Biology and glyphosate resistance in *Chloris truncata* (windmill grass) and *Chloris virgata* (feathertop Rhodes grass) in southern Australia

by

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Abstract

Chloris truncata and *C. virgata*, which are major weeds in cotton and grain crops in the sub-tropical region of Australia, have recently emerged as potential weeds of the future in southern Australia. Glyphosate, an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), is the most widely used non-selective post-emergence herbicide globally. As a result of over-reliance on glyphosate combined with dominance of reduced tillage systems for weed control, glyphosate-resistant populations of *C. truncata* have already been reported in Australia. *C. virgata* is also considered hard to kill with glyphosate, but resistance has not been reported so far in the literature. Studies on growth, development and seed biology of *C. truncata* and *C. virgata* were conducted to better understand the biology of these emerging weed species. Under field conditions, *C. truncata* and *C. virgata* required 748-786 degree-days (Cd) and 1200 Cd respectively to progress from emergence to mature seed production. Freshly produced seeds of *C. virgata* were dormant for about 2 months, whereas 16-40% of seeds of *C. truncata* germinated within a week after maturation. Seed dormancy of *C. virgata* was released by the pre-treatment with 564 mM NaClO for 30 minutes. Exposure to light significantly increased germination of *C. truncata* seed from 0-2% in the dark to 77-84% in the light, and of *C. virgata* seed from 2-35% in the dark to 72-85% in the light. Seeds of these two species could germinate over a wide temperature range (10-40^{o}C), with maximum germination at 20-25^{o}C for *C. truncata* and 15-25^{o}C for *C. virgata*. The predicted base temperature for germination was 9.2-11.2^{o}C for *C. truncata* and much lower 2.1-3.0^{o}C for *C. virgata*. Seedling emergence of *C. virgata* (76% for seeds present on soil surface) was significantly reduced by burial at 1 (57%), 2 (49%) and 5 cm (9%), whereas seedling emergence of *C. truncata* was completely

inhibited by burial of seed even at a shallow depth (0.5 cm). Under field conditions, both *C. truncata* and *C. virgata* seeds persisted in the soil for at least 11 months and seasons with below-average spring-summer rainfall increased seed persistence.

Detailed studies were undertaken to identify glyphosate-resistant populations and to understand the mechanism of glyphosate resistance in *C. truncata* and *C. virgata*. Glyphosate resistance (GR) was confirmed in five populations of *C. truncata* and four populations of *C. virgata*. GR plants were 2.4 to 8.7-fold (*C. truncata*) and 2 to 9.7-fold (*C. virgata*) more resistant and accumulated less shikimate after glyphosate treatment than susceptible (S) plants. The differences in shikimate accumulation indicated that glyphosate did reach the target site but inhibited the EPSPS enzyme of each population differently. Glyphosate absorption and translocation did not differ between GR and S plants of either *C. truncata* or *C. virgata*. Two target-site EPSPS mutations (Pro-106-Leu and Pro-106-Ser) were likely to be the primary mechanism of glyphosate resistance in *C. virgata* but no previously known target-site mutations were identified in *C. truncata*. The *C. virgata* population with Pro-106-Leu substitution was 2.9 to 4.9-fold more resistant than those with Pro-106-Ser substitution. The primary mechanism of resistance to glyphosate in *C. truncata* was a combination of target-site *EPSPS* mutation (Glu-91-Ala) and amplification of the *EPSPS* gene. There were 16 to 48-fold more copies of the EPSPS gene in GR plants compared to S plants, with the number of EPSPS copies found to be variable both between and within populations.

Declaration

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Abbreviations

a.e	acid equivalent
a.i	active ingredient
AGRF	Australian Genome Research Facility
ALS	acetolactate synthase
ANOVA	analysis of variance
Bq	becquerel
C	Celsius
Cd	degree-days
d	day
EPSPS	enzyme 5 - enolpyruvylshikimate - 3 - phosphate synthase
G_{max}	maximum germination
h	hour
HAT	hour after treatment
LD_{50}	lethal dose required for 50% mortality
LSD	least significant difference
MPa	megapascal
NADH	NADH dehydrogenase subunit F
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
QPCR	quantitative real-time PCR
RI	resistance index relative to sensitive biotype
S3P	shikimate - 3 - phosphate

T_{50}	the time required for the germination of half the total germinated seeds
T_b	base temperature
wk	week

Chapter 1: Review of Literature

1.1 Introduction

1.1.1 *Chloris truncata*

C. truncata is found throughout the Australian mainland (Australia's Virtual Herbarium, 2016a). This species is an Australian native, annual or short-lived perennial, stoloniferous C_4 grass (Meredith and Mitchell, 1994; Nightingale et al., 2005). This grass occurs in native vegetation and within the wheat-belt in Western Australia, South Australia, Victoria and New South Wales (Australia's Virtual Herbarium, 2016a; Johnston, 1996; Michael et al., 2012b). Only a few native Australian grass species including *C. truncata*, have ability to establish in disturbed ecosystems, which have regular cultivation, fertiliser inputs, ruminant grazing or crop competition (Kloot, 1985; Michael et al., 2012a). *C. truncata* has been a significant weed in cotton and grain crops in the sub-tropical region of Australia, particularly in no-till agriculture (Werth et al., 2013). It was projected that *C. truncata* could become one of the five most threatening weed species to agriculture in the southwest of Western Australia (Michael et al., 2010). Recently, this species has been ranked as the 7th most important weed of summer fallow based on yield and revenue loss in Australia (Llewellyn et al., 2016).

Due to over-reliance on glyphosate for weed control, resistance to glyphosate was confirmed in *C. truncata* from chemical fallows and roadsides in Australia (Malone et al., 2012; Preston, 2016), making control of this weed in summer fallow situations more challenging. However, the resistance mechanism to glyphosate in this weed species has not been identified yet. The dominance of reduced tillage systems combined with the heavy reliance on glyphosate for weed control in the fallow phase

has also favoured this grass (Werth et al., 2013; Widderick et al., 2014). As a summer-active grass species, *C. truncata* will reduce the potential yield of winter crops by utilising moisture and nutrients that would otherwise be available to the crop, and delay the sowing of crops due to the time taken for weed control in the autumn (Osten et al., 2006). For example, the absence of control of *C. truncata* over summer fallow reduced yield of the following wheat crop in Merredin, Western Australia by 0.3 t ha^{-1} (Borger et al., 2010). In addition, *C. truncata* is a common host for aphids and cereal leaf diseases such as barley yellow dwarf virus and wheat streak mosaic virus (Coutts et al., 2006; Hawkes and Jones, 2005) and some potential insect species such as Rutherglen bug (*Nysius vinitor*) (Danne et al., 2010).

1.1.2 *Chloris virgata*

Chloris virgata Swartz is a warm-season, C_4 annual grass (Halvorson et al., 2003) that is widely distributed globally. It generally grows throughout tropical, sub-tropical and warm temperate regions extending well into the temperate regions, in areas where hot summers are typical (Anderson, 1974). In Australia, this grass is found throughout the mainland (Australia's Virtual Herbarium, 2016b). It has been a major weed in cotton and grain crops in the sub-tropical region of Australia for many years (Werth et al., 2013) and has become an emerging weed in southern Australia over the last five years. *C. virgata* was the third most common weed identified (50% of paddocks) in a summer fallow survey in 2012 (Widderick et al., 2014). This grass is a weed of vineyards and orchards in South Australia and in parts of the Western Australian grain region (Osten, 2012). More recently, this species has been ranked in top four (northern regional ranking) and top ten (national ranking) residual weeds in all crops in Australia (Llewellyn et al., 2016). *C. virgata* is also a host for aphids (Holman, 2009) and diseases such as barley yellow dwarf virus and cereal yellow dwarf virus in the grain-belt of south-western Australia (Hawkes and Jones, 2005).

In the sub-tropical regions of Australia, *C. virgata* has a higher level of tolerance to glyphosate than many other weed species present (Werth et al., 2013; Werth et al., 2011; Widderick et al., 2014). The dominance of reduced tillage systems combined with the heavy reliance on glyphosate for weed control has made it difficult to effectively control this weed species (Werth et al., 2013; Widderick et al., 2014). There is concern that *C. virgata* populations in several locations have become more difficult to control with this herbicide over time and may have evolved resistance to glyphosate.

1.2 Biology and Ecology

1.2.1 Botanical Description and Taxonomy

The genus *Chloris* belongs to the tribe Chlorideae in family Poaceae. This genus has 56 species, including *C. truncata* and *C. virgata* (Anderson, 1974). The genus *Chloris* is characterized by a basic chromosome number of x = 10 (Anderson, 1974; Brown, 1950). *C. truncata* is annual or perennial, 20 to 50 cm tall, stoloniferous; sheaths glabrous, ligules short-ciliate; blades 9 to 17 cm long, 0.2 to 0.3 mm wide, glabrous to scabrous; spikes 5 to 13 in number, 5 to 23 cm long, radiate. The ploidy level of *C. truncata* is tetraploid with 2n = 40 (Anderson, 1974). *C. virgata* is annual, extremely variable in size, ranging from a few centimeters to over a meter; culms usually tufted, several arising from one root system, occasionally stoloniferous with branches capable of rooting at the joints; sheaths usually glabrous; ligules glabrous; blades up to 30 cm long and 1.5 cm wide, usually glabrous with scabrous margins; spikes 4 to 20, 5 to 10 cm long. (Anderson, 1974). *C. virgata* is diploid with 2n = 20 (Brown, 1950; Rice et al., 2015).

1.2.2 Weed Biology and Management

Weed biology relates to plant attributes such as morphology, seed dormancy and germination, physiology of growth, competitive ability and reproductive output.

Improved knowledge of weed biology can facilitate effective weed management in a number of important ways. Studies on population biology, such as seedbank dynamics, for annuals and root reserves, dormancy and longevity of vegetative propagules for perennials can be employed to predict weed infestations and to evaluate sustainable management strategies (Bhowmik, 1997). In addition, seed germination, a process regulated by several environmental factors, such as temperature, light, moisture and soil salinity, is a key event determining the success of a weed species in an agroecosystem (Chachalis and Reddy, 2000; Koger et al., 2004). Therefore, integrated approaches that deplete root reserves or seedbanks by interfering with dormancy or germination requirements have great potential to enhance weed management strategies. Even in the herbicide era, where weed problems including herbicide-resistant weeds persist, weed biology studies remain essential to develop effective weed management approaches (Van Acker, 2009).

In Australia, more research is required in the area of weed biology to improve the understanding of the processes and mechanisms involved in weed dispersal, seed germination and seedling establishment, weed physiology and weed-crop competition (Pratley, 1996). For example, weed biology studies should be more explanatory to understand why weeds respond in the way they do. If these studies stop at the observation and not proceed to an investigation, this will lead to poor insights (Cousens and Mortimer, 1995). Weed management has to address a diversity of species with a wide range of biology traits. Due to this diversity, robust weed management systems that require biological insights into individual species are necessary (Mortensen et al., 2000). Therefore, studying plant growth, development and seed biology of *C. truncata* and *C. virgata* in southern Australia is essential to develop sound management systems for these emerging weed species.

1.2.3 Seed Biology and Ecology

1.2.3.1 Dormancy

Seed dormancy is the failure of an intact viable caryopsis to complete germination when placed under favourable conditions such as moisture, oxygen and temperature (Hilhorst, 2007; Loch et al., 2004). Seed dormancy is common in species belonging to the major C_4 grass genera. Simpson (1990) listed 89 species of C_4 grasses from 24 genera, including *Chloris* (*C. gayana, C. ciliate, C. virgata and C. truncata*), as having one or more forms of dormancy. In dormant warm-season C_4 grass seeds, there are at least two primary dormancy mechanisms; mechanisms based within the embryo covering structures and mechanisms based within the embryo (Adkins et al., 2002). Within the covering structures, mechanisms may involve mechanical resistance (preventing embryo expansion), permeability (preventing water uptake or gaseous exchange) and chemical barriers to germination (prevention of leakage of germination inhibitors and conversely allowing uptake of both germination inhibitors and stimulants) (Bewley et al., 1994). Within the embryo, mechanisms may involve the expression of certain genes, levels of certain plant growth regulators, the activity of important respiratory pathways or the mobilisation and utilisation of food reserves. In addition, some embryos may be too immature to germinate immediately and have to undergo a further growth phase before germination is possible. An individual species could have one or several dormancy mechanisms. The level of dormancy and the actual dormancy mechanisms also can be different within a species distributed over a wide geographic and climatic range (Loch et al., 2004).

After-ripening is loss of dormancy occurring in ‘air-dry’ seeds. This loss of dormancy during after-ripening is a function of time, temperature and moisture content (Bazin et al., 2010) and varies in a predictable manner (Bewley et al., 1994). *C. truncata* seeds have dormancy (after-ripening requirement), which prevents

germination for 3-4 months after initial seed production (Baskin and Baskin, 2004). After-ripening may involve the breakdown of germination inhibitors, an alteration in the form of respiration or an alteration in gene expression (Loch et al., 2004). For example, at 25^{o}C under constant fluorescent light, germination of *C. truncata* increased from 4-9% for 1-3 month-old intact seeds to 30% for 4 month-old-seeds and reached a maximum of 62% for 6-12 month-old-seeds (Borger et al., 2011). Similarly, germination of another *C. truncata* population from central New South Wales increased from 6-7% for freshly harvested seeds to over 80% for 7-8 month-old-seeds at 12 h alternating light/dark and temperature of 30/20^{o}C (Maze et al., 1993). Additionally, it is reported that removal of covering structures (lemma, palea and glumes) increased germination of *C. truncata* seeds (Lodge and Whalley, 1981; Read and Bellairs, 1999). *C. truncata* germination increased significantly by removing the lemma and palea of freshly harvested (Lodge and Whalley, 1981; Maze et al., 1993), 2-month-old (Read and Bellairs, 1999) and 12-month-old seeds (Lodge and Whalley, 1981).

C. virgata seeds are also dormant at maturity. Freshly collected seeds in India required an after-ripening period of about 6 months, and no significant difference in germination was observed for 6, 12 and 18-month-old seeds (Parihar et al., 1998). However, *C. virgata* populations in Central Queensland, Australia required a much shorter after-ripening period of 6 to 10 weeks before seeds germinated (Osten, 2012). In contrast, 98% germination occurred in freshly collected *C. virgata* seeds in northern China (Li et al., 2006). Pre-chilling treatment has been shown to be an effective method to break seed dormancy of *C. virgata* (Osten, 2012; Yu, 1999). Different results among various studies may be due to a wide geographic and climatic distribution range of this species (Anderson, 1974) that could have selected for different levels of dormancy and dormancy mechanisms (Loch et al., 2004). Furthermore*, C. virgata* may possess coat-imposed and endosperm-imposed seed dormancy mechanisms. It has also been

shown that removal of covering structures (lemma, palea and glumes) significantly increased germination of *C. virgata* seeds (Pezzani and Montana, 2006).

1.2.3.2 Effect of Environmental Factors on Germination and Emergence

1.2.3.2.1 Temperature and Light

Seed germination, a process regulated by several environmental factors such as temperature, light, moisture and soil salinity, is a key event determining the success of a weed in an agroecosystem (Chachalis and Reddy, 2000; Koger et al., 2004). Seeds that respond to environmental conditions and alter their germination behaviour are more likely to survive and establish successfully. The timing of germination and emergence is critical for the survival of annual plants (Saatkamp et al., 2009), with temperature and light among the most important environmental signals that regulate germination and emergence of a plant species (Baskin and Baskin, 1998; Schutte et al., 2014). For seeds in the soil, temperature and light are important cues for initiating germination by providing a sense of their position in the soil profile and the occurrence of soil disturbance (Batlla and Benech-Arnold, 2014). *C. truncata* seed can germinate over a broad temperature range of 15-35^{o}C (Lodge and Whalley, 1981; Maze et al., 1993). Within this temperature range, germination of non-dormant *C. truncata* seed was similar across the different temperatures. For example, *C. truncata* germination ranged between 58 to 73% at 17/7, 22/12, 30/20, 35/20 and 40/20^{o}C (Maze et al., 1993). *C. truncata* germination was also similar (57%) at different constant temperatures of 20, 25 and 30^{o}C (Lodge and Whalley, 1981). There have been several studies of the effect of temperature on seed germination of *C. virgata* in China, Qatar and Australia (Bhatt et al., 2016; Fernando et al., 2016; Osten, 2012; Zhang et al., 2012; Zhang et al., 2015; Zhang et al., 2008). Germination of *C. virgata* seed can occur across temperatures from 5 to 40^{o}C and optimum germination temperature is around 25^{o}C (Osten, 2012; Zhang et al., 2015; Zhang et al., 2008). However, there is currently

no information on the base temperature required for germination of Australian populations of *C. truncata* and *C. virgata*.

A light requirement for germination is common among small-seeded species and warm-season grasses (Adkins et al., 2002**;** Grime et al., 1981**;** Milberg et al., 2000). Light can penetrate only a few mm in soil (Benvenuti, 1995; Cussans et al., 1996) so that it cannot reach buried seeds even at shallow depths. Light may stimulate germination by altering the balance of germination promoters and inhibitors in the embryo (Adkins et al., 2002). The light requirement can ensure that germination takes place away from other vegetation and only on or close to the soil surface (Adkins et al., 2002**;** Milberg et al., 2000), which would enhance the probability of seedling survival of small-seeded weed species. It was reported that light had a positive effect on germination of non-dormant *C. truncata* seeds (Maze et al., 1993). In contrast, Lodge and Whalley (1981) found that light had no effect on germination of both 5 and 9-month-old seeds of *C. truncata*; but the final germination achieved in this study was very low (<18%). Pezzani and Montana (2006) also reported a similar level of germination (30-40%) of *C. virgata* seeds under different light conditions including white light, far-red light and complete darkness.

1.2.3.2.2 Osmotic and Salt Stress

An ability to germinate under conditions of moisture stress or high salt content of soils can enable a weed to take advantage of conditions that limit the growth of other species. Germination of *C. truncata* declined with a reduction in water potential (Maze et al., 1993; Watt, 1982). While Watt (1982) found that the lowest water potential for *C. truncata* germination was at -0.5 MPa, Maze et al. (1993) reported that germination ceased below -1.5 MPa. Germination of *C. virgata* in Queensland was completely inhibited at an osmotic potential of -0.6 MPa, which is slightly lower than for *Setaria glauca* (-0.7 MPa)*, Setaria viridis* (-0.7 MPa)*, Digitaria sanguinalis* (-0.8 MPa) and

Eleusine indica (-1.2 MPa) (Masin et al., 2005). Germination of *C. virgata* in Queensland was completely inhibited at 250 mM NaCl (Fernando et al., 2016), while there was more than 80% germination of *C. virgata* from the Songnen Grassland in China at this level of salt stress, where this species is considered a halophyte and a potentially useful grass species for saline areas (Zhang et al., 2012). These differences between studies could reflect adaptation of ecotypes in China to soil salinity. However, the effect of osmotic and salt stress on germination of *C. truncata* and *C. virgata* populations from cooler Mediterranean conditions of the southern and western grain regions of Australia has not been described.

1.2.3.2.3 Chemical Stimulants

Previous studies found that exogenously applied gibberellic acid (GA_3) can overcome dormancy in many species, including a number of C_4 grasses (Groves et al., 1982; Mott, 1978; Mott, 1980). Exogenous GA_3 stimulates germination by mobilizing stored food reserves, which provide substrate for ATP production. This energy then promotes elongation of the embryonic axis, an important early step in germination (Loch et al., 2004). Field application of nitrogen fertilizers can also stimulate germination in some species. Several studies have shown that N-containing compounds are able to overcome embryo dormancy in many seeds. Adkins et al. (1984b) found that the effect of N-containing compounds was dependent on seed age, with promotion limited to partially after-ripened seeds but had no effect on freshly harvested seeds. These chemicals are considered to induce germination, not through the direct action of N, but by their ability to act as electron acceptors (Adkins et al., 1984a). Nitrate has been found to stimulate germination of a number of warm-season C_4 grasses, including *C. gayana* (Adkins et al., 2002; Loch et al., 2004). In addition, sodium hypochlorite (NaOCl) can overcome dormancy in several species. There are two major roles for NaOCl in the termination of dormancy (Hsiao, 1979**;** Hsiao, 1980**;**

Hsiao and Quick, 1984). The first role is that NaOCl may modify the properties of the hull and seed coat membranes in such a way as to increase water uptake by the embryo, leading to the release of dormancy. The second role is that NaOCl could act as an oxidant on vital pre-germination processes within the caryopsis. The prevention of water uptake is not a common dormancy mechanism found in grass species. However, the capacity for gaseous exchange might be limited by the tissues surrounding the embryo (Adkins et al., 2002).

1.2.3.3 Seedbank Persistence

By understanding the factors that control seedbank dynamics, seed production and seed dispersal, it is possible to better manipulate annual weed populations (Bhowmik, 1997). Understanding of the soil seedbank dynamics enables formulation of more effective management strategies for annual weeds (Roger and Martin, 1995). This requires knowledge of the length of seed persistence in the soil seedbank and the periodicity of seedling emergence as influenced by the environment (Roberts and Boddrell, 1985). Seed persistence in the field can be categorized into three groups: transient (< 1 year), short-lived (1 to 5 years) and long-lived (> 5 years) (Thompson et al., 1998). The seeds of many agricultural weeds tend to get buried in the soil by tillage, the seed drill at planting time or other means, but being persistent allows them to survive un-germinated until another soil disturbance event relocates them near the surface (Cavers and Benoit, 1989). The soil disturbance places weed seeds at different depths, which differ in availability of moisture, diurnal temperature fluctuation, light exposure and activity of predators. All these microenvironment attributes have the potential to influence the behaviour of weed seedbanks through influences on dormancy, germination and seedling emergence.

Seed persistence of many annual grass weeds is relatively short (Hurle, 1993). Seed viability of three summer annual grass weeds including *Digitaria sanguinalis*,

Setaria glauca and *S. viridis* buried at 4.0-4.5 cm deep decreased significantly to about 70% after almost 1 year of burial, with no seeds surviving after 3 years. *C. virgata* seeds in Queensland, Australia appear to be short-lived (about 7 to 12 months) suggesting short field persistence, and burying seed deeper than 5 cm significantly reduced germination and viable seeds of the seedbank (Osten, 2012). Emergence of *C. virgata* in fields where deep tillage was applied was much lower than that in fields where crops were grown with zero- or no-till systems (McLean et al., 2014; Widderick et al., 2014). However, seedbank persistence of *C. virgata* under cooler Mediterranean conditions of the southern and western grain regions of Australia has not been reported. In addition, seedbank dynamics of *C. truncata* have not been studied in Australia yet.

1.3 Glyphosate

1.3.1 Glyphosate and Weed Control

Glyphosate is the most widely used non-selective herbicide in the world (Duke and Powles, 2008). There are several main attributes that make glyphosate a valuable herbicide. It provides simple, inexpensive, flexible and effective control of a broad spectrum of weeds in a wide variety of agronomic situations (Baylis, 2000). In almost all countries, the use of this herbicide has continued to increase as its patent has expired and its price has declined. Glyphosate has no soil activity (Baylis, 2000), which allows famers to sow crops shortly after its application (Preston, 2010). In addition, glyphosate has low toxicity to higher organisms including mammals, birds, fish, aquatic invertebrates, and terrestrial invertebrates (such as earthworms and honeybees) (Dill et al., 2010; Duke and Powles, 2008) and no human health safety issues (Williams et al., 2000). All these attributes make glyphosate an ideal herbicide for use by non-professionals (Preston, 2010).

In Australia, glyphosate is widely used in agricultural and nonagricultural systems. In grain production, glyphosate is used to control weeds before sowing annual crops, in summer and winter fallows, between crop rows and in glyphosate-tolerant cotton and canola fields. In horticulture, glyphosate is used to control weeds under trees and vines and for weed control prior to planting horticultural crops. Additionally, glyphosate is used to control woody weeds in forestry and native ecosystems. Glyphosate is widely used by local governments to control weeds along roadsides, on footpaths, around structures and in parks and gardens. It is also the most important herbicide sold for home garden use (Preston, 2010).

1.3.2. Glyphosate Mode of Action

Glyphosate (N - phosphonomethyl glycine) is a phosphonomethyl derivative of the amino acid glycine and has a unique mode of action. Glyphosate inhibits the enzyme 5 - enolpyruvylshikimate - 3 - phosphate synthase (EPSPS) (Amrhein et al., 1980), which is present in plants, fungi and bacteria, but not in animals (Kishore and Shah, 1988). EPSPS is the sixth enzyme of the shikimic acid pathway in which phosphoenolpyruvate (PEP) and erythrose 4 - phosphate are converted to chorismate, the precursor of the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and many aromatic secondary metabolites (e.g., auxins, phytoalexins, anthocyanins, and lignin) (Herrmann and Weaver, 1999). EPSPS catalyses the transfer of the enolpyruvyl moiety from PEP to shikimate - 3 - phosphate (S3P) (Perez-Jones and Mallory-Smith, 2010). Glyphosate is a transition state analogue of PEP and inhibits EPSPS through the formation of an EPSPS - S3P - glyphosate ternary complex, only binding to the enzyme after the formation of EPSPS - S3P binary complex (Alibhai and Stallings, 2001). Therefore, glyphosate acts as a competitive inhibitor with *PEP* as it occupies its binding site (Schonbrunn et al., 2001). EPSPS inhibition by glyphosate prevents the biosynthesis of aromatic amino acids (Perez-Jones and Mallory-Smith,

2010). Glyphosate is the only commercial herbicide that targets EPSPS of all higher plants (Duke and Powles, 2008).

1.3.3 Glyphosate Resistance Mechanisms

1.3.3.1 Absorption and Translocation of Glyphosate

Glyphosate is taken up through plant surfaces, and leaf uptake rates vary considerably between species. Diffusion is the most likely mode of transport across the plant cuticle (Duke and Powles, 2008), which varies in composition and thickness in various plant species. Uptake is also dependent on several interdependent factors, including droplet size and droplet spread, surfactant type and concentration, ionic strength and salt concentration, humidity, and most importantly, glyphosate concentration (Dill et al., 2010). After it is absorbed, the physicochemical properties of glyphosate enable it to be translocated from the leaf via phloem transport to meristematic growing points in the roots and shoots (Dill et al., 2010; Duke and Powles, 2008). The phloem movement of glyphosate and the efficiency of translocation are affected by health and developmental stage of plants, which are often related to environmental conditions (Dill et al., 2010).

