Characterization of Glyphosate Resistance in *Hordeum glaucum* Steud (Barley grass)

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ABSTRACT

Hordeum glaucum Steud. is a major grass weed in South Australia in crops and pastures across the agricultural region. Control of this grass species has become a problem in recent decades as a consequence of the evolution of herbicide-resistance to several of the herbicides frequently used. Glyphosate is used for non-selective control of *Hordeum* species in non-cropped situations, such as fence lines and crop margins, and is also used effectively for pre-sowing knockdown control or spray-topping to stop seed set of *Hordeum* species in pastures. The repeated use of glyphosate has resulted in the evolution of resistance to glyphosate in this species. Glyphosate resistant *H. glaucum* populations were found along fence lines and around stockyards after more than a decade of repeated herbicide application. This study was undertaken to characterise resistance in these populations and investigate the mechanisms responsible for glyphosate resistance. Better understanding of the evolution of resistance to glyphosate in *H. glaucum* will improve resistance management strategies to delay or prevent resistance evolution in this species.

Glyphosate resistant *H. glaucum* populations were collected from non-cropped areas along fence lines and stockyards in the years 2016, 2017 respectively with a subsequent collection in 2019 from the same farm. In a series of dose-response experiments the resistance level of the suspected resistant populations (YP-16, YP-17 and YP-19) and susceptible populations (RW, TW and YN) were investigated. These confirmed glyphosate resistance in these populations with resistance level of 2.8 to 6.6- fold higher than the susceptible populations as determined by ratio of their LD_{50} values. Screening of these populations for mechanisms conferring resistance showed no differences in glyphosate absorption and translocation that could account for resistance and no mutation in the *EPSPS* gene. However, 9-12 fold increase in EPSPS gene copy number in the

resistant populations compared to the susceptible was observed, which is likely to be the basis of resistance in these resistant populations.

The mechanism of gene amplification is known to be influenced by selection pressure and therefore unstable. A study was conducted to determine whether glyphosate selection could alter copy number in *H. glaucum* populations and in their subsequent progenies. Clones were generated from individual plants by splitting plants into two and applying glyphosate on one of the clones of each plant. Results showed that progenies of *H. glaucum* clones exposed to one cycle of glyphosate selection had higher EPSPS gene copies and increased resistance to glyphosate compared the untreated clones. LD_{50} values of treated clones increased by 75% to 79% compared to the untreated clones. Similarly, gene copy numbers of the treated clones increased from 1.5 to 4- fold that of the untreated clones. This suggests that this species responds rapidly to glyphosate selection pressure through increased EPSPS copy number and continued application of glyphosate will increase the level of resistance.

A study into the inheritance pattern of glyphosate resistance in *H. glaucum* found no evidence of a single-gene Mendelian inheritance pattern. F_2 individuals had gene copy numbers ranging from the same as the susceptible parent to more than the resistant parent with no obvious pattern. Elevated EPSPS gene copies were observed in F_2 individuals following glyphosate treatment which may likely influence spread and persistence of resistance.

Results from this study confirm EPSPS gene amplification is the molecular basis of resistance in glyphosate resistant *H. glaucum*. Amplified gene copies in *H. glaucum* are unstable and increases with glyphosate selection and resistance inheritance is complex and non-Mendelian.

PUBLICATIONS ARISING FROM THE THESIS

- Adu-Yeboah P, Malone JM, Fleet B, Gill G, Preston C (2020) EPSPS Gene Amplification Confers Resistance to Glyphosate Resistant Populations of *Hordeum glaucum* Stued (Northern Barley Grass) in South Australia. Pest Management Science 76:1214-1221.

- Adu-Yeboah, P., Malone, J.M., Gill, G. and Preston, C. (2021), Stability of EPSPS gene copy number in *Hordeum glaucum* Steud (barley grass) in the presence and absence of glyphosate selection. Pest Manag Sci. https://doi.org/10.1002/ps.6367.

- Adu-Yeboah P, Malone JM, Gill G, Preston C (2020). Inheritance of resistance to glyphosate in a *Hordeum glaucum* (Northern Barley Grass) population from South Australia. Pest Management Science. Submitted paper.

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ABREVIATIONS

a.e	acid equivalent
a.i	active ingredient
AGRF	Australian genome research facility
ALS	acetolactate synthase
ANOVA	analysis of variance
DAP	day after treatment
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
HAT	hour after treatment
LD50	herbicide dose required for 50% mortality
LSD	least significant difference
PCR	polymerase chain reaction
QPCR	Quantitative polymerase reaction
RI	resistance index relative to sensitive biotype
SEM	standard error of mean
SNP	Single nucleotide polymorphism

Chapter 1 LITERATURE REVIEW

1.1 Introduction

As World population increases, agriculture has revolutionised into large scale mechanised farming where weed control is a major challenge. Farmers are constantly faced with the problem of effectively controlling weeds in their fields to increase productivity and profitability. The introduction of herbicides during the 1940s was therefore a timely development, which was readily adopted by farmers (Ross 1985; Timmons 2005). With herbicides, farmers can selectively control weeds in their crops. Adoption of conservation agriculture practices, such as no-tillage and minimum tillage systems to preserve the soil structure (Lindwall et al. 1994), only became feasible after the availability of herbicides that provide broad-spectrum weed control. However, the intensive use of herbicides has resulted in the evolution of weeds resistant to many of the herbicides currently used. The International Survey of Herbicide Resistant Weeds has already recorded 486 cases of herbicide-resistant weeds globally and this number is still increasing (Heap 2020).

Glyphosate, a systemic post emergent herbicide is one of the most widely used herbicides in the world. Introduced by the Monsanto company in 1974, glyphosate controls a wide range of weeds, has no soil activity and is very effective even when applied at low rates (Franz et al. 1997). The introduction of Roundup Ready crops and reductions in herbicide price increased the area of glyphosate use. This increased use of this herbicide has also increased the potential for selecting glyphosate resistant weeds. Currently 39 weed species have been reported as resistant to glyphosate across the world (Heap 2020). Glyphosate-resistant weeds have been found in cropping situations such as orchards, vineyards, cereals, and pulses as well as in non-crop situations such as fence lines, crop margins, chemical fallows, and irrigation channels and along roadsides. In all these situations, resistance evolved after several years of continuously

applying glyphosate without herbicide rotation or other weed management strategies (Preston et al. 2009).

Hordeum glaucum Steud. syn. *H. murinum* ssp *glaucum*, *Critesion glaucum* (commonly referred to as Northern barley grass) is one of the most important grass weeds in South Australia. These self-pollinating grass species occur mostly in crops and pastures in the grain cropping regions. *H. glaucum* provides early stock feed in autumn after the onset of rains, but seeds of mature plants can injure grazing animals (Warr 1981). On disturbed lands, *H. glaucum* seedlings can establish quickly and seeds are easily dispersed carried by livestock, farm implements and as contaminants of hay and grains (Groves et al. 2003). *H. glaucum* plants effectively compete for resources with field crops and pastures causing large losses in yield, as well as acting as alternative hosts for various pathogens, fungi and cereal diseases (Ali 1981). This weed species has a short-lived seed bank and often occurs in high densities, which may be correlated with its tendency to evolve resistance to herbicides (Kleemann and Gill 2006).

H. glaucum can be difficult to control and the use of herbicides has proved to be the most efficient method of its control in crops (Squires 1963). Even so, farmers are faced with limited options of post-emergent herbicides suitable for the control of this weed in wheat and other cereals because of the risk of injury to the crops. At present, ACCase inhibitors are the main herbicides that provide effective in-crop control of *H. glaucum* in broadleaf crops. Recent introduction of pyroxasulfone has allowed farmers to achieve effective control of *H. glaucum* in wheat. In non-crop situations such as fence lines and crop margins, glyphosate and paraquat provide effective control of *H. glaucum.* The persistent use of these herbicides has however, resulted in the selection of resistant populations. The first incidence of herbicide resistant *H. glaucum* was a paraquat resistant population reported in Victoria, Australia (Warner and Mackie 1983). Subsequently, resistant populations of *Hordeum leporinum* and *H. glaucum* have emerged in pastures, cereals, legumes and other winter pulses across Australia (Heap

2020). In South Australia, growers have been using glyphosate for many years for pre-sowing knockdown control of *H. glaucum* in crops and pastures and for controlling seed set in pastures. Glyphosate is also being intensively relied on as the primary control of *H. glaucum* along fence lines, crop margins and roadsides. Recently some *H. glaucum* populations along crop margins and yards of a cropping field in the Yorke Peninsula, South Australia were observed to survive glyphosate treatment after a decade of persistent use. Preliminary dose response test showed resistance level of 4.5 folds greater than the susceptible populations.

The evolution of glyphosate resistance in *H. glaucum* is of concern because of its importance as a pre-sowing knockdown control and limits the herbicide range of post emergent control of this weed in pastures. The possibility of glyphosate resistant *H. glaucum* species appearing in Roundup Ready crops is high, which may again affect the control options in such situations. It also presents an economic challenge for farmers, as alternative control strategies of resistant species have to be developed which may have repercussions on cost of production. Estimates show that herbicide resistance costs producers from $1.1 to 1.5 billion annually in terms of increased herbicide use and decreased yield and quality (Hugh 2018). As farmers and researchers are now faced with the challenge of finding suitable strategies for controlling glyphosate resistant *H. glaucum*, there is the need to better understand the factors influencing the evolution of resistance to help delay or avoid resistance appearing. This can be achieved by identifying the mechanisms and characteristic that can influence persistence and spread of resistance in a population. Better understanding of the evolution of resistance of *H. glaucum* to glyphosate may improve resistance management by better defining herbicide use patterns to delay or avoid resistance to these herbicides and the formulation of effective management strategies.

This project will focus on investigating the factors and characteristics of *H glaucum* that influences the evolution, persistence and spread of resistance by identifying the mechanisms

of resistance, the effect of selection pressure on resistance and pattern of inheritance in resistant populations.

1.2 Herbicide use and evolution of resistance

Herbicides are chemicals synthesized from various organic or inorganic compounds and used to suppress and kill undesirable plants. Herbicides provide the most economic and effective method of weed control in both crop and non-crop situations and, therefore, have become the most widely used weed management tactic around the world. The introduction of herbicide-tolerant crops has provided farmers with increasing convenience of selectively controlling weeds in fields (Gianessi 2005; Triplett and Dick 2008).

Unfortunately, with the intensive use of herbicides some weed species have evolved resistance to herbicides. Herbicide resistance is the ability of a plant to survive and reproduce after being treated with lethal doses of the herbicide that would normally kill the sensitive ones (Powles and Shaner 2001; Prather et al. 2000). The first incidence of weed resistance was reported in 1970 (Ryan 1970), subsequently worldwide occurrence has increased to a total of 485 (249 dicots and 236 monocots) (Table 2) cases currently recorded (Heap 2020). These resistant species are being identified from fields persistently treated with the same herbicide or herbicides with the same mode of action over years.

The USA still remains the country with the highest number of cases (160) of weed resistance followed by Australia with a total of 88 weed species recorded (Heap 2020). These resistant species are common weeds of crops and pastures and some are noted for having a high tendency of evolving resistance due to their biology. For example, many herbicide resistant weeds belong to the Poaceae family (Matzrafi et al. 2014). Species belonging to this family have been reported as some of the worst herbicide-resistant weeds globally. An example is annual ryegrass (*Lolium rigidum*), which has evolved resistance to many herbicides with different modes of action and developed cross and multiple resistance to many herbicides as well

(Powles et al. 2007; Preston and Powles 2002b). Cross resistance is where a weed population with a single resistance mechanism can evolve resistance to two or more herbicides from different groups and multiple resistance is where the weed population has two or more resistance mechanisms and it's able to withstand many herbicides from different herbicide groups (Powles and Preston 1995). The herbicide groups with the highest number of resistant cases are the ALS inhibitors, photosystem II inhibitors and ACCase inhibitors. In the past decade, herbicides in the glycine group (EPSP synthase inhibitors) are rapidly being selected for resistance although it was initially presumed impossible (Bradshaw et al. 1997). The number of weed species currently with resistance to glyphosate stands at 38 and increasing.

Table 1: Number of weed species resistant to herbicides of different group of action (Heap 2020)

Herbicide group	**HRAC Group**	**Example**	**Total**
ALS inhibitors	B	Chlorsulfuron	159
Photosystem II Inhibitors	C1	Atrazine	73
ACCase inhibitors	A	Sethoxydim	48
EPSP synthase inhibitors	G	Glyphosate	38
Synthetic Auxins	O	2,4-D	36
PSI Electron Diverter	D	Paraquat	32
PSII inhibitor (Ureas and amides	C2	Chlorotoluron	28
PPO inhibitors	E	Oxyfluorfen	13
Microtubule inhibitors	K1	Trifluralin	12
Lipid inhibitors	N	Triallate	10
Carotenoid biosynthesis (unknown)	F3	Amitrole	6

Long chain fatty acid inhibitors	K3	Butachlor	5
PSII inhibitors (Nitriles)	C3	Bromoxynil	4
Carotenoid biosynthesis inhibitors	F1	Diflufenican	4
Glutamine synthase inhibitors	H	Glufosinate-ammonium	3
Cellulose inhibitors	L	Dichlobenil	3
Antimicrotubule mitotic disrupter	Z	Flamprop-methyl	3
HPPD inhibitors	F2	Isoxaflutole	2
DOXP inhibitors	F4	Clomazone	2
Mitosis inhibitors	K2	Propham	1
Unknown	Z	Endothall	1
Cell elongation inhibitors	Z	Difenzoquat	1
Nucleic acid inhibitors	Z	MSMA	1
Total number of resistant biotypes			**485**

Modified from Heap IM, *International Survey of Herbicide-Resistant Weeds (Heap 2020)*. Available at http://www.weedscience.org.

1.3 Factors influencing resistance development

Plants usually evolve resistance to herbicides through the process of natural selection. The evolution of herbicide resistance is a process that can be influenced by the interaction of many factors that may be naturally occurring or human imposed, such as through genetic engineering. Selection for resistance to herbicides in weeds begins when some individual weeds in a population have the inherent ability to survive herbicide application. Populations normally have resistance genes at low frequency and when a herbicide is applied, the susceptible individuals are killed, and the few resistant survivors produce seed (Figure 1). When the same herbicide is repeatedly applied the number of resistant individuals increases. Several factors have been reported to influence the rate of resistance evolution in weeds. These include weed species characteristics (initial frequency of resistance mutation, gene flow by pollen, fitness in the presence or absence of herbicides, seed bank characteristics), herbicide properties

(herbicide mode of action, efficacy and soil residual activity) and management strategies adopted (Diggle and Neve 2001).

1.3.1 Initial gene frequency

Resistance alleles can appear in populations due to spontaneous gene mutations or through gene flow from outside (Jasieniuk et al. 1996). Gene mutations occur normally at very low frequencies in natural plant populations before herbicides are applied. However, new mutations are continuously generated at different rates, which may vary between species and herbicide mode of action. There are suggestions that the rate of increase of genetic variation in a plant may be an evolved response to stress and therefore, may be influenced by environmental factors and management practices (Lynch 2010; Rainey et al. 1993). Although the actual levels of the initial gene frequency needed to confer resistance in a weed population is not known, it was assumed to be approximately 1 $x10^{-6}$ in herbicide resistant weeds (Maxwell and Mortimer 1994a; Merrell 1981). The initial frequency of resistance is important, because it can be used to predict the rate at which a plant evolves resistance to a herbicide. When the initial frequency of resistance in a weed population is low, it delays the evolution of resistance; especially when the herbicides being applied impose less intense selection pressure. For instance, the initial gene frequency for *L. rigidum* resistant to sulfonylurea herbicides was reported to be between 2.2 x 10^{-5} to 1.2 x 10^{-4} (Preston and Powles 2002a). Such high initial gene frequency is consistent with the reports of resistance to these herbicides after just four applications (Gill 1995).

1.3.2 Selection pressure

Selection pressure is one of the main factors that contribute to resistance evolution. This is mainly imposed by the herbicide used. Thus, where genetic variation exists in a weed population, selection pressure imposed by a herbicide favours the evolution of resistance.

Major characteristics of herbicides that contribute to increased selection pressure include herbicide efficacy, mode of action, residual activity and frequency of use (Maxwell and Mortimer 1994a). Herbicides that provide 99% weed control will impose more intense selection pressure than herbicides that provide lower levels of weed control. Depending on the soil and herbicide properties, herbicide residual activity is also known to promote the evolution of herbicide resistance (Jasieniuk et al., 1996; Beyer et al., 1988). Herbicides that persist in soil over extended periods provide higher selection pressure than non-persistent herbicides. Herbicide resistant weeds have been reported from broadacre crops, orchards and vineyards because of the more intense selection pressure imposed by repeated use of the same herbicides in these situations (Heap et al. 2001).

1.3.3 **Gene Flow**

Gene flow is the movement of resistant genes between and within plant populations. This can occur through pollen or seed movement by mechanisms such as wind, animals, water and agricultural implements (Jasieniuk et al. 1996; Maxwell and Mortimer 1994b). Transfer of pollen or seeds from resistant individuals in a susceptible population can provide the initial source of resistance alleles for selection for resistance.

The success of gene flow through pollen movement is influenced by the distance of movement, environmental conditions and mating type of plants (Yerka et al. 2017). High levels of pollen flow always occur near the source and decline rapidly at further distances. Pollen viability is also reported to decline with distance, which affects rates of fertilization. Pollen dispersal through insects occurs in some plant species and can be more efficient. A study on *Raphanus sativa* showed that about 6 to 18% of seeds could be fertilized by pollen from about 100km distance (Ellstrand and Marshall 1985).

Gene flow by seed movement is one of the common ways through which resistant weeds are transferred between fields. For example, in a study of *L. rigidum* populations resistant to glyphosate on fence lines, it was suggested that transfer of seeds through the movement of farm machinery caused resistance to move 50m into crops (Adu-Yeboah et al. 2014). There are also reports of long distance weed seed movements in hay during periods of severe drought.

