Genetic Control of Seed Dormancy in *Lolium rigidum* Gaudin and *Bromus diandrus* Roth

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Faculty of Sciences

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Abstract

Bromus diandrus and *Lolium rigidum* are winter annual grass weeds that have become serious weeds of cereal crops in Australia. Surveys of Australian grain growers over the last 20 years have consistently shown *L. rigidum* to be one of the most troublesome weeds. Characteristics such as staggered seed germination, herbicide resistance and adaptation to environmental stresses have also made *B. diandrus* an increasing problem of crop production systems in Australia. From recent studies, some evidence has emerged of changes in germination behaviour of these weeds species that may be making their management more difficult. Studies of genetic variation in seed dormancy within populations of these weeds may help to better understand adaptive response of these weed species to changes in management practices. Even though several researchers have highlighted the need to understand genetic control of weed seed dormancy, not many researchers have investigated this topic.

Seed dormancy was assessed in five *B. diandrus* populations collected from wheat fields in cropping areas of South Australia in 2017. Seedling emergence pattern showed large differences in seed dormancy among these populations. Time to 50% seedling emergence (t_{50}) for *B. diandrus* populations ranged from 10.3 to 28.5d. Seedling emergence in these populations of *B. diandrus* continued for up to 80d which provided an opportunity to select early and late emerging cohorts within each population in 2018 and 2019. Evaluation of seeds of the progeny of these cohorts in 2019 and 2020 consistently showed significant differences in seed dormancy. Seeds produced by early cohorts had a lower dormancy than seeds of late cohorts. For example, the seeds of the early cohort of Riverton population had t_{50} of 23.5d as compared to 46d for the late cohort. A similar pattern of differences in t_{50} between the seeds of early and late cohorts was also observed in Mallala population. Consistent differences in seed dormancy expression between the early and late cohorts of the same population clearly indicated that the expression of seed dormancy in these cohorts was under strong genetic

control. In order to identify genes associated with differences in seed dormancy, seeds of early and late cohorts of Riverton and Mallala were analysed for the expression of *ABA1* and *GA20ox* genes using qPCR at 30, 60, 90 and 120 days after maturity. Higher *ABA1* and lower *GA20ox* gene expression was observed in seeds of late cohorts (high dormancy) compared to the early emerging cohorts (low dormancy). During the after-ripening period, the expression of *GA20ox* increased and *ABA1* decreased, which was correlated with the loss of seed dormancy in late emerging cohorts. These studies have shown large differences in seed dormancy between individuals present within the same populations of *B. diandrus*. Presence of genetic variation for this important trait could play an important role in their adaptation to escape pre-sowing weed control tactics and become an even greater problem in field crops grown in this region.

Similar studies of seed dormancy were also undertaken on *L. rigidum*. Seeds of seven *L. rigidum* populations were collected in 2017 from wheat fields on three commercial farms in the medium (Paskeville and Roseworthy) and high (Hilltown) rainfall areas of South Australia. Presence of greater than two-fold differences in seed dormancy between populations from the same farm indicates likely influence of management practices on selection for seed dormancy. For example, the time taken to reach 50% (t_{50}) of total seasonal seedling emergence for 3 populations from a single farm at Roseworthy ranged from 11d to 27d. Similarly, t_{50} for 2 populations from a farm at Hilltown varied from 13d for HT1 to 23d for HT2. Occurrence of seedling emergence over a long period in *L. rigidum* populations provided an opportunity to select low (LD) and high dormancy (HD) cohorts. Seeds produced by these cohorts of three *L. rigidum* populations over two years showed significant differences in seed dormancy between them. Seeds of low and high dormancy cohorts of HT2, PSK1 and RAC3 were concurrently analysed for seed dormancy and the expression of *ABA1* and *GA20ox* using qPCR at 30, 60 and 90 days after maturity. Greater *ABA1* and lower *GA20ox* gene expression was observed in seeds of high dormancy cohorts than in low dormancy cohorts. During the after-ripening

period, the expression of *GA20ox* increased and *ABA1* decreased, which was significantly correlated with seed dormancy. The results from this investigation provide evidence for genetic control of seed dormancy in field populations of *L. rigidum*. Presence of heritable genetic variation in seed dormancy in *L. rigidum* populations will allow this weed to rapidly adapt to changes in weed management practices such as delayed sowing of crops.