1.3.3.2 Glyphosate Resistance Mechanisms

Herbicide resistance is defined as “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis” by the Weed Science Society of America (1998, p. 789). To develop effective herbicide resistance management strategies, it is important to understand the processes and means by which weeds withstand labelled herbicide treatments.

In 1996, the first evolved resistance to glyphosate in weed species was reported in *Lolium rigidum* in Australia (Pratley et al., 1996). Since then, there has been a sharp increase in the number of glyphosate-resistant weeds (Heap, 2016), because of the over-reliance on glyphosate for pre-sowing weed control for decades and the introduction of glyphosate-resistant transgenic crops in the mid-1990s (Powles, 2008). To date, glyphosate resistance has been identified in 36 weed species worldwide (Heap, 2016). Resistance mechanisms to glyphosate in weeds include (1) target-site alterations: (1a) target-site mutation (Baerson et al., 2002; Chen et al., 2015; Yu et al., 2015), represented by amino acid substitutions that affect herbicide interactions at the target enzyme; (1b) target-site gene amplification (Gaines et al., 2010; Malone et al., 2016; Salas et al., 2012), where sufficient EPSPS protein is produced so that the shikimate pathway can continue to operate despite the fact that glyphosate inhibits some of the enzyme; and (2) non-target-site mechanisms involving different modes of exclusion from the target site: (2a) reduced glyphosate uptake (De Carvalho et al., 2012; Vila-Aiub et al., 2012), where less glyphosate is absorbed by resistant plants than susceptible plants; (2b) reduced glyphosate translocation (Bostamam et al., 2012; Ghanizadeh et al., 2015; Lorraine-Colwill et al., 2002; Vila-Aiub et al., 2012), where amounts of glyphosate absorbed by both resistant and susceptible plants are similar; however, the absorbed herbicide mostly remains in the treated leaf in resistant biotypes and only a smaller amount of glyphosate was transported to the meristems of the treated plant; and/or (2c) vacuole sequestration (Ge et al., 2010; Ge et al., 2012), where more glyphosate is sequestered in the cell vacuole of resistant plants than in those of susceptible plants.

Target-site mutations that change Pro-106 of EPSPS to Ala, Leu, Ser or Thr have been reported in glyphosate-resistant populations of six different weed species (Gaines and Heap, 2016; Sammons and Gaines, 2014). It was concluded that the Pro101 (position Pro-106 in plant mature EPSPS consensus corresponds to position

Pro-101 in *E. coli*) is not directly involved in molecular interactions with either glyphosate or the substrate PEP, but any mutation at this site would shift other amino acids (Thr-97 and Gly-96) towards the inhibitor molecule resulting in a structural change in the glyphosate-binding site (Healy-Fried et al., 2007). More recently, a double amino acid substitution in a single EPSPS allele (Thr-102-Ile + Pro-106-Ser) was found in glyphosate-resistant *Eleusine indica* populations from Malaysia and China (Chen et al., 2015; Yu et al., 2015). This double amino acid substitution conferred high-level glyphosate resistance (more than 180-fold) (Yu et al., 2015), whereas the single Pro-106 mutations of the six weed species provided moderate resistance (less than10-fold) (Gaines and Heap, 2016).

In 2010, glyphosate resistance due to extensive amplification of the EPSPS gene was first revealed in a population of *Amaranthus palmeri* (Gaines et al., 2010). This mechanism has since been identified in six other weed species: *Lolium perenne ssp. multiflorum* (Salas et al., 2012), *Amaranthus spinosus* (Nandula et al., 2014), *Amaranthus tuberculatus* (Chatham et al., 2015; Lorentz et al., 2014), *Eleusine indica* (Chen et al., 2015), *Kochia scoparia* (Wiersma et al., 2015) and *Bromus diandrus* (Malone et al., 2016). Individuals of the glyphosate-resistant populations contained few to many more copies of the EPSPS gene than did susceptible plants, with the number of EPSPS copies found to be variable both between and within populations. For example, *A. palmeri* from Georgia, North Carolina and New Mexico with 5-160-fold (Gaines et al., 2010), 22-36-fold (Chandi et al., 2012) and 2-8-fold (Mohseni-Moghadam et al., 2013), respectively, more copies of the EPSPS gene; and a 10-36-fold increase in *B. diandrus* (Malone et al., 2016) and a 10-25-fold increase in *L. multiflorum* (Salas et al., 2012). It has been suggested that the effect of additional copies of EPSPS is additive, and additional copies confer higher levels of resistance to glyphosate (Gaines et al., 2010). This amplification of the EPSPS gene produces

sufficient EPSPS protein to enable the shikimate pathway to continue to operate despite glyphosate inhibiting some of the enzyme (Powles, 2010).

In 2010, glyphosate resistance was confirmed in *C. truncata* populations from chemical fallows and roadsides in Australia (Malone et al., 2012; Preston, 2016) because of the widespread reliance on glyphosate for weed control. However, the resistance mechanism to glyphosate in this weed species has not been elucidated. Similar to *C. truncata*, *C. virgata* is seen as a weed that has been hard to kill with glyphosate (Cameron et al., 2012; Werth et al., 2013; Widderick et al., 2014). There is concern that populations in several locations, particularly in summer fallow situations, in southern Queensland and northern New South Wales, Australia, have become more difficult to control with this herbicide over time and may have evolved resistance to glyphosate (Werth et al., 2011). Once glyphosate-resistance populations are found in *C. virgata*, it would be important to understand the mechanism of herbicide resistance so that strategies can be developed to both slow the evolution of resistance and to control existing populations (Wakelin and Preston, 2006).

1.4 Research Objectives

Most Australian studies on *C. truncata* biology so far have been undertaken from a pasture perspective in northern Australia (Lodge, 1981; Lodge and Whalley, 1981; Maze et al., 1993), and not in cooler Mediterranean conditions of the southern and western grain regions. Most seed biology studies on *C. virgata* so far have been undertaken in China, India, Qatar, Honduras (Bhatt et al., 2016; Li et al., 2006; Zelaya et al., 1997) and northern Australia (Fernando et al., 2016). Therefore, the objectives of this research project were to determine plant growth, development and seed biology of *C. truncata* and *C. virgata* in southern Australia, including: (1) growth and development under field conditions with a particularly time required to reach flowering

and seed production; (2) seed dormancy and mechanism; (3) effect of physical environmental factors (temperature, light, osmotic stress, salt stress and burial depth) on seed germination and seedling emergence; and (4) seedbank persistence under field conditions.

Glyphosate resistance was confirmed in *C. truncata* (Malone et al., 2012; Preston, 2016) and *C. virgata* is seen as a weed that has been hard to kill with glyphosate (Cameron et al., 2012; Werth et al., 2013; Widderick et al., 2014); however resistance mechanisms to glyphosate in either *C. truncata* or *C. virgata* have not been elucidated. Therefore, other objectives of this research project were to identify glyphosate-resistant populations and to elucidate possible mechanisms of resistance in *C. truncata* and *C. virgata*. It would be important to understand the mechanism of herbicide resistance so that strategies can be developed to both slow the evolution of resistance and to control existing populations (Wakelin and Preston, 2006).

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Chapter 2: Growth, Development and Seed Biology of Feather Fingergrass (*Chloris virgata*) in Southern Australia

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Tuesday, 7 February 2017

Dear The Duc Ngo,

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Weed Science 2017

Growth, Development, and Seed Biology of Feather Fingergrass (*Chloris virgata*) in Southern Australia

The D. Ngo, Peter Boutsalis, Christopher Preston, and Gurjeet Gill*

Feather fingergrass is a major weed in agricultural systems in northern Australia and has now spread to southern Australia. To better understand the biology of this emerging weed species, its growth, development, and seed biology were examined. Under field conditions in South Australia, seedlings that emerged after summer rainfall events required 1,200 growing degree days from emergence to mature seed production and produced 700 g m^{-2} shoot biomass. Plants produced up to 1,000 seeds $panicle^{-1}$ and more than 40,000 seeds $plant^{-1}$, with seed weight ranging from 0.36 to 0.46 mg. Harvested seeds were dormant for a period of about 2 mo and required 5 mo of after-ripening to reach 50% germination. Freshly harvested seed could be released from dormancy by pretreatment with 564 mM sodium hypochlorite for 30 min. Light significantly increased germination. Seed could germinate over a wide temperature range (10 to 40 C), with maximum germination at 15 to 25 C. At 20 to 25 C, 50% germination was reached within 2.7 to 3.3 d, and the predicted base temperature to germinate was 2.1 to 3.0 C. The osmotic potential and NaCl concentration required to inhibit germination by 50% were −0.16 to −0.20 MPa and 90 to 124 mM, respectively. Seedling emergence was highest (76%) for seeds present on soil surface and was significantly reduced by burial at 1 (57%), 2 (49%), and 5 cm (9%). Under field conditions, seeds buried in the soil persisted longer than those left on the soil surface, and low spring–summer rainfall increased seed persistence. This study provides important information on growth, development, and seed biology of feather fingergrass that will contribute to the development of a more effective management program for this weed species in Australia.

Nomenclature: Feather fingergrass, *Chloris virgata* Sw.

Key words: Base temperature, dormancy, emergence, germination, seedbank persistence, and survival.

Feather fingergrass is a warm-season, C_4, annual grass that is widely distributed globally. It generally grows throughout tropical, subtropical, and warm temperate regions, extending well into temperate regions in areas where hot summers are typical (Anderson 1974). In Australia, this grass has been a major weed in cotton (*Gossypium hirsutum* L.) and grain crops in the subtropical region of Australia for many years (Werth et al. 2013). In a survey of the northern subtropical grain region of Australia in 2008 and 2010, feather fingergrass was ranked in the top 20 weeds, and it would be expected to further increase in dominance in a glyphosate-based system (Werth et al. 2013). It was the third most common weed found (50% of paddocks) in a summer fallow survey in 2012 (Widderick et al. 2014). This grass was a weed of vineyards and orchards in the state of South Australia and in parts of the grain region in the state of Western Australia (Osten 2012). More recently, this species has been ranked in the top 10 (national ranking) and top four (northern regional ranking) residual weeds in all crops in Australia (Llewellyn et al. 2016). Overreliance on glyphosate in reduced tillage systems has favored feather fingergrass, which tends to have a higher level of tolerance to this herbicide relative to other weeds present in the region (Werth et al. 2013; Widderick et al. 2014).

The timing of germination and emergence is critical for the survival of annual plants (Saatkamp et al. 2009), with temperature and light among the most important environmental signals that regulate germination and emergence of a plant species (Baskin and Baskin 1998; Schutte et al. 2014). For seeds in the soil, temperature and light are important cues for initiating germination by providing a sense of their position in the soil profile and the occurrence of soil disturbance (Batlla and Benech-Arnold 2014). There have been several studies of the effect of temperature on seed germination of feather fingergrass in China, Qatar, and Australia (Bhatt et al. 2016; Fernando et al. 2016; Zhang et al. 2012, 2015). However, there

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is currently no information on the base temperature required for germination in Australia.

Seed germination, a process regulated by several environmental factors such as moisture and soil salinity, is a key event determining the success of a weed in an agroecosystem (Chachalis and Reddy 2000; Koger et al. 2004). Seeds that respond to environmental conditions and alter their germination behavior are more likely to survive and successfully establish themselves. The influence of seed age on dormancy release and the effect of burial depth on germination of feather fingergrass in southern Australia is poorly understood. The effect of osmotic stress (Fernando et al. 2016) and salinity (Fernando et al. 2016; Zhang et al. 2012) on germination of feather fingergrass was studied in China and Queensland, Australia. In China, this grass is considered as a halophyte (salt-tolerant) species and as a potentially useful grass species for saline areas (Zhang et al. 2012).

To effectively manage a weed, an understanding of its basic biology is critical. This includes germination conditions, dormancy, seedbank dynamics, growth, and development (Bhowmik 1997; Cousens and Mortimer 1995; Mennan and Ngouajio 2006). An understanding of the particular seed-dormancy mechanisms involved is vital for the development of control strategies for weeds (Adkins et al. 2002). Simpson (1990) listed 89 species of C_4 grasses from 24 genera, including *Chloris*, as having one or more forms of dormancy. In many species, the embryo has the capability to germinate, but dormancy is due to the embryo-covering structures. The mechanisms within the covering structures may involve permeability (preventing water uptake or gaseous exchange) and mechanical (preventing embryo expansion) and chemical barriers to germination (Adkins et al. 2002). It is also important to manage the soil seedbank of weed populations that have developed herbicide resistance (Kleemann et al. 2016) or other difficult to manage weeds. Persistence of the soil seedbank determines the length of time management strategies are required to reduce the population to minimal levels (Matthews 1994). Preliminary reports showed that feather fingergrass seed had short field persistence (10 to 12 mo) in the northern cropping region of Australia (Osten 2012; Widderick et al. 2014). However, seedbank persistence in cooler Mediterranean conditions of the southern and western grain regions of Australia has not been reported.

Most seed biology studies on feather fingergrass so far have been undertaken in China, India, Qatar, Honduras (Bhatt et al. 2016; Li et al. 2006; Zelaya et al. 1997), and northern Australia (Fernando et al. 2016). There is little information on growth, development, and seed biology of feather fingergrass in southern Australia. Therefore, the objectives of this study were to determine (1) growth and development under field conditions, particularly time required to reach flowering and seed production; (2) seed dormancy and mechanism; (3) effect of physical environmental factors (temperature, light, osmotic stress, salt stress, and burial depth) on seed germination and seedling emergence; and (4) seedbank persistence under field conditions.

Materials and Methods

Seed Sources. Mature seeds of feather fingergrass populations were collected from Monash (namely CV1 in June 2013), Hectorville (CV4 in June 2013), Mildura (CV5 in March 2013), and Vintners (CV14 in April 2014) in South Australia; and Emerald (CV12 in January 2011) in Queensland, Australia. Harvested seeds were cleaned and stored in paper bags at room temperature (~20 C). Seeds of these populations were collected from plants grown at a common site (Waite Campus, University of Adelaide, South Australia, Australia), and this seed was used for further studies.

Growth, Development, Seedling Emergence, and Survival

Growth and Development under Irrigated and Rainfed Conditions. A total of 200 seeds each of CV5 and CV12 were sown on January 24, 2014, in 1 m^2 plots with four replicates in randomized complete blocks in a field at Roseworthy, South Australia, Australia. The field soil was a clay loam. Before this experiment was conducted, feather fingergrass had never been observed at the experimental site, which is regularly used for crop production. After sowing, irrigation was applied weekly to prevent water stress. The time taken to reach tillering, panicle emergence and seed production, and panicle number per plant and square meter were recorded. Four panicles of each population were sampled from each replicate and dried at 25 C for 4 wk to calculate seed weight (g 100 $seeds^{-1}$) and number of seeds per panicle and square meter. Shoot biomass at flowering was determined from a quadrat (0.5 by 1 m) each of four replicates, dried in an oven at 70 C for 48 h. Remaining plants were allowed to mature and set seed from April to June 2014. Naturally shed seed germinated after rainfall on November 21 to

November 23, 2014, and January 8 to January 13, 2015. Time taken to reach tillering, panicle emergence, and seed production were determined.

Seedling Emergence and Survival. Field plots with seeds produced from April to June 2014 were used to examine seedling emergence and survival. Four quadrats (0.3 by 0.3 m) were established in the field to investigate the mortality of seedlings that emerged after the rainfall events from January 8 to January 13, 2015 in both low-density (fewer than 1,700 seedlings m^{-2}) and high density (more than 6,200 seedlings m^{-2}) areas of the field. The mortality data were recorded every 2 wk until panicle emergence.

General Germination Test Protocols. Germination was evaluated by evenly placing 25 (or 50) seeds of each population in a 15-cm-diameter petri dish containing two layers of Whatman No. 1 filter paper and moistened with 9 ml of distilled water or a treatment solution (gibberellic acid, polyethylene glycol, and salt). There were four replicates of each population of each treatment. Dishes were sealed within parafilm and placed in an incubator set at 25 C, the temperature previously determined to be in the optimum range for germination of feather fingergrass (Bhatt et al. 2016; Zhang et al. 2012, 2015). The photoperiod was set at 12 h with fluorescent lamps used to produce light intensity of 43 µmol m^{-2} s^{-1}. Seed germination was determined under 12 h alternating light/dark conditions, and germinated seeds (emerged coleoptile, radical >5 mm long) were counted every 2 d for 14 d.

Seed Dormancy. Germination tests began soon after harvest and were repeated at approximately monthly intervals for 11 mo. Previous research had shown that a tetrazolium viability test was ineffective in a closely related small-seeded species, windmill grass (*Chloris truncata* R. Br.) (Farley et al. 2013), which could be due to small seed size and dark seed color. Therefore, maximum germination at 25 C during the after-ripening period was used as the indicator of seed viability. The experiment was conducted with original field-collected seeds of CV1 and CV4 (25 seeds per replicate). As feather fingergrass can produce seeds over several weeks, the experiment was also conducted with freshly harvested seeds of CV1 and CV4 (50 seeds per replicate) grown at the Waite Campus, University of Adelaide, South Australia, Australia, to determine the influence of the timing of seed maturity on the expression of seed dormancy. Seeds that matured in February, March, and April 2015 were collected and stored, and germination was assessed using the general germination test.

Seed Germination with a Pretreatment of NaOCl. Preliminary experiments were conducted to determine a suitable treatment time (30 min) for fresh seed in 564 mM sodium hypochlorite (NaOCl) (White King Premium Bleach; Pental Products, Victoria, Australia). Four replicates of 50 seeds each were used in four different treatments to break dormancy: (1) incubation in 300 ml of 564 mM NaOCl for 30 min followed by rinsing for 30 min in running water and incubation in water; (2) incubation in 300 ml of 564 mM NaOCl for 30 min followed by rinsing for 30 min in running water and incubation in 0.5 mM gibberellic acid (GA_3); (3) incubation in 0.5 mM GA_3; and (4) incubation in water. Freshly harvested seeds of CV12 were used for this experiment, and the experiment was conducted three times with 15-, 30-, and 45-d-old seeds.

Effect of Temperature on Germination. Non-dormant (12-mo-old) seeds (25) of each replicate of CV4 and CV5 were used to examine the effect of temperature on germination. Seeds were incubated at six different constant temperature regimes (10, 15, 20, 25, 30, and 40 C). Germinated seeds were counted and recorded daily for the estimation of base temperature. Germination tests were terminated when no further germination occurred for 7 d, and the maximum germination (G_{max}) was expressed as the percentage of total seeds used.

Effect of Light on Germination. The effect of two light regimes (12 h alternating light/dark and 24 h dark) on germination of nondormant (more than 7 mo old, 50 seeds per replicate) seeds of CV4 and CV5 was examined. The 24 h dark treatment was achieved by wrapping each petri dish in two layers of aluminum foil. The petri dishes of both treatments were only opened after 14 d, and the number of germinated seeds were counted. The experiment was conducted twice.

Effect of Osmotic Stress on Germination. Four replicates of 50 nondormant (12-mo-old) seeds each of CV4 and CV5 were used to investigate the effect of osmotic stress on germination. Solutions with osmotic potentials of 0, −0.1, −0.2, −0.4, −0.6, −0.8, and −1.0 MPa were prepared by dissolving polyethylene glycol 8,000 (BioUltra, 8,000; Sigma) in distilled water as described by Michel (1983). The tests were terminated

when no further germination occurred for 7 d. The experiment was conducted twice.

Effect of Salt Stress on Germination. Salt-stress treatments were applied by using sodium chloride (NaCl) solutions of 0, 20, 40, 80, 160, 250, 320, and 500 mM. This range represents the level of salinity occurring in some soils in southern Australia (Chauhan et al. 2006). Seed source and other methods were similar to those described in "Effect of Osmotic Stress on Germination."

Seedling Emergence from Different Burial Depths. The pot experiment was conducted by placing seed at 0, 1, 2, and 5 cm below the soil surface to assess the impact of seed burial on emergence. Four replicates of 50 seeds (CV4 and CV5) each were placed at the selected depths in pots (15-cm deep by 12-cm diameter). The pots were filled with field soil (clay loam) to 1.5 cm below the rim and randomly placed on a bench. The pots were lightly watered as needed to maintain adequate soil moisture. Four pots without seeds were used as controls to check whether there was any contamination of feather fingergrass seeds in the test soil. The number of emerged seedlings were counted after 2 wk and were expressed as the percentage of total seed input. The experiment was conducted twice: in a glasshouse with natural light and temperature of 25 C and in a growth room set at 12 h photoperiod at 708 µmol m^{-2} s^{-1} and temperature of 25 C.

Seedbank Persistence under Field Conditions. A split-plot design was used for this field experiment at Roseworthy, South Australia, Australia. Main plot treatments (seeds at the soil surface or buried at 5-cm depth) were randomly assigned among the four replicates. Within each main plot, subplots (populations) were randomly assigned. A total of 25 (or 50) seeds from each population (at least 7 mo old) were mixed with soil and placed in a permeable nylon bag (10 by 5 cm).

Seed packages were removed at 0, 2, 4, 6, 8, 10, and 12 mo, and seeds were germinated in an incubator for 14 d. In addition, seeds of the same populations stored in the lab were germinated at the same time as the seeds removed from the field. Seed viability (%) was expressed as germination count of seeds removed from 0 or 5 cm in the field relative to the maximum germination count (as maximum viability) of seeds stored in the lab. The experiment was conducted three times in late July 2013 with CV5, in late August 2014 with CV5 and CV14 (25 seeds per replicate), and in early April 2015 with CV1 and CV4 (50 seeds per replicate).

Statistical Analyses

Estimation of Base Temperature. Several methods to estimate the minimum temperature thresholds for seed germination were compared, and the reciprocal time to 50% of germination was the most statistically robust and biologically relevant method (Steinmaus et al. 2000). A logistic function was used to analyze germination response of each replicate (Prism v. 6.00; GraphPad, La Jolla, CA):

$$Y = 100/\{1+10\hat{}[(\log T_{50}-X)^{*}\text{HillSlope}]\} \quad [1]$$

where Y is the percentage of cumulative germination, X is the time (in hours), germination rate (T_{50}) is the time required for the germination of half the total germinated seeds, and HillSlope describes the steepness of the family of curves.

A linear regression was performed with germination rates of the four replicates against incubation temperature. The base temperature (T_b) was estimated as the intercept of the specific regression line with the temperature axis. As G_{max} of both CV4 and CV5 decreased by more than 50% at 40 C as compared with the optimum temperature, the T_{50} value of this temperature was excluded from the estimation of T_b.

The estimated T_b value was used to calculate the growing degree days (GDD) to tillering, panicle emergence, and seed production by the following equation:

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

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

As there was no experiment (or population) by treatment interaction, data from two experiments (osmotic and salt stress), two experiments and two populations (burial depth), and two populations (seedbank persistence) were combined for analysis.

Exponential models (one-phase decay) were selected as the best fit for survival of emerged seedlings, germination at different concentrations of osmotic potential, and changes in viable seeds (seedbank persistence). A logistic function provided the best fit for germination of seeds after harvest (dormancy) and germination at different concentrations of NaCl.

Table 1. Growth and development under field conditions.

			Thermal time and days from emergence to:[a]					
			Tillering		Panicle emergence		First mature seed	
Condition	Emergence date	Population	Cd	d	Cd	d	Cd	d
Irrigated	January 24, 2014	CV5	261 ± 37ab	12 ± 1.3a	816 ± 61a	45 ± 2.3a	1,145 ± 63a	67 ± 2.4a
		CV12	309 ± 9bc	14 ± 0.3a	1,000 ± 33b	57 ± 1.5b	1,256 ± 33a	77 ± 1.5b
Rainfed	November 23, 2014	CV5	247 ± 29a	14 ± 1.9a	1,741 ± 31c	96 ± 1.7c	2,095 ± 19b	118 ± 1.4c
	January 10, 2015	CV12	330 ± 30c	19 ± 3.2a	1,813 ± 33c	117 ± 3.5d	—	—

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

The Shapiro-Wilk test for normality was used to examine the residuals of the original percentage data. If the residuals failed to meet the assumptions ($P < 0.05$), the original percentage values (response to NaOCl, effect of light) were arcsine transformed before ANOVA. Otherwise, the original percentage values (effect of temperature: G_{max}) were used for ANOVA. Fisher's protected LSD multiple comparisons were employed to differentiate between predicted means, and means were presented as back-transformed data (GenStat 17; VSN International, Herts, UK).

Results and Discussion

Growth, Development, Seedling Emergence, and Survival

Growth and Development. Under field conditions, it took 260 to 330 Cd (12 to 19 d) from seedling emergence to tillering, and this value was similar for both irrigated and rainfed environments. Plants growing under irrigated conditions required 816 to 1,000 Cd and 1,145 to 1,256 Cd to reach panicle and seed maturity stages, respectively (Table 1). This thermal time to panicle emergence of feather fingergrass is similar to that of shattercane [*Sorghum bicolor* (L.) Moench ssp. *arundinaceum* (Desv.) de Wet & Harlan] in a well-watered environment with 861 to 1,126 Cd (Donatelli et al. 1992), but is much greater than that of southern sandbur (*Cenchrus echinatus* L.) with 518 Cd (Machado et al. 2014). However, almost double the thermal time was required to reach the panicle (1,740 to 1,810 Cd) and seed maturity (2,095 Cd) stages in rainfed compared with irrigated conditions (Table 1). This delay in plant development under rainfed conditions may be associated with water stress due to extremely low rainfall over the summer months in 2014 to 2015 (Figure 1). A previous study also found that the thermal time to flowering in shattercane increased with an increase in water stress when transpiration was reduced to 0.5 or less of that in irrigated controls (Donatelli et al. 1992). The delay in flowering due to severe drought stress could be a result of abscisic acid accumulation under water deficit (Blum 1996).