1.3.4 **Fitness**

The relative fitness of plants containing resistance alleles is an important factor that influences the evolution of herbicide resistant weeds. Fitness is a measure of the ability of a plant to survive and reproduce viable offspring relative to another in the natural environment (Maxwell and Mortimer 1994b). It is reported that relative fitness can be influenced by the plant genotype and its interaction with other ecological factors and is therefore not a stable parameter (Frenkel et al. 2017; Warwick and Black 1994). For instance, the relative fitness of a plant with resistance allele will be considerably higher in the presence of herbicides than susceptible plants of the same species. However, when compared in the absence of the herbicide, susceptible individuals may have an increased or equal fitness because of negative impacts of the resistant allele. Therefore, in the absence of herbicide selection pressure, susceptible individuals with a higher fitness penalty can replace less fit resistant individuals. Fitness of herbicide resistant plants normally depends on the mechanism of resistance and the environmental conditions. For example in a recent study, the application of PSII inhibitors gave resistant plants of *Brachypodium hybridum* (target site resistance**)** fitness dominance over the susceptible biotype, however, the absence of herbicide there was an ecological advantage to susceptible plants (Frenkel et al. 2017). For resistance due to target site modification, the point

of mutation or amino acid substitution conferring resistance have also been observed to impact enzyme functionality and associated pleiotropic effects in many resistant individuals examined. For resistance to ALS inhibiting herbicides, fitness penalties have only been examined for the Pro-197 to histidine (His) substitution although several sites of mutation conferring resistance have been reported (Tranel and Wright 2002; Yu et al. 2007). The study showed a fitness cost associated with resistant individuals possessing the Pro-197-His allele compared to susceptible individuals evidenced in a reduction in plant biomass and seed production of the resistant population (Alcocer-Ruthling et al. 1992; Guttieri et al. 1992). Similarly, fitness cost associated with ACCase resistance was found to be specific to resistance endowing mutations where some amino acid substitutions does adversely impact vegetative and reproductive plant traits however some mutations significantly impaired plant growth and seed production (Vila-Aiub et al. 2009). Parameters often assessed for fitness in plants include differences in germination, seed dormancy, emergence, seed longevity, growth and other physiological traits. Plant fitness in the field could be exploited for weed management strategies to reduce the evolution of resistance.

1.4 Glyphosate properties and use

Glyphosate (N-(phosphonomethyl) glycine) is a post-emergent, broad spectrum, systemic herbicide that controls a wide range of weeds in crop and non-cropped situations (Baylis 2000). Glyphosate was discovered by the Monsanto Company in the 1970s (Franz et al. 1997) and later patented and introduced into the market under the brand name Roundup in 1974 (Duke and Powles 2008). Glyphosate is marketed in various salt formulations such as isopropylamine, potassium, diammonium, monoammonium, trimesium and dimethylamine salts in 360, 450, 480 or 540 g/L of active ingredient with or without surfactants (Baird et al. 1971).

Glyphosate has proved to be very effective against a wide range of weeds; especially plants with complex root systems (rhizomes), which are generally difficult to control. As glyphosate has no soil residual activity as due to its rapid degradation by soil microbes, there are no concerns of leaching into nearby water sources (Duke and Powles 2008). The use of glyphosate has increased more than 100-fold in the past few years due to the introduction of glyphosate-tolerant (Roundup ready) crops and the adoption of no-tillage practices by farmers (Myers et al. 2016).

1.5 **Glyphosate mode of action**

Glyphosate inhibits the enzyme EPSPS in the shikimate pathway of plants (Amrhein et al. 1980). This enzyme catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvyl-shikimate-3-phosphate (EPSP) (Steinrücken and Amrhein 1980). Inhibition of this enzyme prevents the synthesis of the aromatic amino acids tyrosine, tryptophan, phenylalanine and other secondary plant metabolites that are essential for plant growth (Dill 2005; Steinrücken and Amrhein 1980). It also increases the accumulation of shikimate in plant tissues, which causes diversion of energy and resources needed for other plant processes (Sikorski and Gruys 1997).

Although plant growth ceases immediately after glyphosate application, yellowing of plant leaves, which is one of the typical symptoms, is normally observed after 2 to 3 weeks (Ashton and Crafts 1973). Glyphosate efficacy and injury symptoms can be influenced by environmental conditions such as temperature, rainfall and humidity (McWhorter and Azlin 1978; Vila-Aiub et al. 2013). In a previous study, the efficacy of glyphosate on common lambsquarters (*Chenopodium album*) control was observed to be influenced by rainfall after application (Sivesind et al. 2011). In another study, control of *Desmodium tortusum* was also observed to be affected by temperature and relative humidity after glyphosate application

(Sharma and Singh 2001). The plant growth stage at the time of application have also been reported to influence glyphosate efficacy (Jordan et al. 1997).

1.6 Glyphosate resistance

The first case of glyphosate resistance was reported in *L. rigidum* in Australia in 1996 (Powles et al. 1998). Subsequently, more resistant weeds have been identified and the number of resistant species has been increasing. Currently 39 weed species have been identified as resistant to glyphosate in over 30 countries (Heap 2020). Majority of these resistant weeds were found in Roundup Ready crops, orchards, vineyards and a few from non-crop situations, such as fence lines. Reports indicate that Roundup Ready crops account for about 98% of area infested with glyphosate resistant weeds globally (Heap 2014). The *Amaranthus* species (*A. palmeri, A. tuberculatus* and *A. hybridus*) are the most widespread glyphosate resistant weeds followed by the *Conyza* species.

Early reports of glyphosate resistance came from developed countries such as the United States, Australia and Canada, where large scale mechanised farming systems are common. However, glyphosate resistance has now been reported in many countries such as China, Portugal and most recently South Korea (Heap 2020).

1.7 Mechanisms of glyphosate resistance

The mechanisms identified to confer resistance to glyphosate can be generally grouped into target-site resistance and non-target site resistance. Target site resistance is where a mutation in the EPSPS gene causes loss of affinity between glyphosate and the protein target site (Kaundun et al. 2017). This mechanism usually confers low to intermediate level of resistance in weeds. An alternative mechanism is over expression of the EPSPS gene by amplification (Gaines et al. 2010). The level of resistance induced by gene amplification mechanisms is suggested to vary and is influenced by the number of gene copies present (Patterson et al. 2017; Pline-Srnic 2006).

Non-target resistance mechanisms limits glyphosate from reaching its target site through either reduced uptake and translocation, vascular sequestration or increased metabolism (Kleinman and Rubin 2017; Preston and Wakelin 2008). This resistance mechanism confers moderate levels of glyphosate resistance (Preston et al. 2009). The most common resistance mechanism identified in glyphosate resistant weeds is reduced translocation/sequestration. There are examples where glyphosate resistant weeds have two of these resistance mechanisms (Preston et al. 2009). Two populations of glyphosate resistant *L. rigidum* (Bostamam 2010) in South Australia, and more recently some populations of *Parthenium hysterophorus* in the Caribbean (Vila-Aiub et al. 2013), were identified with both target site resistance and reduced translocation mechanisms. Three populations of glyphosate resistant *Eleusine indica* from China were also reported to be endowed with both target site mutation and gene amplification resistance mechanisms conferring 13.8 to 28.3 fold resistance (Chen et al. 2015).

1.7.1 Gene amplification as a mechanism of glyphosate resistance

Gene amplification/duplication is another form of a target site resistance mechanism. Since first reported in glyphosate resistant *Amaranthus palmeri* (Palmer amaranth) (Gaines et al. 2010), gene amplification has been documented in several other glyphosate resistant weed species (Heap and Duke 2018; Sammons and Gaines 2014). Gene amplification is the process whereby a segment of DNA is replicated to generate additional copies in the genome of the organism (Flagel and Wendel 2009). The duplicated genes enable the plants to produce sufficient enzyme to maintain the shikimate pathway and continue metabolic activities in the presence of glyphosate (Gaines et al. 2010). The mechanism of gene amplification reportedly can occur through the processes of unequal recombination leading to tandem repeats, meiosis or hybridization errors resulting in aneuploid cells, activities of mobile transposable elements or whole genome duplication (Eichler 2001; Hurles 2004; Pumphrey et al. 2009; Schoenfelder and Fox 2015). Gene amplification mechanism has also been documented in resistance to some

antibiotics, insecticides and fungicides (Bass and Field 2011; Ma and Michailides 2005; Sandegren and Andersson 2009). This mechanism is noted to provide high levels of resistance in weeds and in glyphosate resistant weeds, large copy-number variations in individual populations have been observed with copy numbers ranging from as low as < 3 to > 100 (Heap and Duke 2018). Increased *EPSPS* gene copies are also most often found to positively correlate with the level of resistance (Gaines et al. 2013).

1.8 Inheritance of glyphosate resistance in weeds

The modes of inheritance of glyphosate resistance in weeds studied have typically revealed inheritance patterns linked to an incompletely or a completely dominant single nuclear gene. These patterns of inheritance of resistance is most often observed associated with resistance due to reduced translocation and altered target site mechanisms in weeds (Powles and Preston 2006). This has been demonstrated in *L. rigidum* (Lorraine-Colwill et al. 2001; Preston and Wakelin 2008; Preston et al. 2009; Wakelin and Preston 2006), *C. canadensis* (Zelaya et al. 2004), *E. indica* (Ng et al. 2004), *Erigeron Canadensis* (Ge et al. 2010; Zelaya et al. 2004) and *Lolium perenne* (Jasieniuk et al. 2008). Glyphosate resistance in weeds is not always a single gene trait. There have been rare cases where resistance was found to be controlled by two nuclear genes. In these cases, resistance was found to be conferred by two different mechanisms; reduced glyphosate translocation and a target site mutation where each is controlled at a single locus and acting additively (Okada and Jasieniuk 2017; Simarmata et al. 2005; Yu et al. 2009). Contrary to this inheritance pattern is resistance due to EPSPS gene amplification/duplication. Inheritance of gene amplification mechanism in reported weed species appears to be more complex and variable involving multiple genes. Jugulam et al. (2014) reported that amplified EPSPS copies in *B. scoparia*, were present as a tandem repeat block and inherited as a single locus with Mendelian segregation. However, in some

populations of *A. palmeri* and *B. diandrus* variable EPSPS gene copies in F_2 crosses derived did not conform to the single gene model, whilst in others it was inconclusive (Chandi et al. 2012; Gaines et al. 2010; Giacomini et al. 2019; Malone et al. 2016; Mohseni-Moghadam et al. 2017) . The inheritance of resistance due to gene amplification has therefore been suggested to be influenced by the genomic organisation of EPSPS on the chromosomes and the plant species.

1.9 H. glaucum

H. glaucum (one of the *Hordeum* species present in Australia) is a self-pollinating annual weed species that germinates rapidly in autumn after breaking rains. It is a widespread weed of crops and pastures, but also occurs in non-crop situations, such as along roadsides, margins of cultivated lands and around buildings. *H. glaucum* can provide valuable early stock feed for grazing animals, but becomes undesirable after seed set, because its sharp seeds can penetrate the eyes, skin and wool of sheep resulting in loss of productivity (Campbell et al. 1972). According to Davidson (1977), *H. glaucum* was accidentally introduced to Australia in the eighteenth and nineteenth century as a contaminant of wool and livestock from Europe. Now this weed has naturalised and become a weed of economic importance in Australian agriculture.

The *Hordeum murinum* complex is the most widespread of all the *Hordeum* species (Mizianty 2006). Originally native to the Mediterranean, Western Asia and Europe, this species complex is currently widely distributed in all the states of Australia. The *H. murinum* complex consists of three subspecies: *H. murinum* ssp. *murinum* L (wall barley), *H. murinum* ssp. *leporinum* Link (hare barley) and *H. murinum* ssp. *glaucum* Steud (smooth barley) (Cocks et al. 1976; Morrison 1958). According to their chromosome count, *H. m. murinum* and *H. m. leprinum* are described as tetraploids (2n=28), while *H. m. glaucum* is a diploid (2n=14) (Rajhathy and Morrison 1962). *H. leporinum* is known to dominate in areas with colder climates where

average rainfall exceeds 425mm and are therefore more prevalent in southern Australia. *H. glaucum* however tends to occur in warmer climates and is more prevalent in the semi-arid regions where rainfall is less than 425mm (Cocks et al. 1976; Tozer et al. 2009).

1.9.1 **Description and Biological characteristics**

H. glaucum is a seed-propagated species that grows vigorously on a wide range of soils, but mostly dominates on low-phosphorus soils (Groves et al. 2003). It is a prolific seed producer and establishes well on disturbed lands, such as grazed fields (Halloran and Pennell 1981). Seeds are easily dispersed by attaching to the fleece of grazing animals or as a contaminant of hay or on farm implements. *H. glaucum* germinates rapidly at temperatures ranging from 7-32°C with optimum temperatures between 10-15° C; temperatures above 35°C reduces seed germination (Piggin et al. 1973). *H. glaucum* seeds have a short dormancy period, which is normally lost before autumn, but a few seeds remain dormant to germinate in winter, spring or subsequent seasons. However seed dormancy can be broken and germination stimulated by gibberellic acid (GA) treatment and cold stratification (Fleet and Gill 2012).

H. glaucum plants can grow to about 45 cm in height with well-developed auricles which can be up to 20 mm long. The leaves taper to a pointed tip with parallel veins and twist. The leaves are smooth to hairy and may feel rough to touch. The leaf blade is about 40-200 mm long and 1.5-12 mm wide. The inflorescence is a cylindrical, spike like panicle about 30-100 mm long x 10 mm wide often enclosed by a sheath of leaf. The spikelets are made up of three florets: the central spikelet is stalk-less, and smaller than the laterals. When ripe, the spikelets fall as three units containing one seed. The mature plant averages 27 spikelets/head and about 25 seeds/head (Fig 1). The seed heads are 30-100 mm long. The seeds have rough bristles of varying lengths with 92% fertility (Hitchcock et al. 1969; Whitson et al. 1991).

Figure 1: *Hordeum* spikelet

1.9.2 Ecological impact and management

H. glaucum is a common widespread weed of crops and pastures. It reduces crop yields and reduces crop value by contaminating harvest. It also an alternative host for a number of cereal diseases, such as root lesion nematode in cereals, barley scald, eyespot of wheat, powdery mildew, net blotch and stripe rust in cereals (Cocks and Donald 1973). This reportedly can cause yield loss of up to 80% in ideal conditions (Groves et al. 2003). As an alternative host, it can produce a rapid buildup of numbers of fungi and nematodes causing agents and serve as sources of reinfection of the next season's crops.

Mowing and grazing provides some control of *H. glaucum* in crops and pastures (George 1972). However, herbicides are the most effective tools for control (Squires 1963). Herbicides such as paraquat, diquat, some ACCase-inhibitors (Fops and Dims) and ALS-inhibitors (SUs and Imis) and glyphosate generally provide good control. In a recent study by Shergill et al (2016), application of post emergent herbicides such as propyzamide, pyroxasulfone or imazamox was observed to provide high level control of *H. glaucum* with no crop injury while field pea grain and forage yield was increased.

1.10 Resistance to herbicides in H. glaucum

H. glaucum has evolved resistance to paraquat, diquat, several ACCase- and ALS-inhibiting herbicides (Heap 2020). The first instance of paraquat resistance was reported in a population of *H. glaucum* from a lucerne field where paraquat had continuously been used for 15 years in Victoria, Australia (Warner and Mackie 1983). This population had 250-fold greater resistance than the susceptible populations (Powles 1986). Populations of the related species *H. leporinum* were found to be resistant to some ACCase and ALS-inhibiting herbicides such as flauzifop, butoxydim, quizalofop, chlorosullfuron, sulfosulfuron, imazamox paraquat and diquat (Mathews et al. 2000; Owen et al. 2012a; Shergill et al. 2015; Tucker and Powles 2017; Yu et al. 2007). These resistant populations appeared in pastures and cropping fields where these herbicides had been used to control weeds for 5-15 years.

Some populations of *H. leporinum* and *H. glaucum* with cross-resistance to herbicides have also been reported. Matthews et al. (2000) reported a population of *H. leporinum* resistant to fluazifop with cross-resistance to clethodim and sethoxydim. In Western Australia, two *H. leporinum* populations were also found to be resistant to SUs and IMIS class ALS inhibiting herbicides (Owen et al. 2012b). These populations were highly resistant to sulfosulfuron and sulfometuron, but also showed a low level of resistance to imazamox. Shergill et al (2017) also found some populations of *H. glaucum* in South Australia with cross-resistance to Fops and Dims class of ACCase-inhibiting herbicides and some with multiple resistance to ALS- and ACCase-inhibiting herbicides.

1.11 Research objectives

Glyphosate has been effective in controlling *H. glaucum* in both crop and non-crop situations until recently when some populations suspected to be resistant were observed on a farm in South Australia. These populations had survived glyphosate at the recommended field rate.

Preliminary dose response tests confirmed *H. glaucum* resistance to glyphosate. Initial assessment of the morphological characteristics of the weed suggests that it is *H. glaucum*.

Therefore, to be able to characterise and understand the evolution of glyphosate resistance in *H. glaucum* populations, the objectives of this project are to:

- Screen populations in dose response experiments to determine level of glyphosate resistance.
- Determine the mechanism conferring glyphosate resistance in *H. glaucum* populations identified in a series of laboratory experiments.
- Assess influence of glyphosate selection on resistant populations and transfer of resistance to second generation progenies.
- Determine mode of inheritance of glyphosate resistance in *H. glaucu*m populations.

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Chapter 2 EPSPS GENE AMPLIFICATION CONFERS RESISTANCE TO GLYPHOSATE RESISTANT POPULATIONS OF *HORDEUM GLAUCUM* STUED (BARLEY GRASS) IN SOUTH AUSTRALIA

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EPSPS gene amplification confers resistance to glyphosate resistant populations of *Hordeum glaucum* Stued (northern barley grass) in South Australia

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Abstract

BACKGROUND: Glyphosate failed to control two populations of *Hordeum glaucum* (northern barley grass) along a fence line and around stockyards near Arthurton on the Yorke Peninsula, South Australia after more than a decade of regular use of glyphosate. These were investigated to confirm resistance and to determine resistance mechanisms.

RESULTS: Dose–response experiments confirmed resistance of these populations to glyphosate with resistance levels of 2.8–6.6-fold compared with two susceptible populations. Shikimate assays confirmed resistance to glyphosate with less shikimate accumulation in the resistant populations compared with the susceptible populations. Quantitative PCR of genomic DNA showed increased gene copy number in the resistant populations with 9–11-fold more copies of EPSPS compared with the susceptible populations, suggesting resistance is likely conferred by gene amplification.

CONCLUSION: This study identified the first examples of glyphosate resistance in the grass species *H. glaucum* with resistance associated with *EPSPS* gene amplification.