As water stress during spring time can be a frequent occurrence in Australian grain growing regions, studies were undertaken to determine the impact of moisture stress during reproductive development on seed dormancy in *B. diandrus* and *L. rigidum* populations. Seeds of these populations were also investigated for the expression of genes involved with GA and ABA synthesis. Seeds collected from plants of both species exposed to water stress from GS31 onwards were the most dormant in both weed species. In contrast, plants that were well-watered (control) produced seeds with the lowest dormancy. The seeds collected from the well-watered treatment of both *L. rigidum* and *B. diandrus* populations reached 50% germination at 90 days after maturity (DAM) while the germination of stress at GS31 was approximately 50-67% lower than the well-watered (control) treatment. Water stress treatments altered the expression of *ABA1* and *GA20ox* genes which was correlated with the level of dormancy in seeds of *B. diandrus* and *L. rigidum*. The *ABA1* expression levels were high at 30DAM but decreased during the after-ripening periods. This coincided with the gene expression levels of *GA20ox*, which were low soon after maturity but gradually increased. The 2-fold increase in the *GA20ox* levels in all the populations and stress treatments at 90-150DAM points towards the loss of seed dormancy. The differences observed in the seed germination behaviour of water stressed *vs* non stressed treatments and also the gene expression levels of *ABA1* and *GA20ox* highlighted the fact that effects of water stress on seed dormancy are mediated through these dormancy regulating genes in these two weed species.

PUBLICATIONS ARISING FROM THIS THESIS

- Ramiz Z., Malone J., Preston C., Gill G (2022). Selection for seed dormancy within *Bromus diandrus* Roth populations alters ABA1 and GA20ox gene expression.

- Ramiz Z., Malone J., Preston C., Gill G (2022). Genetic control of seed dormancy in *Lolium rigidum* and its association with *GA20ox* and *ABA1* expression.

- Ramiz Z., Malone J., Preston C., Gill G (2022). Impact of water stress during reproductive development on seed dormancy in *Bromus diandrus* Roth and *Lolium rigidum* Gaudin.

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ABREVIATIONS

DAS- days after sowing

DAM- days after maturity

ABA- Abscisic acid

GA- Gibberellic acid

t_{50}- time to 50% emergence

d- days

h- hours

L- Litre

FC- Field Capacity

HD- High dormancy

LD- Low dormancy

NCBI- National Centre for Biotechnology and Information

Qpcr- Quantitative Polymerase Chain Reaction

SEM- Standard Error of Mean

SE- Standard error

RMSE- Root Mean Square Error

HT- Hill Town

PSK- Paskeville

RW- Roseworthy

RAC- Roseworthy Agriculture College

W- Waite campus

R- Roseworthy campus

Chapter 1: Literature Review

1.1 Introduction

Bromus diandrus Roth is a winter annual weed native to Europe and Mediterranean regions. In Australia, *Bromus diandrus* is found in agricultural regions characterized by wet, cool winters and hot dry summers (Kon and Blacklow 1988). *Lolium rigidum* Gaudin was initially introduced into Australia as a pasture species, but became one of the most problematic weeds of Australian cereal based cropping systems (Powles and Bowran 2000). Both of these grass weed species are highly competitive in wheat and other crops where they can cause a significant yield loss (Gill and Blacklow 1985). In a survey of Australian grain growers conducted by Llewellyn *et al.* (2016), *L. rigidum* was ranked as the most problematic weed in winter cereals based on area infested (8,009,979 ha), yield and annual revenue loss ($93.1m). In the same survey, *B. diandrus* was ranked as the 4th most problematic weed of winter cereal crops in Australia, the area infested by *B. diandrus* was reported to be 1,414,297 ha with an economic yield loss of $22.5 m per year.

Bromus diandrus, a self-pollinating grass species, has thrived as a weed in cereal crops in Australia where it is more difficult to selectively control with herbicides. The increase in frequency of crop production and early sowing of crops since the adoption of conservation tillage or no-till systems has also favoured this weed species (Gill *et al.* 1987). *Bromus diandrus* can be highly competitive with wheat, significantly reducing its growth and yield (Gill *et al.* 1987). Research studies have shown that the decline in wheat yield due to competition with *B. diandrus* was largely associated with its ability to compete aggressively for nutrients in soil during the early stages of crop growth (Gill and Blacklow 1985).

Lolium rigidum is a cross-pollinated species that is widely distributed across different climatic zones of Australia. It has been suggested that such a wide distribution of this weed

species is likely to be associated with its ability to adapt to variable soil types and climatic conditions. Some studies have shown that seeds of populations collected from the high rainfall zones tended to be less dormant as compared to those collected from dry areas (Peltzer and Matson 2002). Presence of different levels of seed dormancy within a weed population can lead to staggered or protracted seed germination during the growing season, which is likely to reduce effectiveness of weed control tactics and allow some weeds to escape control and replenish the soil seed bank.