Under irrigation, feather fingergrass produced 468,000 to 573,000 seeds m^{-2}, with an average seed weight of 0.4 to 0.5 mg (67 to 77 d after sowing) and 619 to 730 g of dry biomass m^{-2} (89 d after sowing) (Table 2). The mean seed weight obtained was lower than that reported (0.63 mg) in north-eastern China (Zhang et al. 2015). The dry biomass and seed production values of feather fingergrass were much higher than those of naturally occurring windmill grass (146 g m^{-2} and 61,000 seeds m^{-2}) in Merredin, Western Australia (Borger et al. 2011). If these large amounts of feather fingergrass seeds were dispersed into cropping fields and successfully established, this summer weed would reduce the potential yield of winter crops by utilizing moisture and nutrients that would otherwise be available to the crop and would delay sowing due to the time needed for weed control in the autumn (Osten et al. 2006).

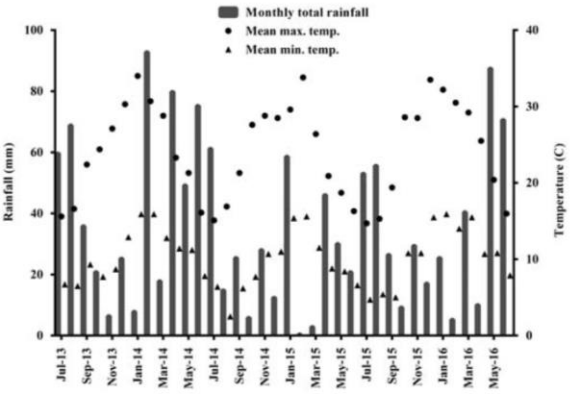

Figure 1. Monthly total rainfall and mean maximum and minimum temperatures from July 2013 to May 2016 at Roseworthy, South Australia, Australia (http://www.bom.gov.au).

Table 2. Biomass, panicle density, and seed production in an irrigated field.

Population	Biomass	Panicle plant^{-1}	Seed panicle^{-1}	Seed m^{-2}	Seed weight
	g m^{-2}	panicle	seed	1,000 seed	mg
CV5	619 ± 53	37.4 ± 6.6	1,071 ± 64	573 ± 43	0.360 ± 0.017
CV12	730 ± 89	42.7 ± 6.7	1,244 ± 72	468 ± 120	0.455 ± 0.020
P[a]	0.223	0.425	0.090	0.202	0.009

[a] P: probability of *t*-test of null hypothesis that mean ± SE of CV5 is equal to mean ± SE of CV12.

Seedling Emergence and Survival. Large numbers of seedlings emerged after the rainfall events from January 8 to January 13, 2015, and seedling survival of this cohort was investigated. Exponential (one-phase decay) models fitted well to seedling density over time in both low- and high-density plots (Figure 2). At 10 d after seedling emergence started, seedling density in the high-density plots was 3.6-fold higher than those in the low-density area. Seedling density decreased rapidly by 50% in both low- and high-density plots at 10 to 12 d (half-life) after emergence. Self-thinning of seedlings reduced the difference between the high- and low-density patches with time, particularly beyond 50 d after emergence (Figure 2). This self-thinning is likely to be mainly due to drought stress, as there was little rain from February to March 2015 (Figure 1). Drought was also the major cause of mortality in seedling populations of 20 other species establishing under natural conditions (Moles and Westoby 2004). The self-thinning of feather fingergrass in this study provides additional evidence for the operation of density-dependent population regulation in plants (Weller 1987; Yoda et al. 1963). Plants that survived until April 5, 2015, received rainfall from April 6 to April 27, 2015 with a total of 45.6 mm (Figure 1), which allowed them to produce seeds. Results of this study provide evidence for the adaptation of feather fingergrass to the dry summer conditions of South Australia, where despite these conditions, plants were able to emerge, survive, and produce a large amount of seed to complete their life cycles.

Seed Dormancy. Germination response to time after harvest fitted well to a logistic model ($R^2 = 0.90$ to 0.98). Under room storage conditions, freshly harvested seeds did not germinate until they had after-ripened for about 2 mo (Figure 3). The dormancy pattern was not affected by the times of seed maturation. Seeds that had matured in February, March, and April 2015 had very similar dormancy patterns (Figure 3b and c). Germination of fresh seed was nil. Seeds required about 5 mo after maturity to achieve more than 50% germination. Germination reached a maximum at 8 to 9 mo after maturity (Figure 3). Feather fingergrass in Central Queensland also required an after-ripening period of 6 to 10 wk to germinate (Osten 2012); however, 39%, 50%, and 98% of fresh seeds collected in Honduras, Qatar, and northern China, respectively, could germinate (Bhatt et al. 2016; Li et al. 2006; Zelaya et al. 1997). The differences in level of dormancy among studies may be due to the wide geographic and climatic range of this species, which could have selected for different levels of dormancy and dormancy mechanisms in different regions (Loch et al. 2004).

Response of Dormant Seeds to NaOCl. Germination of fresh dormant seeds was significantly ($P \leq 0.01$) increased by treatment with NaOCl. NaOCl increased germination of fresh seeds (15, 30, and 45 d old) by 10-fold, whereas GA_3 alone could not overcome the dormancy in feather fingergrass. Furthermore, treatment with NaOCl followed by GA3 did not increase seed germination as compared with

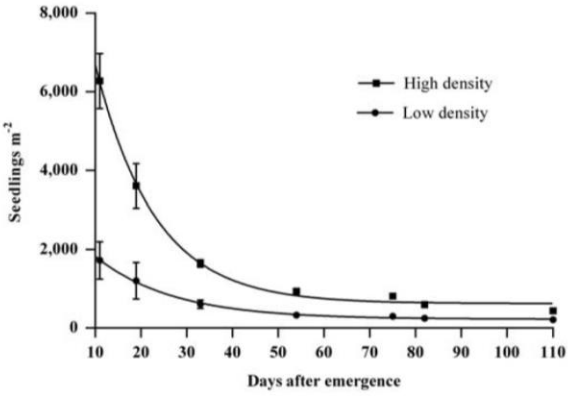

Figure 2. Survival of seedlings following rainfall January 8 – 13, 2015. The fitted lines represent one-phase decay exponential model: $Y = 2891.2*\exp(-0.05805*X) + 225.8$ ($R^2 = 0.59$) for low-density; and $Y = 13188.7*\exp(-0.07738*X) + 623.3$ ($R^2 = 0.92$) for high-density plots. Each data point represents the mean of four replicates, and the vertical bars are SE of the mean.

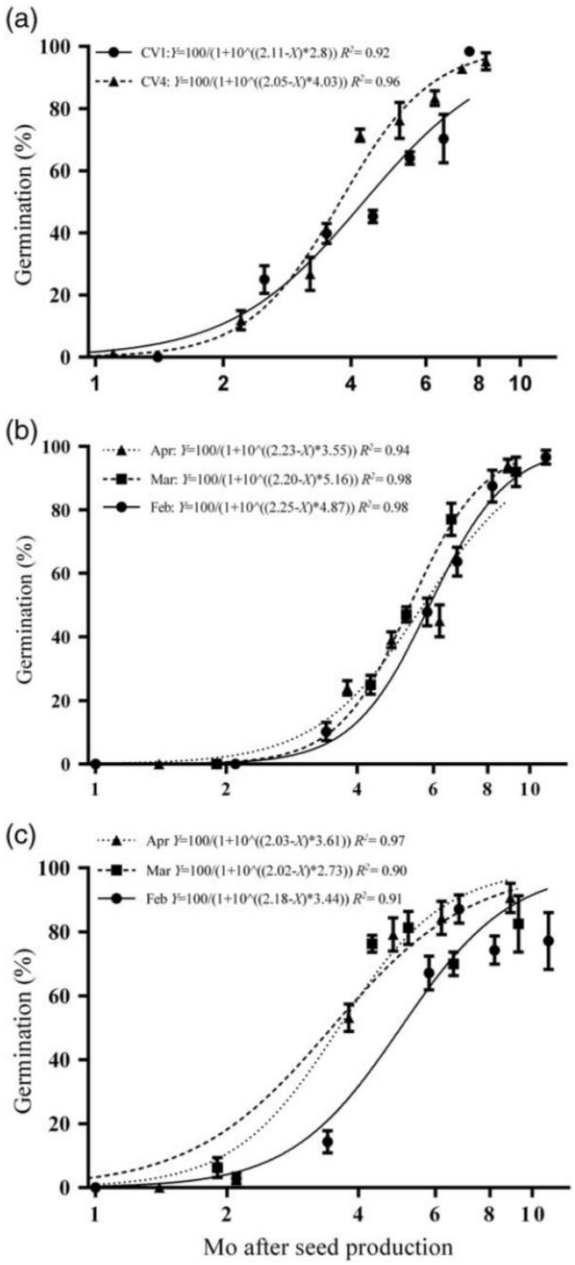

Figure 3. Dormancy pattern of CV1 and CV4 collected in June 2013 (a); CV1 (b) and CV4 (c) matured in February, March, and April 2015. Each data point represents the mean of four replicates, and the vertical bars are SE of the mean.

NaOCl alone (Table 3). Hsiao and Quick suggested two major roles for NaOCl in the termination of dormancy (Hsiao 1979, 1980; Hsiao and Quick 1984). First, NaOCl may modify the properties of the hull and seed coat membranes in such a way as to increase water uptake by the embryo, leading to the release from dormancy. Second, NaOCl could act as oxidant on vital pregermination processes within the caryopsis. The prevention of water uptake is not a common dormancy mechanism found in grass species. However, the capacity for gaseous exchange might be limited by the tissues surrounding the embryo (Adkins et al. 2002). The dormancy mechanism of feather fingergrass in this study could be based on embryo-covering structures of the seed.

Effect of Temperature on Germination. Seed could germinate across a wide range of temperatures from 10 to 40 C. The optimum temperatures for maximum germination (G_{max}) ranged from 15 to 30 C (unpublished data). Based on G_{max}, germination was significantly inhibited at 40 C. Previously, it was reported that feather fingergrass could germinate across temperatures of 5 to 40 C (Zhang et al. 2012, 2015), and germination was similar among three temperature regimes 15/25, 20/30, and 25/35 C (Bhatt et al. 2016).

Seed germinated faster as temperature increased (Figure 4). The optimum temperatures for both G_{max} and germination rate (T_{50}) were 20 to 25 C. It required about 2.7 to 3.3 d for 50% germination at 20 to 25 C. Our results are consistent with previous studies in China, which reported 1 to 3 d to reach half of the maximum germination (Li et al. 2006; Liu et al. 2003).

By plotting temperature against the inverse of time to 50% germination, the base temperature (T_b) for germination was estimated to be 2.1 to 3.0 C (Figure 5). This estimate of T_b is consistent with the findings of Zhang et al. (2015), who reported that more than 80% of feather fingergrass seed could germinate at 5 C, which is difficult to reconcile with their estimate of 7.0 C for T_b. Our estimated T_b value for feather fingergrass is much lower than those of four other annual summer grass weeds, namely green foxtail [*Setaria viridis* (L.) Beauv.], yellow foxtail [*Setaria pumila* (Poir.)], large crabgrass [*Digitaria sanguinalis* (L.) Scop.], and goosegrass [*Eleusine indica* (L.) Gaertn.] with T_b for germination of 6.1, 8.3, 8.4, and 12.6, respectively (Masin et al. 2005). With rapid germination and a low base temperature, feather fingergrass can germinate and emerge under field conditions after rainfall events in South Australia that would maintain adequate soil moisture for a few days only in spring. Within this study period, suitable conditions for seed germination and seedling establishment were created by rainfall events in summer in 2014 (November 21 to 23) and summer (January 8 to 13) and autumn

Table 3. Germination response of freshly harvested seeds to NaOCl and GA_3 treatments.

	Germination (%) of seeds of different age[a]		
Treatment	15 d	30 d	45 d
H_2O	5.0 ± 1.3a	4.0 ± 1.4a	4.5 ± 2.5a
0.5 mM GA_3	6.5 ± 3.3a	10.5 ± 2.2a	4.0 ± 1.4a
564 mM NaOCl for 30 min → H_2O	54.0 ± 3.6b	56.5 ± 5.7b	74.5 ± 5.7b
564 mM NaOCl for 30 min → 0.5 mM GA_3	61.5 ± 4.6b	48.5 ± 1.5b	67.5 ± 2.2b

[a] Values (mean ± SE) within a column followed by different letters are significantly different (Fisher's protected LSD test: $P \leq 0.01$).

(April 6 to 8) of 2015 (Table 1 and Figure 1; emergence data in April 2015 not shown).

Effect of Light on Germination. Germination was significantly stimulated by light, but the light effect was variable across populations and years (Table 4). In 2014 germination increased from 17% to 35% in the dark to 72% to 78% in the light. Similarly, in 2015 germination increased from 2% to 33% in the dark to 70% to 85% when exposed to light (Table 4). Similar results were also found in feather fingergrass in Queensland, Australia (Fernando et al. 2016). Light requirement for germination is common among small-seeded species and warm-season grasses (Adkins et al. 2002; Grime et al. 1981; Milberg et al. 2000). Light may stimulate germination by altering the balance of germination promoters and inhibitors in the embryo (Adkins et al. 2002). The light requirement can ensure that germination takes place away from other vegetation and only on or close to the soil surface (Adkins et al. 2002; Milberg et al. 2000), which would enhance the probability of seedling survival of such small-seeded weed species.

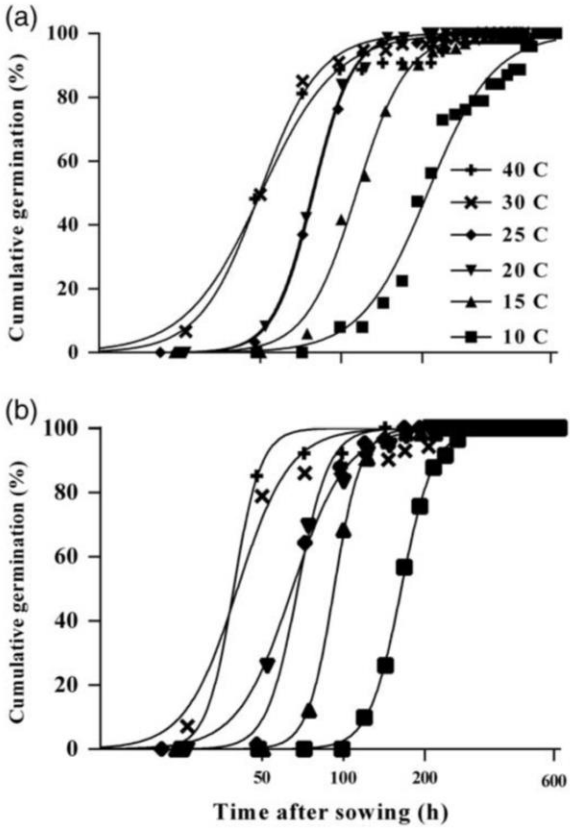

Figure 4. Cumulative germination of CV4 (a) and CV5 (b) at different temperatures. Each data point represents the mean of four replicates, and curves were fitted using Equation 1. R^2 of the fitted curves for both CV4 and CV5 ranged from 0.92 to 0.99.

Effect of Osmotic Stress on Germination. Germination decreased exponentially with increased osmotic potential and was completely inhibited at −1.0 MPa (Figure 6). Similarly, for other annual summer grasses, the base water potentials for

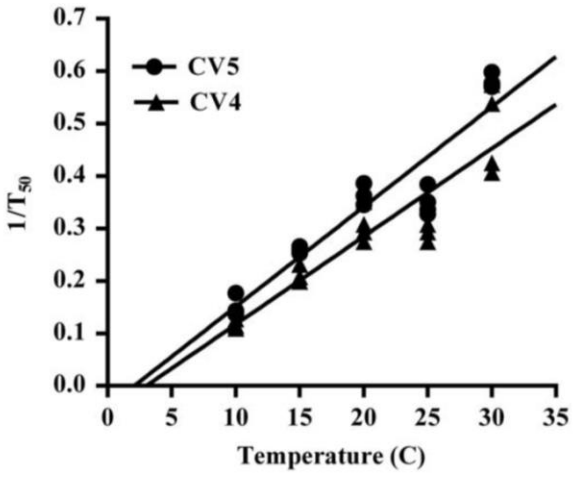

Figure 5. Base temperature (T_b) estimation of CV4: $Y = 0.01678*X - 0.05059$ ($R^2 = 0.93$); and CV5: $Y = 0.01906*X - 0.03959$ ($R^2 = 0.90$). Each data point represents a replicate.

Table 4. Effect of light on germination.[a]

Year	Population	Germination (%)	
		24 h dark	12 h light/dark
2014	CV4	35.0 ± 3.4b	78.0 ± 1.2cd
	CV5	17.0 ± 5.3a	72.0 ± 6.5c
2015	CV4	2.3 ± 1.6a	84.7 ± 4.0d
	CV5	33.0 ± 2.1b	70.0 ± 2.5c

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

germination of yellow foxtail, green foxtail, large crabgrass, and goosegrass were −0.7, −0.7, −0.8, and −1.2 MPa, respectively (Masin et al. 2005). In another study, base water potential for rigid ryegrass (*Lolium rigidum* Gaudin) in South Australia was −1.2 MPa (Chauhan et al. 2006). The osmotic potential required for 50% inhibition of the maximum germination was −0.16 to −0.20 MPa (Figure 6), which was higher than that of feather fingergrass (−0.09 MPa) in Queensland, Australia (Fernando et al. 2016). This means feather fingergrass has a similar base water potential to other grass weed species, is not drought tolerant at germination, and only germinates when there is adequate soil moisture (osmotic potential less than −1.0 MPa).

Effect of Salt Stress on Germination. Germination response to NaCl stress fitted well to a logistic model ($R^2 = 0.92$ to 0.94). Germination was not inhibited below 40 mM NaCl. The concentration of NaCl required to inhibit germination by 50% was estimated to range from 90 to 120 mM, which was

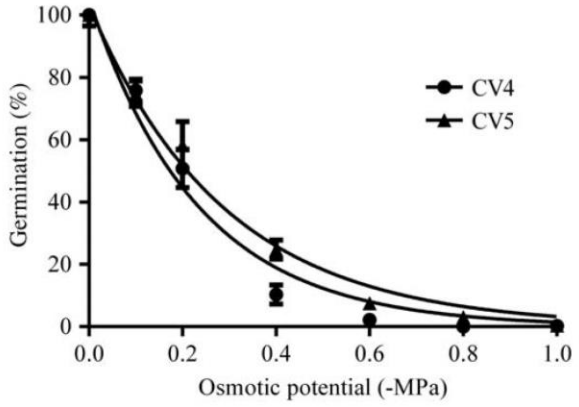

Figure 6. Effect of osmotic potential (MPa) on germination at 25 C and 12 h alternating fluorescent light/dark. The fitted line represents an exponential model: $Y = 105.2*\exp(-4.286 *X)$ ($R^2 = 0.93$) for CV4; and $Y = 103.2*\exp(-3.459*X)$ ($R^2 = 0.92$) for CV5. Each data point represents the mean of two experiments pooled with four replicates. Vertical bars are SE of the mean.

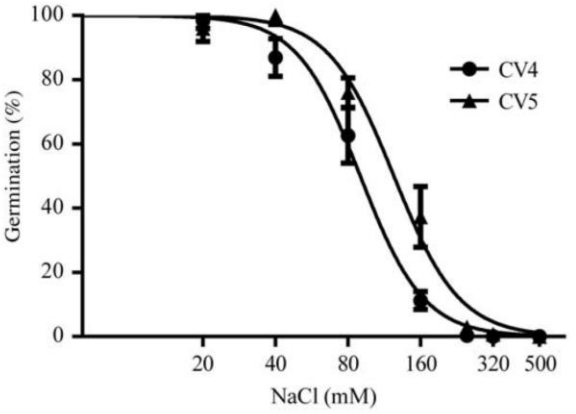

Figure 7. Effect of salt stress (NaCl) on germination at 25 C and 12 h alternating fluorescent light/dark. The fitted line represents a logistic response equation: $Y = 100/\{1 + 10\text{^}[(1.954 - X)* - 3.298]\}$ ($R^2 = 0.94$) for CV4; and $Y = 100/\{1 + 10\text{^}[(2.094 - X)* - 3.193]\}$ ($R^2 = 0.92$) for CV5. Each data point represents the mean of two experiments pooled with four replicates. Vertical bars are SE of the mean.

much higher than that of feather fingergrass (less than 50 mM) in Queensland, Australia (Fernando et al. 2016). Seed germination was completely inhibited at 320 to 500 mM NaCl (Figure 7). At 250 mM NaCl, there was less than 3% germination in this study (Figure 7), which is much lower than that of a population (more than 80%) from the Songnen Grassland in China at this level of salt stress (Zhang et al. 2012). These differences between studies could reflect adaptation of ecotypes in China to soil salinity.

Seedling Emergence from Different Burial Depths. Seedling emergence response to different burial depths was linear ($R^2 = 0.99$), decreasing significantly as the depth of seed burial increased (Figure 8). Similarly, 2-cm depth of seed burial had a significant impact on the emergence of feather fingergrass in Queensland (McLean et al. 2014). Seedling emergence was highest for seeds on the soil surface (76%), but reduced significantly to 57%, 49%, and 9% for seeds buried at 1, 2, and 5 cm, respectively (Figure 8). This reduction in seedling emergence is consistent with the stimulation of seed germination by exposure to light observed in this study (Table 4). Ability of some seeds of this species to germinate and emerge in the dark at shallow burial depth (1 and 2 cm) could be an advantage in fields used for crop production where seeds are likely to be buried by tillage or a seed drill at planting time. In fact, seeds buried at shallow depth could

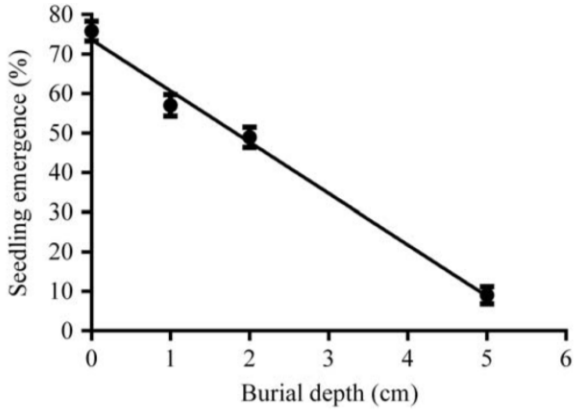

Figure 8. Linear relation between seedling emergence (CV4 and CV5) and burial depths. Each data point represents the mean of two experiments and two populations pooled with four replicates. Vertical bars are SE of the mean. Estimated equation for regression line $Y = -12.97^{*}X + 73.63$ ($R^2 = 0.99$).

have a higher probability to germinate and emerge, because after each rainfall event, the soil is likely to remain moist for longer at depth than on the surface. Therefore, weed seeds present on the soil surface over hot summer months are likely to desiccate faster than seeds buried at shallow depths.

Deep burial (5 cm) significantly reduced seedling emergence (9%) compared with seeds on the soil surface (76%). As seeds are small (0.3 to 0.4 mg), they are unlikely to have sufficient energy reserves or coleoptile length to emerge from deep burial. Therefore, emergence in fields where deep tillage was applied was likely to be much lower than in fields where crops were grown with zero- or no-till systems (McLean et al. 2014; Widderick et al. 2014). Widespread adoption of no-till cropping systems in Australia is likely to favor invasion by feather fingergrass.

Seedbank Persistence under Field Conditions. Under field conditions, viability of seeds decreased exponentially with time (R2 = 0.71 to 0.93), and was influenced by the amount of summer rainfall received over the 3 yr (Figure 9). In 2013 to 2014 and 2014 to 2015 experiments, seeds at 5-cm depth initially lost their viability faster than those left on the soil surface. At 2 mo after seed burial, seed viability at 5-cm depth decreased to about 20%, whereas the number of viable seeds on the soil surface remained high (50% to 75%) (Figure 9a and b). Some seeds could have germinated in the dark under more favorable moisture conditions, and this could have contributed to the faster decline of viable seeds buried at 5 cm. Seeds placed on the soil

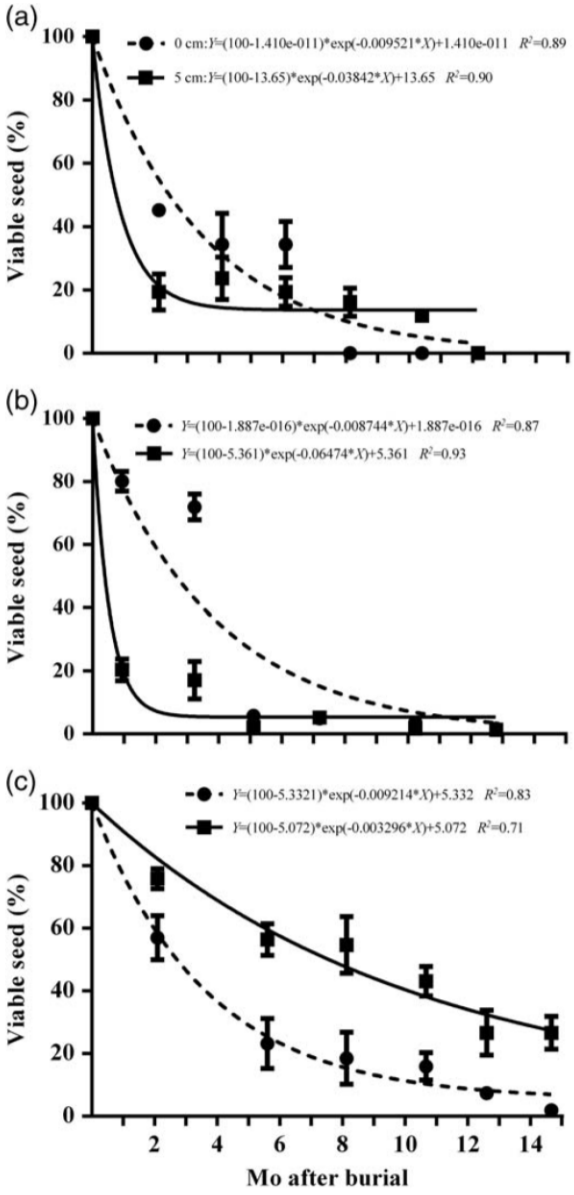

Figure 9. Changes in viability of seeds at the soil surface compared with seeds at 5-cm burial depth, conducted in July 2013 for CV5 (a), in August 2014 for CV5 and CV14 (b), and in April 2015 for CV1 and CV4 (c). Each data point represents the mean of four replicates (a) or two populations pooled with four replicates (b and c). Vertical bars are SE of the mean.

surface would have dried faster and maintained greater seed viability. Seed viability of the surface-stored seeds decreased rapidly from 34% to 0% from January to March (8 mo after burial) 2014 and from 71% to 5% from December 2014 to January 2015 (5 mo after burial) (Figure 9a and b). This rapid loss of viability in

surface seeds was associated with 92 mm rainfall in February 2014 and 58 mm in January 2015 (Figure 1). After these heavy rainfall events, favorable moisture and temperature conditions could have stimulated seed germination in the field and reduced the viable seedbank.