Keywords: *Hordeum glaucum*; glyphosate resistance; *EPSPS*; gene amplification

1 INTRODUCTION

Hordeum glaucum Stued. (syn. *H. murinum* ssp. *glaucum*, *Critesion glaucum*) is a widespread annual winter-growing grass species in South Australia. It is commonly a weed of pastures where it serves as early feed for grazing animals, but the seeds cause damage to stock.[1] Recently, there have been some reports of increased infestations of this weed in cropping fields.[2] This species has a widespread distribution across the grain-cropping regions of South Australia, such as the Eyre Peninsula and Upper North regions. *H. glaucum* is a vigorous and competitive grass species,[3] which can also serve as a host to a number of diseases of cereal crops.[4] *H. glaucum* is also a common weed in non-crop situations along fences and yards, around buildings, and along roadsides and railways. Seedlings of this self-pollinating species can estab-lish rapidly on a wide range of soils and it often occurs in high densities. Plants of *H. glaucum* can produce a large number of seeds that are readily dispersed by attaching to the fleece of grazing animals or as contaminants of hay.[5] Good control of *H. glaucum* is mostly achieved with herbicides, however, in many situations there are limited options available for its selec-tive control without damaging desirable species. Pre-sowing herbicides such as paraquat, diquat and some selective post-emergent herbicides from Acetyl co-enzyme A carboxy-lase (ACCase)-inhibiting subgroups aryloxyphenoxypropionate (Fops), cyclohexanedione (Dims), and acetolactate synthase (ALS)-inhibiting subgroups imidazolinones (Imis) and sulfony-lureas (SUs) can generally provide effective control of *H. glaucum*. However, resistance to these herbicides has been reported in *H. glaucum* populations in South Australia.[6–11]

Glyphosate is a non-selective post-emergent herbicide used extensively to control annual and perennial weeds in both crop and non-crop situations.[12] Glyphosate has become a popular weed control tool in agricultural systems owing to its broad spectrum weed control, high efficacy and low cost com-pared with other herbicides. With the adoption of minimum or no-tillage practices by many farmers, and the increase in area under glyphosate-tolerant crops, the use of glyphosate has increased more than 100-fold in recent years.[13] Glyphosate targets the enzyme 5-enolpyruvylshikimate-3-phosphate synthase in the shikimic acid pathway of plants, which is responsible for the synthesis of the aromatic amino acids tyrosine, tryptophan and phenylalanine.[14] The inhibitory effect of this herbicide on the

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production of amino acids essential for plant growth eventually leads to plant death.[15]

With the continuous and extensive use of glyphosate, resistance to this herbicide has become widespread in many weed species across the world. Currently 43 weed species have been reported to have evolved resistance to glyphosate[16] either through a muta-tion in the *EPSPS* gene,[17,18] amplification of the *EPSPS* gene[19,20] or reduced herbicide uptake and translocation.[21,22] Although reports indicate that ~ 98% of glyphosate-resistant weeds evolve from use in glyphosate-tolerant crops[23] where selection intensity is high due to several applications each season, resistance has also been reported from non-crop situations, such as fence lines, where glyphosate is usually applied only once annually.[24] Examples of weed species that have evolved resistance to glyphosate are ryegrasses (*Lolium* spp.), amaranths (*Amaranthus* spp.), fleabanes (*Conyza* spp.) and ragweeds (*Ambrosia* spp.). To date, there have been no reported cases of glyphosate resistance in *H. glaucum* anywhere in the world.[16] In South Australia, glyphosate is generally used for pre-sowing knockdown or spray topping control of *H. glaucum* in pastures, along fence lines and crop margins.

Glyphosate resistance along fence lines and crop margins is a significant problem in Australia. Currently, 92 sites have been confirmed with at least one being identified each year, with widespread resistance in *Lolium* species.[24] Fence lines, crop mar-gins and other non-cropped areas are characterized by little or no competition from crops or other weed species. Therefore, plants that escape herbicide treatment in these situations can grow vigorously and set a large amount of seeds. Because fence lines are mostly adjacent to crop fields, seeds from resistant plants can easily be transferred into cropped areas by farm operations.[25]

This study aims to determine whether populations of *H. glaucum* collected from a fence line and around stockyards on a farm near Arthurton on the Yorke Peninsula of South Australia were resistant to glyphosate and also to determine the resistance mechanisms present.

2 MATERIALS AND METHODS

2.1 Plant material

Resistant populations of *H. glaucum* (YP1 and YP2) were collected from non-cropped area along fences and around stockyards, respectively, near Arthurton, South Australia (34°14′44.2″S 137°44′32.6″E) following reports of multiple control failures with glyphosate. Seeds of the fence line population were collected in 2016 and a preliminary test confirmed resistance to glyphosate. In 2017, seeds were collected from around sheep-handling yards where glyphosate had failed to control *H. glaucum* on the same farm. Susceptible populations (RW and TW) were collected from pasture fields in Roseworthy (34°31′38.8″S 138°41′09.1″E) and Two Wells (34°35′46″S 138°30′27.57″E), South Australia which had normal sensitivity to glyphosate.

2.2 Seed germination and plant growth

Seeds for dose –response experiments were germinated in plas-tic trays (33 × 28 × 5 cm) containing standard potting mix and later transplanted into 9.5 × 8.5 × 9.5 cm punnet pots (Masrac Plastics, Adelaide, South Australia) containing standard potting mix (produced by steaming 540 L cocoa peat, 220 L of water and 60 L of sand for 1 h).[26] Each pot had nine plants of the same population and was replicated three times for each herbi-cide rate, arranged in a randomized complete block design. The plants were maintained outdoors for the entire duration of the experiments and watered as needed at the University of Adelaide Waite Campus.

2.3 Dose–response experiment

The plants were treated with glyphosate (540 g a.e. ha^{-1} Roundup Nufarm, Laverton, Victoria, Australia) at three- to four-leaf stage at rates ranging from 0 to 2160 g a.e. ha^{-1}. Herbicide was applied using a laboratory moving boom with output volume of 118 L ha^{-1} at a pressure of 250 kPa and a speed of 1 m s^{-1} using a Tee-Jet 001 nozzle (Tee-jet 110u flat fan Spraying Systems, Wheaton, IL, USA). Plants were returned outdoors and herbicide response assessed after 21 days. Plants showing active growth with new tillers were counted as survivors and plants showing severe chlorosis to com-plete mortality were considered dead.[27] Mortality data was anal-ysed using PriProbit (v. 1.63) [28] and the herbicide rate required for 50% mortality (LD_{50}) was calculated from the probit curves. Curves were back-transformed for plotting using GraphPad soft-ware (GraphPad Inc., San Diego, CA, USA). The resistance index (R/S) was calculated for each population as the ratio of the LD_{50} of the resistant population to the LD_{50} of the standard susceptible population. The experiment was repeated three times.

The sensitivity of *H. glaucum* populations to four other herbicides with different modes of action was also assessed. At the three-to four-leaf stage, plants were treated with field recommended rates of the herbicides using the method described earlier. Plants were assessed visually after 21 days and scored dead if showing chlorotic symptoms and no new regrowth. There were three repli-cations of five plants for each treatment.

2.4 Shikimate assay

Shikimate accumulation in leaves was assessed following the methods of Ngo *et al.*[29] with some modifications. Five leaf discs (2 mm) were excised from the basal half of fully expanded leaf from plants of both susceptible and resistant populations. Leaf discs were placed into a single well of a 96-well flat-bottomed microplate containing 100 μL glyphosate stock solution at the rates of 0, 50, 200, 500, 1000 μmol L^{-1} glyphosate and 10 mmol L^{-1} phosphate buffer (pH 7) with three replications. Plates were covered and incubated under light at 65 μmol m^{-2} s^{-1} (Fluval LED A3981; Rolf C. Hagen Corp., Mansfield, MA, USA) and a temperature of 25 °C for 16 h. Following incubation, 0.06 mol L^{-1} HCl was added to each well, and the samples were frozen and thawed through five cycles at −80 °C for 15 min followed by 60 °C for 15 min until the leaves turned brown. A 25-μL aliquot of solution was transferred from each well into new microplates. Immediately after, 100 μL of 0.25% (w/v) periodic acid and 0.25% (w/v) sodium *m*-periodate solution were added to each well and incubated for 60 min at room temperature. The reaction was terminated by adding of 100 μL of quench buffer (0.6 mol L^{-1} NaOH, 0.22 mol L^{-1} Na_2SO_3) into each well. A standard curve for shikimic acid was developed at the same time for each reaction using final concentrations of 1, 2.5, 5, 10, 25 and 50 μ mol L^{-1}. A 150 μL aliquot was transferred from each well to a fresh microplate and absorbance was measured at 380 nm using a microplate manager (Benchmark Plus, Bio-Rad Labora-tories, Inc., Hercules, CA, USA) spectrometer. The mean value of optical density measured in the controls of each population was subtracted from those measured in the glyphosate treatments as background. Shikimate levels were expressed as nM of shikimic acid accumulated per cm^{-2} of leaf area using the standard curve. Shikimic accumulation at different glyphosate concentrations was then fitted to an exponential model using GraphPad Prism (v. 7; GraphPad Software, La Jolla, CA, USA).

2.5 *EPSPS* gene sequencing

Fully expanded leaves from both resistant and susceptible plants were collected and immediately frozen in liquid nitro-gen. Genomic DNA was extracted using Isolate II plant DNA extraction kit (Bioline, Alexandria, New South Wales, Australia) following the manufacturer's instructions. A conserved region of the *EPSPS* gene was amplified by polymerase chain reaction (PCR) following a standard protocol.[30] Each 25 μL reaction mix contained ~ 20 ng genomic DNA, 12.5 μL (2×) MyFi mix reaction buffer (containing MyFi DNA polymerase, dNTPs, $MgCl_2$) and 0.4 μM each of gene-specific primers with forward primer AW1 5′-AACAGTGAGGAYGTYCACTACATGCT-3′ and reverse primer AW2 5′-CGAACAGGTAGGGCAMTCAGTGCCAAG-3′ as reported previously.[17] Amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler Gradient; Eppen-dorf, Hamburg, Germany) with PCR conditions as follows: 3 min denaturing at 95 °C; 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, 45 s elongation at 72 °C and a final extension for 7 min at 72 °C. PCR products were prepared with 1 × Ficoll loading dye (15% w/v Ficoll 4000, 0.25% w/v bromophenol blue 0.25% w/v xylene cyanol FF) and visualized on 1.5% agarose gels stained with 1 × SYBR® Safe DNA gel stain. Samples were elec-trophoresed in 1 × TAE buffer, 40 mmol L^{-1} Trizma base, 1 mmol L^{-1} Na_2EDTA (pH to 8) with glacial acetic acid at 100 V and pho-tographed under 302 UV light. The sizes of the DNA fragments were estimated by comparing to a standard DNA ladder (EasyLad-der I, Invitrogen Victoria, Melbourne, Australia) with known band sizes. Using the same primers in the amplification process, DNA sequencing was conducted by the Australian Genome Research Facility, Australia. Nucleotide sequences were assembled and analysed using the ContigExpress VectorNTi (11.5) and AlignX software programs (Invitrogen).

2.6 ^{14}C absorption and translocation from leaves

Glyphosate absorption and translocation from leaves was studied following procedures described in Wakelin *et al.*[22] with modifica-tions. Seeds had their husks removed, were placed on agar (0.6%) and incubated in a germination cabinet with 12:12 h light/dark photoperiod (30 mmol m^{-2} s^{-1}) at 20 °C during the day and 15 °C at night. Seedlings were transplanted and grown hydroponically in a nutrient solution.[31] Five plants of each resistant and sus-ceptible population were transplanted into a sealed black plas-tic container (270 × 190 × 95 mm) filled with Hoagland solution and supported with a layer of black polypropylene beads. Plants were maintained in a growth room with a 12:12 h photoperiod at 20 °C during the day and 15 °C at night; 300 μmol m^{-2} s^{-1} light intensity during the day. At the three- to four-leaf stage, plants were treated with 125 g ha^{-1} of glyphosate and immedi-ately after radiolabelled ^{14}C-glyphosate (0.5 μL) was applied to the middle of the second leaf of each plant. Each aliquot 0.5 μL of ^{14}C-glyphosate solution contained 0.5 kBq of radioactivity and 0.0136 mmol of glyphosate. The specific activity of ^{14}C-glyphosate (phosphonomethyl-^{14}C) (American Radiolabelled Chemicals, St Louis, MO, USA) was 0.167 GBq $mmol^{-1}$. Each treated leaf was marked for easy identification and applications were completed within 30 min after herbicide application.

Plants were harvested 48 h after treatment (HAT) and sectioned: treated leaf lamina, untreated leaf lamina, roots and sheaths of treated leaf and untreated leaves (stem). In a preliminary experi-ment, glyphosate absorption and translocation was observed to have stabilized by 48 HAT and therefore harvesting in subsequent experiments was done at 48 HAT. Unabsorbed radioactivity on the treated leaves was removed by washing each leaf in 5 mL of 1% Triton X-100 solution (Sigma-Aldrich, Sydney, NSW, Australia). Plant samples were dried for 7 days at room temperature and later combusted in an automatic preparation and oxidization system (Sample Oxidiser 307; Perkin Elmer, Shelton, CT, USA) for 1 min. Radioactive CO_2 released from the combusted samples was collected in 14 mL scintillation fluid (Carbo-Sorb E: Permaflour E+, 1:1 v/v; Canberra Packard, Groningen, The Netherlands), and radioactivity quantified using liquid scintillation spectroscopy (Beckman Coulter, Fullerton, CA, USA). Radioactivity in the wash solutions was also quantified using liquid scintillation spectroscopy follow-ing the addition of 5 mL of Ultima Gold XR scintillation fluid (Can-berra Packard) to the 5 mL of leaf wash solution. The amount of ^{14}C-glyphosate in each plant part was expressed as a percentage of the amount absorbed.

This experiment was repeated. All percentage data were arcsine transformed before analysis. Results of the two experiments were not significantly different as determined by the non-significant interaction in the two-way analysis of variance (ANOVA); therefore, the data were pooled for analysis by one-way ANOVA (Genstat) and means were separated using Fisher's unprotected LSD multiple comparisons at $P \leq 0.05$ (Genstat 17; VSN International, Hemel Hempstead, UK).

2.7 *EPSPS* gene copy number and expression analysis

The *EPSPS* copy number and expression of the resistant and susceptible populations relative to a reference gene was deter-mined by using quantitative PCR (qPCR) following the method

Table 1. Primers and probes used in gene copy number determination

Primer name	Primers/probes sequences 5′ – 3′
EPSPS-F	CCAAGAATGAGGGAGCGACCTAT
EPSPS-R	CAGTGCCAAGGAAACAATCAACA
EPSPS Probe	TGGTGACTTAGTTGTCGGTTTGAAGCA + *FAM*
ALS -F	GCCCAAGCCACCATCTAC
ALS-R	CGCCACCAACATACAGAATTGG
ALS Probe	TGAATCGCTTGAGCAGGTCCTGC + TET

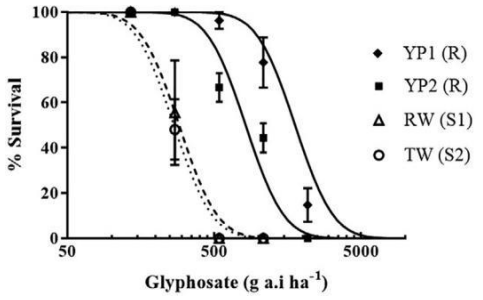

Figure 1. Response of resistant [YP1 (◇); YP2 (■)] and susceptible [RW (△); TW (○)] populations of *Hordeum glaucum* to glyphosate. Each data point is a mean of three replicates and error bars are standard error of means (SEM). Lines are probit curves calculated for each popula-tion with probits transformed back to % survival. Probit curves are YP1 $y = -15.870 + 4.885 \times \log(\text{dose})$; YP2 $y = -14.230 + 4.885 \times \log(\text{dose})$; RW $y = -12.017 + 4.885 \times \log(\text{dose})$; TW $y = -11.846 + 4.885 \times \log(\text{dose})$.

described previously by Malone *et al.*[20] Genomic DNA used for *EPSPS* gene copy number analysis was the same as that used for *EPSPS* gene sequencing described above. The gene copy num-ber was determined by calculating the ratio of *EPSPS* gene copy number to acetolactate synthase (*ALS*) gene copy number. A KAPA PROBE FAST Universal (KAPA Biosystems, Wilmington, MA, USA) assay using Dual-Labelled BHQ FRET probes (Bioresearch Tech-nologies, Petaluma, CA, USA) was used. *EPSPS* primers and probes designed by Malone *et al.*[20] and ALS control gene primers and probes (Table 1) were designed with different fluorophores so the genes could be assayed independently in one qPCR reaction. Genomic DNA templates (~ 20 ng) were amplified in a 10 μL reac-tion mixture containing 5 μL of SsoFast Probe Supermix (Bio-Rad, Gladesville, NSW, Australia), 1 μmol L^{-1} of *EPSPS* and *ALS* forward and reverse primers, 0.3 μM of *EPSPS*-FAM and *ALS*-TET probe, and run on a RG3000 Rotor-Gene real-time thermal cycler with the fol-lowing parameters: 3 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 16 s at 60 °C, acquiring at 510 nm (*EPEPS*-FAM) and at 555 nm (*ALS*-TET). Primer efficiencies were calculated to range from > 98% to < 102%. A modified version of the C_T ($2 = {}^{-}{}^{CT}$) method was used to analyse data from the quantitative PCR experiments.[19,32] Relative quantification of genomic *EPSPS* was cal-culated as $C_T = (C_T{}^{ALS} - C_T{}^{EPSPS})$. Genomic *EPSPS* copy number increase was expressed as $2^{-}{}^{CT}$. Results were expressed as fold increase in genomic *EPSPS* copy number relative to *ALS*. Standard curves were used to calculate the amount of *EPSPS* and *ALS* in pop-ulations. The ratio of *EPSPS* to *ALS* was calculated for each qPCR, and the average and the standard deviation of the duplicate qPCRs were recorded for each population.

For gene expression, total RNA was extracted from ten and five individual plants of each of the two resistant and susceptible pop-ulations respectively. cDNA was synthesized using the Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instructions. A dilution series of four concentrations of cDNA samples was used to construct a standard curve. The same primers used in the gene copy analysis were in the gene expression assays. Quantitative real-time PCR was performed as described above to amplify cDNA templates in a 10 μL reaction mixture using Syber-Green master mix (Bio-Rad) at the above thermoprofile conditions to obtain the product melt curve. The same relative quantification calculation was used for fold increase in *EPSPS* expression.

