%20study.pdf

Werth J (2015) The Future of Australian Cotton in Terms of Herbicide Resistance. Cotton Collective, Narrabri, August 2015.

David Thornby and Jeff Werth presented on BYGUM, HRMS, Weed Ecology and Non-glyphosate tactics at the ICAN/Cotton Info Weeds Masterclasses in Goondiwindi and Griffith on June 2nd and 7th.

Jeff Werth participated in the Workshops on weed management for growers in conjuntions with ICAN/Cotton Info at Emerald, St George, Goondiwindi, Dalby and Pittsworth.

The project team has presented findings at the Northern Region Weed Research forums held each year.

B. Have you developed any online resources and what is the website address?

1. The Barnyard Grass Understanding and Management (BYGUM) Model

   This tool provides a means to combine biological, agronomic and economic factors to examine summer grass management strategies, and compare new tacitics. It can be downloaded from the CottonInfo Website.

   https://www.cottoninfo.com.au/barnyard-grass-understanding-and-management-bygum

2. Weeds of Australian Cotton App

   This app is a tool for cotton growers and theirs advisors, designed to assist in identifying 50 key weed species. It's available on both apple and android smart phone devices and can be downloaded for free

Part 4 – Final Report Executive Summary

Farming systems in cotton are changing and weed management is again becoming more complex. The rise of herbicide resistance, particularly to glyphosate and the imminent introduction of new herbicide tolerant traits to dicamba and glufosinate has changed the way weeds need to be managed. The project team undertook to pre-empt these changes.

Three new modelling and decision support products were created for industry. The BYGUM decision support tool predicts economic outcomes from summer grass weed management strategies. The tool can be used to create and compare five-year rotations of cotton, grains and fallows, including cover crops, demonstrating the effectiveness, cost and efficiency of herbicide and non-herbicide weed control. BYGUM has been used in workshops and to create extension materials, and has been downloaded by over 300 unique users to date. The Weeds of Australian Cotton ID app allows identification of 50 key weed species in cotton fields. The app uses the Lucid framework and an extensive image library. The app is among the first to include cotyledon shape as a factor for identification, meaning weeds can be identified while still small enough to control effectively. It is free to use and available in Apple and Google app stores for use offline, and via the Identic Lucid Key library for use on desktop and laptop computers.

The Diversity model is a world-first multi-herbicide, multi-species, polygenetic model of herbicide resistance evolution. It determines and quantifies how much diversity is enough, to slow or prevent evolution towards resistance. We used the model to assess the resistance potential of weed management under new triple stack systems, such as Xtendflex® cotton. Our results suggest three key points:

1. That these systems are substantially more diverse than Roundup Ready, and with the right extra tactics can be the basis of long-term effective weed management;
2. Glyphosate, glufosinate and dicamba alone or with minimal extra modes of action are incapable of controlling our existing glyphosate resistant grasses and fleabane—systems are likely to fail due to poorly controlled resistant populations long before new resistances have time to develop;
3. The 2+2+0 strategy is predicted to remain effective, but modelling multiple species at once reminds us that both grasses and broadleaves need multiple effective options in the system.

Genetic exploration of the mechanisms of glyphosate resistance in key weed species led to the discovery of the key role of ploidy and gene copy number in the evolution of glyphosate resistance. Our work on gene expression in fleabane and the genome assembly showed that there are many copies of the target site *EPSPS* gene in this species. This makes it very hard for fleabane to evolve target site resistance to glyphosate because many copies have to have a mutation, instead, species with many copies of the target site gene have to evolve non-target-site resistance and this is more difficult for the weed because it usually involves more than one mutation. Feathertop Rhodes grass was found to be diploid with one copy of the *EPSPS* gene, and this species evolved resistance over 10 times by target site mutations. This understanding explains why it took sowthistle so long to develop resistance to glyphosate, and allows us to make predictions about herbicide resistance evolution in the future. Diploid species with one copy of the target site gene will be more likely to readily evolve resistance to a herbicide than polyploid species or those diploid species with multiple copies of the target site gene.

Work on the population genetics of four key species led to some surprising results. Fleabane is considered a well-dispersed species, but had strong regional genetic structure indicating that wind dispersal may play less of a role than previously expected. Windmill grass and feathertop Rhodes showed very little evidence for outcrossing, but there may have been some admixture in the past. Outcrossing is important because it affects the ability of a weed to develop resistance to multiple modes of action (MOA). Fleabane and sowthistle had evidence for some outcrossing in the genetic data, but we were unable to find experimental evidence for outcrossing in 200 offspring of each species. This highlights how very low levels of outcrossing might still play an important role in the evolution of resistance in species like fleabane that we had previously thought to be only self-pollinating.

In windmill grass and feathertop Rhodes grass, their highly selfing reproductive mode can be used in the fight against herbicide resistance. These species are less able to 'stack' resistance to different modes of action.. Our work shows, however, that feathertop has evolved resistance multiple times and how these have spread across the cotton system, and that almost 1/3 of windmill grass populations are now resistant. Each glyphosate resistant individual has the potential to evolve resistance to a second MOA. and our work highlights the importance of controlling glyphosate resistant populations to avoid multiple MOA resistance. Overall, the population genetics work emphasises the importance of the '0' in the 2+2+0 strategy survivor control is essential to prevent the spread of resistance and to avoid multiple MOA resistance.

Studies on the growth and development of awnless barnyard grass, feathertop Rhodes grass, windmill grass, fleabane and sowthistle were conducted. In general, with the summer grasses, plants that emerged at the start of summer grew larger and produced more seed than those emerging later. This is where the focus of control should be for the greatest impact. However, it is important to note that plants emerging later still produce seed and need to be controlled. Sowthistle now has the ability to emerge and grow well throughout the year. Fleabane also appears to be adapting to warmer temperatures, readily producing seed throughout spring, summer and autumn.

The addition of glufosinate and dicamba has the potential to improve control, particularly on the five key species tested. When glufosinate was used, as a double knock partner, effective control was achieved in both glyphosate-resistant and susceptible populations tested. The glufosinate double knock should prove an effective option in Xtendflex® cotton.

Research on cover crops was hampered with dry conditions, and as the result the effects on weed emergence were limited. However, growers have shown cover crops to be an effective option provided they start with a clean crop and ensure that the cover provided is adequate and evenly spread.

The 2+2 and 0 was shown to be an effective management strategy for long-term resistance management. Research also concluded that additional options will provide more effective control in years with more rainfall events and subsequent emergences.