Information on seed ecology and biology is vital for understanding the adaptation of weeds to environmental factors and weed control tactics. Such information could aid in designing effective integrated weed control strategies. The key objective of this research project is to investigate genetic factors responsible for regulating seed germination characteristics of *L. rigidum* and *B. diandrus* populations present in crop production fields. Such studies are expected to enhance our understanding of adaptation of these weed species to cropping systems and weed management practices. This project aims to understand genetic variation in seed dormancy present within populations of these two weed species and role of the expression of genes known to influence seed dormancy. Water stress during spring is an important determinant of crop yields in this environment, which also affects weed seed production. This project seeks to determine the effect of water stress during reproductive development on seed dormancy and expression of target genes associated with seed dormancy.

1.2 *Lolium rigidum*

The genus *Lolium* comprises of 8 species that are native to Europe, temperate Asia, North Africa and the North Atlantic islands. *Lolium rigidum*, commonly known as annual ryegrass was originally cultivated as a pasture species in Australia, but quickly became a noxious grass weed of field crops in South Australia (Kloot 1983). *Lolium rigidum* is considered one of the most troublesome and prevalent weeds of cereal crops grown in Mediterranean climates

including Australia, Argentina, France, Iraq, Portugal and Spain (Gill 1996). In wheat in Spain, it has been shown to cause up to 80% yield loss (González-Andújar and Fernández-Quintanilla 2004).

1.2.1 Biology and Ecology

Kloot (1983) described *L. rigidum* as a hairless bright green plant with narrow shiny leaves that have long auricles and wide ligules. The below ground plant part is often reddish purple and, upon crushing, the seedlings ooze a clear sap. Mature plants usually reach up to a height of 900 mm, the flowering stems are flat with a length of up to 300 mm. Each spikelet has 3 to 9 flowers and the length of the husk is almost equal to the length of the spikelet. The seeds are straw coloured and flat, averaging 4 to 6 mm long and the seed embryo is visible through the outer layers. The seeds are attached securely to the flower stem and a significant force is needed to separate them.

1.2.2 Weedy traits

Many factors contribute to the weediness of *L. rigidum* in southern Australian cropping systems. These include:

Large seed production: *Lolium rigidum* can produce large numbers of viable seed with the potential to produce over 1000 seeds/plant (Reeves and Smith 1975). It has been reported that under irrigated conditions, weed density of 100 plants/m^2 can produce up to 31,000-45,000 seeds/ m^2 in a wheat crop (Rerkasem *et al.* 1980).

High seed survival over summer and autumn: short-lived primary seed dormancy prevents germination after sporadic summer rainfall events and temperature is the major regulator of loss in dormancy (Steadman *et al.* 2003b). Between 40-80% of seeds germinate at the break of the season when conditions are favourable for plant establishment and survival. In addition to this, Gramshaw (1976) reported that potential seed germination rate is increased whenever

seeds are wetted substantially, thus wetting and drying cycles associated with summer rains will affect seedling recruitment in autumn.

Staggered germination: *Lolium rigidum* can germinate in several cohorts throughout the growing season. Genetic factors responsible for the establishment of distinct cohorts of these weed species will be a major focus of this project. Gramshaw (1972) demonstrated that near total germination of the seed bank can occur within 4-6 days under non-limiting moisture conditions, with temperatures cycling between 12 and 24ºC and exposure to light for 12 hours a day. However, meteorological data for many South Australian localities indicates that autumn rain is rarely continuous for more than 2-3 days. Late cohorts are less important for crop competition and yield loss, but contribute to seed bank persistence by avoiding control and producing seed. As most of previous studies on seed dormancy were undertaken 40-50 years ago, it is feasible that *L. rigidum* populations may have evolved to exhibit greater dormancy and staggered germination.

Dark dormancy: 10-20% of the seed population can exhibit dark dormancy (Gramshaw and Stern 1977), whereas seeds exposed to light conditions at the soil surface do not exhibit this dormancy characteristic. This component of the seed bank slowly loses its dormancy and progressively germinates with shallow burial. This may be important for short term persistence of the seed bank. However, the non-dark dormant component of the seed bank tends to have a higher rate of germination under dark conditions than light conditions (Gramshaw 1976). Therefore, cultivation and subsequent burial of seeds may increase germination rates of the non-dark dormant component.

Crop-weed competition and herbicide resistance: *Lolium rigidum* competes with wheat as early as the two leaf stage in heavy infestations and can cause significant yield loss in a wide range of crops (Gill 1996). Several studies of herbicide resistance in *L. rigidum* reported that

this weed has developed resistance to multiple herbicide groups (Broster and Pratley 2006; McGillion and Storrie 2006; Boutsalis *et al.* 2012; Heap 2022).