In the 2013 to 2014 and 2014 to 2015 experiments, seed viability both on the soil surface and at depth was almost completely lost (0% to 3.4% viable seed) after 12 mo (Figure 9a and b). These results are consistent with previous reports from the northern cropping region of Australia, where seeds of feather fingergrass persisted only for 10 to 12 mo, irrespective of burial depth (Osten 2012; Widderick et al. 2014). However, seed persistence in the 2015 to 2016 experiment was much greater than in 2013 to 2014 and 2014 to 2015. In 2015 to 2016, seeds buried at 5 cm had greater viability in all assessments than those placed on the soil surface (Figure 9c). Viability of seeds removed at 8 mo after placement was 18% for those on the soil surface compared with 54% for those at 5-cm depth. After 14 mo, seed viability on the soil surface was almost completely lost (2% viable seed), whereas seeds buried at 5 cm still had 25% viability (Figure 9c). An extremely dry summer in 2015 to 2016 could have increased seed persistence compared with 2013 to 2014 and 2014 to 2015, when significant rainfall events occurred (Figure 1). These differences in seed persistence between years could be associated with greater seed germination and seed decay in wet and warm conditions than in dry and warm conditions. Our results suggest that feather fingergrass populations are unlikely to develop persistent seedbanks and could be reduced quickly if no further seeds are added to the seedbank by dispersal. Our results also indicate that an adequate seedbank can be present in spring to early summer (September to December) or over summer (September to February) for the recruitment of seedlings of this weed species in southern Australia. As shown earlier, plants can emerge, establish, and produce seeds when temperature and moisture are favorable, as was the case after spring (2014) and summer (2015) rainfall events (Table 1 and Figure 1). Plants have high fecundity, with up to 1,000 seeds panicle^{-1} and more than 40,000 seeds plant^{-1} (Table 2). Consequently, even a small seedbank can lead to successful colonization of agricultural and nonagricultural land.

Feather fingergrass has several characteristics that enable it to survive and persist in the Mediterranean environment of South Australia. It has rapid germination and a low base temperature (2.1 to 3.0 C), so it can germinate and emerge under field conditions after rainfall events in spring, summer, and autumn in South Australia. It has a short period to maturity, requiring 300 and 1,200 Cd from seedling emergence to tillering and mature seed stages, and high fecundity. Germination of this small-seeded species was stimulated by light, and seedling emergence was highest for seeds present on the soil surface, but declined significantly for seeds buried at 1, 2, and 5 cm. Seeds were dormant for about 2 mo after maturity; however, seeds buried at 5-cm depth remained viable for more than 14 mo, whereas seeds on the soil surface lost viability after 12 mo. Low rainfall over the spring and summer in the third year of this study extended seedbank persistence beyond 14 mo, especially for the seeds buried at 5-cm depth. These characteristics also make feather fingergrass ideally suited to the no-till farming systems widely adopted in southern Australia, and it is likely this species will become a problem in such production systems.

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Chapter 3: Plant Development and Seed Biology of Windmill Grass (*Chloris truncata*) in Southern Australia

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Monday, 20 February 2017

Dear The Duc Ngo,

Ngo, T.D, Boutsalis, P., Preston, C and Gill, G. (2017) 'Plant development and seed biology of windwill grass (*Chloris truncata*) in southern Australia, ***Weed Science,*** **Cambridge University Press**

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The article has not yet been published but was accepted by ***Weed Science*** on 19 February 2017.
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Contribution to the Paper	Planned the study, conducted all experiments, analysed and interpreted data, wrote the manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

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

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Contribution to the Paper	Supervised development of work, reviewed the studies, helped in data interpretation and edited the manuscript.		
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Plant development and seed biology of windmill grass (*Chloris truncata*) in southern Australia

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Windmill grass is a major weed in agricultural systems in northern Australia and it is now become more common in southern Australia. Little information is available on the biology of this weed species in southern Australia so studies were conducted to investigate plant development and seed biology. Under irrigated field conditions in South Australia, windmill grass required 748 to 786 degree days from emergence to mature seed production. Freshly harvested seed had low dormancy with 16 to 40% germination. Seeds required light exposure for germination and less than 2% germination was observed in complete darkness. Seed could germinate over a wide temperature range (10 to 40 C) with maximum germination at 20 to 25 C. At 25 to 30 C, 50% germination occurred within 27.3 to 45.5 h and the predicted base temperature for germination of the two populations investigated ranged from 9.2 to 11.2 C. The NaCl concentration and osmotic potential required to inhibit germination by 50% were 51 to 73 mM and -0.27 MPa. Seedling emergence was completely inhibited by burial of seed, which is consistent with its absolute requirement for light exposure to begin germination. Under field conditions, there was no clear effect of burial depth on seed viability in the first two years with average rainfall and seeds were completely non-viable after 12 mo. However, in the 3rd year with low spring-summer rainfall, buried seeds (37% viability after 14 mo) persisted longer than those left on the soil surface

(6% viability after 14 mo). This study provides important information on plant development and seed biology of windmill grass, which will contribute to the development of a management program for this weed species in southern Australia.

Nomenclature: Windmill grass, *Chloris truncata* R. Br.

Keywords: Base temperature, dormancy, emergence, germination, seedbank persistence.

Windmill grass (*Chloris truncata* R. Br) is a warm season, C_4, annual or short-lived perennial grass native to Australia (Mitchell, 1994; Nightingale et al., 2005). In Australia, some native grass species have the ability to establish in disturbed ecosystems that have regular cultivation, fertiliser inputs, ruminant grazing or crop competition (Kloot, 1985; Michael et al., 2012a). This includes windmill grass which has been a significant weed in cotton (*Gossypium hirsutum* L.) and grain crops in the sub-tropical region of Australia, particularly in no-till agriculture cropping systems (Werth et al., 2013). In the Western Australian grain belt, this species was found at 12% of the sites in a field survey of summer fallow sites (Michael et al., 2010a). It was projected that windmill grass could become one of the five most threatening weed species to agriculture in the southwest of Western Australia (Michael et al., 2010b). More recently, this species has been ranked as the 7th most important weed of summer fallow by yield loss (36,871,000 kg) and revenue loss (9.1 million AUD) in Australia (Llewellyn et al., 2016).

As a result of an overreliance on glyphosate for weed control, resistance to glyphosate was confirmed in windmill grass in chemical fallow sites and roadsides in Australia (Malone et al., 2012; Preston, 2016) making control of this weed in summer

fallow situations more difficult. The dominance of reduced tillage systems combined with the over-reliance on glyphosate for weed control in the fallow phase of crop rotations has also favoured this grass in northern Australia (Werth et al., 2013; Widderick et al., 2014). As a summer-annual grass species, windmill grass will reduce the potential yield of winter crops by utilising moisture and nutrients that would otherwise be available to the crop, and delay sowing due to the time taken for weed control in the autumn (Osten et al., 2006). For example, the absence of management of windmill grass over summer fallow reduced yield of the following wheat crop in Merredin, Western Australia by 0.3 ton ha^{-1} (Borger et al., 2010).

Seed germination, a process regulated by several environmental factors, such as temperature, light, moisture and soil salinity, is a key event determining the success of a weed species in an agroecosystem (Chachalis and Reddy, 2000; Koger et al., 2004). The timing of germination and emergence is critical for the survival of annual plants, with temperature and light among the most important environmental signals that regulate germination and emergence of a plant species (Baskin and Baskin, 1998; Saatkamp et al., 2009; Schutte et al., 2014). For seeds in the soil, temperature and light are important cues for initiating germination by providing a sense of their position in the soil profile and the occurrence of soil disturbance (Batlla and Benech-Arnold, 2014). In Australia, there have been several studies of the effect of temperature and light on germination of windmill grass in New South Wales (Lodge and Whalley, 1981; Maze et al., 1993). However, there is currently no information available on the base temperature required for germination of windmill grass in Australia. In addition, seeds that respond to environmental conditions and alter their germination behaviour are more likely to survive and establish successfully. The effect of burial depth and other factors that may impact germination of windmill grass in southern Australia are poorly understood.

To effectively manage a weed, an understanding of its basic biology is critical. This includes germination conditions, dormancy, seedbank dynamics and plant development (Bhowmik, 1997; Cousens and Mortimer, 1995; Mennan and Ngouajio, 2006). An understanding of the particular seed-dormancy mechanisms involved can also be useful for the development of control strategies for weeds (Adkins et al., 2002). Simpson (1990) listed 89 species of C_4 grasses from 24 genera, including *Chloris*, as having one or more forms of dormancy. In many species, the embryo has the ability to germinate, but dormancy is caused by embryo covering structures. The mechanisms within the covering structures may involve permeability (preventing water uptake or gaseous exchange), mechanical (preventing embryo expansion), and chemical barriers to germination (Adkins et al., 2002). Furthermore, it is important to manage the soil seedbank of weed populations that have developed herbicide resistance (Kleemann et al., 2016), such as windmill grass. Persistence of the soil seedbank determines the length of time management strategies are required to reduce the population to minimal levels (Matthews, 1994). A preliminary report showed that windmill grass seed had short field persistence (12 mo) in the northern cropping region of Australia (Widderick et al., 2014). However, the seedbank persistence of windmill grass in cooler Mediterranean conditions of the southern and western grain regions of Australia has not been reported.

The objectives of this study were to determine (1) plant development under field and controlled conditions (time required to reach panicle emergence and seed maturity); (2) seed dormancy; (3) effect of physical environmental factors (temperature, light, salt stress, osmotic potential and burial depth) on germination and seedling emergence; and (4) seedbank persistence under field conditions.

Materials and Methods

Seed Sources. Mature seeds of two windmill grass populations were collected in June 2013 from Buchfelde (CT2) and Smithfield (CT3) in South Australia, Australia. Harvested seeds were cleaned and stored in paper bags at room temperature (~20 C). Seeds of these populations were grown at a common site (Waite Campus, University of Adelaide, South Australia, Australia) and the seeds collected from these plants were used for further studies.

Plant Development in an Irrigated Field and Pots. A total of 200 seeds (more than 6 mo old, average germination of 77% for lab tests) from each of the CT2 and CT3 populations were mixed with sand and scattered on surface on 24 January 2014 in 1 m^2 plots with four replicates in randomized complete blocks at Roseworthy (-34.524807, 138.686362), South Australia, Australia. The field soil was a clay loam. Before conducting this experiment windmill grass had never been observed at the experimental site, which is regularly used for crop production. After sowing, irrigation was applied weekly to prevent water stress. Ten tagged seedlings in each replicate were used to assess the time taken to reach tillering, panicle emergence and seed production.

Field collected seeds of CT2 and CT3 populations were germinated in an incubator set at 25 C to produce seedlings. These windmill grass seedlings were transplanted into pots eight different times at 3 to 4 week intervals from 3 October 2013 to 8 March 2014 to determine the influence of growing season on the timing of panicle emergence. Ten seedlings (one leaf) of each population (CT2 and CT3) were grown in four pots (25-cm deep by 18-cm diameter) containing standard potting mix (Boutsalis

et al., 2012) and kept outdoors at Waite Campus, University of Adelaide, South Australia, Australia (34.970302ºS, 138.639700ºE). Plants were watered as required to maintain adequate soil moisture. The time taken to reach panicle emergence was recorded.

Standard Germination Test Protocols. Effects of seed dormancy, temperature, light, and salt or osmotic potential on seed germination were evaluated by these protocols. Germination was evaluated by evenly placing seeds of each population in a 15-cm-diameter petri dish containing two layers of Whatman No. 1 filter paper and moistened with 9 ml of distilled water or a treatment solution (e.g., polyethylene glycol and salt). There were four replicates of each treatment. Dishes were sealed with parafilm and placed in an incubator (Cat. No R1235D, Ser. No SO 17; S.E.M. Pty Ltd, Magill, SA, Australia) set at 25 C, the temperature previously determined to be in the optimum range for germination of windmill grass (Lodge and Whalley, 1981; Maze et al., 1993; Michael et al., 2012b). The photoperiod was set at 12 h with fluorescent lamps used to produce light intensity of 43 µmol m^{-2} s^{-1}. Germinated seeds (emerged coleoptile, radical over 5 mm long) were counted every two days for 14 d.

Seed Dormancy. Covering structures (lemma and palea) of windmill grass seeds turn black at maturation. Mature seeds were collected and experiments started within a week after seed collection, and repeated at approximately monthly intervals for 12 mo. The Tetrazolium seed viability test is considered unreliable for windmill grass (Farley et al., 2013) and was not used in this study. Germination was calculated as a percentage of total seeds used in each test. The experiment was conducted with the original field collected seeds of CT2 and CT3 in June 2013 (25 seeds each replicate). As windmill grass can produce seeds over several weeks, the experiment was also

conducted with seeds that were freshly harvested in November and December 2014 to determine the influence of the timing of seed maturity on germination response.

Effect of Temperature on Germination. Seeds (9 mo old, 25 seeds each replicate) of CT2 and CT3 populations were used to examine the effect of temperature on germination. Seeds were incubated at six different constant temperature regimes (10, 15, 20, 25, 30 and 40 C). Germinated seeds were counted and recorded daily, and germination tests were terminated when no further germination occurred for 7 d. The maximum germination (G_{max}) was expressed as the percentage of total seeds used.

Effect of Light on Germination. The effect of two light regimes (12 h alternating light/dark and 24 h dark) on germination of CT2 and CT3 populations (5 to 7 mo old, 50 seeds each replicate) was investigated in an incubator set at 25 C. The 24 h dark treatment was achieved by wrapping each petri dish in two layers of aluminium foil. The petri dishes of both treatments were only opened after 14 d, and the number of germinated seeds were counted. The experiment was repeated.

Effect of Salt Stress and Osmotic Potential on Germination. Four replicates of 50 seeds (≥8 mo old) each of CT2 and CT3 were used to investigate the effect of salt stress (or osmotic potential) on germination. Salt stress treatments were applied by using sodium chloride (NaCl) solutions of 0, 20, 40, 80, 160, 250 and 320 mM. This selected range represents the level of salinity occurring in some soils in southern Australia (Chauhan et al., 2006b). Solutions with osmotic potentials of 0, -0.1, -0.2, -0.4, -0.6, -0.8 and -1.0 MPa were prepared by dissolving polyethylene glycol 8000 (BioUltra, 8,000; Sigma-Aldrich, Castle Hill, NSW, Australia) in distilled water as

described by Michel (1983). The tests were terminated when no further germination occurred for 7 d. The experiments were repeated.

Seedling Emergence from Different Burial Depths. A pot experiment was conducted by placing seed at 0, 1, 2 and 5 cm below the soil surface to assess the impact of seed burial on emergence. Fifty seeds of each population (CT2 and CT3) were placed at a single depth in each pot (15-cm deep by 12-cm diameter) with four replicates. The pots were filled with field soil (clay loam), sieved through a 2-mm diameter sieve, to 1.5 cm below the rim and randomly placed on a bench. The pots were lightly watered as needed to maintain adequate soil moisture. Four pots without seeds were used as controls to check if there was any contamination of the test soil with seeds of this grass species. There was no emergence of windmill grass from the control pots during the course of the experiment, confirming that there was no contamination. The number of emerged seedlings was counted after 14 d, and was expressed as the percentage of total seed input. The experiment was conducted in a greenhouse with natural light and temperature of 25 C, and repeated in a growth room set at 12 h photoperiod at 708 µmol m^{-2} s^{-1} and temperature of 25 C.

Seedbank Persistence under Field Conditions. A field experiment was conducted at Roseworthy (-34.525252, 138.686388), South Australia, Australia to determine seedbank persistence of windmill grass. The experiment was a split plot design with main plot treatments (two burial depths at 0 and 5 cm) randomly assigned among the four replicates. Within each main plot, subplots (populations) were randomly assigned. A total of 25 (Experiment 1: July 2013 to July 2014 and Experiment 2: August 2014 to September 2015) or 50 (Experiment 3: April 2015 to June 2016) seeds from each population were mixed with soil (clay loam) and placed in a permeable nylon bag (10

by 5 cm). As previously described, there was no contamination of the test soil with windmill grass seeds.

Seed bags from two burial depths in four replicates were removed at 0, 2, 4, 6, 8, 10 and 12 mo following burial. The exhumed seeds were germinated in an incubator set at 25 C 12 h light/ dark cycle for 14 d. In addition, seeds of the same populations stored in the lab were germinated at the same time as the seeds exhumed from the field. Seed viability (%) was expressed as germination count of exhumed seeds buried at 0 or 5 cm in the field relative to the maximum germination count (as maximum viability) of seeds stored in the lab during the course of the experiment. The experiment was conducted three times in time and space.

Statistical Analyses. *Estimation of base temperature.* Among several methods used to estimate the minimum temperature thresholds for seed germination, the reciprocal time to 50% of germination has been shown to be the most statistically robust and biologically relevant method (Steinmaus et al., 2000). A logistic function was used to analyse germination response of each replicate (GraphPad Prism ver 6.00, La Jolla California, USA):

$$Y = 100/\{1 + 10\text{^}[(\text{Log}T_{50} - X)*\text{HillSlope}]\} \quad [1]$$

where Y is the percentage of cumulative germination, X is the time (h), germination rate (T_{50}) is the time required for the germination of half the total germinated seeds, and HillSlope describes the steepness of the family of curves.

A linear regression was performed with the reciprocal of the T_{50} against incubation temperature. The base temperature (T_b) was estimated as the intercept of the specific regression line with the temperature axis.

The estimated T_b value was used to calculate the growing degree days (GDD) to tillering, panicle emergence and seed production by the following equation:

$$\text{GDD (degree days, Cd)} = \Sigma\,[(T_{max} + T_{min})/2 - T_b] \qquad [2]$$

where T_{max} is the daily maximum air temperature, T_{min} is the daily minimum air temperature, and T_b is the estimated base temperature for seed germination of each population.

For the plant development (pot study), seed dormancy and seedbank persistence experiments there was no population by treatment interaction, so data were pooled across populations. For the salt stress experiments there was no experiment by treatment interaction, so data were pooled across experiments. For the osmotic potential experiments there was no experiment and population by treatment interaction, so data were pooled across experiments and populations. Logistic models were fitted to data of germination at different concentrations of NaCl and different osmotic potentials (GraphPad Prism ver 6.00, La Jolla California, USA).

The Shapiro-Wilk test for normality was conducted to investigate the distribution of percentage data. As the percentage data (effect of temperature: G_{max} and effect of light) was not normally distributed (P <0.05), the original percentage values were arcsine transformed before ANOVA analysis. Fisher's protected LSD multiple comparisons were employed to differentiate between predicted means, and means were presented as back-transformed data (GenStat 17; VSN International, Herts, UK).

Results and Discussion

Plant Development in an Irrigated Field and Pots. Under irrigated field conditions, windmill grass required 272 (21 to 23), 518 to 522 (43 to 45) and 748 to 786 degree days (Cd) (74 to 75 d) from seedling emergence to reach tillering, panicle emergence and mature seed stages (Table 1). The thermal time to panicle emergence of windmill grass is similar to that of southern sandbur (*Cenchrus echinatus* L.) in a well-watered environment with 518 Cd (Machado et al., 2014). However, windmill grass in another study conducted in Merredin, Western Australia under glasshouse conditions required a greater thermal time from emergence to the start of seed production (970 Cd) (Michael et al., 2012b) than that of CT2 and CT3 populations in this study. The observed differences between the two studies may be related to the differences between populations or environments for this parameter.

In the pot studies, the thermal time to panicle emergence increased from 442 Cd for plants transplanted on 3 October 2013 to 629 Cd for plants transplanted on 8 January 2014, whereas the number of days to panicle emergence decreased from 55 to 35 d over this period (Figure 1). However, the thermal time to panicle emergence increased from 621 to 964 Cd for plants transplanted from 5 February 2014 to 8 March 2014 with a corresponding increase in days to panicle emergence from 52 to 191 over this period. Plants transplanted on 8 March 2014, when day length was less than 12 h and temperatures were cooler, remained vegetative over winter and did not produce panicles until 15 September 2014 (the following spring). This indicates that windmill grass can behave as an annual or a short-lived perennial in South Australia.

Seed Dormancy. Freshly harvested seed had low dormancy, with 16 to 40% germination at the optimum temperature (Figure 2). Lower germination (9%) was observed for freshly harvested seed in a study of windmill grass in New South Wales, Australia (Lodge and Whalley, 1981). In the present study, germination response was not affected by the timing of seed maturity. Seeds that had matured in June 2013, November 2014 and December 2014 had similar germination patterns. Seed required approximately one mo after maturity to achieve 43 to 56% germination. Germination reached a maximum (73 to 83%) at 5 to 7 mo after maturity (Figure 2). Other windmill grass populations in Western Australia and New South Wales, Australia also took more than 6 mo after seed production to achieve a maximum germination of 70% and 80%, respectively (Borger et al., 2011; Maze et al., 1993). These findings indicate that windmill grass can germinate soon after seed maturity whenever temperature and moisture are suitable, but some seed will not germinate for several months allowing it to avoid complete depletion of seedbank in adverse conditions over the summer.

Table 1. Growing degree days and days from emergence to tillering, panicle emergence, and first mature seed of two windmill grass populations (CT2 and CT3) in an irrigated field (2014).

	Tillering		Panicle emergence		First mature seed	
	Cd	d	Cd	d	Cd	d
CT2	272.3 ± 5.9	22.8 ± 0.8	521.9 ± 6.6	43.3 ± 0.5	786.1 ± 34.0	73.8 ± 2.5
CT3	272.0 ± 10.0	21.0 ± 1.1	518.4 ± 19.7	44.5 ± 1.6	747.5 ± 18.8	75.3 ± 1.8
P[a]	0.978	0.232	0.873	0.471	0.359	0.640

[a] P: probability of *t-test* of null hypothesis that mean ± SE of CT2 is equal to mean ± SE of CT3.

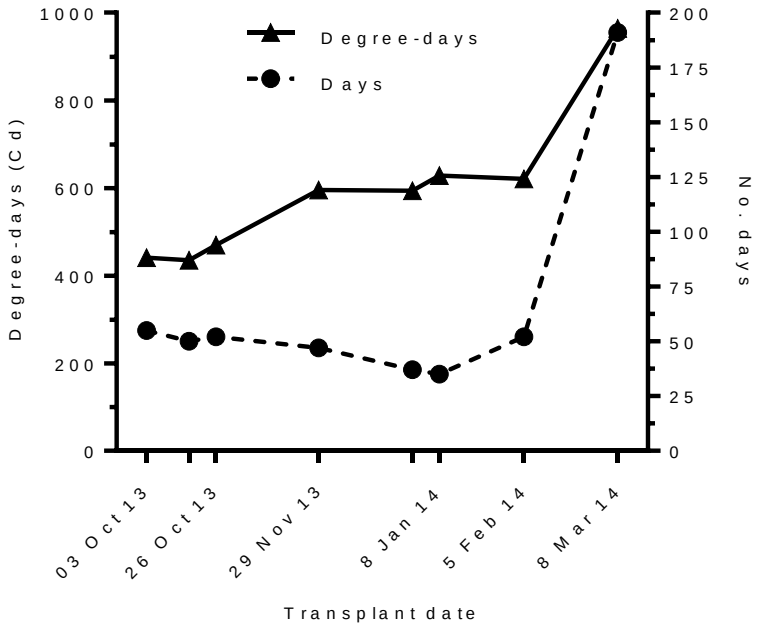

Figure 1. Growing degree-days and number of days from one leaf stage to panicle emergence of windmill grass populations at different transplant dates.

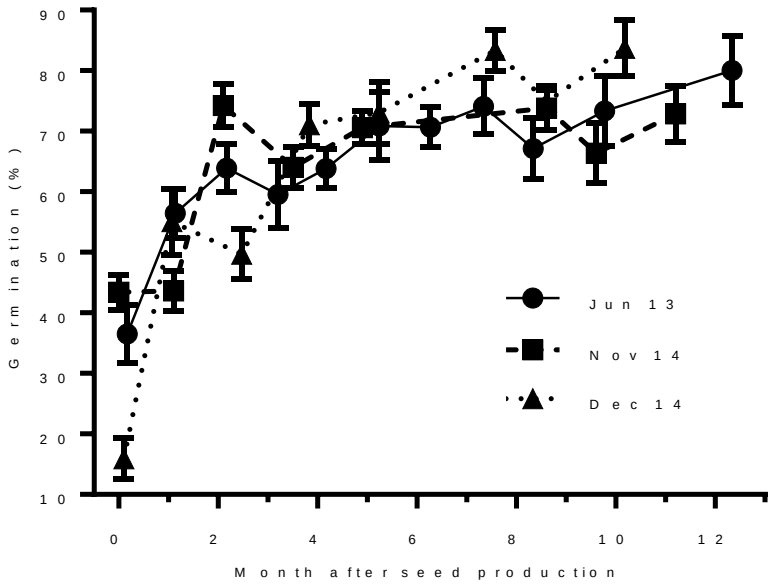

Figure 2. Germination response of CT2 and CT3 populations of windmill grass from matured seed collected in June 2013, November 2014 and December 2014. Each data point represents the mean of two populations pooled with four replicates. Vertical bars are SE of mean.

Effect of Temperature on Germination. Windmill grass seed could germinate across a wide range of temperatures from 10 to 40 C. The optimum temperatures for maximum germination (G_{max}) ranged from 15 to 30 C, with highest G_{max} of 70% (CT2) and 99% (CT3) at 25 C. G_{max} was significantly inhibited to 0 (CT3) and 5% (CT2) at 10 C, and to 13% (CT2) at 40 C (Table 2). This is consistent with previous studies that reported the optimum range for germination of windmill grass was 15 to 35 C (Lodge and Whalley, 1981; Maze et al., 1993; Michael et al., 2012b). Our results also show large variation in the tolerance of some populations (CT3) to germinate under high temperature conditions (G_{max} of 85% at 40 C) (Table 2).