3 RESULTS

3.1 Dose–response experiments

The dose –response experiments confirmed resistance of the *H. glaucum* populations (YP1 and YP2) to glyphosate. Whereas the two susceptible populations were completely killed at the recommended rate of 540 g ha^{-1}, the resistant populations required much higher rates to achieve control (Fig. 1). In all three experiments, the effective dose required to achieve 50% con-trol (LD_{50}) in the susceptible populations ranged from 207 to 317 g ha^{-1} (Table 2). YP1 population required between 971 and 1772 g ha^{-1} glyphosate to achieve 50% control giving this popu-lation a resistance level of 4 – 6.6-fold higher than the susceptible populations. YP2 population had LD_{50} values of between 738 and 869 g ha^{-1} giving it a resistance level of 2.8 –3.6-fold higher than the susceptible populations. Weed control with glyphosate has been reported to be influenced by environmental conditions[27] and therefore the variations observed in the LD_{50} rates may be the result of prevailing environmental conditions at the different times the experiments were conducted. However, the response of the resistant populations to glyphosate was different to that of the susceptible populations in all experiments.

Glyphosate-resistant *H. glaucum* population YP 1 was tested with other commonly used herbicides with different modes of action and showed susceptibility to selected ACCase and ALS herbicides; whereas for paraquat 41% of the treated plants survived (Table 3). This suggests YP 1 may also have resistance to paraquat, but can be controlled by other herbicides.

3.2 Shikimate assay

Glyphosate inhibits the enzyme *EPSPS* in the shikimic pathway of plants, which leads to massive accumulation of shikimate.[33] As reported in several previous studies, glyphosate-resistant weeds tend to accumulate less shikimate compared with suscep-tible populations.[29,34,35] According to Shaner *et al.*,[33] if shikimate accumulation in a glyphosate-susceptible population is higher than the resistant populations at both low and high rates of glyphosate, it indicates that the resistant population likely con-tains a target site mechanism (gene mutation or amplification) or an unknown mechanism. In this study, shikimate accumula-tion in the susceptible populations (RW and TW) increased with glyphosate concentration until it plateaued at 300 μM (Fig. 2). The level of shikimate accumulation in the resistant populations (YP1 and YP2) was considerably less than that of the susceptible populations. The two susceptible populations required between 40 and 50 μM glyphosate for 50% accumulation of shikimate (Table 4); however, YP2 required 127 μM and YP1 ~ 675 μM for 50% accumulation of shikimate. Based on shikimate accumulation, YP2 was 2.7-fold resistant to glyphosate and YP1 14.3-fold resis-tant. The shikimate assay confirmed glyphosate resistance in the glyphosate-resistant populations and that YP1 was more resistant than YP2. It also indicated that a target site mechanism was likely.

3.3 *EPSPS* sequencing

Previous studies have shown that point mutations in the *EPSPS* gene at codon 106 can endow resistance to glyphosate (Pro106).[17,36–38] Recently a rarer mutation at codon 102 (Thr102)

Table 2. Glyphosate dose required for 50% mortality (LD_{50}) of resistant and susceptible *Hordeum glaucum* populations with confidence intervals in parenthesis. RI is the ratio of LD_{50} of resistant and susceptible populations

Population	Experiment 1		Experiment 2		Experiment 3	
	LD_{50}	RI	LD_{50}	RI	LD_{50}	RI
YP1(R)	971.9 (811.2, 1164.6)	4.7	1246.9 (1066.45, 1465.1)	4.0	1772.6 (1492.1, 1861.2)	6.6
YP2(R)	738.7(616.8, 884.7)	3.6	869.2 (742.9, 1014.7)	2.8	818.2 (695.2, 963.2)	3.1
RW(S)	224.5 (187.2, 269.1)	1	317.8 (269.0, 375.1)	1	288.3 (244.7, 339.4)	1
TW(S)	207.4 (172.7, 248.5)	–	308.8 (261.30, 364.5)	–	266.1 (225.7, 313.2)	–

Table 3. Sensitivity of glyphosate-resistant and -susceptible *Hordeum glaucum* populations to herbicides with different modes of action. R denotes a resistance status and S, susceptible status

				% Survival	
Herbicide chemical class	Active ingredient	Herbicide mode of action	Rate (g ha^{-1})	Resistant	Susceptible
Aryloxyphenoxypropionate	Quizalofop	Inhibition of ACCase	99.5	0	0
Sulfonylurea	Mesosulfuron	Inhibition of ALS	30	0	0
Cyclohexanedione	Clethodim	Inhibition of ACCase	240	0	0
Bipyridyl	Paraquat	Inhibition of photosystem I	250	41	0

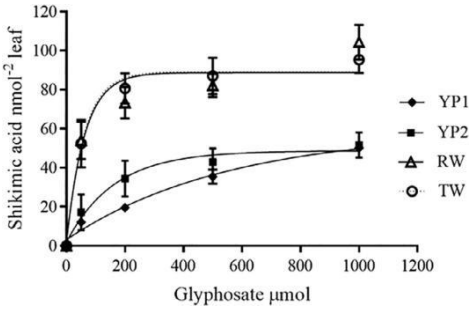

Figure 2. Accumulation of shikimate in leaf discs of resistant [YP1 (___); YP2 (___)] and susceptible [RW (___);TW(___)] *Hordeum glaucum* populations. Each data point represents the mean of two experiments pooled with six replicates, and the vertical bars are the standard error of the mean (n = 6). The curves were fitted using the equation

YP1 $y = 551.9 \times [1 - \exp(-0.0018 \times x)]$, $R^2 = 0.92$: YP2 $y = 162.9 \times [1 - \exp(-0.0061 \times x)]$, $R^2 = 0.56$; RW $y = 62.0 \times [1 - \exp(-0.0048 \times x)]$, $R^2 = 0.76$; TW $y = 58.83 \times [1 - EXP(-0.0049 \times x)]$, $R^2 = 077$.

Table 4. Effective glyphosate concentration resulting in 50% shikimate accumulation in resistant and susceptible populations of *Hordeum glaucum*. Standard error in parentheses. Resistance factor calculated by the R/S ratio using the average of the susceptible populations

Population	IC50 (µM)	Resistance factor
YP1 (R)	675.9 (±12.71)	14.3
YP2 (R)	127.6 (± 9.59)	2.7
RW (S)	51.1 (± 7.04)	1
TW (S)	43.6 (±7.01)	1

that, in combination with a mutation at Pro106, gives higher levels of resistance has also been observed in some populations of *Eleusine indica*.[18] To determine if a mutation in the *EPSPS* gene confers glyphosate resistance in the *H. glaucum* populations, a ~ 330-bp DNA fragment of the conserved *EPSPS* gene encompassing the amino acid positions (^{95}LFLGNAGTAMRPL^{107}) was analysed from the resistant and susceptible populations. Comparison of the sequences (data not shown) showed the same amino acid sequence for the susceptible (RW and TW) and resistant populations (YP1 and YP2). No nucleotide change at Pro106 or Thr102 was identified in either resistant population that would lead to a mutation in the *EPSPS* gene. The absence of a mutation in any of the position in the *EPSPS* gene associated with resistance to glyphosate implies that resistance in these *H. glaucum* populations is not conferred by the target site mutation.

3.4 ^{14}C absorption and translocation

This study was undertaken to determine whether differential absorption and translocation was associated with resistance to glyphosate in the resistant populations. Previous studies report high retention of radiolabelled glyphosate applied to the treated leaves of resistant plants compared with susceptible populations.[22,39] Results from this study showed no differences in the amount of glyphosate absorbed by both susceptible and resistant populations after 48 h of treatment. ^{14}C-glyphosate applied to the leaves of resistant and susceptible populations was translocated in largest proportion to the roots and leaf sheaths (stem) 48 HAT (Table 5), with 18 – 19% retained in the treated leaf lamina of resistant plants, which was not significantly different from the 21 – 24% retained in the treated leaf lamina of the susceptible populations. Similarly ^{14}C-glyphosate translocated to untreated leaves was less in both susceptible and resistant populations. Resistant populations accumulated between 7.5% and 7.8% herbicide in untreated leaves, which was not significantly different from the 3.1 – 3.8% in untreated leaves of the susceptible populations. The only difference between the populations in glyphosate translocation was that resistant population YP2 accumulated significantly more glyphosate in the roots compared with resistant population YP1 and susceptible population TW (Table 5). This is unlikely to account for the expression of resistance in this population.

3.5 *EPSPS* gene copy number and expression

EPSPS relative gene copy number estimates of populations YP1, YP2, RW and TW obtained by qPCR, and measured against an internal reference gene, *HvALS* showed differences between the resistant and susceptible populations. Susceptible individuals contained between 0.8 and 1 copy of *EPSPS* relative to the control gene, whereas resistant individuals contained a much higher number of *EPSPS* copies ranging from 9 to 11.9 (Fig. 3). Slight variability in the copy number of different individuals of both YP populations was observed. This may be due to variation of qPCR for the single copy gene or to some differences between the resistant individuals.

EPSPS gene expression relative to ALS was also investigated using qPCR on cDNA. *EPSPS* expression in resistant individuals was found to be increased with levels ranging from 4.0- to 18.4-fold that of susceptible individuals. There was, however, no positive correlation between genomic copy number and cDNA expression in individual resistant populations (Fig. 4). Lower copy number individuals displayed higher *EPSPS* expression levels and vice versa in both resistant populations. This pattern of *EPSPS* activity displayed in these populations is not yet discernible. However, expression levels in YP1 populations were on average higher than for the YP2 population, which is similar to that observed for gene copy numbers.

Table 5. ^{14}C glyphosate absorption and translocation in glyphosate susceptible and resistant populations of *H. glaucum* populations 48 HAT

		^{14}C glyphosate present at 48 HAT				
Population	*n*	Absorption (% applied)	Treated leaf lamina	Untreated leaf lamina	Leaf sheath (stem)	Roots
YP1(R)	10	65.7 ± 6.7 a	17.7 ± 4.1 a	7.8 ± 1.8 a	36.9 ± 3.8 a	37.6 ± 2.9 ab
YP2(R)	10	72.6 ± 6.1 a	19.2 ± 3.7 a	7.5 ± 1.6 a	32.0 ± 3.4 a	41.3 ± 2.6 b
RW(S)	10	72.2 ± 6.4 a	20.8 ± 3.9 a	3.8 ± 1.7 a	37.3 ± 3.6 a	38.2 ± 2.8 ab
TW(S)	10	70.3 ± 6.4 a	23.9 ± 3.9 a	3.1 ± 1.7 a	40.7 ± 3.6 a	32.3 ± 2.8 a
p		0.856	0.680	0.1333	0.361	0.126

Mean (± standard error) values followed by the same letter are not significantly different at $P \leq 0.05$. *n*, number of individual plants used to calculate the statistics for each population. The data presented are representative of two experiments conducted with similar results. R, resistant; S, susceptible; HAT, hours after treatment.

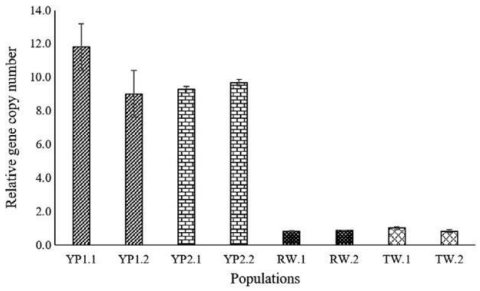

Figure 3. Relative EPSPs gene copy number estimates of glyphosate-resistant (YP1 and YP2) and -susceptible (RW and TW) *Hordeum glaucum* populations obtained by qPCR measured against internal reference gene *H. vulgare* acetolactate synthase (*HvALS*) at a copy of 1. Vertical bars represent the standard error of means (SEM) from each population with two replicates.

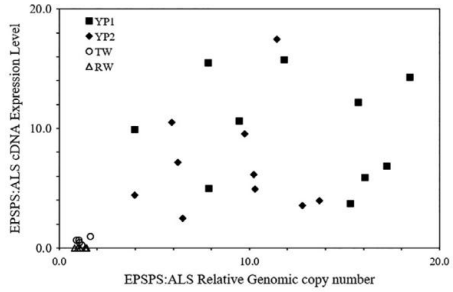

Figure 4. Correlation of EPSPS genomic copy number and the expression levels relative to ALS for glyphosate-resistant populations YP1 (■); YP2. (◆) and glyphosate-susceptible populations RW (○); TW (△). Increase in *EPSPS* genomic copy number is not positively correlated with expression levels.

4 DISCUSSION AND CONCLUSION

This study investigated the first known instance of glyphosate resistance in *H. glaucum*. Populations of *H. glaucum* have evolved resistance to paraquat, diquat and other herbicides in the ACCase-inhibiting (Fops and Dims) and ALS-inhibiting (Imis and Sus) groups with reports of cross and multiple resistance to many of these herbicides.[7,8] The evolution of resistance to multiple her-bicides in this weed species limits control options. Glyphosate is one of the herbicides used for non-selective control of *H. glaucum* and other weeds in non-cropped areas, such as fence lines and crop margins, and is also used effectively for spray-topping to stop seed set of grasses in pastures. The repeated use of glyphosate for the control of *H. glaucum* populations in these situations has provided the selection pressure for the development of resistance to glyphosate. The *H. glaucum* populations used in this study were found along fence lines and around stockyards on the same farm. Dose –response experiments confirmed resistance of the *H. glaucum* populations to glyphosate (Fig. 1) with a resistance level 2.8 –6.6-fold that of the susceptible populations. Although both populations were sampled from the same farm, population YP1 had a higher LD_{50} than YP2.

Shikimate accumulation experiments confirmed resistance to glyphosate in YP1 and YP2, with YP1 considerably more resis-tant than YP2 (Table 3). However, shikimate accumulated in both resistant and susceptible plants indicating that glyphosate is able to inhibit EPSPS, albeit to different extents (Fig. 3). Shikimate has been widely used as a marker of glyphosate resistance,[40] and it has been suggested that the pattern of shikimate accumulation can provide information about the mechanism of resistance to glyphosate.[33,34,40] In the case of YP1 and YP2, shikimate accumu-lation was less at both low and high glyphosate concentrations, suggesting a mechanism associated with *EPSPS*.[40]

To determine the mechanisms responsible for resistance in these populations, screening for the three known resis-tance mechanisms (reduced absorption and translocation of glyphosate; target site mutation; and *EPSPS* gene amplification) in glyphosate-resistant weeds was conducted. No differences in glyphosate absorption and translocation that could account for resistance were observed between the resistant and suscep-tible populations. Similarly, no mutation was observed in the ~ 330 bp DNA fragment of the amplified region of the *EPSPS* gene. However, qPCR identified 9 –11-fold increase in *EPSPS* gene copy number, which is likely to be the basis of resistance in these resistant populations. A similar range of *EPSPS* gene copies have been reported in other glyphosate-resistant weed species such as *Amaranthus tuberculatus*,[41] *Amaranthus palmeri*,[19] *Lolium multiflorum*[35] and *Kochia scoparia*.[42] In many instances, a positive relationship between gene copy number and resistance level has been observed.[35,43] Increased cDNA expression (4 – 18-fold) in resistant populations was also observed with no direct correlation to gene copy numbers. It was observed that some individuals with high copy numbers had low cDNA expression and some with low copy numbers exhibited slightly higher expression levels. In *A. palmeri* and *L. multiflorum*, a positive correlation was reported between gene copy number and expression.[19,35] However, in *B.*

diandrus, no direct correlation was observed where populations with higher copy numbers exhibited low expression.[20] In some *Conyza* species, slight increases in *EPSPS* expression without increases in copy numbers have been reported.[44,45] It is not yet understood why the number of cDNA copies can be so widely different from the number of genomic copies of *EPSPS* in *H. glaucum*; however, this may be influencing the relative levels of resistance to glyphosate between the two resistant populations and may provide clues to the evolution of gene amplification.

EPSPS gene amplification is becoming a common glyphosate resistance mechanism identified in weed populations from situa-tions where glyphosate is applied intensively, such as transgenic crops and other crop situations.[46] The majority of examples of *EPSPS* gene amplification were reported from the USA[19,35,41,42] and a few from Australia.[20,29] This mechanism usually provides a higher level of glyphosate resistance compared with target site muta-tions. Herbicide applications on fence lines and crop margins in Australia are usually done once annually late in spring. The selec-tion intensity imposed by a single annual application is lower than situations where several applications may occur each year but is still sufficient to select for resistance. However, the gene copy numbers identified in these populations are lower than reported in other species such as *A. palmeri* with 5 to > 160 gene copy numbers,[19,47] *Bromus diandrus* up to 25 copy numbers[20] and *Chlo-ris truncata* with 16 –25 copy numbers.[29] There is not always a clear relationship between *EPSPS* copy number and the level of glyphosate resistance.[20,41,42] The location and expression of these *EPSPS* genes may be variable[48] between species and individuals. For example, in the present work, the level of gene amplification in the two populations was similar (Fig. 2); however, YP1 was more resistant to glyphosate than YP2.

The number of weed species evolving resistance to glyphosate is increasing with at least one new species with resistant popu-lations being confirmed each year.[16,24] This is the first report of glyphosate resistance in *H. glaucum* in which two populations had evolved resistance along fence lines and stockyards after more than a decade of persistent use of glyphosate. The results from this study suggest that resistance is due to a gene amplification mech-anism. The fact that weed populations in situations with low selec-tion intensity are being selected for resistance mechanisms similar to those that occur in situations with high selection intensity con-firms our initial suggestions that selection intensity may not influ-ence the resistance mechanism selected in weed populations.[49] The reasons why YP1 is more resistant to glyphosate than YP2 is unknown, but may be due to differential expression of the ampli-fied *EPSPS* alleles. The genetic inheritance of copy number and fitness penalty that may be associated with *EPSPS* gene amplifi-cation in glyphosate-resistant *H. glaucum* is yet not known. How-ever, previous studies have reported no fitness cost associated with glyphosate resistance in other weed species with high gene copy numbers.[50]

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DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

CP and GG conceived the research. BF conducted initial screening and supplied seed samples. CP, GG, JMM and PAY designed all experiments. PAY and JMM conducted experiments and data collection. CP, GG, JMM and PAY analysed data. PAY, CP, GG and JMM wrote the manuscript. Manuscript has been read, edited and approved by all authors.

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Chapter 3 STABILITY OF EPSPS GENE COPY NUMBER IN *HORDEUM GLUACUM STEUD* (BARLEY GRASS) IN THE PRESENCE AND ABSENCE OF GLYPHOSATE SELECTION.

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Stability of EPSPS gene copy number in *Hordeum glaucum* Steud (barley grass) in the presence and absence of glyphosate selection

Patricia Adu-Yeboah,* Jenna M Malone, Gurjeet Gill and Christopher Preston

Abstract

BACKGROUND: Gene amplification has been shown to provide resistance to glyphosate in several weed species, including *Hordeum glaucum* populations in South Australia. The stability of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene copies in resistant populations in the presence or absence of glyphosate selection has not been determined.