1.3 *Bromus diandrus*

Bromus diandrus (great brome or ripgut brome) in Australia has been reported to inhabit croplands, pastures, wastelands, hilltops, roadsides, coastal sand dunes and even parks and reserves (Kon and Blacklow 1998). In Australia, *B. diandrus* is dispersed between latitudes 23º and 44º S, in regions that receive annual rainfall greater than 250 mm, a minimum of four months growing period and a mean July temperature of at least 15°C. *Bromus diandrus* can be found across southern Australia, from far eastern Queensland to south-west Western Australia (Kon and Blacklow 1998).

1.3.1 Biology and Ecology

Although native to the Mediterranean and European regions (Kon and Blacklow 1988), plants of the genus *Bromus* can be found across the temperate regions of the world. The species of this genus that have been introduced in Australia include: *B. rigidus, B. diandrus, B. rubens, B. madritensis, B. tectorum* and *B. sterilis* (Kon and Blacklow 1998). However, *B. diandrus* is regarded as the most serious weed of crops and pastures in Australian cropping systems (Williams 1986).

1.3.2 Weedy traits

Competitiveness with crops and herbicide resistance: Bromus diandrus has been reported to be one of the most competitive grass weeds of wheat. Research in Western Australia showed that 100 plants/m^2 of *B. diandrus* reduced wheat grain yield by 30% (Williams 1986). Populations of *B. diandrus* have evolved resistance to herbicides from Groups 1, 2 and 9 (Heap 2022), which reduces herbicide options available to control this species.

Stress tolerance and prolific seed production: This species tolerates drought, phosphorus deficiency, and shows better response to nitrogen than wheat. As a result, providing nitrogen

to the crop may enhance *Bromus diandrus* growth and increase competition with wheat (Reitano and Watts 2006). Seed production can vary from 600 to more than 3000 seeds/plant with a seed mass of 10-13 mg/seed (Kon and Blacklow 1988). The ability to shed a large proportion of seed before crop harvest is another distinguishing feature (Kon and Blacklow 1988), which would reduce the effectiveness of harvest weed seed capture and makes *B. diandrus* more difficult to manage.

Produce contamination and an alternate host to pathogens of cereals: In cropping situations *B. diandrus* contaminates grain (Michael *et al.* 2010). In pastures the seeds contaminate wool, damage hides and meat, and cause injury to livestock by entering their eyes, mouth, feet and intestines. Left uncontrolled in fallow or pasture phases, *B. diandrus* will host and carry over cereal diseases and pests. Diseases include ergot (*Claviceps purpurea*), take-all (*Gaeumannomyces graminis*), powdery mildew (*Erysiphe graminis*), septoria glume blotch (*Leptosphaeria nodorum*), black stem rust (*Puccinia graminis*), brown rust (*Puccinia recondita*), barley net blotch (*Pyrenophora teres*), sharp eyespot (*Rhizoctonia solani*), bunt (*Tilletia caries*) and cereal yellow dwarf virus. Pests include cereal cyst nematode (*Heterodera avenae*) and root-knot nematodes (Amor 1982; Kon and Blacklow 1998).

1.4 Seed dormancy

Seed dormancy has been described as "one of the least understood phenomena in seed biology" (Finch-Savage and Leubner-Metzger 2006), and remains confusing despite much recent progress. This confusion reflects the likelihood that dormancy is not a single phenomenon, but a condition with many contributing causes. Dormancy is the inability of mature, viable seed to germinate under conditions that would otherwise be ideal for germination. Dormancy is a complex phenomenon, which is generally regarded as a seed characteristic (Vleeshouwers *et al.* 1995).

Seed dormancy is common in wild plants, where it may ensure the ability of a species to survive unfavourable conditions, decrease competition between individuals of the same species, or prevent germination out of season. In contrast, domesticated species have been selected for fast and uniform germination, followed by rapid seedling establishment, to achieve good crop yield. However, complete lack of seed dormancy even in crop species is not desirable, because it may result in quality downgrade due to pre-harvest sprouting, a serious problem in cereals including rice, wheat, barley, and maize (Bewely and Black 1994).