Windmill grass germinated more rapidly as temperature increased. The optimum temperatures for both G_{max} and germination rate (T_{50}) of windmill grass were 25 to 30 C. It required about 27.3 to 45.5 h for 50% germination at 25 to 30 C. (Table 2). By plotting temperature against the inverse of time to 50% germination, the base temperature (T_b) for germination was estimated to be 9.2 to 11.2 C (Figure 3). This estimated T_b value is consistent with previous findings where windmill grass germination was near zero at 5 to 10 C (Lodge and Whalley, 1981; Maze et al., 1993). The estimated T_b value of windmill grass is similar to that of three other annual summer grass weeds including yellow foxtail (*Setaria pumila* (Poir.) Roemer & J.A. Schultes.), large crabgrass (*Digitaria sanguinalis* (L.) Scop.), and goosegrass (*Eleusine indica* L.) with T_b for germination of 8.3, 8.4 and 12.6 C, respectively; however higher than that of green foxtail (*Setaria viridis* L.) with T_b of 6.1 C (Masin et al., 2005). With rapid germination (T_{50} = 27.3 to 45.5 h) and T_b of 9.2 to 11.2 C, windmill grass is likely to germinate and emerge under field conditions after rainfall events that would maintain adequate soil moisture for only a few days in spring (6 to 29 C), summer (12 to 34 C) and autumn (9 to 29 C) (Figure 4) in South Australia. Germination in spring would make weed control difficult, as cereal crops are still growing in the fields at this time (Borger et al., 2011).

Table 2. Effect of temperature on germination rate (T_{50}, R^2 from Equation 1) and maximum germination (G_{max}) of CT2 and CT3 populations of windmill grass under 12 h alternating light/dark conditions.

	CT2			CT3		
Temp	T_{50}	R^2	G_{max}	T_{50}	R^2	G_{max}
C	h		%	h		%
10	-	-	5.0 ± 1.0a	-	-	0.0 ± 0.0a
15	99.0 ± 2.2b	0.99	62.0 ± 1.2cd	228.3 ± 10.1c	0.97	95.0 ± 1.9cd
20	52.1 ± 1.9ab	0.98	67.0 ± 2.5de	99.2 ± 4.9b	0.98	98.0 ± 2.0cd
25	40.4 ± 3.0a	0.99	70.0 ± 1.2e	47.3 ± 3.1a	0.97	99.0 ± 1.0d
30	27.3 ± 0.6a	0.98	58.0 ± 3.5c	45.5 ± 1.3a	0.99	92.0 ± 3.7bc
40	49.2 ± 1.8ab	0.96	13.0 ± 1.0b	60 ± 0.7a	0.98	85.0 ± 1.0b

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

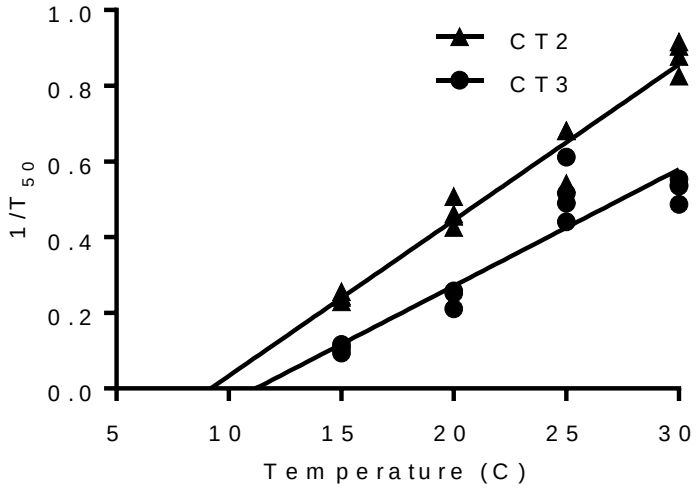

Figure 3. Base temperature (T_b) estimation for windmill grass seed germination. The lines are: CT2: Y = 0.041*X - 0.378 (R^2 = 0.98), and CT3: Y = 0.031*X - 0.344 (R^2 = 0.92). Each data point represents a replicate.

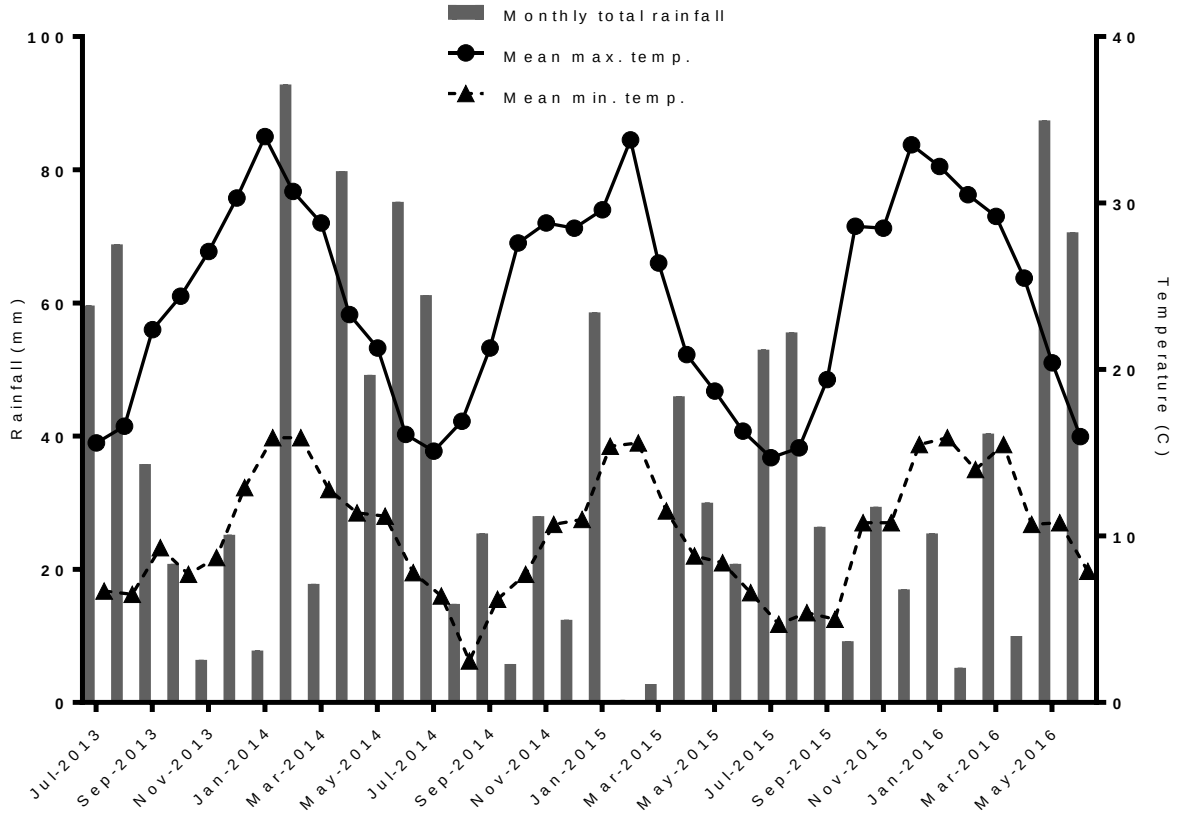

Figure 4. Monthly total rainfall, mean maximum and minimum temperatures from July 2013 to May 2016 at Roseworthy, South Australia, Australia, the period covered by the field experiments (http://www.bom.gov.au).

Effect of Light on Germination. Germination was strongly stimulated by light. In 2014, germination increased from 2% in the dark to 44 to 77% when seeds were exposed to light. Similarly in 2015, germination increased from 0 to 0.5% in the dark to 63 to 83% in the light (Table 3). Maze et al. (1993) also reported that less than 5% windmill grass seeds germinated in the dark, while there was more than 80% germination in the light. Light requirement for germination is common among small-seeded species and warm season grasses (Adkins et al., 2002; Grime et al., 1981; Milberg et al., 2000). Light may stimulate germination by altering the balance of germination promoters and inhibitors in the embryo (Adkins et al., 2002). The light requirement can ensure that germination takes place away from other vegetation and only on, or near the soil surface (Adkins et al., 2002; Milberg et al., 2000), which would

enhance the probability of seedling survival of small-seeded weed species, such windmill grass which had average seed mass of 0.31 to 0.33 mg seed^{-1} in the present study. Management practices such as tillage (or the seed drill at planting time), narrow crop row spacing and use of cover crops could reduce germination and emergence of windmill grass under field conditions.

Table 3. Effect of light on germination of CT2 and CT3 populations of windmill grass at 25 C.

Year	Population	Germination (%)	
		24 h dark	12 h light/dark
2014	CT2	2.0 ± 1.2 a	44.0 ± 3.7 b
	CT3	2.0 ± 1.2 a	77.0 ± 3.0 d
2015	CT2	0.0 ± 0.0 a	63.0 ± 3.6 c
	CT3	0.5 ± 0.5 a	83.7 ± 3.6 e

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

Effect of Salt Stress and Osmotic Potential on Germination. A logistic model was fitted to germination response to NaCl concentration (R^2 = 0.94 to 0.95). Seed germination was greater than 50% up to a NaCl concentration of 40 mM. The concentration of NaCl required to inhibit germination by 50% was estimated to range from 51 to 73 mM (Figure 5). The NaCl concentration that completely inhibited germination of windmill grass was 250 mM NaCl, and similar to that of annual sowthistle (*Sonchus oleraceus* L.) (Chauhan et al., 2006a), but slightly lower than that of rigid ryegrass in South Australia (*Lolium rigidum* Gaudin), which required 320 mM for complete inhibition of germination (Chauhan et al., 2006b).

A logistic model was fitted to the germination response to osmotic potential of the germination medium (R^2 = 0.95). Germination was inhibited by 50% at -0.27 MPa and completely at -0.8 MPa (Figure 6). Similarly, the base water potentials for germination of yellow foxtail, green foxtail, large crabgrass were -0.7, -0.7, -0.8 MPa, respectively (Masin et al., 2005). This indicates that windmill grass has a similar base water potential to other summer grass weed species and is not drought tolerant at germination, and will only germinate when there is adequate soil moisture (osmotic potential less than -0.8 MPa).

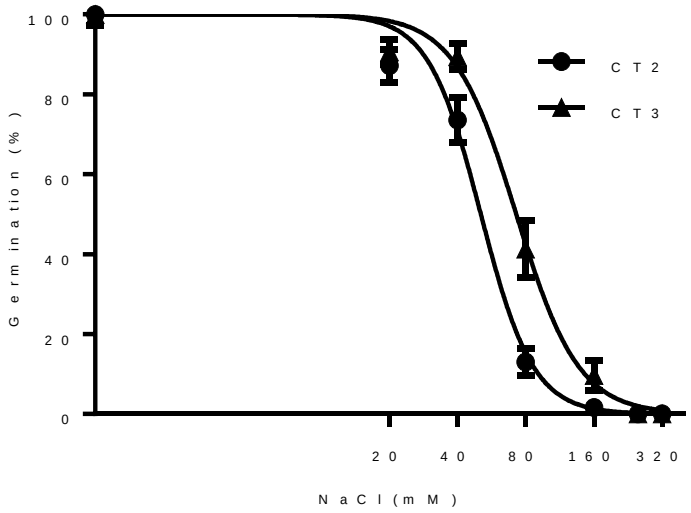

Figure 5. Effect of salt stress (NaCl) on germination of CT2 and CT3 populations of windmill grass at 25 C and 12 h alternating fluorescent light/dark. Each data point represents the mean of two experiments pooled with four replicates. Vertical bars are SE of mean. The fitted line represents a logistic response equation: *Y* = 100/{1 + 10^[(1.704 - *X*)*-3.750]} (R^2 = 0.95) for CT2; and *Y* = 100/{1 + 10^[(1.861 - *X*)*-3.150]} (R^2 = 0.94) for CT3.

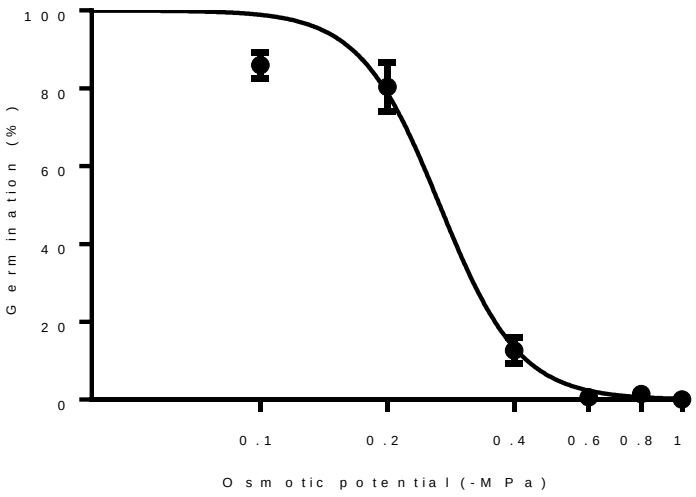

Figure 6. Effect of osmotic potential on germination of CT2 and CT3 populations of windmill grass at 25 C and 12-h alternating light/dark. Each data point represents the mean of four replicates from two experiments and two populations pooled. Vertical bars are SE of mean. The fitted line represents a logistic response equation: Y = 100/{1 + 10^[(2.425 - X)*-4.587]} (R^2 = 0.95), where X is log(osmotic potential) as kPa.

Seedling Emergence from Different Burial Depths. Seedling emergence was completely inhibited by burial regardless of depth. Emergence of seedlings from seeds on the soil surface was 57.3±5.7 to 69.8±4.3% for CT2 and 66.5±8.5 to 78.4±2.6% for CT3, while seedling emergence was zero for all seeds buried at 0.5, 1, 2 or 5-cm depth (data not shown). The lack of seedling emergence with burial is consistent with other findings of this study that windmill grass seed required light for germination (Table 3). Light can penetrate only a few mm in soil (Benvenuti, 1995; Cussans et al., 1996), so there would be no light to stimulate germination of buried seed; therefore, seedling emergence was not observed even at shallow burial depth (0.5 cm). Tillage that buries seed is likely to inhibit germination and widespread adoption of no-till cropping in Australia is likely to favour windmill grass invasion. These results are consistent with a

previous study in which all types of tillage treatments reduced emergence of windmill grass compared to the no-till treatment (Widderick et al., 2014).

Seedbank Persistence under Field Conditions. Under field conditions, seed viability was reduced with time (Figure 7), and was influenced by the amount of summer rainfall received over the three years (Figure 4). In Experiment 1 (July 2013 to July 2014), viability of seeds on the soil surface and at 5-cm depth decreased by 50% after 6 mo, and was completely lost after 12 mo (Figure 7a). Seeds in Experiment 2 (August 2014 to September 2015) initially lost viability faster than those in Experiment 1. After 2 mo, seeds on the soil surface and at 5-cm depth lost 50% of viability in Experiment 2. In this year, viability of buried seed was lost completely after 12 mo while there was 8% viable seed remaining on the soil surface (Figure 7b). Widderick et al. (2014) also found that less than 1% of windmill grass seed persisted up to 12 mo after burial at 2 cm and 10 cm in the northern cropping region of Australia. As windmill grass seedbank was found to only persist for 12 mo, prevention of seed set in windmill grass populations with herbicides, tillage or mowing could deplete their seedbanks rapidly.

Conversely, seedbank persistence in Experiment 3 (April 2015 to June 2016) was much greater than that in Experiment 1 and Experiment 2. Seeds buried at 5 cm in Experiment 3 had greater viability in all assessments than those placed on the soil surface. Viability of seeds exhumed at 8 mo after burial was 70% for those at 5-cm depth as compared to 56% for those on the soil surface. After 14 mo, buried seed still had 37% viability whereas seed viability on the soil surface was almost completely lost (6% viable seed) (Figure 7c). An extremely dry spring and summer in 2015 to 2016 could have increased seedbank persistence compared to 2013 to 2014 and 2014 to 2015 when significant rainfall events occurred (Figure 4). These differences in seedbank persistence between years could be associated with less seed germination

and less seed decay in dry and hot conditions than would occur in wet and warm conditions. Our results show that an adequate surface seedbank can be present in spring to summer (September to February) for seedling recruitment of this weed species in most seasons in southern Australia.

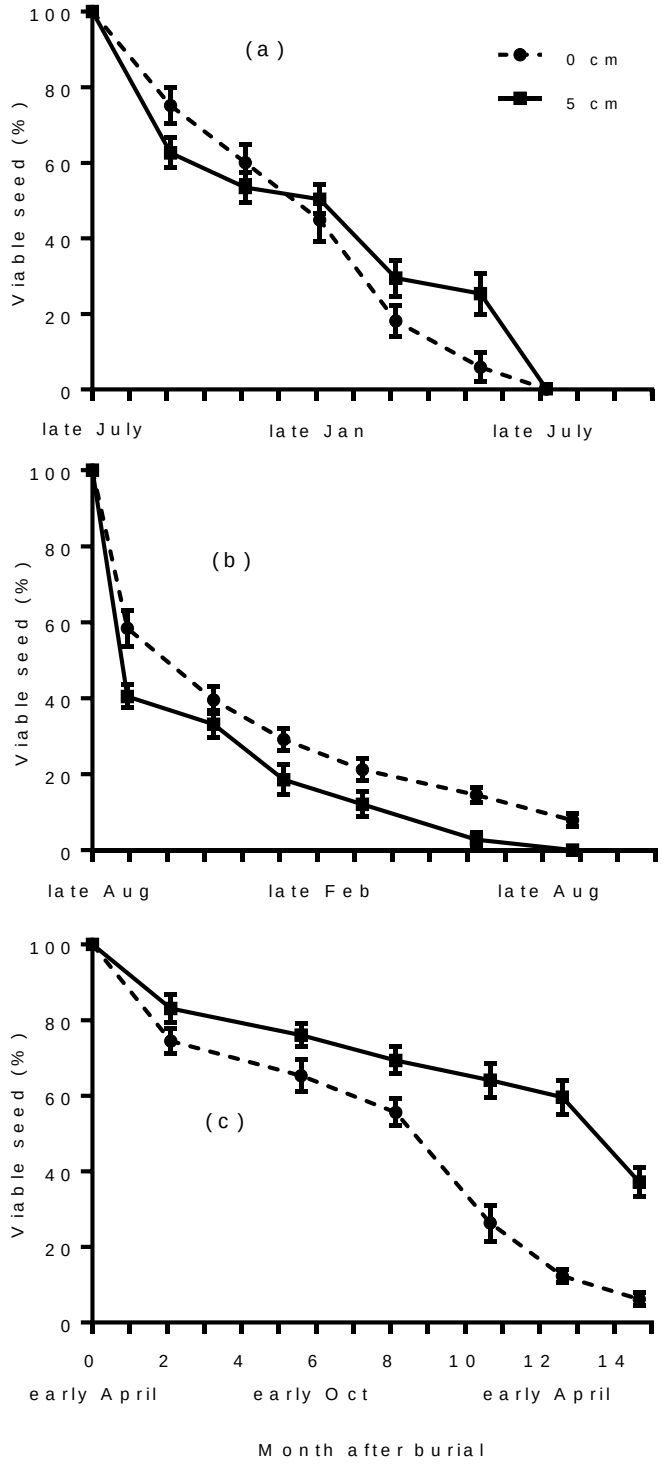

Figure 7. Changes in viability of seeds of CT2 and CT3 populations of windmill grass at the soil surface compared with seeds at 5-cm burial depth; Experiment 1 (a): July 2013 to July 2014, Experiment 2 (b): August 2014 to September 2015, and Experiment 3 (c): April 2015 to June 2016. Each data point represents the mean of two populations pooled with four replicates. Vertical bars are SE of mean.

Windmill grass has several characteristics that enable it to survive and persist in the Mediterranean environment of South Australia. It has rapid germination (27.3 to 45.5 h for 50% germination) and a base temperature of 9.2 to 11.2 C, so can germinate and emerge under field conditions after rainfall events in spring, summer and autumn in South Australia. It has a short period to maturity requiring 780 Cd from emergence to mature seed stage. Germination of this small-seeded species required light, so seedling emergence occurred only for seeds present on the soil surface. Seed has low dormancy, however seeds on the soil surface and buried at 5 cm depth remained viable for more than 11 mo. Low rainfall over spring and summer in the 3rd year of this study extended seedbank persistence beyond 14 mo, especially for the seeds buried at 5-cm depth. These characteristics make this grass species ideally suited to the no-till farming system widely adopted in southern Australia, and it is likely this species will become a problem in such production systems. The demonstration that windmill grass seeds persist for about 12 mo in an average season offers the opportunity to eradicate this weed species from a field, provided there is no further seed invasion. Control of new seed production for a period of 12 mo or more should exhaust the soil seed bank. Windmill grass seed requires soil temperatures above 9 C and sufficient moisture to germinate. This allows a focus on periods during the year when seedlings may establish and need to be controlled. Lastly, seed germination in windmill grass has an absolute requirement for light. Therefore, burial to depths greater than 0.5 cm, through strategic inversion tillage for example, could completely inhibit germination.

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Chapter 4: Target Site Mutations Conferring Resistance to Glyphosate in Feathertop Rhodes Grass (*Chloris virgata*) Populations in Australia

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Target-site mutations conferring resistance to glyphosate in feathertop Rhodes grass (*Chloris virgata*) populations in Australia

The D Ngo,* Mahima Krishnan, Peter Boutsalis, Gurjeet Gill and Christopher Preston

Abstract

BACKGROUND: *Chloris virgata* is a warm-season, C_4, annual grass weed affecting field crops in northern Australia that has become an emerging weed in southern Australia. Four populations with suspected resistance to glyphosate were collected in South Australia, Queensland and New South Wales, Australia, and compared with one susceptible (S) population to confirm glyphosate resistance and elucidate possible mechanisms of resistance.

RESULTS: Based on the rate of glyphosate required to kill 50% of treated plants (LD_{50}), glyphosate resistance (GR) was confirmed in four populations of *C. virgata* (V12, V14.2, V14.16 and V15). GR plants were 2–9.7-fold more resistant and accumulated less shikimate after glyphosate treatment than S plants. GR and S plants did not differ in glyphosate absorption and translocation. Target-site EPSPS mutations corresponding to Pro-106-Leu (V14.2) and Pro-106-Ser (V15, V14.16 and V12) substitutions were found in GR populations. The population with Pro-106-Leu substitution was 2.9–4.9-fold more resistant than the three other populations with Pro-106-Ser substitution.

CONCLUSION: This report confirms glyphosate resistance in *C. virgata* and shows that target-site EPSPS mutations confer resistance to glyphosate in this species. The evolution of glyphosate resistance in *C. virgata* highlights the need to identify alternative control tactics.

Keywords: *Chloris virgata*; glyphosate resistance; EPSPS; target-site mutation

1 INTRODUCTION

Glyphosate is the most widely used non-selective herbicide globally.[1] Glyphosate provides simple, inexpensive, flexible and effective control of a broad spectrum of weeds in a wide variety of agronomic situations.[2] Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS),[3] which is a key enzyme in the shikimate pathway in plants, fungi and bacteria, but not in animals.[4] Glyphosate normally has no soil activity,[2] which allows famers to sow crops shortly after its application.[5]

The widespread reliance on glyphosate for pre-sowing weed control for decades and the more recent introduction of glyphosate-resistant transgenic crops in the mid-1990s has resulted in a sharp increase in the number of glyphosate-resistant weeds.[6] To date, glyphosate resistance has been documented in 35 weed species worldwide.[7] Mechanisms that have contributed to resistance to glyphosate in weeds include: (1) target-site alterations (target-site mutation,[8,9] target-site gene amplification[10,11]) resulting in reduced inhibition of enzyme activity by glyphosate; (2) non-target-site mechanisms involving different modes of exclusion from the target site, such as reduced glyphosate uptake[12,13] and/or reduced glyphosate translocation.[12,14–16]

Feathertop Rhodes grass (*Chloris virgata* Swartz) is a warm-season, C_4, annual grass that has been considered a major weed in cotton (*Gossypium hirsutum* L.) and grain crops in the subtropical region of Australia for many years,[17] and has become an emerging weed in southern Australia over the last 5 years. In a 2008 and 2010 survey of the northern subtropical grain region of Australia, *C. virgata* was ranked as one of the 20 most important weed species.[17] *C. virgata* was the third most common weed species identified in 50% of paddocks in a summer fallow survey in 2012.[18] More recently, *C. virgata* has been ranked in the top 10 (national ranking) and top 4 (northern regional ranking) weeds in all crops in Australia.[19] This weed species was identified in vineyards and orchards in South Australia and in the Western Australian grain-growing region 5 years ago,[20] and its abundance has continued to increase.

In Australia, *C. virgata* is considered hard to kill with glyphosate.[17,18] The dominance of reduced-tillage systems combined with the heavy reliance on glyphosate for weed control has made it difficult to control this weed species effectively.[17,18] Even though *C. virgata* has greater tolerance to glyphosate than

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most other local weed species, there is concern that populations in several locations have become more difficult to control with this herbicide over time and may have evolved resistance to glyphosate. Once resistance to glyphosate is confirmed in *C. virgata*, it would be important to understand the mechanism of herbicide resistance so that strategies can be developed both to slow the evolution of resistance and to control existing populations.[21]

The objectives of this study were to identify glyphosate-resistant populations and to understand the mechanism of glyphosate resistance in *C. virgata*.