RESULTS: Applying glyphosate to a cloned plant resulted in an increase in resistance and *EPSPS* copy number in the progeny of that plant compared to the untreated clone. The LD_{50} (herbicide concentration required for 50% mortality) increased by 75% to 79% in the progeny of the treated clones compared to the untreated in both populations (YP-17 and YP-16). *EPSPS* copy number estimates were higher in treated individuals compared to untreated individuals with an average of seven copies compared to six in YP-16 and 11 compared to six in YP-17. There was a positive correlation ($R^2 = 0.78$) between *EPSPS* copy number and LD_{50} of all populations.

CONCLUSION: *EPSPS* gene copy number and resistance to glyphosate increased in *H. glaucum* populations under glyphosate selection, suggesting the number of *EPSPS* gene copies present is dependent on glyphosate selection.

Supporting information may be found in the online version of this article.

Keywords: gene amplification; glyphosate resistance; 5-enolpyruvylshikimate-3-phosphate synthase; gene copy stability

1 INTRODUCTION

Hordeum glaucum Steud. syn. *H. murinum* ssp *glaucum*, *Critesion glaucum* (commonly referred to as Northern barley grass) is one of the most important grass weeds in South Australia. This species is widespread in crops and pastures across the grain-growing regions and is also common on roadsides, along fence lines and around livestock enclosures.[1] *Hordeum glaucum* is a major competitor of cereal crops that reduces crop yields,[2,3] serves as a host for pathogenic fungi and nematodes,[4] disperses rapidly and is difficult to control due to the limited herbicide options available.[3,5] *H. glaucum* has evolved resistance to a range of the most commonly used herbicides, including the acetyl-coenzyme A carboxylase, acetolactate synthase and photosystem I-inhibiting herbicides.[6–10] Recently, resistance to glyphosate has occurred in this species as a result of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene amplification.[11]

Since the first identification of *EPSPS* gene amplification in glyphosate-resistant *Amaranthus palmeri* (palmer amaranth),[12] gene amplification has been identified in numerous glyphosate-resistant weed species.[13,14] Gene amplification is the process whereby a segment of DNA is replicated to generate additional copies in the genome of the organism.[15] The duplicated gene copies can evolve new functions to tolerate extreme conditions, which are seen as an adaptive response to unfavourable stressors.[16–18] It is now evident that amplification of gene copies is a form of an adaptive mechanism that enables plasticity in organisms and contributes to genetic diversity.[19–21] Gene amplification has been documented in resistance to some antibiotics, insecticides and fungicides.[22–24] In glyphosate resistance, duplication of the *EPSPS* gene targeted by glyphosate enables the plants to produce sufficient enzyme to maintain the shikimate pathway and continue metabolic activities in the presence of glyphosate.[12,25] In all cases reported, copy-number variations in individual populations have been observed, with copy numbers ranging from as low as <3 to >100.[13]

Understanding of the precise mechanism(s) of gene duplication conferring herbicide resistance is limited; however, events leading to initial duplication of gene copies have been attributed to unequal recombination leading to tandem repeats, meiosis or hybridization errors resulting in aneuploid cells, activities of mobile transposable elements or whole genome duplication.[26–30] Recent investigations into some populations of *Kochia scoparia* have identified the involvement of mobile genetic elements (transposons)

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near the EPSPS locus hypothesized to facilitate the tandem duplication event in resistant populations.[31]

Currently *EPSPS* gene amplification is known to occur through tandem repeats as well as on nonchromosomal DNA elements. Resistance to xenobiotics through gene amplification is thought to be unstable and mainly influenced by selection pressure. Therefore, when selection is withdrawn it is possible that the extra gene copies produced will be lost or be deactivated to form pseudogenes.[32,33] This has been demonstrated in some eukaryotes in resistance to some drugs for cancer treatment and in some examples of insecticide resistance.[34,35] The stability of *EPSPS* gene copies in glyphosate resistant weeds is still not known. A fascinating question is whether resistant weeds lose *EPSPS* gene copies in the absence of glyphosate selection considering previously observed instability of *EPSPS* gene copies in cell cultures.[36–40] In this study we took advantage of the fact that grass plants can be physically divided into nearly identical tillers to investigate the effect of glyphosate selection on amplified *EPSPS* gene copies in glyphosate-resistant *H. glaucum* populations.

1 MATERIALS AND METHODS

1.1 Source and characterization of plant material

Seeds of resistant *H. glaucum* populations were originally collected from areas along fences and stockyards near Arthurton, South Australia (34°14^{0}44.2^{00} S, 137°44^{0}32.6^{00} E) in 2016 (YP-16) and 2017 (YP-17),[11] with a further collection made from the same farm in 2019 (YP-19). The susceptible populations (RW, TW and YN) were collected from pastures at Roseworthy (−34°31^{0}38.8^{00} S 138°41^{0}09.1^{00} E), Two Wells (−34°35^{0}46^{00} S, 138°30^{0}27.57^{00} E) and Yaninee (−32°56^{0}59.99^{00} S, 135°15^{0}60.00^{00} E), South Australia, with no known prior exposure to glyphosate. Previous screening and dose–response experiments conducted with glyphosate (540 g a.e. ha^{-1} Roundup Nufarm, Laverton, Victoria, Australia) confirmed resistance and susceptibility to glyphosate.[11] Populations, however, showed no resistance to any of the acetyl coenzyme A carboxylase (ACCase), acetolactate synthase (ALS) and photosystem II (PSII) inhibiting herbicides.[11] Seeds sampled were cleaned, bagged separately and kept in a 9 °C cold room to be used in the subsequent experiments.

1.2 Generation of clones and herbicide treatment

Seeds of resistant (YP-16 and YP-17) populations were germinated in separate plastic trays (33 × 28 × 5 cm) containing standard potting mix (mixture of 540 L of cocoa peat, 220 L of water, 60 L of sand and steamed for 1 h).[41] After germination, seedlings were transplanted into 12 punnet pots (9.5 × 8.5 × 9.5 cm) (Masrac Plastics, South Australia) for each population, with three plants per pot. At the four to five tiller stage, plants were uprooted and tillers were separated to generate two clones of 10 individual plants for each population. This was done by carefully excising tillers while retaining some roots on each clone. The shoots were then trimmed back (3/4 of shoot length) to reduce excessive water loss and then planted in separate trays of a 20-cell punnet propagation square pot (5 × 5 × 7 cm) filled with standard potting mix. Planting was arranged in a grid pattern, individually labelled for easy identification, and maintained outside. When the plants had regrown, fresh leaf tissue was collected from five individuals of each population to assess *EPSPS* gene copy numbers of the first-generation plants before herbicide application. One clone from each individual was treated with glyphosate at 405 g ha^{-1} (540 g a.e. ha^{-1} WeedMaster Argo, Nufarm, Laverton, Victoria, Australia) using a laboratory moving-boom with output volume of 118 L ha^{-1} at a pressure of 250 kPa and a speed of 1 ms^{-1} with a double Tee-Jet 001 nozzle (Tee-jet 110u flat fan Spraying Systems, Wheaton, IL, USA) and the other clone was left untreated. Plants were maintained outdoors after herbicide treatment. Previous tests had shown that this rate of the herbicide was sufficient to kill susceptible plants, but not resistant ones. The plants were later repotted separately into 5-L pots, placed 1.5 m apart and maintained outside until maturity to bulk up seeds. Seeds were bagged separately and stored under normal room temperature for subsequent experiments.

A separate experiment was conducted under two different temperatures to assess whether temperature stress would influence glyphosate resistance and *EPSPS* copy number in the progeny of *H. glaucum* populations. Plants were grown in different growth chambers under a low-temperature condition of 20/18 °C day/night and a 12 h photoperiod at 553 µmol m^{-2} s^{-1}, and a high-temperature condition of 30/28 °C day/night with the same light intensity and photoperiod. Seeds were again separately bulked to generate second-generation individuals.

1.3 Dose–response experiment on progeny of clones

Seeds from the glyphosate-treated and untreated clones of five individuals of the resistant (YP-16 and YP-17) and susceptible (RW, TW and YN) populations were selected and germinated in plastic trays (33 × 28 × 5 cm) containing standard potting mix. Seedlings were later transplanted into punnet pots (9.5 × 8.5 × 9.5 cm) with nine plants of the same population per pot and replicated three times for each herbicide rate, arranged in a randomised complete block design. The plants were maintained outdoors between March and July 2020 at the University of Adelaide Waite Campus.

At the three to four leaf stage, the plants were treated with glyphosate at rates ranging from 0 to 2160 g ha^{-1} as described above. Three susceptible populations (TW, RW and YN) were treated with rates ranging from 0 to 540 g ha^{-1}. The plants were returned outdoors and herbicide response assessed after 21 days. Plants showing active growth with new tillers were counted as survivors and plants showing severe chlorosis to complete mortality were considered dead.[42] Freshly regenerated leaf tissues of surviving individuals were collected at the 540 g ha^{-1} rate for DNA extraction and copy number assessment.

Dose–response experiments were repeated and data pooled with six replicates and 45–54 plants per individual population. Mortality data was analysed using PriProbit (version 1.63)[43] and the dose of herbicide required to kill 50% of the plants (LD_{50}) calculated from the probit curves. The curves were back-transformed for plotting using GraphPad Software (GraphPad Inc., San Diego, CA, USA). The resistance index (R/S) was calculated for each population as the ratio of the LD_{50} of the resistant population to the LD_{50} of the standard susceptible population.

1.4 DNA extraction and *EPSPS* copy number determination

Genomic DNA was extracted from resistant and susceptible plants using Isolate II plant DNA extraction kit (Bioline, Alexandria, New South Wales, Australia) and *EPSPS* copy number assessed via quantitative polymerase chain reaction (qPCR). The primer sets EPSP-F (5^{0}-CCAAGAATGAGGGAGCGACCTAT-3^{0}) × EPSP-R (5^{0}-CAGTGCCAAGGAAACAATCAACA-3^{0}), ALS-F (5^{0}- GCCCAAGCCACCATCTAC-3^{0}) × ALS-R (5^{0}-CGCCACCAACATACAGAATTGG-3^{0}) and probes EPSPS (TGGTGACTTAGTTGTCGGTTTGAAGCA + FAM) × ALS

(TGAATCGCTTGAGCAGGTCCTGC + TET) were used for quantitative PCR on genomic DNA. Gene copy number was estimated by calculating the ratio of *EPSPS* gene copy number to the control gene *ALS*. *ALS* was selected as a reference gene because the *ALS* copy number is not expected to vary across *H. glaucum* individuals and has been used in similar studies previously.[12,44,45] Genomic DNA templates (~5 ng) were amplified in a 10 µL reaction mixture containing 5 µL of SsoFast Probe Supermix (Biorad, Gladesville, New South Wales, Australia), 1 µM of *EPSPS* and *ALS* forward and reverse primers, and 0.3 µM of *EPSPS*-FAM and *ALS*-TET probe, and run on a RG3000 Rotor-Gene real-time thermal cycler with the following parameters: 3 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 16 s at 60 °C, acquiring at 510 nm (*EPEPS*-FAM) and at 555 nm (*ALS*-TET). Primer efficiency curves were generated for each primer set using a dilution series (1×, 1/2×, 1/4× and 1/8×) of susceptible genomic DNA. Primer efficiencies ranged from >98% to <102%. A modified version of the ΔC_T ($2 = {}^{-\Delta\Delta CT}$) method was used to analyse data from the qPCR experiments.[12,46]

1.1 cDNA cloning for SNP identification

RNA was extracted from five individual plants of each of the two resistant and one susceptible populations using the Plant RNeasy Mini Kit (Qiagen) (Melbourne, Victoria, Australia). cDNA was synthesized using the Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instruction. To obtain genomic sequence for Single-nucleotide polymorphism (SNP) identification, an approximately 1200 bp fragment of the *EPSPS* gene was amplified for SNP detection. Amplification was carried out in 25 µL of reaction containing 1 µL of cDNA, 12.5 µL (2×) of MyFi mix reaction buffer (containing MyFi DNA polymerase, dNTPs, $MgCl_2$) and 0.4 µM of each primer with forward primer AW1 5^0-AACAGTGAGGAYGTYCACTACATGCT-3^0 and reverse primer AW2 5^0-CGAACAGGTAGGGCAMTCAGTGCCAAG-3^0. Amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) with PCR conditions as follows: 3 min denaturing at 95 °C, 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, 45 s elongation at 72 °C and a final extension for 7 min at 72 °C. Fragments were purified via gel extraction (QIAquick Gel Extraction kit, Qiagen, Valencia, CA, USA) before cloning using the Topo TA cloning kit (Life Technologies) according to the manufacturer's instructions. White colonies with putative inserts were used as templates for PCR amplification before a library of positive colonies were created on standard LB/kan plates and plasmid DNA of positive clones (20 of each population), isolated using Qiagen Plasmid Mini kit from the regrown streaked colonies. Plasmids were sequenced using the standard M13 vector primers. Sequences were analysed using Geneious 8.1.9 (Java Version 1.7.0_51-b13 (64 bit) to identify any SNPs between populations.

1.2 Resistance and *EPSPS* copy numbers in *H. glaucum* collected from 2016 to 2019

The level of glyphosate resistance in *H. glaucum* populations collected in 2016, 2017 and 2019, as well as *EPSPS* copy number, was assessed. Populations were screened with glyphosate in a dose–response experiment and the *EPSPS* copy number of five individual plants from each collection determined as described above.

2 RESULTS

2.1 Glyphosate response of treated and untreated second-generation clones

Dose–response experiments were conducted on the progeny of the glyphosate-treated and untreated individuals to determine any changes in response to glyphosate. The susceptible populations were controlled by low rates of glyphosate with LD_{50} values between 123 and 206 g ha^{-1}. The resistant populations required much higher rates of glyphosate for 50% mortality (Table 1). Generally, progeny of the treated clones of both resistant populations showed higher resistance to glyphosate compared with the untreated clones (Fig. 1). Progeny of treated clones of population YP-16 needed glyphosate doses of 823 to 1011 g ha^{-1} for 50% control equating to a resistance index of 3.9 to 4.9 compared with the progeny of untreated clones that had LD_{50} values between 679 and 757 g ha^{-1}, giving a resistance index of 3.3 to 3.7.

Table 1. Glyphosate dose required for 50% mortality, LD_{50} (with 95% CI in parenthesis) of the progeny of treated and untreated clones of YP-16 and YP-17 and for three susceptible populations of *H. glaucum*

	Treated		Untreated	
Population/individual	LD_{50}	RI	LD_{50}	RI
YP16-1	823.1 (651.3, 1040.3)	3.9	678.6 (537.7, 856.7)	3.3
YP16-2	864.5 (676.0, 1106.7)	4.2	757.4 (600.6, 955.5)	3.7
YP16-3	1011.4 (799.8, 1279.2)	4.9	749.6 (592.8, 947.8)	3.6
YP16-4	917.4 (728.0, 1156.3)	4.4	682.7 (542.2, 860.0)	3.3
YP16-5	883.4 (701.1, 1113.3)	4.2	706.1 (560.6, 889.5)	3.4
YP17-1	1252.9 (1071.6, 1461.8)	6.1	963.0 (815.7, 1133.1)	4.7
YP17-2	1195.7 (1022.5, 1394.8)	5.8	767.3 (643.4, 911.2)	3.7
YP17-3	1177.3 (1005.7, 1375.7)	5.7	1004.5 (853.37, 1178.4)	4.9
YP17-4	1565.7 (1344.6, 1824.6)	7.6	1029.3 (875.4, 1206.4)	4.9
YP17-5	1177.9 (1006.8, 1374.6)	5.7	1041.0 (885.71219.6)	5.0
RW-S1	206 (186.6, 228.3)	1	–	–
TW-S2	141(126.5, 157.6)	–	–	–
YN-S3	123 (107.4, 140.9)	–	–	–

Data are results of two experiments pooled with six replicates (N = 45–54).
RI, ratio of LD_{50} of resistant and susceptible populations.

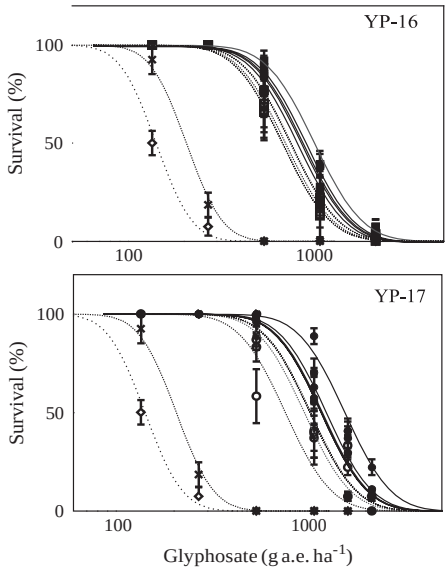

Figure 1. Dose response of progeny of treated and untreated [T (—); UT (– –)] YP-16 and YP-17 individuals and two susceptible RW-S (×) and TW-S (◊) *H. glaucum* populations. Each data point is a mean of six replicates and error bars are SEM. Lines are probit curves calculated for each individual with probits back-transformed to percentage survival.

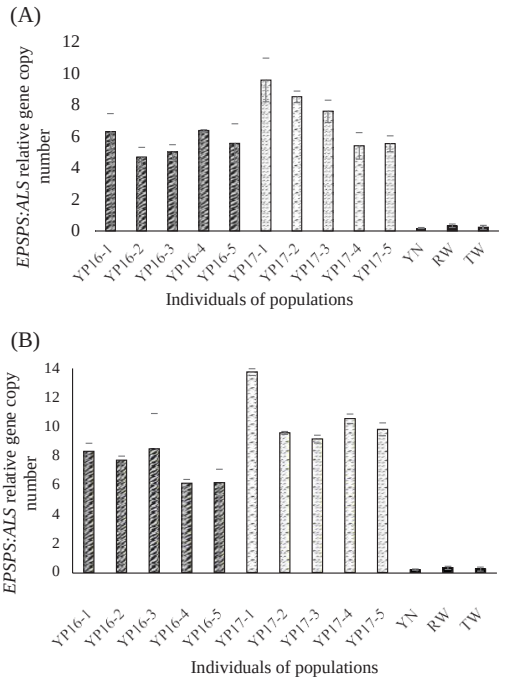

Figure 2. Relative *EPSPS:ALS* gene copy number estimates of resistant (YP-16 and YP-17) and susceptible (RW, TW and YN) individuals of *H. glaucum* populations before (A) and after (B) glyphosate treatment. Data obtained by qPCR measured against internal reference gene *H. vulgare* acetolactate synthase (*HvALS*). Vertical bars represent the standard error of means from each population with two replicates.