The weed seed bank pathway depends on seed dormancy, an attribute with high plasticity in weeds and with tremendous adaptive value to regulate the population in a cropping system (Baskin and Baskin 2006; Westwood *et al.* 2018; Schwartz-Lazaro and Copes 2019; Batlla *et al.* 2020). Potential regulators of dormancy have been recognized as substances whose accumulation in seeds tends to be associated with the depth of dormancy. In order to understand seed dormancy and germination it is essential to differentiate between dormancy (which is considered to be induced during seed maturation on mother plant) and germination. These are two different physiological processes that are dependent on each other (Bentsink *et al*. 2018).

Baskin and Baskin (1998) and Baskin *et al.* (2000) developed a classification system which includes five types of seed dormancy: physiological, morphological, morpho-physiological, physical and a combinational of physical and physiological. All categories of dormancy cause an interruption between seed shedding or dispersal and germination, but the underlying causes can differ (Finch-Savage and Leubner-Metzger 2006).

1) Physiological dormancy

Seed dormancy and germination are induced and regulated by environmental factors, such as light, moisture, temperature and nutrients, and genetic factors, such as seed coat components and the concentration of abscisic acid (ABA) and gibberellic acid (GA). Physiological dormancy is commonly present in major agricultural weeds including *B. diandrus* and *L. rigidum*. This type of dormancy depends on seasonal cueing and allows seeds to remain viable in soil until favourable environmental conditions appear for germination (Batlla *et al.* 2020). Therefore, dormancy release is dependent on seasonal variation and it is relieved during the season preceding emergence (i.e. winter for summer annuals as their germination occurs in spring and summer in winter annuals as their germination occurs in autumn) (Batlla and Benech-Arnold 2010).

2) Morphological dormancy

In morphological dormancy (MD) the seeds have an underdeveloped, but well distinguished, embryo (i.e. cotyledon, hypocotyl and radicle can be differentiated) (Baskin and Baskin, 1998). Embryos in seeds with morphological dormancy are not physiologically dormant and do not need a dormancy-breaking pre-treatment for germination; their only requirement is time to complete maturation (of embryo) and then the radicle protrudes.

3) Morpho-physiological dormancy

Seeds with this kind of dormancy have an underdeveloped embryo with a physiological component of dormancy. Thus, in order to germinate they require a dormancy-breaking pre-treatment. In seeds with morpho-physiological dormancy (MPD), embryo growth/radicle emergence requires a considerably longer period of time than in seeds with morphological dormancy.

4) Physical dormancy

Physical dormancy occurs due to impermeable layers of palisade cells in the seed or fruit coat (Baskin *et al.* 2000). Physical dormancy breaking treatment usually involves physical damage to the seed coat through which water enters the embryo and dormancy is lost (Baskin *et al.* 2000). The maternal environment during seed development such as water stress, temperature and relative humidity have a significant effect on physical dormancy (Fenner 1991).

5) Combinational dormancy

In seeds with combined dormancy (physical + physiological dormancy), the seed (or fruit) coat is water impermeable and, in addition, the embryo is physiologically dormant (Baskin and Baskin, 1998). This type of dormancy could be broken through acid scarification and then treating the seeds with KNO3 and GA3 (Wei *et al.* 2010).

1.4.1 Genetic variation in seed dormancy

Genetic variation in seed dormancy has been reported to occur both between and within species (Baskin and Baskin 1998). The depth of dormancy in several species usually varies with environmental characteristics like altitude and latitude (Baskin and Baskin 1998), which is anticipated to reflect adaptation to diverse environments (Alonso-Blanco *et al*. 2009). The genetic basis of seed dormancy is regarded as an important trait for adaptive evolution of plants, therefore studies involving the mapping of Quantitative trait loci (QTL) has been used to investigate the genetic basis of seed dormancy in *Arabidopsis thaliana* (Van der Schaar *et al*. 1997; Alonso-Blanco *et al*. 2003; Clerkx *et al.* 2004; Bentsink *et al*. 2010). These studies reported that there are several loci that encode dormancy regulating proteins that have been identified by genetic studies (Bentsink *et al.* 2018). Mutations in these loci cause pleiotropic effects, signifying that the occurrence of dormancy is due to the major effect of these regulators (Finkelstein *et al.* 2008).

Some genetic studies involving ABA and GA biosynthesis and signalling mutants have revealed that these two hormones play key role in dormancy induction and release (Finkelstein *et al.* 2008). Genetic studies have discovered that ABA produced by maternal tissues or provided exogenously is not adequate to cause dormancy induction (Finkelstein *et al.* 2002; Finch-Savage and Leubner-Metzger 2006), suggesting that there is a form of embryo-controlled dormancy reliant on ABA synthesis in the embryo or endosperm. Gibberellin (GA) on the other hand is responsible for dormancy regulation and germination (Finch-Savage and Leubner-Metzger 2006). GA accumulation is associated with dormancy release, but treating seeds alone with GA doesn't usually break seed dormancy in some species (Jacobsen *at al.* 2002). A decrease in expression levels of ABA are pre-requisite before the increase of GA levels can cause dormancy release (Bewley 1997; Jacobsen *et al*. 2002).