2 EXPERIMENTAL METHODS

2.1 Plant materials

Seeds of 36 *C. virgata* populations were collected from Queensland, New South Wales, Victoria and South Australia. These populations were screened for glyphosate response at two rates: 300 and 600 g a.e. ha^{-1} in a preliminary experiment in 2014. Of these, 30% of the populations showed 80–88% reduction in shoot biomass at 300 g a.e. ha^{-1} of glyphosate and were classified as susceptible (S); CV7 (Table 1) was part of this group. A similar number of weed populations showed no reduction in shoot biomass even at 600 g a.e. ha^{-1} and were classified as suspected resistant (SR); V12, V14.16, V15 and V14.2 (Table 1) were part of this group. Most populations were collected from sites where glyphosate was commonly used, such as chemical fallows, roadsides and vineyards. As *C. virgata* seeds are readily dispersed by wind, it was not possible to determine the exact exposure history of these populations to glyphosate. Seeds were germinated on agar (6 g L^{-1}) in an incubator set at 12 h alternating fluorescent light/dark temperatures of 25 °C. One-leaf seedlings were transplanted into 0.55 L square pots (Masrac, Dry Creek, SA, Australia) containing standard potting mix.[22]

In the whole-plant dose response experiments, the original field-collected seeds of five populations were germinated, and nine seedlings were transplanted into four replicate pots for each herbicide rate. Plants were grown outdoors and watered as required at the Waite Campus, University of Adelaide, South Australia.

For the shikimate assay, and absorption and translocation experiments, the original CV7 (S) seeds and seeds of survivors of glyphosate treatment of V15 (375 g a.e. ha^{-1}) and V14.2 (1500 g a.e. ha^{-1}) populations were used. Plants were grown in a growth chamber set at a 12 h photoperiod at 708 μmol m^{-2} s^{-1} light intensity and a temperature of 25 °C.

To identify target-site EPSPS mutations, untreated plants of the CV7 population and survivors of glyphosate treatment of V12 and V14.16 (500 g a.e. ha^{-1}), V15 (375 g a.e. ha^{-1}) and V14.2 (1500 g a.e. ha^{-1}) populations were used for the DNA extraction.

2.2 Whole-plant dose response to glyphosate

Glyphosate (Glyphosate 540; Nufarm, Laverton North, Vic, Australia) was applied to plants at the 4–5-leaf stage. The doses of glyphosate used for CV7 plants were: 0, 125, 250, 500, 1000, 2000 and 4000 g a.e. ha^{-1}; for V12, V14.16 and V15 plants: 0, 500, 1000, 2000, 4000, 6000 and 8000 g a.e. ha^{-1}; for V14.2 plants: 0, 1000, 2000, 4000, 6000, 8000 and 12 000 g a.e. ha^{-1}. The glyphosate treatments were applied using a laboratory, moving-boom, twin-nozzle sprayer Hardi ISO F-110-01 standard flat fan (Hardi, Adelaide, SA, Australia) with a water volume of 118 L ha^{-1}, a pressure of 250 kPa, a boom speed of 1 m s^{-1} and a boom height of 40 cm. Plants that had green leaves and produced new tillers were recorded as alive 28 days after glyphosate treatment.

Table 1. The locations in Australia of *C. virgata* populations used in this study

Population	Collection date	Origin	Situation
CV7 (S)	March 2013	Mildura, Vic	Vineyard
V12 (SR)	January 2011	Emerald, Qld	Chemical fallow
V14.16 (SR)	June 2014	Borah Creek, NSW	Chemical fallow
V15 (SR)	April 2012	Pym St Adelaide, SA	Roadside
V14.2 (SR)	March 2014	McLaren Vale, SA	Roadside

Based on the results of a preliminary experiment in February 2015, the whole-plant dose response experiments were conducted twice in November 2015 (experiment 1) and in January 2016 (experiment 2). A normal distribution function and an all-or-nothing model were employed to analyse the mortality data, and LD_{50} values (lethal dose required for 50% mortality) and their 95% fiducial limits were calculated (PriProbit v.1.63).[23]

2.3 Glyphosate absorption and translocation

Glyphosate absorption and translocation of *C. virgata* were studied using the method described by Wakelin *et al.*,[24] with modifications. Preliminary experiments with harvesting times at 24, 48 and 72 h after treatment (HAT) were conducted, and absorption and translocation had stabilised by 48 HAT, so this time was used to sample plant tissue to investigate glyphosate absorption and translocation of *C. virgata* populations. Twelve seedlings at the 1–2-leaf stage from each population were grown in a hydroponic medium in a black plastic container (26 × 19 × 9 cm). Six seedlings of similar size at the 4–5-leaf stage from each population were selected to treat with glyphosate. To prepare the hydroponic medium, the container was filled with black polypropylene beads, and then Hoagland's nutrient solution was added.[25]

Plants were sprayed with 250 g a.e. ha^{-1} of glyphosate. Within 10 min of the glyphosate application, each plant was treated with 1 μL of radiolabelled ^{14}C-glyphosate as a single droplet to the midpoint of the adaxial surface of the third fully expanded leaf. Radiolabelled ^{14}C-glyphosate solution was made up in 250 g a.e. ha^{-1} of commercial glyphosate formulation (Roundup PowerMax; Nufarm). The ^{14}C-glyphosate contained in a 1 μL droplet was approximately 227 Bq, with 12.53 mM of glyphosate.

Six plants of each population were harvested 48 HAT. The harvested plants were divided into four sections: treated leaf blade; untreated leaf blade; leaf sheaths and apical meristem; roots. To remove unabsorbed ^{14}C-glyphosate, the treated leaf was submerged for 5 s in 5 mL of 0.1% Triton X-100 (Sigma-Aldrich; Castle Hill, NSW, Australia) solution inside a 20 mL glass vial. Then, 8 mL of Ultima Gold XR scintillation fluid (PerkinElmer, Waltham, MA) was added into each glass vial containing leaf wash solution. Each plant section was transferred into a combusto-cone (PerkinElmer, Waltham, MA) and dried in a paper envelope at room temperature.

Each sample was separately combusted in an automatic preparation and oxidation system (Sample Oxidizer 307; PerkinElmer, Shelton, CT). The emitted $^{14}CO_2$ was trapped in 14 mL of scintillation mixture of 7 mL Carbo-Sorb E and 7 mL Permafluor E+ (PerkinElmer, Shelton, CT). The radioactivity of the wash solution and each separate plant section was quantified by a liquid scintillation analyser (Tri-card 2100TR; Packard Bioscience Company; Meriden, CT).

The total amount of ^{14}C-glyphosate recovered in a single plant was calculated as the sum of the total amount absorbed in the whole plant and the amount in the treated leaf wash solution. The amount of ^{14}C-glyphosate in each plant section was expressed as the percentage of total ^{14}C-glyphosate absorbed in the whole plant. Percentage values were arcsine transformed before ANOVA analysis. Fisher's protected LSD multiple comparisons were employed to differentiate between predicted means, and means were presented as back-transformed data (GenStat 17; VSN International, Hemel Hempstead, Herts, UK). The experiment was conducted twice. As there was no experiment by treatment interaction, the data was pooled for analysis.

2.4 Shikimate assay

The shikimate assay was conducted using the method described by Shaner *et al.*,[26] with modifications.

Ten leaf discs (3.5 mm diameter) were excised from a single fully expanded leaf of each plant from the susceptible CV7, resistant V15 and V14.2 populations. These leaf discs were treated with one of five glyphosate rates, with two discs placed into a single well of a 96-well flat-bottomed microtitre plate, each well containing 0, 50, 200, 500 or 1000 μM of glyphosate (Roundup PowerMax; Nufarm) and 10 mM of phosphate buffer (pH 7). The plates were incubated under light at 65 μmol m^{-2} s^{-1} (Fluval LED A3981; Rolf C. Hagen Corp., Mansfield, MA) and a temperature of 25 °C for 16 h.

Following 16 h incubation, 0.06 M of HCl was added to each well, and the samples were frozen and thawed through two cycles at −80 °C for 15 min, followed by 60 °C for 15 min until the leaf tissue was no longer green. From each well, 25 μL was transferred into new empty microtitre plates to measure shikimate levels. Similarly, 25 μL of shikimic acid solution at each concentration of 1, 2.5, 5, 10, 25 and 50 μM was added into empty wells as standards. The 100 μL solution of 0.25% (w/v) periodic acid and 0.25% (w/v) sodium *m*-periodate was added to each well for both extract and standard shikimic acid standards. The samples were incubated for 60 min at room temperature. After sample incubation, 100 μL of made quench buffer (mixture of 0.6 M of NaOH and 0.22 M of Na_2SO_3) was added into each well to terminate the reaction. A 150 μL aliquot was taken from each well to measure absorbance at 380 nm with a double-beam spectrometer (Cintra10 UV; GBC, Melbourne, Vic, Australia).

The mean value of optical density measured in the controls of each population was subtracted from those measured in the glyphosate treatments as background. A shikimate standard curve was developed, and shikimate levels were expressed as nM of shikimic acid accumulated cm^{-2}.

The experiment was conducted twice, and five leaves from five plants from each population were used as replicates. As there was no experiment by treatment interaction, the data was pooled. Data of shikimic acid accumulation at different glyphosate concentrations was best fitted to an exponential model (one-phase association) using GraphPad Prism v.6.00 (GraphPad Software, La Jolla, CA).

2.5 EPSPS gene sequencing

Young green leaf tissue was sampled separately from five plants of each population, and DNA was extracted using Isolate II Plant DNA kit (Bioline, Alexandria, NSW, Australia) in accordance with the manufacturer's instructions.

For polymerase chain reaction (PCR) amplification, a standard 25 μL PCR reaction mix contained: 20–40 ng of DNA, 1× high-fidelity PCR buffer [600 mM of Tris-SO4 (pH 8.9), 180 mM of $(NH4)_2SO_4$], 0.4 mM of dNTP mixture, 4 mM of $MgSO_4$, 0.4 μM of each specific primer, AW1-Fwd (5′-AACAGTGAGGAYGTYCACTACATG CT-3′) and AW2-Rev (5′-CGAACAGGAGGGCAMTCAGTGCCAAG-3′), 1 U of MyFi Taq DNA polymerase (Bioline) and 5% of dimethyl sulphoxide. The forward and reverse primers were used for amplification of a 500 bp fragment of the EPSPS gene.

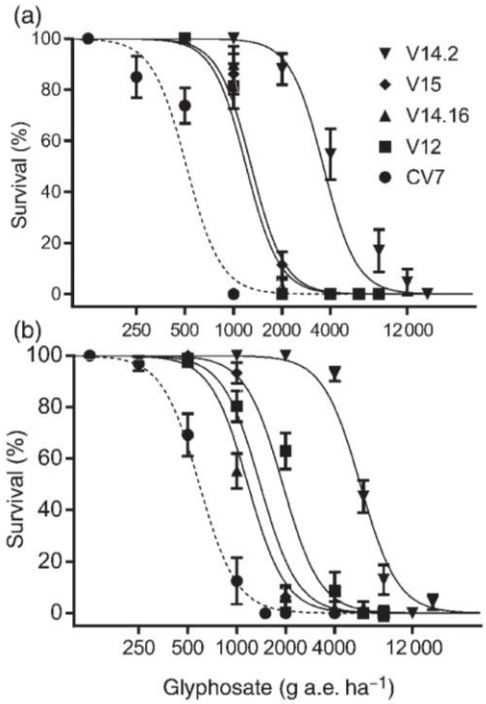

Figure 1. Glyphosate dose response experiments of *C. virgata* populations (susceptible CV7 and resistant V14.2, V12, V15 and V14.16) on 2 November 2015 (a) and 19 January 2016 (b). Each data point is the mean of four replicates, and the vertical bars are standard error of the mean. The curves were fitted using the equation $Y = 100*[1 - \text{NORMSDIST}(B + A*X)]$, where Y (% survival) is back-transformed from mortality (expressed as normal equivalent deviates) and X is log(dose). See Table 2 for parameters A and B (± SE) of the equations.

An automated DNA thermal cycler (Eppendorf Mastercycler® Gradient; Eppendorf, Hamburg, Germany) was used for amplification with the following cycle parameters: 1 min denaturing at 95 °C, 41 cycles of 15 s denaturing at 95 °C, 15 s annealing at 57 °C and 1 min elongation at 72 °C and a final extension for 10 min at 72 °C.

PCR products were examined on 1.0% agarose gels stained with 1× SYBR® Safe DNA gel stain. Samples were electrophoresed in 1× TAE buffer [40 mM of Trizma base, 1 mM of Na_2EDTA (pH to 8) with glacial acetic acid] at 120 V and photographed under UV light ($\lambda = 302$ nm). DNA fragment sizes were estimated by comparison with known size bands of Easy Ladder (Bioline).

PCR products were sequenced with the same primers as used for amplification at Australian Genome Research Facility Ltd (AGRF). ClustalW alignment was employed to compare sequencing data of a susceptible population, four suspected resistant populations and the known EPSPS susceptible sequences of goosegrass [*Eleusine indica* (L.) Gaertn.] AJ417034[8] (Geneious 8.1.3, http://www.geneious.com).[27]

Table 2. Parameters (± SE) of the equations[a] used to calculate the glyphosate doses required for 50% mortality (LD_{50}) (g a.e. ha^{-1}) of a susceptible (S) and four resistant (R) *C. virgata* populations with 95% fiducial limits in parentheses and resistance index (RI) of the R populations compared with the S population. Experiments 1 and 2 were conducted in November 2015 and January 2016 respectively

	Experiment 1[b]			Experiment 2[c]		
Population	*B*	LD_{50}	RI	*B*	LD_{50}	RI
V14.2 (R)	−20.19 ± 1.24	4458 (3963, 5005)	8.7 (8.6, 8.7)	−19.37 ± 1.69	5786 (4912, 6771)	9.7 (8.9, 10.7)
V15 (R)	−17.31 ± 1.07	1345 (1182, 1524)	2.6 (2.6, 2.6)	−16.20 ± 1.41	1402 (1138, 1725)	2.4 (2.3, 2.5)
V14.16 (R)	−17.27 ± 1.06	1325 (1151, 1523)	2.6 (2.5, 2.6)	−15.81 ± 1.38	1179 (942, 1474)	2.0 (1.9, 2.0)
V12 (R)	−17.09 ± 1.04	1226 (1089, 1380)	2.4 (2.4, 2.4)	−16.92 ± 1.47	1933 (1593, 2341)	3.3 (3.1, 3.5)
CV7 (S)	−15.00 ± 0.92	515 (455, 583)	–	−14.28 ± 1.27	594 (460, 760)	–

[a] For $Y = B + A*X$, where Y is mortality (expressed as normal equivalent deviates) and X is log(dose).
[b] For experiment 1, parameter A is 5.53 ± 0.33 and the same for all populations.
[c] For experiment 2, parameter A is 5.15 ± 0.44 and the same for all populations.

3 RESULTS

3.1 Whole-plant dose response to glyphosate

Resistance to glyphosate was confirmed in four populations of *C. virgata*: V14.2, V15, V14.16 and V12, with the resistance index (RI, LD_{50} relative to the sensitive biotype) for the populations ranging between 2- and 9.7-fold (Fig. 1 and Table 2). The susceptible CV7 population was completely controlled at 1000–1500 g a.e. glyphosate ha^{-1}, with an LD_{50} value of 515–594 g a.e. ha^{-1}. V14.2 was the most resistant population and required 9000–12 000 g a.e. ha^{-1} of glyphosate for its control. The RI of the V14.2 population (8.7–9.7-fold) was 2.9–4.9-fold higher than that of the three other resistant populations. The V15, V14.16 and V12 populations had a similar level of resistance to glyphosate, with an RI of 2–3.3-fold, and were controlled at 2400–3900 g a.e. ha^{-1}. For each population, LD_{50} in experiment 1 was similar to that in experiment 2 (Fig. 1 and Table 2).

3.2 Glyphosate absorption and translocation

Absorption and translocation of ^{14}C-glyphosate were determined in susceptible CV7 and two resistant V15 and V14.2 populations. There was no difference in absorption of ^{14}C-glyphosate between the susceptible and resistant plants, with 58–62% of the applied ^{14}C-glyphosate being absorbed into leaf tissue by 48 HAT (Table 3). The amount of absorbed ^{14}C-glyphosate in treated leaves in the susceptible plants was similar to that in the resistant plants (31–38%). Furthermore, a similar amount of ^{14}C-glyphosate (25–29%) was translocated into stems in the susceptible and resistant plants. However, the amount of ^{14}C-glyphosate in untreated leaves and roots of the resistant plants was slightly higher (1–8%) than in the susceptible plants.

3.3 Shikimate assay

A significant difference in shikimate accumulation was observed between susceptible (CV7) and resistant (both V15 and V14.2) plants (Fig. 2). Shikimate accumulation in the susceptible plants was significantly higher than in the V15 and V14.2 plants at all glyphosate concentrations used. Furthermore, shikimate accumulation in the V15 plants was significantly greater than that in the V14.2 plants at every concentration of glyphosate used. Shikimate accumulation in CV7, V15 and V14.2 plants reached a maximum of 214, 146 and 132 nmol cm^{-2} leaf respectively (Fig. 2). The calculated IC_{50} values (glyphosate concentration to accumulate half of the maximum shikimic acid) of V14.2 (449 μM) and V15 (149 μM) plants were 7.7- and 2.5-fold higher, respectively, than those of the susceptible CV7 plants (58 μM). This difference in shikimate accumulation is consistent with the whole-plant dose response, where LD_{50} values of the resistant V14.2 and V15 populations were 8.7–9.7-fold and 2.4–2.6-fold higher than those of the susceptible CV7 population (Table 2).

3.4 EPSPS gene sequencing

To determine whether a target-site modification at Pro-106 was present in the glyphosate-resistant populations, a 500 bp EPSPS DNA fragment of the EPSPS gene was sequenced from five individual plants each of a susceptible population (CV7) and four resistant populations (V14.2, V15, V14.16 and V12). The predicted amino acid sequence of the susceptible CV7 population was similar to the consensus sequence. A single nucleotide substitution of CCG to CTG at codon 106 was identified in the V14.2 population. The nucleotide change of C to T at the second base of codon 106 would result in an amino acid substitution from proline to leucine. This is the most infrequently reported mutation of the EPSPS Pro-106

Table 3. ^{14}C-glyphosate absorbed and distribution of absorbed radiolabel from the treated leaf to other plant parts of a susceptible (S) and two resistant (R) populations of *C. virgata* 48 h after treatment. Means (± SE) within a column followed by the same letter are not significantly different (Fisher's protected LSD test, $P \leq 0.05$)

		Translocation (% absorbed)			
Population	Absorption (% applied)	Treated leaf/ leaf blade	Untreated leaf/ leaf blade	Stem/ leaf sheath	Root
CV7 (S)	58.1 ± 1.4 a	38.1 ± 1.9 a	2.0 ± 0.2 a	28.8 ± 1.6 a	31.1 ± 1.4 a
V15 (R)	61.2 ± 2.5 a	36.9 ± 2.3 a	3.2 ± 0.5 b	24.9 ± 1.2 a	35.1 ± 1.8 ab
V14.2 (R)	61.6 ± 2.1 a	31.2 ± 2.2 a	4.2 ± 0.3 c	25.6 ± 1.3 a	39.0 ± 1.1 b

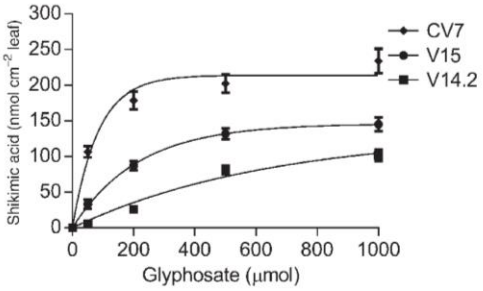

Figure 2. Shikimic acid accumulation of leaf discs from one susceptible (CV7) and two resistant (V15, V14.2) *C. virgata* populations at different glyphosate concentrations. Each data point represents the mean of two experiments pooled with five replicates, and vertical bars are standard error of the mean. The curves were fitted using the equation $Y = 214*[1 - \exp(-0.01182*X)]$, $R^2 = 0.83$ for CV7; $Y = 146.2*[1 - \exp(-0.004658*X)]$, $R^2 = 0.88$ for V15; $Y = 133.2*[1 - \exp(-0.001545*X)]$, $R^2 = 0.87$ for V14.2.

Table 4. Nucleotide and predicted amino acid sequence of EPSPS DNA isolated from a susceptible and four resistant populations of *C. virgata*

Amino acid number[a]	104	105	106	107	108
Amino acid	Met	Arg	Pro	Leu	Thr
Consensus sequence	ATG	CGG	CCG	TTG	ACA
CV7 (S)	ATG	CGG	CCG	TTG	ACA
V14.2 (R)	–	–	Leu CTG	–	–
V15 (R)	–	–	Ser TCG	–	–
V14.16 (R)	–	–	Ser TCG	–	–
V12 (R)	–	–	Ser TCG	–	–

[a] Amino acid assignments are based on the plant EPSPS numbering system used by Padgette *et al.*[44]

mutations.[28] Additionally, another single nucleotide substitution of CCG to TCG at codon 106 was found in the three other resistant populations (V12, V15 and V14.16), predicting a substitution from proline to serine at codon 106 (Table 4). Both Pro-106-Leu and Pro-106-Ser mutations always displayed a clear single peak at the mutation site in the sequence chromatograms (data not shown), indicating that the individuals examined were likely to be homozygous for these mutations.

4 DISCUSSION

4.1 Whole-plant dose response

In the dose response experiments reported here, glyphosate field rates recommended for the control of summer-growing grass weed species in Australia under field conditions (500–600 g a.e. ha^{-1}) are unlikely effectively to control susceptible *C. virgata* (LD_{50} of 515–594 g a.e. ha^{-1}). However, resistant populations required higher doses than the susceptible population for control (Table 2). These results support field observations of high innate glyphosate tolerance in *C. virgata*.[17,18]

Glyphosate efficacy can be reduced by a number of environmental conditions, including high air temperature,[29] low soil moisture content[30] and low relative humidity.[31,32] *Echinochloa colona* control with glyphosate was greater at 20–25 °C than at 30–35 °C.[33–35] This difference in plant response was related to reduced glyphosate absorption in *E. colona* when air temperature increased.[33] Similarly, two other grass species (*Sorghum halepense* and *Lolium rigidum*) that had higher levels of glyphosate resistance at higher temperatures were identified.[36] Under well-watered conditions of the present study, glyphosate efficacy on each population was similar in the two experiments (Table 2).

4.2 Absorption and translocation of glyphosate

Reduced glyphosate uptake and/or translocation mechanisms have been reported to contribute to glyphosate resistance in several weed species.[12–16,37] When resistance was related to reduced translocation of glyphosate, this herbicide mostly remained in the treated leaves of resistant plants and a smaller amount was transported to the shoot meristem of the treated plant.[12,14–16] In the present study, the amount of absorbed ^{14}C-glyphosate in susceptible and resistant plants was similar, and the radiolabel remaining in the treated leaves of the susceptible and resistant plants at 48 HAT was also similar. A slightly higher amount of ^{14}C-glyphosate in the untreated leaves (1–2%) and roots (4–8%) of resistant plants compared with susceptible plants was unlikely to contribute to resistance to glyphosate (Table 3). This suggests that glyphosate resistance in the two resistant populations (V15 and V14.2) was not due to alteration in glyphosate absorption or translocation.

4.3 Shikimate assay related to whole-plant resistance

The shikimate assay using excised leaf discs eliminates the contributions of absorption through the leaf cuticle and translocation of glyphosate to the leaf cells in assessing whether glyphosate inhibits EPSPS. The leaf disc assay is only affected by the ability of glyphosate to penetrate into the chloroplast and the tolerance of the target enzyme.[33] There was significantly greater shikimate accumulation in plants of CV7 than in V15 and V14.2 at all glyphosate concentrations used, indicating that glyphosate did reach the target site and inhibited the EPSPS enzyme of each population differently.[1] This resulted in higher IC_{50} values of V14.2 (449 μM) and V15 (149 μM) compared with CV7 (58 μM) plants (Fig. 2). The shikimate accumulation assay has been shown to differentiate between susceptible and glyphosate-resistant plants in which resistance is due to an alteration of EPSPS,[26] including target-site mutation and target-site gene amplification. Thus, significantly greater IC_{50} values of resistant plants (V14.2 and V15) compared with susceptible CV7 plants could be related to target-site mechanisms.

4.4 Target-site EPSPS mutations conferring glyphosate resistance

Four target-site EPSPS mutations at Pro-106 (Pro-106-Ser, Pro-106-Thr, Pro-106-Ala and Pro-106-Leu) have been previously reported in glyphosate-resistant populations of six different weed species.[28,38] Healy-Fried *et al.*[39] concluded that the Pro101 (position Pro106 in plant mature EPSPS consensus corresponds to Pro101 in *E. coli*) is not directly involved in molecular interactions with either glyphosate or the substrate PEP, but any mutation at this site would shift other amino acids (Thr-97 and Gly-96) towards the inhibitor molecule, resulting in a structural change in the glyphosate-binding site. A double amino acid substitution in a single EPSPS allele (Thr-102-Ile + Pro-106-Ser) was also found in glyphosate-resistant *Eleusine indica* populations from Malaysia and China.[9,40] This double amino acid substitution conferred high-level glyphosate resistance (more than 180-fold),[9] whereas the single Pro-106 mutations of the six weed species provided

moderate resistance (less than tenfold).[38] Here, we identified two target-site EPSPS mutations, including Pro-106-Leu in the resistant V14.2 population and Pro-106-Ser in the other three resistant populations (V14.16, V15 and V12). These mutations conferred moderate resistance to glyphosate at the whole-plant level in the four resistant populations of *C. virgata*: 8.7–9.7-fold for Pro-106-Leu mutation and 2.4–2.6-fold for Pro-106-Ser mutation (Table 2). This is the first report of target-site EPSPS mutations conferring glyphosate resistance in *C. virgata*.

In a previous study, single-site Pro-106 EPSPS mutations were introduced into *E. coli* EPSPS by replacing Pro-101 with serine or leucine. The Pro-101-Ser mutation was more sensitive to glyphosate than the Pro-101-Leu mutation. The K_i value (for glyphosate) increased from 0.4 μM for the wild type to 5.5 μM for Pro-101-Ser and 66 μM for Pro-101-Leu.[39] Similarly, K_i values of maize wild-type EPSPS, maize Pro-106-Ser and maize Pro-106-Leu were 0.5, 1.0 and 28.6 μM of glyphosate respectively.[41] In these two studies, mutant enzymes were 2.5–14-fold (Pro-101/106-Ser) and 70–165-fold (Pro-101/106-Leu) less sensitive to glyphosate than the wild-type EPSPS. In addition, the Pro-101/106-Leu mutant enzyme was 12–28-fold more tolerant to glyphosate than the Pro-101/106-Ser enzyme. The findings of these two studies with mutant EPSPS are consistent with those of our shikimate assay in which the IC_{50} value of the V14.2 plants (Pro-106-Leu) was threefold higher than that of the V15 plants (Pro-106-Ser) (Fig. 2). As a result, LD_{50} values of the V14.2 population (Pro-106-Leu) were 2.9–4.9-fold higher than those of the three other populations (V15, V14.16 and V12) with Pro-106-Ser mutations in the two whole-plant dose response experiments (Table 2).