Similarly, the progeny of treated clones of population YP-17 required glyphosate dose between 1178 and 1566 g ha^{-1} to achieve 50% mortality (LD_{50}), whilst the progeny of untreated clones had LD_{50} values of 767 to 1041 g ha^{-1}, giving resistance indexes of 5.7 to 7.6 and 3.7 to 5.0, respectively. These results show a 25–40% increase in resistance to glyphosate of the progeny of treated individuals compared to progeny of untreated individuals.

1.1 *EPSPS* copy number in the progeny of treated and untreated clones

EPSPS gene copy numbers were assessed in five individuals of the clones and progeny of the clones using qPCR. *EPSPS* gene copies relative to *ALS* ranged from 0.5 to 1 for the susceptible plants, whereas gene copy numbers in resistant individuals prior to glyphosate application ranged from 5 to 9 (Fig. 2). There was a general trend of higher *EPSPS* copy number in the progeny of glyphosate-treated clones of both YP-16 and YP-17 compared to the untreated clones. Progeny of treated clones of YP-16 had copy numbers ranging from 6 to 8 compared to 4 to 7 copies for the untreated clones, indicating an increase in *EPSPS* copy number following one cycle of recurrent selection. Similarly progeny of treated clones of population YP-17 had copy numbers ranging from 9 to 14 compared with 5 to 8 copies for the untreated clones also showing an increase in *EPSPS* copy number. An association between resistance level and gene copy number was observed (R^2 = 0.78, P = 0.001) for all populations where populations with high LD_{50} showed higher copy numbers (Fig. 3). A paired-sample *t*-test showed significant differences in the copy number between treated and untreated individuals of both populations (Table 2).

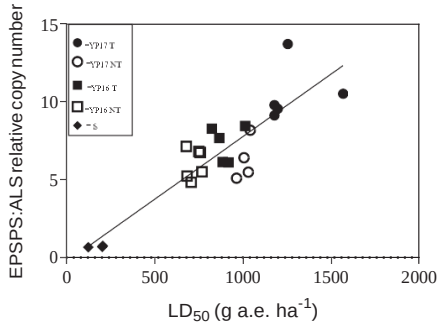

Figure 3. Linear regression of LD_{50} against *EPSPS* gene copy number of progeny of treated and untreated second clones of YP-17(T ●; UT ○), YP-16 (T ■; UT □) and susceptible (S ◆) populations (Y = 0.008043 × X − 0.2743, R^2 = 0.78, P = 0.001, N = 23.

1.2 Effect of temperature stress on glyphosate response of progenies

There is some evidence that temperature influences the level of glyphosate resistance in several weed species.[47–50] We therefore used high temperature (30 °C/28 °C) as a stress to see if it would change the level of glyphosate resistance in the progeny of stressed individuals. In glyphosate-resistant *H. glaucum*

Table 2. Paired sample *t*-test of copy numbers of treated and untreated second-generation individuals of *H. glaucum* populations

Population	Individuals	Mean	*P* value	SD	CI	R^2
YP-16	Treated	7.32	0.0011	0.31	0.79–1.56	0.947
	Untreated	6.15				
YP-17	Treated	10.52	0.021	2.68	1.07–7.73	0.771
	Untreated	6.31				

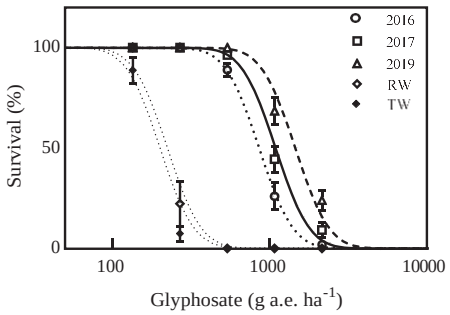

Figure 4. Dose–response curves showing survival of glyphosate susceptible (♦; ◊) and resistant *H. glaucum* populations collected from 2016 (○), 2017(□) and 2019(Δ). Each data point represents the mean percentage survival of two experiments pooled ±SE each with three replicates per treatment.

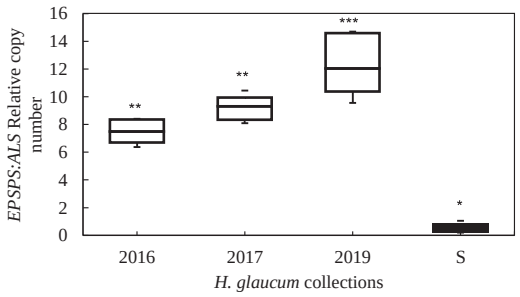

Figure 5. Box and whiskers plot of *EPSPS:ALS* relative gene copy number in *H. glaucum* populations collected in 2016, 2017, 2019 and susceptible populations (S). The line in the box represents the mean value and the whiskers the highest and lowest values obtained. Number of individuals = 5. *Significant differences between populations at $P < 0.0001$.

populations YP-16 and YP-17 there was no effect of high-temperature stress on glyphosate resistance or *EPSPS* gene copies in individual clones or their progenies compared with those under normal growing conditions (Table S1 and Fig. S1).

1.1 Identifying SNPs between populations

Seeds of populations YP-16 and YP-17 were originally collected from adjacent noncropped areas along fences and livestock enclosures in 2016 and 2017, respectively. Despite the similar location, YP-17 was more resistant and had higher *EPSPS* copy number than YP-16. To assess whether these two collections had similar or different genetic backgrounds, ~1200 bp of cDNA of the *EPSPS* gene was sequenced to identify single nucleotide polymorphisms that may indicate any possible differences in genetic background between the populations. Sequence data analysed showed no SNPs between the two resistant populations YP-17 and YP-16, however, eight SNPs were observed at different positions in the gene sequence within the susceptible population RW (Table S2), suggesting that YP-16 and YP-17 may be closely related to each other compared to RW. Six of these substitutions were silent mutations, but missense mutations were observed at positions 154 and 369, resulting in changes from asparagine to aspartic and alanine to valine, respectively. As these substitutions occur only in the susceptible population, and were found to occur in other species, they are unlikely to be related to resistance.

1.2 *EPSPS* copies and resistance in *H. glaucum* populations collected over the years

The glyphosate resistance levels (Fig. 4) and *EPSPS* copy numbers (Fig. 5) in *H. glaucum* populations collected from the same field in 2016, 2017 and 2019 were estimated. Results show *H. glaucum* collected in 2016 had an average of 7 *EPSPS* relative copies and required a glyphosate dose of 862 g ha^{-1} for 50% mortality (Table 3). Collections from 2017 and 2019 had average relative copy numbers of 9 and 12, respectively, and also had LD_{50} values of 1095 and 1461 (Table 3). This shows a progressive increase in resistance level and *EPSPS* copy numbers in collections from the site between 2016 and 2019.

Table 3. LD_{50} values (with 95% CI in parenthesis) of *H. glaucum* populations treated with glyphosate from 2016 to 2019 and susceptible populations (RW and TW)

Collection year	LD_{50} (g ha^{-1})	R/S ratio of (LD_{50})
2016	862.2 (777.2–956.4)	3.8
2017	1095.2 (987.1–1215.3)	4.8
2019	1461.3 (1321.9–1623.0)	6.5
RW (S)	201 (193.5–260.0)	–
TW (S)	204 (173.6–233.5)	–

R/S ratios were calculated as the ratio of LD_{50} values of resistant and susceptible populations. Data are means of two experiments, each with three replicates ($N = 54$).

2 DISCUSSION

There is evidence that gene amplification occurs through adaptive processes where genes duplicate to evolve new functions, providing an immediate adaptive advantage.[51–53] Amplification of *EPSPS* genes in glyphosate-resistant weeds has been shown to be the mechanism for survival in resistant weeds. It is unclear whether plants with multiple copies existed prior to glyphosate

selection or whether amplification is triggered under intense selection.[15,54] Dose–response experiments and quantitative PCRs showed an increase in glyphosate resistance and *EPSPS* gene copy numbers in the progeny of glyphosate-selected clones of resistant *H. glaucum* populations exposed to one cycle of recurrent selection in comparison to those that were not treated with glyphosate. While EPSPS expression levels were not assessed in this study, a previous investigation has shown a general increase in EPSPS expression with increased gene copies in these populations, which is likely to account for the higher resistance level displayed.[11] This is a clear indication that glyphosate selection can increase copy number and the level of resistance in this species. This is supported by the trend of copy number increase and glyphosate resistance observed in *H. glaucum* populations collected from the field between 2016 and 2019. The sequencing evidence suggested that YP-16 and YP-17 were closely related and the only likely difference was an additional year of glyphosate use.

The *EPSPS* copy number of progeny from untreated clones ranged between 4- and 8-fold compared to the susceptible populations, similar to the range of copy numbers in the original plants (Fig. 2). This suggests that over one generation, glyphosate application led to an increase in copy number, rather than loss of copies in the absence of herbicide. This effect was specific for glyphosate stress, as high-temperature stress did not change *EPSPS* copy number or glyphosate tolerance.

In *K. scoparia* a progressive increase in *EPSPS* copies and glyphosate resistance were identified in collections made from farms across the US Great Plains from 2007 to 2012.[55] This was explained as a possible adaptative process exhibited in *K. scoparia* that may have resulted from unequal crossing over of homologs within amplified regions in response to glyphosate selection.[55] Therefore, under continued selection pressure, *EPSPS* sequence homology between duplicated segment acts as a substrate for unequal crossover, leading to an increase in gene copies of progenies of the next-generation numbers and this continues until the gain in additional copies reaches a plateau.[20,51,53]

While unequal crossing over may provide one explanation for the increase in *EPSPS* copy number in *H. glaucum* populations in this study, an alternative mechanism may be selection on somatic mosaicism in cells by glyphosate application.[56–59] In *A. palmeri*, the extra copies of the *EPSPS* genes are located on extra chromosomal circular DNA (eccDNA) molecules.[30,60,61] These eccDNA molecules can be transmitted to daughter cells during mitotic and meiotic cell division and are associated with variations in *EPSPS* gene copies in somatic cells of *A. palmeri*.[30] The eccDNAs appear to replicate autonomously and segregate unequally during the cell cycle and thereby produce variations in somatic cells for resistance evolution.[30,60] As all cells will not have the same number of copies of *EPSPS*, those with higher numbers of *EPSPS* copies are more likely to survive. In grasses, in particular, this may result in new tillers containing cells with higher copy number on average.

It has been proposed that the stability of *EPSPS* gene copies can be influenced by the location and genomic organisation of the gene copies or the mechanism(s) of *EPSPS* amplification. For example, gene copies arranged in tandem repeats are subject to epigenetic control that can influence stability of the *EPSPS* gene copies.[62–64] However, where gene copies are controlled by eccDNA, stability may be dependent on the successful transfer of any genomic modifications to the next generation.[51] The precise mechanism(s) leading to further duplication and gain of gene copies in glyphosate-selected clones is still unknown, but the gene copy increase in the progeny of treated plants suggests that somehow amplified gene copies in soma cells may be successfully transmitted to germ cells of progenies. This can be described as a form of evolutionary adaptation that can drive the spread and persistence of glyphosate resistance in this species. Understanding the structural features and genomic organisation of the *EPSPS* gene copies in *H. glaucum* will give more insight into the mechanisms of this process.

Glyphosate resistance and *EPSPS* copy number both increased in *H. glaucum* with a single round of glyphosate selection. This suggests that glyphosate resistance will continue to increase in these populations so long as glyphosate is used. Therefore, glyphosate mixtures are unlikely to be a long-term strategy for managing resistance in this species and strategies that do not involve the use of glyphosate should be preferred.

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AUTHOR CONTRIBUTION STATEMENT

CP, JMM and GG conceived the research. CP, GG, JMM and PAY designed all experiments. PAY conducted experiments and data collection. PAY and JMM analysed data. PAY, CP, GG and JMM wrote the manuscript. The manuscript has been read, edited and approved by all authors.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Chapter 4 INHERITANCE OF RESISTANCE TO GLYPHOSATE IN A *HORDEUM GLAUCUM* (NORTHERN BARLEY GRASS) POPULATION FROM SOUTH AUSTRALIA

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Inheritance of Resistance to Glyphosate in *Hordeum glaucum* from South Australia

Short running title: Inheritance of glyphosate resistance in *Hordeum glaucum*

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CP, JMM, GG and PAY conceived the research. CP, GG, JMM and PAY designed all experiments. PAY conducted experiments and data collection. PAY analysed data. PAY, CP, GG and JMM wrote the manuscript. Manuscript has been read, edited and approved by all authors.

Headings

Abstract

BACKGROUND: *Hordeum glaucum* Steud. is an important grass weed species in South Australia that has evolved resistance to glyphosate. This study investigated the mode of inheritance of glyphosate resistance in this species.

RESULTS: Hand-pollination of glyphosate susceptible and resistant populations generated two F_1 individuals, selfed to produce F_2 progenies. In dose response experiments, the F_2 progenies showed intermediate response between the two parent populations. High variation in *EPSPS* gene copies was observed among F_2 individuals, with some individuals possessing more gene copies than the resistant parent population. No evidence of a Mendelian single gene pattern of inheritance was observed.

CONCLUSION: Inheritance of gene amplification in *H. glaucum* is non-Mendelian.

Keywords: *EPSPS*, gene amplification, copy number, glyphosate resistance, inheritance

1 INTRODUCTION

Glyphosate (N-(phosphonomethyl) glycine) is the most widely used herbicide in world agriculture, largely owing to the adoption of minimum tillage systems and extensive use in glyphosate tolerant crops [1]. Glyphosate is a broad spectrum herbicide that works by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), an enzyme in the shikimate pathway, and disrupting the synthesis of aromatic amino acids essential for plant growth [2]. As a consequence of the continued reliance on glyphosate for weed management, numerous weed species have evolved resistance to glyphosate [3]. Currently glyphosate resistance has been documented in 50 different weed species across the world [4]. As the incidence of glyphosate resistance increases, there are efforts to understand the evolution of resistance in weed species and the mechanisms conferring resistance in species identified. The mechanisms responsible for glyphosate resistance reported to date include enhanced detoxification, vacuole sequestration, reduced herbicide uptake and translocation, mutated *EPSPS* or *EPSPS* gene amplification [5, 6]. Although the mechanisms associated with resistance in several glyphosate resistant weed species are well documented, less information is available on the inheritance of some of these resistance mechanisms. It is important to understand the genetic basis and mode of inheritance of resistance in weeds species as this is essential in predicting the likelihood of spread and persistence of resistant weed species [7-9].

Glyphosate resistance traits are mostly encoded by a single nuclear gene of complete or partial dominance, commonly associated with resistance due to mutation in the EPSPS gene or altered herbicide absorption and translocation mechanisms [10, 11]. There have been rare cases where resistance was inherited as a two gene trait. In such instances, resistance was found to be due to two different mechanisms, each controlled at a single locus and acting additively[12-14]. An exception to resistance inherited as one or two genes is glyphosate resistance based on EPSPS

gene amplification. The inheritance of resistance due to *EPSPS* gene amplification appears to be more complex and variable depending on the resistant species. For example, in *Bassia scoparia* and *Lolium perenne*, inheritance of the gene amplification mechanism was found to be consistent with a single gene model with Mendelian segregation [15, 16]. Further studies into the genomic organisation of gene copies in *B. scoparia* found that they occur in the form of a tandem repeat block on the homologous chromosomes, possibly resulting in single gene inheritance. In *Amaranthus palmeri*, however, the amplified gene copy mechanism was found to be inconsistent with the Mendelian segregation and did not fit a single gene model. Similar observations were reported in other *A. palmeri* populations and in *Bromus diandrus* where the F_2 progenies did not segregate phenotypically or with EPSPS gene copies [17 18, 19]. Koo et al. [20] reported that amplified EPSPS copies in *A. palmeri* exist in the form of extrachromosomal circular DNA (eccDNA) randomly distributed within different cells, which may be contributing to the confounding segregation patterns observed in *A. palmeri* and possibly in other species.

Recently, the first known case of glyphosate resistance in *Hordeum glaucum* Steud. *syn. H. murinum ssp glaucum, Critesion glaucum* was identified in South Australia[21]. Studies conducted showed glyphosate resistance in *H. glaucum* populations was due to increased *EPSPS* gene amplification [21]. Compared to the susceptible population, the resistant populations had 9 to 11- fold EPSPS gene copies. Inheritance of glyphosate resistance associated with *EPSPS* gene amplification has varied depending on the weed species and the evolution of resistance is known to be partly dependent on the heritability of the resistance trait. The inheritance of glyphosate resistance in *H. glaucum* is not yet known. This study was therefore conducted to investigate the mode of inheritance of glyphosate resistance in *H. glaucum*.

2 MATERIALS AND METHODS

2.1 Plant material and generation of F_1 families

The *H. glaucum* populations used in this study were a glyphosate resistant (GR) Yorke Peninsula population and a glyphosate susceptible (GS) Roseworthy population from South Australia. Resistant plants, surviving a commercially relevant rate of glyphosate (540 g a.e. ha^{-1} WeedMaster Argo, Nufarm, Laverton, Victoria, Australia), and untreated susceptible plants were retained and grown to maturity to produce bulked seeds for use in the subsequent experiments.

Plants to be used as the resistant male parents in the crosses were selected after going through two cycles of recurrent selection with glyphosate, while the populations used as the susceptible female parents had no prior exposure to glyphosate. Seeds of resistant and susceptible populations were germinated separately in plastic trays (33 cm x 28 cm x 5 cm) containing standard potting mix (mixture of 540 L cocoa peat, 220 L of water, 60 L of sand and steamed for 1 hr) [22] and transplanted into 5-L pots (one plant per pot) at the one leaf stage for hand crossing. *H. glaucum* is a self-pollinating species, therefore crosses were made by emasculating flowers on the susceptible plants (female parent) and hand pollinating using pollen from the resistant plants (male parent). Because of the minute structure of the florets, emasculation and hand pollination were performed under a magnifying lens. Glassine bags were placed over the female spikes immediately after pollination and allowed to mature. The process yielded two F_1 seeds (out of > 500 crosses) attempts on reciprocal crosses yielded no successes and was not further pursued.

2.2 Generation of F_2 families

F_2 families were generated from each individually selfed F_1 progeny. F_1 seeds from successful crosses were germinated on agar (0.6%) and incubated in a germination cabinet with 24 h dark periods at 15 ^oC/10 ^oC temperatures. After germination, seedlings were transplanted into

separate 5-L pots maintained until maturity and allowed to self-pollinate to generate F_2 seeds. Seeds from individual plants were collected and bagged separately for subsequent experiments.