1) Abscisic Acid (ABA) in the embryo

Many previously undertaken studies indicate that ABA is responsible for inducing seed dormancy during embryo maturation (Finkelstein *et al.* 2002; Nambara and Marion-Poll 2003; Kermode 2005; Finch-Savage and Leubner-Metzger 2006; Feurtado and Kermode 2007). Increase in ABA during seed maturation is correlated with the beginning of seed dormancy and results in inhibition of germination (Finkelstein *et al.* 2008). Dormancy decreases in seeds that are ABA-deficient due to mutations or chemical inhibition of biosynthesis, while overexpression of ABA biosynthetic enzymes results in enhancement of dormancy (Nambara and Marion-Poll 2003; Lin *et al.* 2007). ABA is known as an inhibitor of germination and its biosynthesis plays a vital role in the induction of primary dormancy (Bewley *et al*. 2013). Biochemical, genetic and genomics analyses have revealed the molecular basis of ABA metabolism and action (Figure. 1) (Tuan *et al*. 2018). Alterations in the ABA biosynthetic pathway can significantly influence seed dormancy and germination (Holdsworth *et al.* 2008; Nambara *et al.* 2010). Some fundamental genes/enzymes have been categorized for their

functional roles in ABA biosynthesis and catabolism and in regulating seed germination and dormancy (Nambara *et al.* 2010). Loss of function in ABA biosynthetic genes, comprising *ZEP*/*ABA1* (Koornneef *et al.* 1982), *NCED*s (Lefebvre *et al.* 2006; Frey *et al.* 2012), *ABA2*/*GIN1*/*SDR1*, or *ABA3/LOS5* (Xiong *et al.* 2001), leads to reduction in dormancy, while overexpression of ABA biosynthetic enzymes maximizes the dormancy (Lin *et al.* 2007).