In conclusion, two target-site EPSPS mutations (Pro-106-Leu and Pro-106-Ser) were identified in four glyphosate-resistant populations of *C. virgata* (V14.2, V15, V14.16 and V12) from three Australian states: South Australia, Queensland and New South Wales. These EPSPS mutations were likely to be the primary mechanism of glyphosate resistance, while reduced glyphosate uptake and translocation were not observed in the four resistant populations. *C. virgata* has a level of natural tolerance to glyphosate and therefore requires high rates to control it in the field. This research was unable to determine why this species is poorly controlled by glyphosate in the field. It was not the result of reduced glyphosate absorption or translocation, as the amount of herbicide absorbed and the amount translocated out of the treated leaf were similar to those observed in other grass weed species.[14,15,24] One possibility is that more glyphosate may be sequestered in the vacuole in this species, as has been observed in some glyphosate-resistant weeds.[42,43] The presence of moderate tolerance to glyphosate in this weed species would allow for the selection of relatively weak target-site mutations at Pro-106. While susceptible plants may not be controlled adequately at normal field rates, they are likely to be significantly damaged (data not shown), but individuals with Pro-106 mutations would be favoured in the field under intense glyphosate pressure. The evolution of glyphosate resistance in *C. virgata* means that strategies reliant on glyphosate alone or glyphosate in mixtures will not be successful, and other management strategies will have to be adopted.

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Chapter 5: EPSPS Gene Amplification Conferring Resistance to Glyphosate in Windmill Grass (*Chloris truncata*) in Australia

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EPSPS gene amplification conferring resistance to glyphosate in windmill grass (*Chloris truncata)* in Australia

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Abstract

BACKGROUND: Five glyphosate resistant populations of *Chloris truncata* originally collected from New South Wales were compared to one susceptible (S) population from South Australia, Australia to confirm glyphosate resistance and elucidate possible mechanisms of resistance.

RESULTS: Based on the amounts of glyphosate required to kill 50% of treated plants (LD_{50}), glyphosate resistance (GR) was confirmed in five populations of *C. truncata* (A536, A528, T27, A534 and A535.1). GR plants were 2.4 to 8.7 fold more resistant and accumulated less shikimate after glyphosate treatment than S plants. There was no difference in glyphosate absorption and translocation between GR and S plants. The *EPSPS* gene did not contain any point mutation that has previously been associated with resistance to glyphosate. The resistant plants (A528 and A536) contained up to 32-48 more copies of the *EPSPS* gene than the susceptible plants

CONCLUSION: This study has identified *EPSPS* gene amplification contributing to glyphosate resistance in *C. truncata*. In addition, a Glu-91-Ala mutation within *EPSPS* was identified that may contribute to glyphosate resistance in this species.

Keywords: dose response; *Chloris truncata*; glyphosate resistance; *EPSPS* gene amplification; *EPSPS* copy number; shikimate assay, target-site mutation

1 INTRODUCTION

Glyphosate [*N-(phosphonomethyl)glycine*] is the most widely used non-selective post-emergence herbicide globally.[1] It provides simple, inexpensive, flexible and effective control of a broad spectrum of weeds in a wide variety of agronomic situations.[2] Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*),[3] which is a key enzyme in the aromatic amino acid biosynthesis pathway in plants, fungi and bacteria.[4] This herbicide is considered to be relatively environmentally friendly.[2] Additionally, crops can be sown shortly after glyphosate application[5] because the herbicide normally has no soil activity.[2] Glyphosate is the only herbicide that targets *EPSPS* of all higher plants, with no analogs or alternative chemical classes targeting this enzyme having been commercialized.[1]

There has been a sharp increase in the number of glyphosate resistant weeds[6] due to a widespread reliance on glyphosate for pre-sowing weed control for decades, and the more recent introduction of glyphosate resistant transgenic crops in the mid 1990's.[7] To date, glyphosate resistance has been identified in 36 weed species worldwide.[6] Glyphosate resistance mechanisms in weeds include (1) target-site alterations: (1a) target-site mutation,[8-10] represented by amino acid substitutions that affect herbicide interactions at the target enzyme; (1b) target-site gene amplification,[11-13] where sufficient EPSPS protein is produced so that the shikimate pathway can continue to operate despite the fact that glyphosate inhibits some of the enzyme; and

(2) non-target-site mechanisms involving different modes of exclusion from the target site: (2a) reduced glyphosate uptake,[14, 15] where glyphosate is absorbed less by resistant plants than susceptible plants; (2b) reduced glyphosate translocation,[14, 16-18] where amounts of glyphosate absorbed by both resistant and susceptible plants are the same; however, the absorbed herbicide mostly remained in the treated leaf in resistant biotypes and a small amount of glyphosate was transported to the meristems of the treated plant; and/or (2c) and vacuole sequestration,[19, 20] where more glyphosate is sequestered in the cell vacuole of resistant plants than in those of susceptible plants.

Windmill grass (*Chloris truncata* R. Br) is an Australian native, warm season, C_4, annual or short-lived perennial grass that is a significant weed in cotton and grain crops in the sub-tropical region of Australia, particularly in no-till agriculture.[21] This species is ranked as the 7th most important weed of summer fallow by yield and revenue loss in Australia[22] and has now spread to southern Australia. As a result of over-reliance on glyphosate for weed control, glyphosate resistant populations were confirmed in *C. truncata* from chemical fallows and roadsides in Australia.[23, 24] However, the mechanism of resistance to glyphosate in this weed species has not been elucidated. By understanding the mechanism of herbicide resistance, strategies can be developed to both slow the evolution of resistance and to control existing populations.[25]

The objective of this study was to identify the mechanism of glyphosate resistance in *C. truncata.*

2 EXPERIMENTAL METHODS

2.1 Plant materials

Seeds of 42 *C. truncata* populations were collected from Queensland, New South Wales, Victoria and South Australia, Australia. In a preliminary experiment in 2014, these populations were screened for glyphosate response at 330 and 660 g a.e ha^{-1} (Touchdown HiTech; Syngenta, NSW, Australia), which are within the recommended rate range for *C. truncata*. Of these 42 populations, 10 showed 19-52% survival and 3-14% biomass (compared to the control of the same population) at 660 g a.e ha^{-1} glyphosate were classified as susceptible (S); a susceptible population (CT3), which was collected on roadside from Smithfield, South Australia, Australia, was part of this group. There were 18 populations with 100% survival and 30-100% biomass at 660 g a.e ha^{-1} and were classified as suspected resistant (SR). Of these SR populations, five were collected from chemical fallow situations at Trangie (T27), Dubbo (A528, A534, A535.1) and Narromine (A536) New South Wales, Australia, and were used in this study. Seeds of all populations were germinated on agar (6 g/L) in an incubator set at 12 h alternating fluorescent light/dark temperature of 25°C. One-leaf seedlings were transplanted into 0.55-L square pots (Masrac, South Australia, Australia) containing standard potting mix.[26]

For experiments of absorption and translocation, the shikimate assay, *EPSPS* gene sequencing, and *EPSPS* gene copy number, the original CT3 (S) seeds and A528 and A536 (R) seeds of survivors of glyphosate treatment (5000 g a.e ha^{-1}) were used. Plants were grown in a growth chamber set at 12 h photoperiod at 708 µmol m^{-2} s^{-1} light intensity and temperature of 25°C.

2.2 Whole-plant dose response to glyphosate

Seeds of the six populations (five SR and S control) were germinated and 9 seedlings were transplanted into four replicate pots for each herbicide rate. Plants were grown outdoors and watered as required at the Waite Campus, University of Adelaide, South Australia, Australia. Glyphosate (Touchdown HiTech; Syngenta, NSW, Australia) was applied to plants at the four-five leaf stage. The doses of glyphosate used for CT3 (S) plants were: 0, 125, 250, 500, 1000, 1500, 2000 and 4000 g a.e ha^{-1}; and A536, A528, T27, A534 and A535.1 plants were treated with 0, 500, 1000, 2000, 2500, (or 3000), 4000, (or 5000), 6000, 8000 and 10000 g a.e ha^{-1}. The glyphosate treatments were applied using a laboratory, moving-boom, twin-nozzle sprayer Hardi ISO F-110-01 standard flat fan (Hardi, Adelaide, South Australia) with water volume of 118 L ha^{-1}, pressure of 250 kPa, boom speed of 1 m s^{-1} and 40 cm boom height. Plants that had green leaves and produced new tillers 21 days after glyphosate treatment were recorded as alive.

The whole-plant dose response experiments were conducted three times in November 2014 (Experiment 1), in February 2015 (Experiment 2) and in November 2015 (Experiment 3). The mortality data was subjected to probit analysis, and LD_{50} values (lethal dose required for 50% mortality) and their 95% fiducial limits were calculated (PriProbit ver.1.63).[27]

2.3 Glyphosate absorption and translocation

Glyphosate absorption and translocation in *C. truncata* was studied using the method described by Wakelin *et al*.,[28] with modifications. In preliminary experiments, plants were harvested at 24, 48 and 72 h after treatment (HAT). However, absorption and translocation was found to have stabilised by 48 HAT, so only this harvesting time was used in subsequent experiments. Twelve seedlings at the one-two leaf stage from

each population (CT3, A528 and A536) were grown in hydroponic medium (Hoagland's nutrient solution[29]) in a black plastic container (26 × 19 × 9 cm) filled with black polypropylene beads. At the four-five leaf stage the seedling number was reduced to six seedlings per population, selecting seedlings of similar size, before glyphosate treatment.

Plants were sprayed with 250 g a.e ha^{-1} glyphosate. Within 10 min of the glyphosate application, each plant was treated with 1 µL of radiolabelled ^{14}C-glyphosate as a single droplet to the midpoint of the adaxial surface of the third fully expanded leaf. Radiolabelled ^{14}C-glyphosate solution was made up in 250 g a.e. ha^{-1} commercial glyphosate formulation (Roundup PowerMax; Nufarm, Vic, Australia). The ^{14}C- glyphosate contained in a 1 µL droplet was approximately 227 Bq, with 12.53 mM glyphosate.

Plants were harvested 48 HAT and the harvested plants divided into four sections: treated leaf blade, untreated leaf blade, leaf sheaths and apical meristem, and roots. To remove unabsorbed ^{14}C-glyphosate, the treated leaf was submerged for 5 s in 5 mL of 0.1% Triton X-100 (Sigma-Aldrich; NSW, Australia) solution inside a 20 mL glass vial. Then, 8 mL of Ultima Gold XR scintillation fluid (PerkinElmer, Waltham, MA, USA) was added into each glass vial containing leaf wash solution. Each plant section was transferred into a combusto-cone (PerkinElmer, Waltham, MA, USA) and dried at room temperature.

Each sample was separately combusted in an automatic preparation and oxidization system (Sample Oxidizer 307, PerkinElmer; Shelton, USA). The emitted $^{14}CO_2$ was trapped in 14 mL of scintillation mixture consisting of 7 mL Carbo-Sorb E and 7 mL Permafluor E+ (PerkinElmer; Shelton, USA). The radioactivity of the wash solution and each separate plant section was quantified by a liquid scintillation analyser (Tri-card 2100TR, Packard Bioscience; Meriden, USA).

The total amount of ^{14}C-glyphosate recovered in a single plant was calculated as the sum of the total amount absorbed in the whole plant and the amount in the treated leaf wash solution. The amount of ^{14}C-glyphosate in each plant section was expressed as the percentage of total ^{14}C-glyphosate absorbed in the whole plant. The experiment was conducted twice. As there was no experiment by treatment interaction, the data was pooled for analysis.

The Shapiro-Wilk test (GenStat 17, VSN International; Herts, UK) for normality was conducted in order to investigate the distribution of the data. The data for absorption and radioactivity levels within the treated leaf blade, leaf sheaths and apical meristem, and roots were normally distributed (P >0.05), whereas the data from the untreated leaf blade were arcsine square root transformed prior to analysis. Means were separated using Fisher's protected LSD multiple comparisons at a 5% level of probability (GenStat 17, VSN International; Herts, UK).

2.4 Shikimate assay

The shikimate assay was conducted using the method described by Shaner *et al*.,[30] with modifications. Ten leaf discs (3.5 mm diameter) were excised from a single fully expanded leaf of each plant from the susceptible (CT3), and resistant (A528 and A536) populations. Two discs were placed into a single well of a 96-well flat-bottomed microtitre plate, with each well containing one of five glyphosate rates; 0, 50, 200, 500 or 1000 µM (Roundup PowerMax; Nufarm, Vic, Australia) and 10mM of phosphate buffer (pH 7). The plates were 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^{o}C for 16 h.

Following incubation, 0.06 M HCl was added to each well, and the samples frozen and thawed through two cycles at -80^{o}C for 15 min followed by 60^{o}C for 15 min

until the leaf tissue was no longer green. From each well, 25 µL was transferred into new empty microtitre plates to measure shikimate levels. Similarly, 25 µL of shikimic acid solution at concentrations of 1, 2.5, 5, 10, 25 and 50 µM was added into empty wells as standards. Then 100 µL of 0.25% (w/v) periodic acid and 0.25% (w/v) sodium m-periodate solution was added to each well for both extract and shikimic acid standards. The samples were incubated for 60 min at room temperature before the reaction was terminated by the addition of 100 µL of quench buffer [0.6M NaOH, 0.22M of Na_2SO_3] into each well. A 150 µL aliquot was taken from each well to measure absorbance at 380nm with a double-beam spectrometer (Cintra10 UV, GBC; Melbourne, Vic, Australia).

The mean value of optical density measured in the controls of each population was subtracted from those measured in the glyphosate treatments as background. A shikimate standard curve was developed, and shikimate levels were expressed as nM of shikimic acid accumulated per cm^{-2} leaf area.

The experiment was conducted twice, and five leaves from five plants of each population used as replicates. As there was no experiment by treatment interaction, the data was pooled. Data of shikimic acid accumulation at different glyphosate concentrations was best fitted to an exponential model (one phase association) using GraphPad Prism version 7.02 (GraphPad Software; La Jolla, California, USA).

2.5 *EPSPS* gene sequencing

Young green leaf tissue was sampled from five plants of each population (CT3, A528 and A536), and DNA extracted using the Isolate II Plant DNA kit (Bioline, Alexandria, NSW, Australia) in accordance with the manufacturer's instructions.

For polymerase chain reaction (PCR) amplification, a standard 25 µL PCR reaction mix contained: 20-40 ng DNA, 1× high-fidelity PCR buffer [600mMTris-SO4

(pH 8.9), 180mM $(NH4)_2SO_4$], 0.4 mM dNTP mixture, 4 mM MgSO4, 0.4 µM of each specific primer (Table 1), 1 unit of My fi Taq DNA polymerase (Bioline, Alexandria, NSW), and 5% of dimethyl sulfoxide. The forward and reverse primers were used for amplification of a 422 bp fragment of the *EPSPS* gene.

An automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Eppendorf; Hamburg, Germany) was used for amplification with the following cycle parameters: 1 min denaturing at 95°C; 40 cycles of 15 s denaturation at 95°C, 15 s annealing at 59°C and 1 min elongation at 72°C, and a final extension for 10 min at 72°C.

PCR products were examined on 1.0% agarose gels stained with 1× SYBR® Safe DNA gel stain (Life Technologies; Mulgrave, Vic, Australia). Samples were electrophoresed in 1× TAE buffer [40mM Trizma base, 1mM Na_2EDTA (pH to 8) with glacial acetic acid] at 120 V and photographed under UV light (λ=302 nm). DNA fragment sizes were estimated by comparison with known size bands of the Easy Ladder (Bioline; Alexandria, NSW, Australia).

PCR products were sequenced with the same primers used for the amplification at the Australian Genome Research Facility Ltd (AGRF). ClustalW alignment was employed to compare sequencing data of a susceptible, two resistant populations and the known *EPSPS* susceptible sequences of *Eleusine indica* (L.) Gaertn. AJ417034 [8] using the Geneious software package (Geneious 8.1.3, http://www.geneious.com).[31]

2.6 *EPSPS* gene copy number

Genomic DNA (CT3, A528 and A536) used for *EPSPS* gene copy number analysis was the same as that used for *EPSPS* gene sequencing described above. DNA samples from five plants of each population were used in quantitative real-time PCR (QPCR) to measure genomic *EPSPS* copy number. The copy number was

determined by calculating the ratio of *EPSPS* to internal control genes, acetolactate synthase (*ALS*) and NADH dehydrogenase subunit F (*NADH*), for which copy number is not expected to vary across *C. truncata* individuals.

A KAPA PROBE FAST Universal (KAPA Biosystems, Wilmington, MA) assay using Dual-Labelled BHQ FRET probes (Bioresearch Technologies, Petaluma, CA) (Table 1) was used. *EPSPS* and control gene probes were designed with different fluorophores so the genes could be assayed independently in one QPCR reaction. QPCR reactions of 10 µl contained 5 µl of 2× KAPA PROBE FAST QPCR master mix universal, 200 nM CtEPSPS and either CtALS or CtNADH forward and reverse primers (Table 1), 100 nM CtEPSPS-FAM and either CtALS-TET or CtNADH-TET probe and ~100 ng gDNA from individuals of susceptible (CT3) or resistant (A528 and A536) populations. QPCR experiments were assembled by hand, in triplicate and run on a RG6000 Rotor-Gene real-time thermal cycler (Corbett Research, Mortlake, NSW, Australia) with the following parameters: 3 min at 95°C followed by 40 cycles of 2 s at 95°C, 20 s at 60°C, acquiring at 510 nm (CtEPEPS-FAM), and at 555 nm (CtALS-TET) and (CtNADH-TET).

Primer efficiency curves were produced for each primer set using a 2-fold serial dilution of genomic DNA templates, ranging from 19 ng to 150 ng. Primer efficiencies were calculated to be >98% - <102%. A modified version of the $2^{-\Delta\Delta Ct}$ method was used to analyse data from the quantitative PCR experiments.[13, 32] Relative quantification of genomic *EPSPS* was calculated as ΔCt = (Ct, *ALS* – Ct, *EPSPS*). Genomic *EPSPS* copy number increase was expressed as $2^{\Delta Ct}$. For each individual sample, there were three technical replicates, and the average increase in genomic *EPSPS* copy number and standard error of mean were calculated for each sample. Results were expressed as fold increase in genomic *EPSPS* copy number relative to *ALS*.

Table 1. Primers and probe sequences used in *EPSPS* gene sequencing and copy number determination analysis		
Gene		Primer/Probe Sequence 5′ – 3′
For sequencing		
EPSPS	Primer_F	AACAGTGAGGAYGTYCACTACATGCT
	Primer_R	CGAACAGGAGGGCAMTCAGTGCCAAG
For QPCR		
CtEPSPS	Probe	CCGTAACTGCCGCTGGAGGAAA -*FAM*
	Primer_F	TGCGACCGTTGACAGCAG
	Primer_R	CACCACAGTTTGGCTACTAAGCAT
CtNADH	Probe	ACCCGATGCTATGGAAGGACCCA -*TET*
	Primer_F	CTGCACAATTCCCTCTTCACGTATG
	Primer_R	CTGCGTGTATAAGAGCCGAAATGG
CtALS	Probe	CCAATCGTCGAGGTCACCCGCT -*TET*
	Primer_F	GACTCCATCCCCATGGTC
	Primer_R	CGAGGTAGTTGTGCTTGGTG

3 RESULTS

3.1 Whole-plant dose response to glyphosate

Glyphosate resistance was confirmed in five populations of *C. truncata* (A536, A528, T27, A534 and A535.1) with the resistance index (RI, LD_{50} relative to the susceptible biotype) for the populations ranging between 2.4-8.7 fold (Figure 1 and Table 2). The susceptible CT3 population was controlled at 1000-3000 g a.e glyphosate ha^{-1}, with an LD_{50} value of 538-1386 g a.e ha^{-1}. A536 was the most resistant population, with RI of 4.0-8.7 fold, and required 8000-11000 g a.e ha^{-1} glyphosate for its control. The four other resistant populations had LD_{50} values from 1856 to 4737 g a.e ha^{-1} and RI from 2.4 to 6.7 fold (Figure 1 and Table 2). Among the three experiments, the LD_{50} of each population was higher in Experiment 2 than in Experiment 1 and 3, except for population T27.

As A536 and A528 were the two most resistant *C. truncata* populations in Experiment 1, they were chosen for subsequent experiments to identify possible resistance mechanisms to glyphosate.

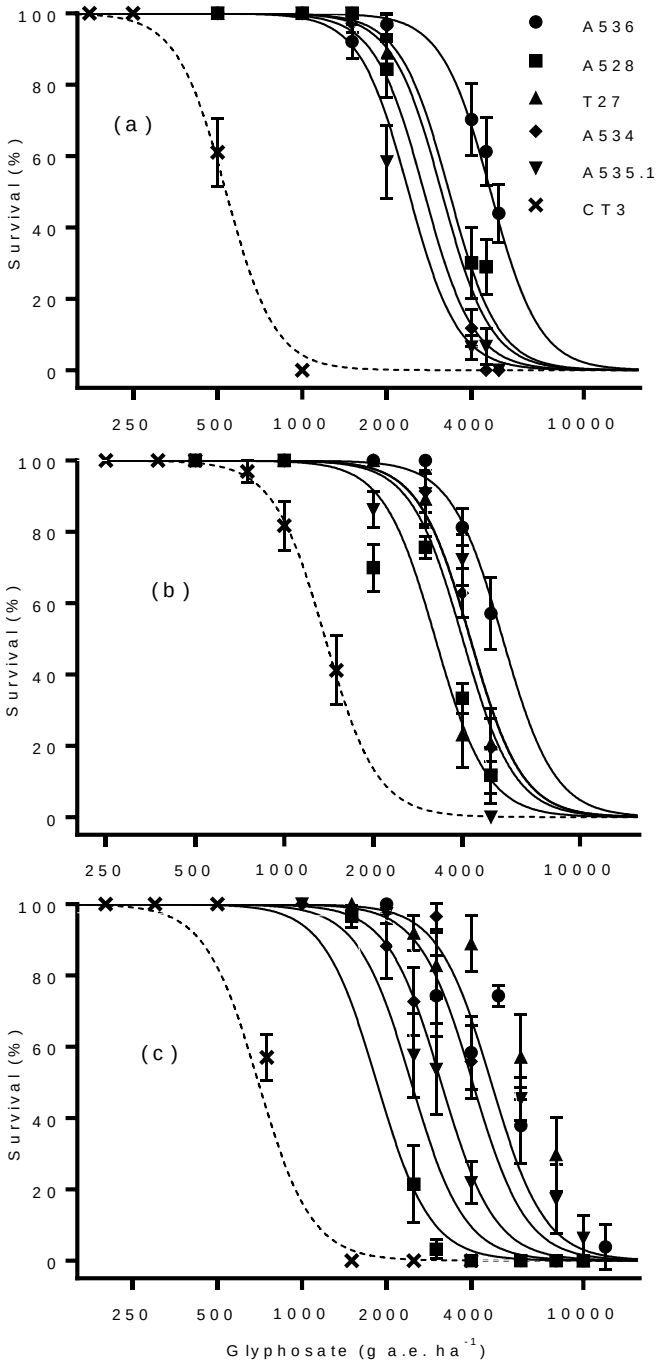

Figure 1. Glyphosate dose response experiments of *C. truncata* populations (susceptible CT3 and resistant A536, A528, T27, A534 and A535.1) in November 2014 (a), February 2015 (b) and November 2015 (c). Each data point is the mean of four replicates, and the vertical bars are standard error of mean. The curves were fitted using an equation: $Y = 100*[1 - NORMSDIST(B + A*X)]$, where Y (% survival) is back transformed from mortality (expressing as normal equivalent deviates), and X is log(dose).

Table 2. Glyphosate dose required for 50% mortality (LD_{50}) (g a.e ha^{-1}) of a susceptible (S) and five resistant (R) *C. truncata* populations with 95% fiducial limits in parentheses and resistance index (RI) of the R populations compared with the S population. The experiments 1, 2 and 3 were conducted in November 2014 (21), February 2015 (21) and November 2015 (21) respectively.

Population	Experiment 1		Experiment 2		Experiment 3	
	LD_{50}	RI	LD_{50}	RI	LD_{50}	RI
A536 (R)	4692 (4312, 5119)	8.7	5514 (4759, 6504)	4.0	4070 (3651, 4524)	5.8
A528 (R)	3344 (3025, 3693)	6.2	3265 (2900, 3671)	2.4	1856 (1588, 2154)	2.6
T27 (R)	3143 (2641, 3782)	5.8	4274 (3806, 4827)	3.1	4737 (4211, 5333)	6.7
A534 (R)	2724 (2464, 3004)	5.1	4288 (3785, 4891)	3.1	3144 (2809, 3516)	4.5
A535.1 (R)	2399 (2176, 2644)	4.5	4034 (3570, 4583)	2.9	2461 (2196, 2750)	3.5
CT3 (S)	538 (477, 609)	-	1386 (1197, 1624)	-	703 (604, 821)	-

3.2 Glyphosate absorption and translocation

Absorption and translocation of ^{14}C-glyphosate was determined in susceptible CT3 and two resistant A528 and A536 populations. There was no difference in absorption of ^{14}C-glyphosate between the susceptible and resistant plants, with 47-49% of the applied ^{14}C-glyphosate being absorbed into leaf tissue by 48 HAT (Table 3). The amount of absorbed ^{14}C-glyphosate in treated leaves of the susceptible plants was similar to that of the resistant plants (42-44%). Additionally, a similar amount of ^{14}C-glyphosate was translocated into stems (21-24%) and roots (24-30%) of the susceptible and resistant plants. The only significant difference between either of the resistant populations and the susceptible was the amount of ^{14}C-glyphosate in untreated leaves of A536 plants being higher than that of the susceptible plants.