2.3 Testing for inheritance of resistance

A dose–response experiment was conducted on the susceptible and resistant parent populations and the two F_2 progenies. Seeds were germinated in plastic trays (33 cm x 28 cm x 5 cm) containing standard potting mix and later transplanted at one leaf stage into punnet pots (9.5 cm x 8.5 cm x 9.5 cm) (Masrac Plastics, South Australia). There were nine seedlings per pot replicated three times for each herbicide rate. At the three to four leaf stage the plants were treated with glyphosate at rates ranging from 0 to 2160 g a.e. ha^{-1}. Herbicide was applied using a laboratory moving boom with output volume of 118 L ha^{-1} at a pressure of 250 kPa and a speed of 1 m s^{-1} using a Tee-Jet 001 nozzle ((Tee-jet 110u flat fan Spraying Systems, Wheaton, IL). The plants were returned outdoors and visually scored for survival 28 days after treatment. Plants with new green leaf tissue were classified as resistant (R) and those that had died or with highly necrotic tissues as susceptible (S). The experiment was repeated three times between March and August 2020. There was no significant difference in response between repeated experiments as determined by two-way ANOVA, therefore data were pooled for further analysis. Mortality data from dose response experiments were analysed using PriProbit (version 1.63) [23] and the herbicide rate required for 50% mortality (LD_{50}) calculated from the probit curves. The curves were back transformed for plotting using GraphPad Software (GraphPad Inc., San Diego, CA). The resistance index (RI) was calculated for each population as the ratio of the LD_{50} of the resistant population to the LD_{50} of the standard susceptible population.

2.4 EPSPS copy number determination in F_1 and F_2 progenies

Relative *EPSPS* gene copy number was determined in the parental lines, F_1 individuals and F_2 progenies by quantitative PCR (qPCR) using genomic DNA (gDNA). For the F_2 individuals, copy number was assessed before herbicide treatment (UT) and in regenerated green leaf tissue (T) 48 days after herbicide treatment for plants surviving glyphosate treatment at 270 g ha^{-1} and 540 g ha^{-1}. Genomic DNA was extracted from the two F_1 individuals and individuals of F_2 plants untreated (F_2-1-UT (n=51): F_2-2- UT (n= 46) and after herbicide treatment (T) (F_2-1-T (n=46); F_2-2-T (n=34) using Isolate II plant DNA extraction kit (Bioline, Alexandria, New South Wales, Australia). qPCR was performed using primer sets EPSP-F (5′-CCAAGAATGAGGGAGCGACCTAT-3′) x EPSP-R (5′-CAGTGCCAAGGAAACAATCAACA-3′), ALS-F (5′-GCCCAAGCCACCATCTAC -3′) x ALS-R (5′- CGCCACCAACATACAGAATTGG -3′) and probes EPSPS (TGGTGACTTAGTTGTCGGTTTGAAGCA + *FAM*) x ALS (TGAATCGCTTGAGCAGGTCCTGC + TET) [21]. qPCR reactions of 10 µL contained a master mix of 5 µL of 2x SensiMix II Probe (Meridian Bioscience, Bioline Alexandria, New South Wales, Australia), 1 µM of *EPSPS* and *ALS* forward and reverse primers, 0.3 µM of *EPSPS*-FAM and *ALS*-TET probe, 10 ng of gDNA and 3 µL of di H_2O. qPCR experiments were assembled in a duplicate run and repeated. Each run was conducted on a RG3000 Rotor-Gene real-time thermal cycler with the following parameters: 3 min at 95 ^oC, followed by 45 cycles of 15s at 95 ^oC and16 s at 60 ^oC, acquiring at 510 nm (*EPEPS*-FAM) and at 555 nm (*ALS*-TET). Primer efficiency curves were generated for each primer set using a four dilution series (1x, 1/2x, 1/4x and 1/8x) concentration of susceptible genomic DNA, Primer efficiencies were calculated to range from >98% to <102%. A modified version of the ΔC_T ($2^{=-\Delta CT}$) method was used to analyse data from the quantitative PCR experiments [24, 25]. Relative quantification of genomic *EPSPS* was calculated as $\Delta C_T = (C_T^{\ ALS} - C_T^{\ EPSPS})$. Genomic *EPSPS* copy number increase was expressed as $2^{-\Delta CT}$. Results were expressed as fold increase in genomic *EPSPS* copy number relative to *ALS*. Standard curves

were used to calculate the amount of *EPSPS* and *ALS* in populations. Relative gene copy number was estimated by calculating the ratio of *EPSPS* gene copy number to the control gene *Hv*ALS. The ratio of *EPSPS* to *ALS* was calculated for each qPCR, and the average and the standard deviation of the duplicate qPCRs were recorded for each sample.

3 RESULTS

3.1 Inheritance of resistance

The F_2 populations (F_2.1 and F_2.2) from the two individually selfed F_1 progenies and their parent populations were assessed in a dose response experiment to determine the segregation pattern of the F_2 populations. The GS population had 100 % mortality at 540 g ha^{-1} glyphosate, while the GR population had no mortality at this rate (Table 2.) The GR population required 1364 g ha^{-1} glyphosate for 50% mortality, which is about 6-fold higher than the LD_{50} of 237g ha^{-1} for the GS population (Table 1). Survival of the F_2 populations was intermediate between the two parents. At 540 g ha^{-1} glyphosate survival of the two F_2 populations were 86% and 81% respectively (Table 2). The response of the F_2 population to glyphosate was compared to the expected response for a single dominant gene assuming a 3:1 ratio. However, the F_2 progenies did not show a single step decline in plant survival as usually expected in segregation pattern for monogenic inheritance (Figure 1). Instead, there was more survival than expected at 270 g ha^{-1} and less than expected at 540g ha^{-1} and 1080 g ha^{-1} glyphosate, suggesting the inheritance of resistance to glyphosate in this *H. glaucum* population does not follow a single-gene segregation pattern.

Table 1: Estimated glyphosate dose required for 50% mortality (LD_{50}) of glyphosate susceptible (GS) and resistant (GR) *H. glaucum* populations with confidence intervals in parenthesis. RI is the ratio of LD_{50} of resistant and susceptible populations.

Families	LD_{50} (g ha^{-1})	RI
GS-♀	236.6 (236.6-236.6)	-
GR-♂	1363.9 (1262.8-1474.5)	5.8
F_2-1	769.8 (696.7 - 851.1)	3.2
F_2-1	690.8 (622.1 – 767.4)	3.0

Table 2: Percentage survival of parent *H. glaucum* population (GS and GR) and F2 progenies after glyphosate treatment.

Populations	Plants treated	Herbicide rate g ha^{-1}		
		270	540	1080
			%	
GS-♀	81	7 b	0 c	0
GR-♂	81	100 a	100 a	77a
F_2-1	81	94 a	86 b	28b
F_2-2	81	90 a	81b	25b

Means followed by the same letter are not significantly different according to Fisher's Protected LSD test ($P \leq 0.05$).

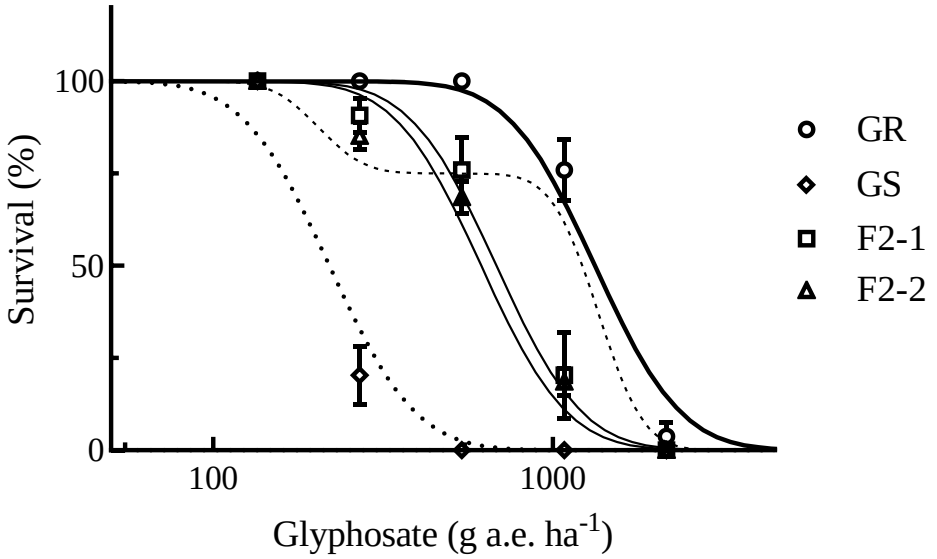

Figure 2: Dose response of susceptible (□), resistant (○) and F2 (Δ: ◊) populations of H. glaucum to glyphosate. Each data point represents the mean percentage survival of three experiments pooled with 81 individuals and nine replicates expressed as a percentage of the untreated controls. The vertical bars are the standard error of the mean ± SE. The dashed line is the expected response for a single dominant allele and was calculated by summing 0.75 x the response of the resistant population and 0.25 x the response of the susceptible population.

3.2 Relative Gene copies in F_2 progenies

The relative gene copies of the parent plants (GS and GR), F_1 (F_1.1 and F_1.2) and F_2 (F_2.1 and F_2.2) individuals were assessed using qPCR. Copy number in the F_2 individuals was assessed before (glyphosate untreated -UT) and after (glyphosate treated -T) herbicide treatment. The

glyphosate susceptible plants (GS) had an average of 1 (n = 5) gene copy which was consistent throughout the runs. Relative *EPSPS: ALS* gene copies in the resistant individuals ranged from 11 to 14 (n = 5) with an average copy of 12. Both F_1 progenies (F_1.1 and F_1.2) contained an average of 7 EPSPS gene copies each, which was intermediate between the two parent populations (Figure 2). Relative *EPSPS: ALS* gene copy of the F_2 individuals assessed before herbicide application (F_2.1-UT and F_2.2-UT) ranged from 1 to 23 and 1 to 15 respectively, with average copies of 9 and 6 (Figure 2). For F_2.1-UT (n = 51) approximately 18% of individuals had < 2 gene copies, 24 % had 2-8 gene copies; 45% had 8-14 gene copies and 14% had > 14 copies (Figure 3a). Similarly, for the F_2.2-UT individuals (n = 45), 23% of individuals had < 2 gene copies, 56% contained 2-8 gene copies; 16% had 8-14 gene copies and 5% had > 14 gene copies (Figure 3b). After the F_2 individuals were exposed to herbicide treatment, an increase in *EPSPS* gene copies was observed with every surviving F_2 individual containing 2 to 26 copies with an average copy number of 15. This suggests that individuals with less than 2 gene copies did not survive the herbicide treatment. F_2.1-T (n = 45) individuals contained gene copies ranging from 2 to 26 with 13% of individuals with 2-8 gene copies, 31% with 8-14 gene copies and 56% individuals with > 14 gene copies (Figure 3a). Similarly, *EPSPS* gene copies of F_2.2-T (n = 33) individuals ranged from 2-23 with 6% of individuals with 2-8 gene copies, 27% with 8-14 gene copies and 67% containing > 14 gene copies (Figure 3b). From this, it is clear there is an unpredictable pattern of *EPSPS* gene copy transmission observed with or without glyphosate selection, which does not conform to Mendelian inheritance in that some F_2 individuals have more gene copies than the resistant parent. It is also clear that the F_2 individuals can gain gene copies under selective pressure.

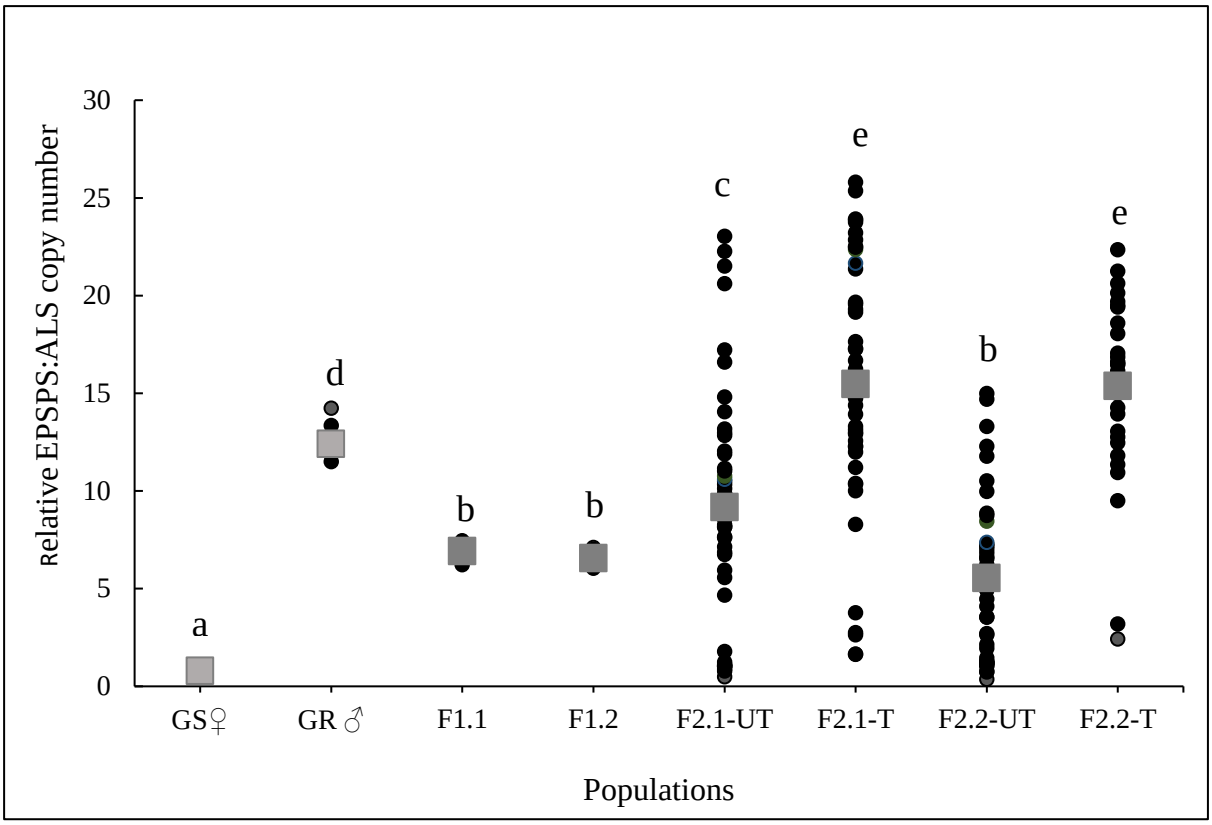

Figure 2: Genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative to acetolactate synthase (ALS) in *H. glaucum* populations (●); glyphosate susceptible (GS), resistant (GR), F_1 and F_2 populations. Gene copies in F_2 individuals were assessed before (UT) and after (T) herbicide treatment. Relative EPSPS: ALS genomic copy number of F_2.1 individual before herbicide treatment (n = 51) ranged from 1 to 23 and after herbicide treatment (n = 45) ranged from 2 to 26. Copies of F_2.2 individuals before herbicide treatment (n = 44) ranged from 1 to 15 and after herbicide treatment (n = 33) ranged from 2 to 22. Grey square (■) represents means of EPSPS relative gene copy number for each population. Means followed by the same letter are not significantly different according to the Fisher protected LSD test at $P \leq 0.05$ on EPSPS copy number.

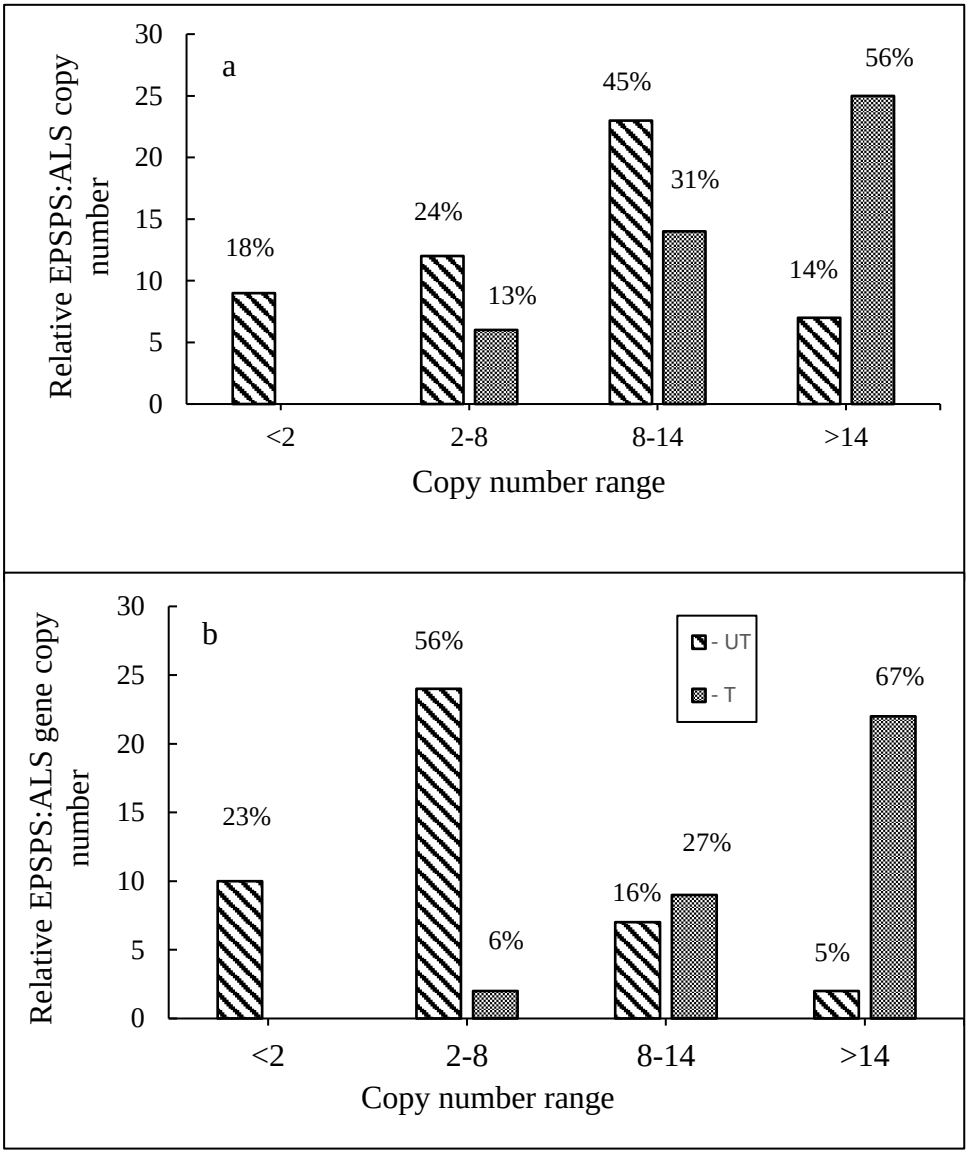

Figure 4: Relative EPSPS: ALS gene copy number range in the untreated (UT) and Treated (T) F2-1 (a) and F2-2 (b) individuals