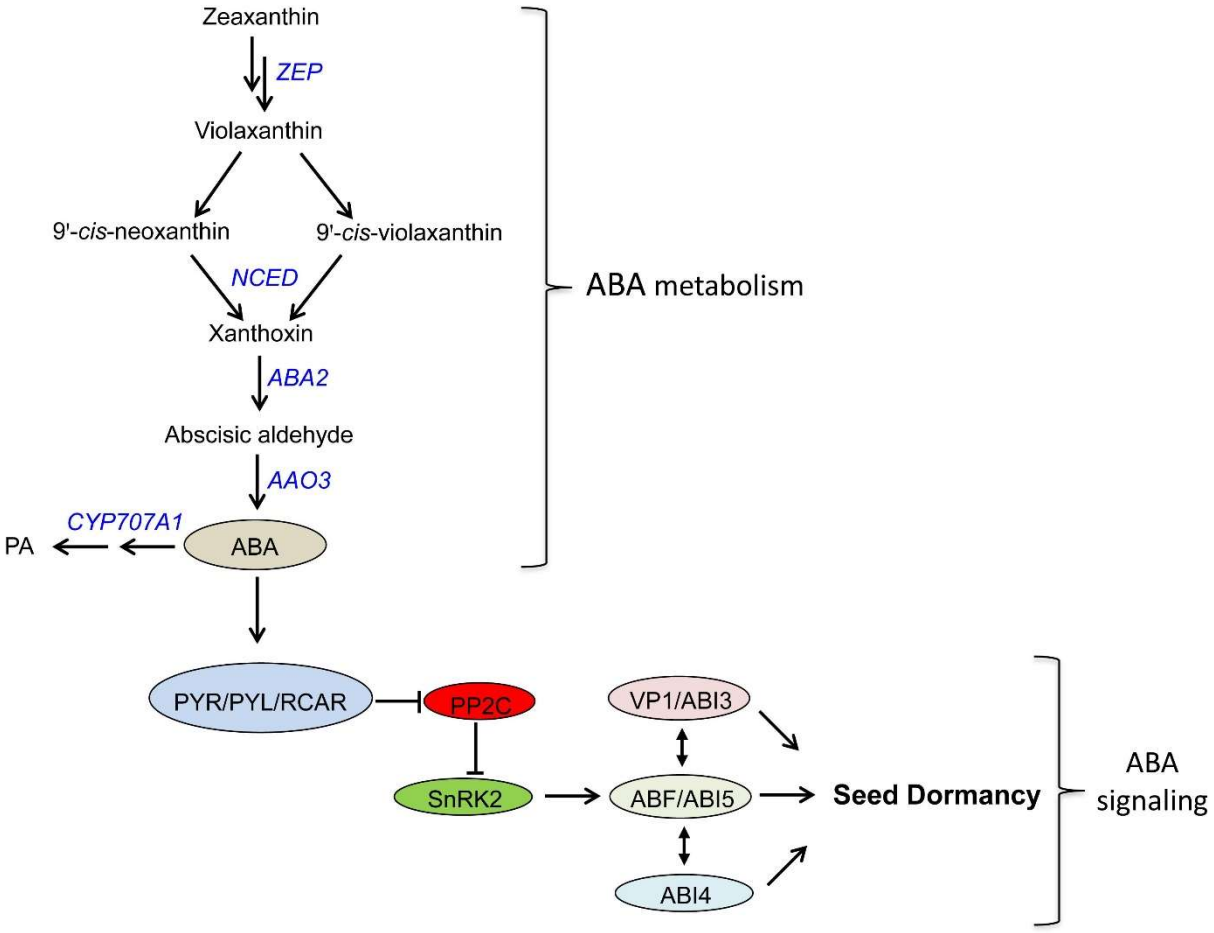


Figure 1. ABA metabolism and signalling pathway in plants (Tuan *et al.* 2018).

2) Seed Coat Components

In various species, the embryo has the ability to germinate, but dormancy is caused by one or more of the tissues that surround the embryo. Such tissues can act as: a) permeability obstructions inhibiting gaseous exchange or water uptake; b) mechanical barriers preventing embryo development; or c) a source of germination inhibitors. Moreover, such tissues can prevent the leaching out of germination inhibitors from embryo tissues during the imbibition process, thereby indirectly prolonging dormancy (Adkins *et al*. 2002). Environmental conditions during seed maturation can have an impact on seed coat hardness in weeds such as black medic (*Medicago lupulina* L.) or the effect of temperature on dormancy in wild oat (Fenner and Thompson 2005). Seed dormancy can also occur due to genetic variation in the structure and pigmentation of the seed coat or adjoining layers (pericarp of the grain) (Groos *et al.* 2002; Sweeney *et al.* 2006; Debeaujon *et al.* 2007). The testa (protective outer covering of the seed) protects the embryo from environmental damage and restricts its growth prior to germination. Seed coat pigments are generally phenolic compounds such as flavonoids. These flavonoids are antioxidants, which limits oxygen availability and constrains metabolic processes of after-ripening and germination, such as oxidative degradation of ABA (Finkelstein *et al.* 2008).

1.4.2 Environmental factors affecting seed dormancy

Seed dormancy helps in the maintenance of the soil seed bank. This results in high weed seed plasticity and supports adaptation of a weed population in a cropping system (Baskin and Baskin 2006; Westwood *et al.* 2018; Schwartz-Lazaro and Copes 2019; Batlla *et al.* 2020). Along with the genetic and development factors, the environment in which the mother plant produces seeds also has a vital influence on seed dormancy (Fenner 1991). The environmental factors which regulate weed seed dormancy are: temperature, moisture, light, pH and nutrient availability.

1) Temperature

Seeds have the ability to adjust their dormancy status by detecting various environmental cues. Among these environmental signals, temperature is of utmost importance across the species, with a difference of as little as 1°C having significant consequences for seed dormancy (Springthorpe and Penfield 2015). High temperatures help in dormancy release of winter annuals, while in summer annuals dormancy is broken by low temperatures (Baskin and Baskin 1985). Soil temperature primarily influences seed germination and dormancy by affecting equally the frequency of germination in non-dormant seeds and the capacity of germination by regulation of seed dormancy (Bouwmeester and Karssen 1992).

Studies have revealed that three cardinal temperatures (minimum, maximum and optimum) define the range of temperature (T) at which seeds of certain species can germinate (Bewley and Black 1994). The minimum or base temperature (Tb) is the lowest T at which germination can occur, the maximum or ceiling temperature (Tc) is the highest T at which seeds can germinate and the optimum temperature (To) is the T at which germination is most rapid. Seed germination rate varies with increasing temperature, it is rapid in the optimal temperature range and slows down above the optimum temperature (Alvarado and Bradford 2002). As seasonal variation controls the temperature, seeds such as *A. thaliana* have temporal sensing signals which determine the temperature fluctuations and alter their seed dormancy status (Nakabayashi and Leubner-Metzger 2021).