Table 3. ^{14}C-glyphosate absorbed and distribution of absorbed radiolabel from the treated leaf to other plant parts of a susceptible (S) and two resistant (R) populations of *C. truncata* 48 h after treatment. Means (± standard error) within a column followed by the same letter are not significantly different (Fisher's protected LSD test P ≤ 0.05).

Population	Absorption (% applied)	Translocation (% absorbed)			
		Treated leaf / leaf blade	Untreated leaf / leaf blade	Stem / leaf sheath	Root
CT3 (S)	48.9 ± 2.8 a	44.3 ± 2.4 a	2.3 ± 0.5 a	23.6 ± 1.1 a	29.8 ± 1.6 a
A528 (R)	47.2 ± 2.1 a	41.9 ± 1.9 a	3.6 ± 0.7 ab	22.6 ± 1.0 a	31.8 ± 1.4 a
A536 (R)	48.2 ± 2.9 a	43.0 ± 3.0 a	5.4 ± 0.9 b	21.4 ± 1.7 a	24.4 ± 1.7 a

3.3 Shikimate assay

Shikimate accumulates in plants when *EPSPS* is inhibited by glyphosate. This is because shikimate-3-phosphate, a substrate in the reaction catalysed by *EPSPS*, converts to shikimate and accumulates faster than it can be consumed in other metabolic pathways.[33] A significant difference in shikimate accumulation was observed between susceptible CT3 and resistant (A528 and A536) plants (Figure 2). Shikimate accumulation in the susceptible plants was significantly higher than in the A528 and A536 plants at all glyphosate concentrations used. Shikimate accumulation in CT3, A536 and A528 plants reached a maximum of 263,192 and 191 nmole cm^{-2} leaf, respectively, before plateauing out. The calculated IC_{50} values (glyphosate concentration to accumulate half of the maximum shikimic acid) of A528 (400 µM) and A536 (251 µM) plants were 13.3 and 8.3-fold higher, respectively, than that of the susceptible CT3 plants (30 µM) (Figure 2).

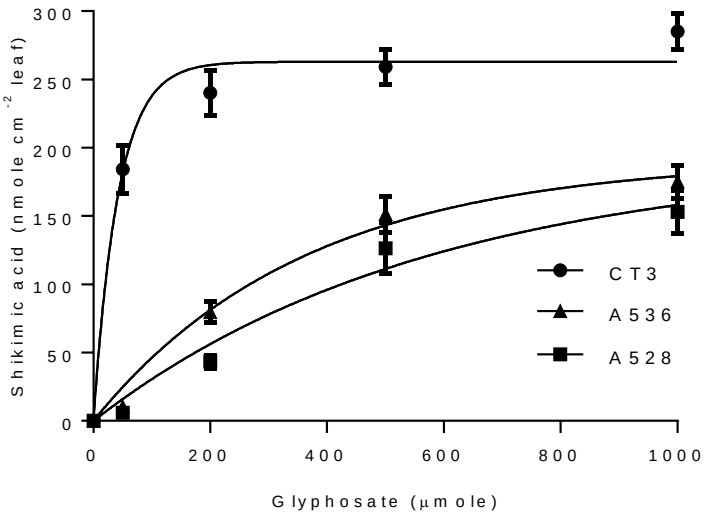

Figure 2. Shikimic acid accumulation of leaf discs from one susceptible (CT3) and two resistant (A528, A536) *C. truncata* populations at different glyphosate concentrations. Each data point represents the mean of two experiments pooled with five replicates, and vertical bars are standard error of mean. The curves were fitted using an equation: $Y = 262.9*[1 - EXP(-0.0235*X)]$ $R^2 = 0.85$ for CT3, $Y = 192.1*[1-EXP(-0.0011731*X)]$ $R^2 = 0.76$ for A528 and $Y = 191.3*[1-EXP(-0.002763*X)]$ $R^2 = 0.87$ for A536.

3.4 *EPSPS* gene sequencing

To determine the possibility of a resistance mechanism based on target-site mutation, a 422-bp fragment of the *EPSPS* gene covering the most common mutation site, Pro-106, was sequenced from five individual plants each of the susceptible (CT3) and two resistant (A528 and A536) populations. The partial *EPSPS* sequence of the resistant plants did not reveal any mutation at Pro106 (Table 4). However, a single nucleotide substitution of GAG to GCG at codon 91 was detected in both resistant populations. The nucleotide change of A to C at the second base of the codon 91 would result in an amino acid substitution from glutamic acid to alanine (Table 4).

Table 4. Nucleotide and predicted amino acid sequence of *EPSPS* DNA isolated from a susceptible and two resistant populations of *C. truncata*.

Amino acid number*	89	90	91	92	93	104	105	106	107	108
Amino acid	Ala	Lys	Glu	Glu	Val	Met	Arg	Pro	Leu	Thr
Consensus sequence	GCG	AAA	GAG	GAA	GTG	ATG	CGA	CCG	TTG	ACA
CT3 (S)	GCG	AAA	GAG	GAA	GTG	ATG	CGA	CCG	TTG	ACA
A528 (R)	GCG	AAA	G**C**G Ala	GAA	GTG	ATG	CGA	CCG	TTG	ACA
A536 (R)	GCG	AAA	G**C**G Ala	GAA	GTG	ATG	CGA	CCG	TTG	ACA

*Amino acid assignments are based on the plant *EPSPS* numbering system used by Padgette *et al.*[59]

2.5 *EPSPS* gene copy number

QPCR was used to measure relative genomic copy numbers of the *EPSPS* gene relative to the *ALS* gene in resistant and susceptible individuals. Genomic *EPSPS* copy numbers relative to the *ALS* gene ranged from 0.92 to 1.08 (n=5) for susceptible plants, whereas relative copy numbers for resistant plants (A536 and A528) were much higher with 28.04 ± 1.37 and 32.34 ± 5.4, respectively (Figure 3). The highest relative copy numbers of *EPSPS* found in a single plant of A536 and A528 were 32.2 and 48.0 fold. Similar results were obtained when *NADH* was used as the control in place of *ALS* (data not shown).

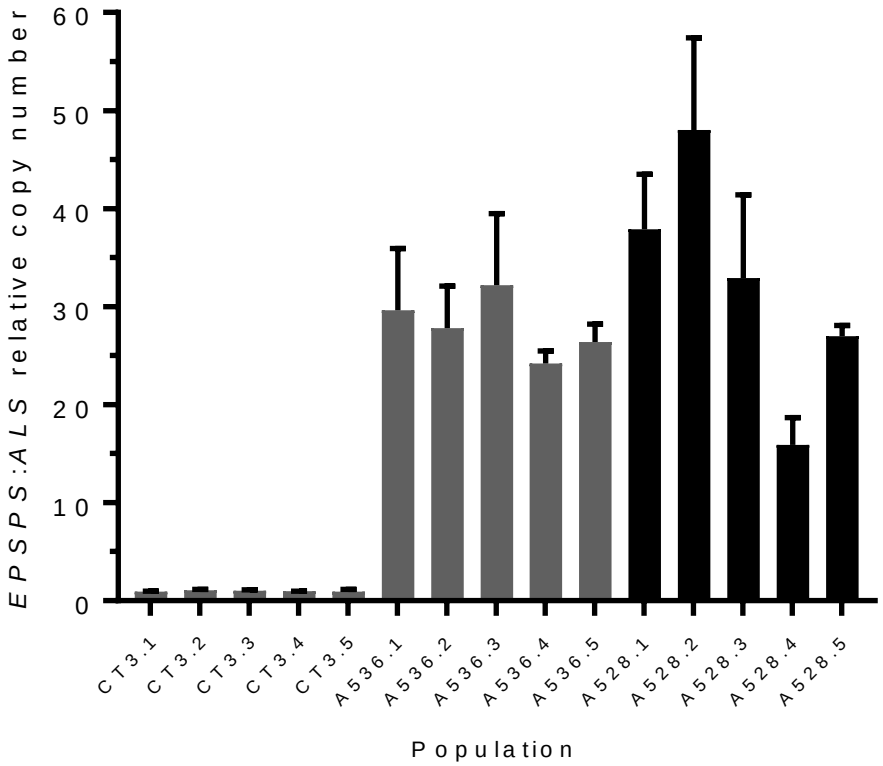

Figure 3. Genomic *EPSPS* gene copy number relative to the *ALS* gene in five individuals each of the susceptible (CT3) and resistant (A536 and A528) populations of *C. truncata*. Vertical bars are standard error of mean for three replicates.

4 DISCUSSION

4.1 Whole-plant dose response

In the dose response experiments, glyphosate field rates recommended for the control of *C. truncata* in Australia under field conditions (330-665 g a.e ha^{-1}) are unlikely to effectively control the susceptible population (LD_{50} of 538-1386 g a.e ha^{-1}). However, higher doses than that of the susceptible population were required for the control of resistant populations (Table 2). In addition, only half of the applied ^{14}C-glyphosate (47-49%) was absorbed into the susceptible and resistant plants by 48 HAT (Table 3). This low absorption may contribute to higher tolerance of *C. truncata* (S population) to glyphosate than other local weeds. Glyphosate absorption in *C. truncata* in this study was lower than that reported in *Lolium rigidum*[18] (76-78%). Glyphosate is taken up through plant surfaces, and leaf uptake rates vary considerably between species. Diffusion is the most likely mode of transport across the plant cuticle,[1] which varies in composition and thickness in various plant species. These results support field observations of high glyphosate tolerance[21, 34] in *C. truncata* and studies reporting resistance to glyphosate in *C. truncata* populations.[23, 24]

Efficacy of glyphosate can be reduced by several environmental conditions, such as high air temperature[35], low soil moisture content[36] and low relative humidity.[37, 38] Glyphosate application at 20-25◦C has been shown to control *Echinochloa colona* better than at 30-35◦C.[39-41] A reduction in glyphosate absorption in *E. colona* when air temperature increased[39] explained the difference in the plant response. In two other grass species (*Sorghum halepense* and *Lolium rigidum*) higher levels of glyphosate resistance have also been reported at higher temperatures.[42] Under the well-watered conditions of the present study, the higher glyphosate efficacy especially on the susceptible population seen in Experiment 1 and 3 compared with Experiment 2 (Table

2) could be due to differences in environmental conditions such as air temperature and relative humidity.

4.2 Absorption and translocation of glyphosate

Reduced glyphosate uptake and/or translocation has been identified as the mechanism of resistance to glyphosate in several weed species.[14-18, 43] Where reduced translocation of glyphosate occurred, glyphosate mostly remained in the treated leaves of resistant plants and a smaller amount of glyphosate was transported to the shoot meristem of the treated plant.[14, 16-18] In the present study, the amount of absorbed ^{14}C-glyphosate in susceptible and resistant plants was similar at 48 HAT, and the radiolabel remaining in the treated leaves of the susceptible and resistant plants was also similar. The higher amount (5.4%) of ^{14}C-glyphosate in the untreated leaves of the resistant A536 plants compared to that of susceptible plants (2.3%) is considered unlikely to contribute to glyphosate resistance (Table 3). This suggests that resistance in populations A528 and A536 is not due to altered glyphosate absorption or translocation.

4.3 Shikimate assay

To assess whether glyphosate inhibits *EPSPS in vivo*, a shikimate assay using excised leaf discs was conducted. Using excised leaf discs in the shikimate assay eliminates the contributions of absorption through the leaf cuticle and translocation of glyphosate to the leaf cells. The leaf disc assay is only affected by the ability of glyphosate to penetrate into the chloroplast and the tolerance of the target enzyme.[39] Shikimate accumulation in plants of CT3 (S) was significantly greater than that of A258 and A536 at all glyphosate concentrations used (Figure 2). This indicated that insufficient glyphosate was present at the *EPSPS* enzyme of the resistant populations

to completely inhibit the shikimate pathway.[30] Therefore, the significant differences in shikimate accumulation between resistant and susceptible plants in this study could be related to a target site mutation within EPSPS,[9, 25] *EPSPS* gene amplification[11, 13] or vacuole sequestration. [19, 20]

4.4 Target-site *EPSPS* mutation

In *EPSPS* sequencing of A528 and A536 populations, a mutation at Pro106 was not identified; however a substitution of the second base of the codon 91 was observed (Table 4). This amino acid is in one of the two known conserved regions of *EPSPS* located between positions 90 and 110, and between 175 and 200 in the mature *EPSPS* enzyme in plants[44]. Amino acid substitutions at positions 101, 102, 106, 144 and 192 can be introduced into the conserved regions of wild-type *EPSPS* enzymes to yield glyphosate-tolerant *EPSPS* enzymes in several plant species[44-46]. A highly conserved region of *EPSPS* of *Escherichia coli* and *Salmonella typhimurium* was also described from amino acid 86 through 131 (position 91 in plant mature *EPSPS* consensus corresponds to position 86 in *E. coli*) [47]. However, Padgette[48] reported that the highly conserved region of *EPSPS* of *E. coli* was shorter from amino acid 90 through 102. To date, single amino acid substitutions at Pro-106 and a double amino acid substitution of Thr-102 + Pro-106 in *EPSPS* are the only known naturally occurring mutations conferring glyphosate resistance in several weed species[9, 10, 49, 50]. A mutation at position 91 of *EPSPS* is not present in any submitted sequences of plant *EPSPS* in the National Center for Biotechnology Information database[51]. Thus, the amino acid substitution from glutamic acid to alanine at position 91 may contribute to resistance in the *C. truncata* populations (A528 and A536) in the present study.

4.5 *EPSPS* gene amplification conferring glyphosate resistance

Glyphosate resistance due to extensive amplification of the *EPSPS* gene was first observed in a population of *Amaranthus palmeri* in 2010.[13] This mechanism has since been identified in six other weed species: *Bromus diandrus*[11], *Eleusine indica*[10] *Amaranthus tuberculatus*,[52, 53] *Kochia scoparia*,[54] *Amaranthus spinosus*[55] and *Lolium perenne ssp. multiflorum*.[12] In the present study, individuals of the glyphosate resistant populations contained 16-48-fold more copies of the *EPSPS* gene than did susceptible plants, with the number of *EPSPS* copies found to be variable both between and within populations. This variability was also found in glyphosate resistant plants of other species with this mechanism including *A. palmeri* from Georgia, Carolina and New Mexico with 5-160-fold,[13] 22-36-fold[56] and 2-8-fold[57] respectively more copies of the *EPSPS* gene; and two grass weed species with a 10-36-fold increase[11] in *B. diandrus* and a 10-25-fold increase[12] in *L. multiflorum*.

It has been suggested that the effect of additional copies of *EPSPS* is additive, and additional copies confer higher levels of resistance to glyphosate.[13] This amplification of the *EPSPS* gene produces sufficient EPSPS protein that the shikimate pathway can continue to operate despite glyphosate inhibiting some of the enzyme.[58] This was observed in the present study where shikimate accumulation in resistant plants with a relative *EPSPS* copy number of 16-48 was significantly lower than that of susceptible plants (Figure 2). These two resistant populations had a similar level of *EPSPS* gene amplification, ranging from 28.04 ± 1.37 (A536) to 32.34 ± 5.4 (A528); however, the LD_{50} of A536 was 1.4-2.2-fold higher than that of A528 in the whole-plant dose response experiments. This difference in whole plant response may be due to difference in *EPSPS* expression between and within populations as was observed in

B. diandrus.[11] This possibility of difference in *EPSPS* expression between and within *C. truncata* populations remains to be determined.

Resistance to glyphosate in the A258 and A536 populations of *C. truncata* from New South Wales is primarily due to a combination of target-site *EPSPS* mutation (Glu-91-Ala) and amplification of the *EPSPS* gene. This is the eighth weed species where amplification of the *EPSPS* gene has been identified. It is not yet known whether increased *EPSPS* gene copy number is stably transmitted to the next generation of plants. The evolution of *EPSPS* gene amplification in glyphosate resistant *C. truncata* populations is not yet fully understood.

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Chapter 6: Conclusions and Recommendations

C. truncata and *C. virgata* have several characteristics that enable them to survive and persist in the Mediterranean environment of South Australia, Australia. These two grass species have rapid germination (time to reach 50% germination: 1.1-2.0 day for *C. truncata*, and 2.7-3.3 day for *C. virgata*) and base temperatures (T_b for germination) of 9.2-11.2^{o}C for *C. truncata* and 2.1-3.0^{o}C for *C. virgata*, so they can germinate and emerge under field conditions after rainfall events in spring, summer and autumn in South Australia. These two species have a short period to maturity, requiring 780 Cd (*C. truncata*) and 1200 Cd (*C. virgata*) from emergence to mature seed stage, and have high fecundity. Germination of *C. truncata* required light, so seedling emergence occurred only for seeds present on the soil surface. Germination of *C. virgata* was also stimulated by light and seedling emergence was highest for seeds present on the soil surface, but declined significantly for seeds buried at 1, 2 and 5 cm. Freshly produced seeds of *C. virgata* required an after-ripening period about 2 months to begin germination, whereas 16-40% of freshly produced seeds of *C. truncata* germinated within a week after maturation. However, seeds of both *C. truncata* and *C. virgata* remained viable for more than 11 months when present on the soil surface and buried at 5 cm depth. Low rainfall over spring and summer in the 3rd year of this study extended seedbank persistence of both *C. truncata* and *C. virgata* beyond 14 months, especially for the seeds buried at 5-cm depth. These characteristics make *C. truncata* and *C. virgata* well suited to the no-till farming system (i.e. no deep burial) widely adopted by the growers in southern Australia, and it is likely these species will become more problematic in such production systems.

To effectively control problem weeds such as *C. truncata* and *C. virgata*, all phases of the life cycle should be managed using a range of chemical and non-chemical methods. Management practices should aim to deplete the seedbank, control seedlings and small plants, stop seed set and prevent new seeds entering from outside the system (Osten, 2012). The demonstration that *C. truncata* and *C. virgata* seeds persist for about 12 months in an average season offers the opportunity to eradicate these weed species from a field, provided there is no external seed invasion. Prevention of new seed production for a period of 12 months or more should exhaust the soil seedbank of *C. truncata* and *C. virgata*. As seeds of *C. virgata* have a base temperature of 3^{o}C, they are likely to germinate earlier in spring than *C. truncata* seeds that require soil temperatures above 9^{o}C to germinate. This information allows farm managers to focus on periods during the year when seedlings may establish and need to be controlled. Lastly, seed germination has an absolute requirement for light in *C. truncata* or was stimulated by light in *C. virgata*. Therefore, burial to depths greater than 0.5 cm (*C. truncata*) or 5 cm (*C. virgata*), through strategic inversion tillage for example, could completely inhibit germination.

Two target-site EPSPS mutations (Pro-106-Leu and Pro-106-Ser) were identified in four glyphosate-resistant populations of *C. virgata* (V14.2, V15, V14.16 and V12 with LD_{50} from 2- to 9.7-fold) from South Australia, Queensland and New South Wales. The *C. virgata* population (V14.2) with Pro-106-Leu substitution was 2.9- to 4.9-fold more resistant than those with Pro-106-Ser substitution (V15, V14.16 and V12). These EPSPS mutations were likely to be the primary mechanism of glyphosate resistance, because reduced glyphosate uptake and translocation were not observed in the four resistant populations. *C. virgata* has a level of natural tolerance to glyphosate and therefore requires high rates to control it in the field. The mechanism

for high natural tolerance to glyphosate in this species is unclear but it was not due to reduced glyphosate absorption or translocation, as the amounts of herbicide absorbed and the amount translocated out of the treated leaf were similar to those observed in other grass weed species (Bostamam et al., 2012; Ghanizadeh et al., 2015; Wakelin et al., 2004). One possibility is that more glyphosate may be sequestered in the vacuole in this species, as has been observed in some glyphosate-resistant weeds (Ge et al., 2010; Ge et al., 2012). This presence of moderate natural tolerance to glyphosate in this weed species would allow for the selection of the relatively weak mechanism of target site mutations at Pro-106. While susceptible plants may not be controlled adequately at normal field rates, they are likely to be significantly damaged but individuals with Pro-106 mutations would be favoured in the field under intense glyphosate pressure.

In *C. truncata*, resistance to glyphosate in the A258 and A536 populations from New South Wales is primarily due to amplification of the EPSPS gene. These two resistant populations had a similar level of EPSPS gene amplification, ranging from 28.04±1.37 (A536) to 32.34±5.4 (A528); however, the LD_{50} of A536 (4.0-8.7-fold) was 1.4- to 2.2-fold higher than that of A528 (2.4-6.2) in the whole-plant dose response experiments. This difference in whole plant response may be due to difference in EPSPS expression between and within populations as was observed in *B. diandrus* (Malone et al., 2016). This possibility of difference in EPSPS expression between and within *C. truncata* populations remains to be determined. Furthermore, it is not yet known whether increased EPSPS gene copy number is stably transmitted to the next generation of plants.

The evolution of EPSPS gene amplification in glyphosate-resistant populations of *C. truncata* and target-site EPSPS mutations in *C. virgata* resistant populations is

not yet fully understood. Main factors that influence the evolution of herbicide resistance in weeds include genetic mutations, intensity of selection pressure by herbicides, initial frequency of resistant alleles, fitness of resistant plants, gene migration, and inheritance of resistance and features of the weed seedbank (Jasieniuk et al., 1996). Previous studies have revealed that resistance to herbicides in general and to glyphosate in particular in plants has been controlled by a single gene in most examples and herbicide resistance due to multiple genes tends to be less common (Darmency, 1994; Gasquez, 1997; Mohseni-Moghadam et al., 2013). Furthermore, glyphosate resistance is inherited from a nuclear gene mutation (Jasieniuk et al., 1996) rather than from a maternally inherited trait as in triazine resistance (Darmency and Pernes, 1985; Gasquez and Darmency, 1983).

The evolution of glyphosate resistance in *C. truncata* and C*. virgata* means that strategies reliant on glyphosate alone will not be successful and other management tactics will need to be adopted. Both *C. truncata* and *C. virgata* are difficult to control with herbicides and there are very few herbicides specifically registered for the control of these weeds in Australia (Borger et al., 2010; Borger et al., 2011; Street, 2011; Widderick et al., 2011). Herbicide efficacy on weeds is well known to be affected by plant growth stage. Mature plants are usually more tolerant of herbicides than the younger plants. For example, *C. truncata* plants at the 2-4 leaf stage were completely killed by glyphosate 1080 g a.i. or paraquat 270 g a.i./diquat 230 g a.i. ha^{-1}, whereas these herbicides were ineffective on mature plants (Borger et al., 2010). Similarly, about 99% *C. virgata* was controlled by glyphosate at the 2-3 leaf stage, while the herbicide efficacy was reduced to 79% and 30% at the 5 leaf stage and at early-late tillering respectively (Widderick et al., 2011). A double-knock approach (i.e. an ACCase-inhibiting herbicide followed by paraquat) should be utilised for the effective

fallow control of these two weeds (Cameron et al., 2012; Widderick et al., 2011), because the individual products are not effective on their own. The double knock tactic can reduce the reliance on glyphosate, help prevent or significantly delay glyphosate resistance evolution and aid in the management of these problematic weeds by greatly reducing seed set (Widderick et al., 2011). For instance, mature *C. truncata* plants under field conditions were successfully killed by glyphosate 540 g a.e. ha^{-1} followed by paraquat 135 g a.i./diquat 115 g a.i. ha^{-1} (Borger et al., 2010). In addition, certain ACCase-inhibiting herbicides, such as quizalofop-p-ethyl and haloxyfop-methyl, show good promise to control *C. truncata* (Borger et al., 2010; Street, 2011) and can be used as an important component of the first application in the double knock. However, Widderick et al. (2011) found that the double knock was only effective (99% control) when applied to seedlings of *C. virgata*. The efficacy of double knock on *C. virgata* was reduced to 90% when applied at early tillering stage, to 9% when applied to late tillering plants and 0% when applied to tussock plants.

Environmental conditions also affect the efficacy of herbicides. If a weed has been stressed by heat or moisture, it will be harder to kill than a plant at an equivalent growth stage that has never been stressed (Borger et al., 2011). For example, Cameron et al. (2012) reported that a double knock can give good levels of control of *C. truncata*, if applied to plants that are actively growing and not stressed. Therefore, weeds in summer fallow such as *C. truncata and C. virgata* should be treated with herbicide 7-21 days after rainfall (Borger et al., 2011). The amounts of glyphosate absorbed in *C. truncata* (47-49%) and *C. virgata* (58-62%) were similar to those observed in other grass weed species (Bostamam et al., 2012; Ghanizadeh et al., 2015; Wakelin et al., 2004). It is necessary to identify a suitable surfactant or adding more surfactant to the spray solution to increase absorption of glyphosate.

In our pot studies, pre-emergence herbicides such as pendimethalin applied on field soil in winter still effectively controlled germination and seedling emergence of both *C. truncata* and *C. virgata* six month later in summer (data not shown). Therefore, field experiments should be conducted to confirm this result because preliminary field trials showed this herbicide to be extremely effective against these weed species.

Efficacy of glyphosate can be reduced by several environmental conditions, such as high air temperature (Caseley, 1972), low soil moisture content (Waldecker and Wyse, 1985) and low relative humidity (Hallgren, 1988; McWhorter et al., 1980). Among three whole-plant dose response to glyphosate experiments, the LD_{50} of each *C. truncata* population was higher in Experiment 2 than in Experiment 1 and 3 (Table 2, Chapter 5). As we did not investigate the biochemical basis of the temperature and air humidity dependence of glyphosate resistance, further research is required to identify the effect of temperature and air humidity on efficacy of glyphosate.

The partial EPSPS sequence (422-bp) of resistant populations (A528 and A536) in *C. truncata* did not reveal any mutation at Pro106. However, plants of the both resistant populations had a single nucleotide substitution of GAG to GCG at codon 91, which was not present in the susceptible population (CT3). The nucleotide change of A to C at the second base of the codon 91 would result in an amino acid substitution from glutamic acid to alanine (Table 4, Chapter 5). At this stage it is difficult to determine physiological significance of this mutation. There is a need to sequence the full EPSPS gene of *C. truncata* and *C. virgata* to determine whether there are any mutations at other positions that were not included in our sequencing studies.

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