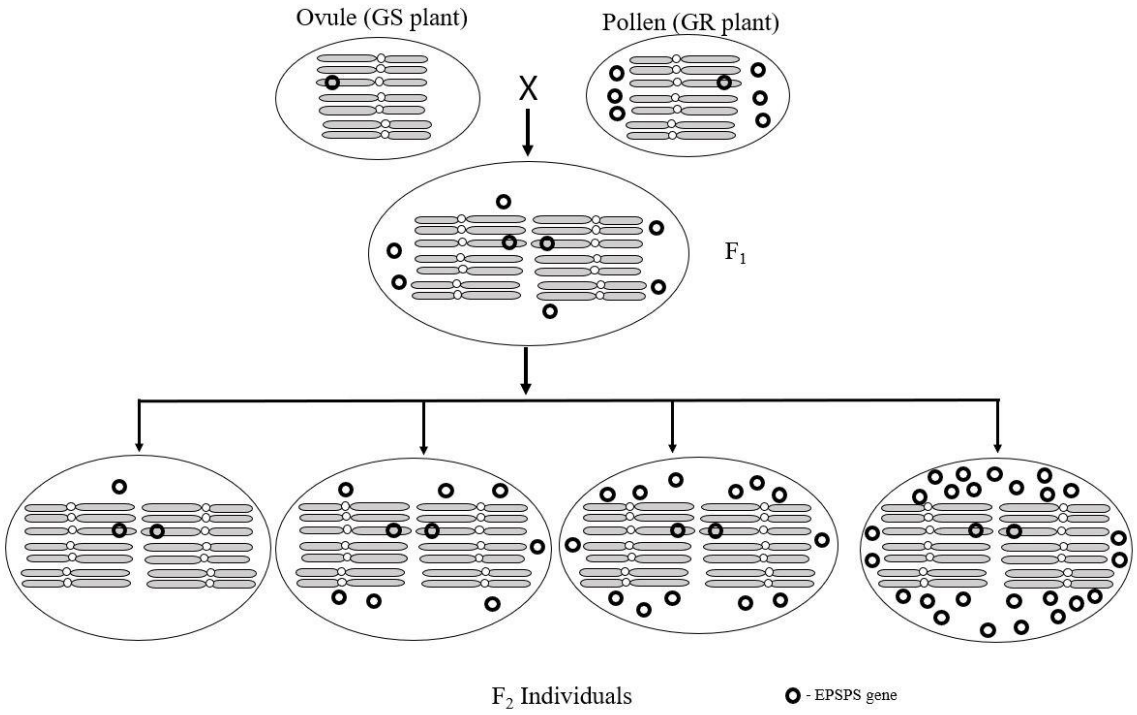

Figure 3: A schematic diagram illustrating how EPSPS gene copies are transmitted to progenies in glyphosate resistant *H. glaucum* population. F_2 individuals displaying varying EPSPS gene copies.

4 DISCUSSION

Genetic inheritance for the most identified glyphosate resistance mechanisms have shown segregation of resistance traits as single dominant or semi dominant loci [6]. Even for the complex mechanism of gene amplification, there has been evidence suggesting resistance segregating as a single locus in *K. scoparia*, although variation in gene copy number may occur from generation to generation through unequal crossing over [15]. In this study glyphosate resistance appears to be segregating in the F_2 populations under nuclear control with high dominance over susceptibility, which is consistent with most previously documented inheritance patterns for glyphosate resistance [6, 26]. However, response of the F_2 population to glyphosate concentrations showed an inheritance pattern that did not conform to a single gene Mendelian model.

Varying *EPSPS* copy numbers were observed in the progenies with or without glyphosate selection. Although the parent resistant population had an average of 12 *EPSPS* gene copies, F_1 progenies ended up with half the number of copies of the parent resistant population. A possible explanation may be that individual pollen from this plant may have ended up with half the copies of the parent following mitosis, which was subsequently transmitted to the F_1 progenies. On the contrary F_2 individuals showed variations in gene copies of individuals ranging between the parent population with some individuals possessing much higher gene copies than the resistant parent population. Although most of the F_2 individuals showed a range of *EPSPS* gene copies that fell between the two parental gene copies, copy numbers were not clustered around the parental gene copy numbers and the means as would be expected if amplified *EPSPS* gene copies are inherited from each parent as independent additive alleles at quantitative trait loci (Figure 4). These findings suggest a possible role of amplifiable *EPSPS* genes on eccDNA. These gene copies associated with eccDNA autonomously replicate during mitosis and meiosis and are then transmitted to the subsequent generation in an unpredictable pattern [20, 27].

This unpredictable pattern of inheritance displayed in *H. glaucum* agrees with other observations on inheritance associated with the gene amplification mechanism [17-19, 25, 28]. In all these cases, the genomic organisation of the *EPSPS* gene copies appears to influence inheritance in resistant species accounting for the non-Mendelian pattern of inheritance usually observed [15, 25, 29-31]. In *A. palmeri* amplified *EPSPS* copies were found dispersed randomly throughout the genome [25]. Koo et al [20] later found the EPSPS associated with eccDNAs from one cell to the next within the same plant and they appear to replicate autonomously and segregate unequally during the cell cycle. Thus, this extrachromosomal distribution of amplified *EPSPS* gene copies appears to be responsible for high copy number variations observed in *A. palmeri* and perhaps *B. diandrus* and their complex inheritance pattern. The mechanism that leads to independent replication of the extrachromosomal DNA is still not known. However this ability to self-replicate appears to benefit the plant by allowing faster evolutionary adaptation in response to stress [18] .

Elevated *EPSPS* gene copies were generally observed in all F_2 individuals following exposure to glyphosate selection. This may be explained as possibly due to a mechanism of selection on somatic mosaicism in cells observed with *EPSPS* gene copies associated with eccDNA [32-34]. According to Koo et al [20] the evolutionary dynamics of eccDNAs suggest that the collection of smaller eccDNAs from different genomic regions can recombine and evolve into large eccDNA organelles under strong selection pressure. This eccDNA behaviour during cell division could result in a rapid increase in copy number under selection pressure from glyphosate application. Molin et al [35] equally observed an increase in EPSPS and other genes in glyphosate resistant populations of *A. palmeri* following glyphosate treatment when compared to the susceptible population. The upregulated genes is predicted to influence adaptation to xenobiotic pressures and ensure genomic plasticity [35]. The increased EPSPS gene copies in F_2 individuals in response

to glyphosate treatment observed is likely to increase adaptation to stressors and provide survival advantage over other individuals in a population.

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Disclosure statement

The authors declare no conflict of interest.

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Chapter 5 GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

Herbicide use in modern agriculture has made a large contribution to world food production by effectively controlling weeds in fields and improving crop yields. However, a challenge to the effective and sustainable use of herbicides has been the rapid evolution of resistance to herbicides. At present 513 weed species have been reported with resistance to one or more herbicides (Heap 2020). As the incidence of herbicide resistance increases, efforts have been made in understanding the factors that influence evolution of resistance in weeds, keenly focusing on identifying the mechanisms and genes responsible for resistance. Herbicide resistance in weeds is reported to be conferred by two main mechanism types; non-target site and target site resistance mechanism (Beckie et al. 2000; Délye et al. 2013). Non-target site mechanisms are mechanisms that dilute available herbicide concentration and prevents its inhibitory effects at the target site. These include detoxification of herbicide (Christopher et al. 1991; Tal et al. 1995), sequestration into plant vacuoles (Ge et al. 2010) and reduced herbicide uptake and translocation (Lorraine-Colwill 2002; Wakelin et al. 2004). This mechanism confers resistance to several herbicide mode-of-action groups (Powles and Yu 2010). Target site mechanisms are conferred by alterations in the target site gene that prevents the herbicide from fully inhibiting the enzyme. This includes alterations to the amino acid sequence of the target site protein as well as mechanisms that result in higher concentrations of target site protein in cells. This mechanism type has been associated with resistance to PS II (Thiel and Varrelmann 2014), ALS (Tranel and Wright 2002), ACCase (Kaundun 2014) and EPSPS (Sammons and Gaines 2014) inhibiting herbicides.

Glyphosate is one of the most extensively used herbicides in agriculture and horticulture systems as well as in non-farmed areas globally (Duke and Powles 2008) . Glyphosate inhibits the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) an important

enzyme in the shikimate pathway and disrupts the synthesis of aromatic amino acids, eventually causing plant death (Amrhein et al. 1980). Initially evolution of glyphosate resistance was considered unlikely. However, extensive use of glyphosate for controlling large numbers of weeds over large areas and the introduction of glyphosate-tolerant crops imposed intensive selection pressure on weeds, resulted in the evolution of glyphosate resistant weeds. Currently glyphosate resistance has been documented in 50 different weed species across the world and increasing with at least one case confirmed each year (Heap 2020).

Hordeum glaucum Steud is a common weed of pastures where they serve as early stock feed (Warr 1981), however, in recent years increased infestations in crop fields have been observed as well (Fleet and Gill 2010). In South Australia, this species is widespread across the grain cropping regions in the Eyre Peninsula and Upper North regions. Glyphosate is one of the herbicides primarily used for pre-sowing control of *H. glaucum* in crops and for control along fence lines and crop margins. It is also widely used to control seed set of *H. glaucum* in pastures. The repeated use of glyphosate has resulted in resistance evolving in some populations of *H. glaucum* in non-crop areas. The presence of resistant *H. glaucum* populations in these situations poses a major threat to successful control and the risk of resistance moving into neighbouring crop fields. Therefore, this study was undertaken to investigate the first known instance of glyphosate resistance in *H. glaucum* and determine the factors that could influence resistance evolution, spread and persistence of glyphosate resistant *H. glaucum*.

Suspected glyphosate resistant *H. glaucum* plants were collected from along fence lines and livestock enclosures in 2016 (YP 2) and 2017 (YP 1) on the same farm after surviving glyphosate application. The response of the suspected glyphosate resistant *H. glaucum* populations to varying glyphosate rates was assessed in a detailed dose response study. Results confirmed resistance of both *H. glaucum* populations to glyphosate with resistance level ranging between 2.8 to 6.6-fold that of the susceptible populations. Herbicide selection pressure

is one of the main factors that contribute to resistance evolution (Maxwell and Mortimer 1994; Maxwell et al. 1990a; Maxwell et al. 1990b). The continued use of glyphosate for the control of *H. glaucum* populations in these situations provided the selection pressure for the development of resistance to glyphosate.

Following the confirmation of glyphosate resistance in these populations, studies were undertaken to determine the mechanism conferring resistance in these populations. Populations were screened for non-target site and target site resistance mechanisms. Altered glyphosate translocation is a common resistance mechanism in many reported glyphosate resistant weeds (Powles and Preston 2006; Shaner 2009). The glyphosate absorption and translocation pattern observed in these *H. glaucum* populations was similar to more typical patterns of glyphosate translocation and no different to that of susceptible populations, indicating that resistance is not due to this mechanism. Similarly, no mutation in the EPSPS gene likely to confer resistance was observed. However, qPCR identified 9 - 11 fold increase in EPSPS gene copy number, suggesting that the mechanism of resistance in these population may be due to EPSPS gene amplification.

Gene amplification refers to duplication a segment of DNA containing genes to generate additional copies in the genome of the organism and can cause increased expression of the amplified gene (Flagel and Wendel 2009). Since first reported in *Amaranthus palmeri* (Gaines et al. 2010), *EPSPS* gene amplification has been reported in several other glyphosate-resistant weed species such as *Amaranthus tuberculatus* (Chatham et al. 2015), *Kochia scoparia* (Wiersma et al. 2015), *Lolium perenne* (Salas et al. 2012), *Bromus diandrus* (Malone et al. 2016) and *Chloris truncata* (Ngo et al. 2018). The copy number range observed in glyphosate-resistant *H. glaucum* is similar to those observed in *B. scoparia*, *B. diandrus* and *C. truncata* populations, with approximately 15 to 25-fold, 10 to 36-fold and 16 to 25-fold increases in their genomic EPSPS genomic copy numbers, compared to the susceptible

populations, respectively (Malone et al. 2016; Ngo et al. 2018; Wiersma et al. 2015). This shows that glyphosate resistant populations can have less than the proposed 30 to 50 EPSPS genomic copies to survive recommended field concentration glyphosate (Gaines et al. 2011).

Cases of resistance to xenobiotics as a result of gene amplification have also been reported in a number of prokaryotic and eukaryotic organisms (Vel'kov 1982). Gene amplification has been shown to be a mechanism of adaptive evolution that enables organisms to survive adverse conditions (Hastings 2007; Romero and Palacios 1997; Taylor and Raes 2004). Under selective pressure, certain genes are duplicated to provide greater adaptation to stress and for the organism to continue normal metabolic functions (Jugulam and Gill 2018; Patterson et al. 2017; Pettersson et al. 2009). However, this process is thought to be unstable and can be reversed when selective pressure is withdrawn (Field and Blackman 2003; Sharma and Schimke 1994). This has been reported in some drugs used in cancer treatment and some insecticides (Kim et al. 1998; Raymond et al. 1998; Sharma and Schimke 1994). The stability of amplified gene copies in pesticides resistance especially in glyphosate resistant weeds is still unknown. To effectively manage glyphosate resistance due to gene amplification, it is necessary to also investigate the fate of amplified genes in the absence of glyphosate selection. An experiment was therefore conducted to assess EPSPS gene copy numbers in populations and their subsequent progenies in the presence or absence of glyphosate selection. Tillers of individual resistant plants were separated to generate two clones, where one clone was treated with glyphosate and other was left untreated. A dose response study and EPSPS copy number assessment was later conducted on second generation progenies (generated from self-pollinated individual clones) from the clones to assess for resistance level and EPSPS gene copies. Results from this experiment showed that glyphosate resistance and EPSPS gene copies increased in *H. glaucum* individuals and second generation progenies following a single cycle of recurrent selection compared to their untreated individuals. There was a 75% to 79% increase in

glyphosate concentration required for 50% mortality of second generation progenies of the treated individuals compared to progenies of the untreated. Similarly, EPSPS gene copies in the progenies of treated clones increased by 2 to 4-fold compared to the untreated. Similar observations were reported by Jugulam et al (2014), where an increase in gene copies and resistance was observed in glyphosate resistant populations of *K. scoparia* with continued glyphosate selection. This suggests that EPSPS gene copies in glyphosate resistant *H. glaucum* may not be stable and glyphosate resistance and amplified gene copies will continue to increase in these populations, so long as glyphosate is used. On the contrary withdrawal of glyphosate application can slow the evolution and spread of resistance. This has implications for resistance management in this weed species, in that any long term control strategy should not involve use of glyphosate.

To have greater understanding of the evolution and spread of resistance to glyphosate in *H. glaucum*, another experiment was conducted to investigate the inheritance pattern of the gene amplification mechanism in this weed species. F_1 progenies were generated by hand crossing resistant *H. glaucum* individuals to susceptible. The F_2 individuals were generated from self-pollinated F_1 progenies. A detailed dose response on the F_2 individuals showed the F_2 individuals were not as resistant as the parent resistant population, and the calculated LD_{50} for both F_2 progenies showed an intermediate response between the parent populations suggesting that resistance may not be completely dominant over susceptibility. High variation in EPSPS gene copies were observed in F_2 individuals assessed before and after glyphosate treatment, with some individuals having gene copies more than the GR parent population. EPSPS gene copies in glyphosate untreated F_2 individuals (F_2.1-UT and F_2.2-UT) ranged from 1 to 15 and 1 to 23 gene copies respectively. However, EPSPS gene copies increased in the F_2 individuals following glyphosate treatment with every individual possessing 2 to 26 gene copies with some individuals having higher copies that the parent resistant population. High

variation in EPSPS gene copies was similarly reported in F_2 progenies of A. *palmeri* and *B. diandrus* populations (Chandi et al. 2012; Gaines et al. 2010; Giacomini et al. 2019; Malone et al. 2016; Mohseni-Moghadam et al. 2017). Likewise, the phenotypic and genotypic segregation analysis conducted on F_2 progenies did not conform to the single gene model. This suggest that inheritance of glyphosate resistance in *H. glaucum* follows a non-Mendelian pattern similar to inheritance patterns reported in some populations of *A. palmeri* and *B. diandrus* (Chandi et al. 2012; Gaines et al. 2010; Giacomini et al. 2019; Malone et al. 2016; Mohseni-Moghadam et al. 2017). This inheritance pattern observed has consequences for the evolution and spread of resistance in this weed species. This is because individual plants with high gene copies are likely to spread rapidly in a population as progenies from crosses with a susceptible are likely to be more resistant than the susceptible parent. This may be less of an issue for self-pollinated species, such as *H. glaucum*, but may not be the case under continued glyphosate selection.

5.2 Conclusion

This research project provides valued insights into the first case of evolved resistance to glyphosate in *H. glaucum* population by characterising resistant populations and investigating factors that could potentially influence the evolution, spread and persistence of resistance in this species. The study confirmed evolved resistance of *H. glaucum* to glyphosate and further identified the mechanism of conferring resistance as EPSPS gene amplification. EPSPS gene amplification is becoming one of the common mechanisms associated with resistance to glyphosate many weed species. However, unlike some other weed species, low numbers of EPSPS gene copies was sufficient for individuals to survive field recommended glyphosate concentrations. The study also showed that selection pressure is one of the factors that strongly influences resistance and copy number increase in this population. Therefore, continued glyphosate selection can rapidly drive the evolution and spread of resistant

individuals in a population, which has implications for control strategies. Studies on the inheritance of resistance in this species showed a complex and unpredictable pattern elsewhere observed with resistance due to gene amplification. The complexities of the inheritance of EPSPS gene copies and the stability of gene copies in resistant plants have almost always been linked to the organisation of EPSPS gene copies in the genome. Similarly, the precise mechanisms leading to the initial duplication event is also associated with the cytogenetics of the gene copies. Based on these findings, it will be prudent to investigate the genomic organisation of the EPSPS gene copies in glyphosate resistant *H. glaucum* populations as well as the fitness cost associated with resistance in this species. Evaluating methods and formulation of strategies to effectively slow or control evolution of glyphosate resistance in this species is also of importance. However, based on our findings, strategies to effectively manage glyphosate resistant *H. glaucum* in a population should not involve glyphosate either in herbicide mixtures or knock down treatments.

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