2) Soil moisture

Of the several environmental aspects that regulate seed behaviour under field conditions, soil moisture is the basic parameter impacting the seed dormancy status of many plant species (Benech-Arnold *et al.* 2000; Batlla *et al.* 2004). The relative seed dormancy is effected by the environmental conditions experienced by the seeds developing on the parent plant (Batlla *et al.* 2020). *Sinapis arvensis* (L.) produced less dormant seeds from mother plants under water stress

(Wright *et al.* 1999). Similar results have been reported in the winter annual grass species *Avena fatua* and summer perennial *Sorghum halepense* (Peters 1982; Benech-Arnold *et al*. 1992). However, adequate water potential has been reported to increase the production of dormant *Alopecurus myosuroides* seeds (Swain *et al*. 2006).

3) Light

The reaction of seeds to light signals is dependent on phytochromes that consist of a group of proteins acting as sensors to changes in light conditions. Cancellation of dormancy by light is mediated by the phytochromes that have two mutually photo-convertible forms: Pfr (considered the active form) with maximum absorption at 730 nm and Pr (inactive form) with maximum absorption at 660 nm. The photo conversion of phytochrome in the red light (R)-absorbing form (Pr) to the far red light (FR)-absorbing form (Pfr), has been identified as part of the germination induction mechanism in many plant species (Gallagher and Cardina 1998). Therefore, light appears in some cases to function as a dormancy breaking signal when deeply buried seeds are moved to shallower soil depths, whereas in other cases exposure of seeds to light may inhibit germination in various ways depending on the species and circumstances (Travlos *et al.* 2020).

4) Nitrates and gases

The results from some studies highlight the effect of environmental factors, such as nitrates and gases, on the regulation of weed seed dormancy (Bewley and Black 1982; Benech-Arnold *et al.* 2000). Hilhorst and Karssen (1988) illustrated that sufficient light exposure and nitrate availability improves the germination ability of *Sisymbrium officinale* (L.) Scop. While in the case of *A. thaliana*, nitrates alter light-induced germination to some extent (Derkx and Karssen 1994). The seeds of summer annual species, *S. officinale*, showed increased sensitivity to nitrates and lost dormancy during the winter season (Hilhorst 1990). Although the mechanisms by which nitrates stimulate dormancy loss remain unclear, it is acknowledged that they may

affect the functionality of the cell membrane (Karssen and Hilhorst 1992). The evaluation of the effects of nitrates in regulating germination in weed seeds and weed emergence is an area of interest for weed scientists and research needs to be carried out on this topic.

Oxygen and carbon dioxide are two of the most major biologically active gases in soil. Oxygen concentration in soil air does not usually fall below 19% (Benech-Arnold *et al.* 2000). During storage of seeds in soil, oxygen can have both detrimental and beneficial effects on the dormancy status of weed seeds. In a study carried out by Symons *et al.* (1986), secondary dormancy was induced in the seeds of *A. fatua* by hypoxia. In other studies, carbon dioxide concentrations between 0.5 and 1% have been reported to break seed dormancy in the seeds of *Trifolium subterraneum* (L.) and *Trigonella ornithopoides* (L) (Ballard 1958; 1967).

1.4.3 Dormancy release and germination

Dormancy release is attained by various mechanisms that comprise complex interactions with the environment mediated by plant hormones and other small molecules, to select environments for germination that are most favourable for plant survival.

1) Gibberellins

Gibberellins [e.g., gibberellic acid (GA)] are a family of 136 tetracyclic diterpenes, a small group of which are active as plant hormones and recognized to stimulate seed germination in many plant species. The most active GA depends on the species (Thomas *et al.* 2005). Gibberellins stimulate germination by inducing hydrolytic enzymes that weaken the barrier tissues, such as the endosperm or seed coat, inducing mobilization of seed storage reserves, and stimulating expansion of the embryo (Bewley and Black 1994). GA may also stimulate germination via the transition from embryonic to vegetative development, in part mediated by the chromatin remodelling factor PICKLE (PKL) (Hirayama and Shinozaki 2007). The balance between the GA biosynthesis and inactivation determines the level of biologically active GAs in plants (Yamaguchi 2008). The biosynthesis of GA is controlled by the catalytic reactions of

GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) (Figure. 2), while the inactivation of GA is regulated by GA 2-oxidase (GA2ox) (Finch-Savage and Leubner-Metzger 2006). The enhanced expression of GA20ox and GA3ox genes is reported to induce germination/dormancy loss in cereal crops (Gubler *et al.* 2008; Liu *et al.* 2014), which is highly relevant for the grass weed species *B. diandrus* and *L. rigidum* investigated in this project.

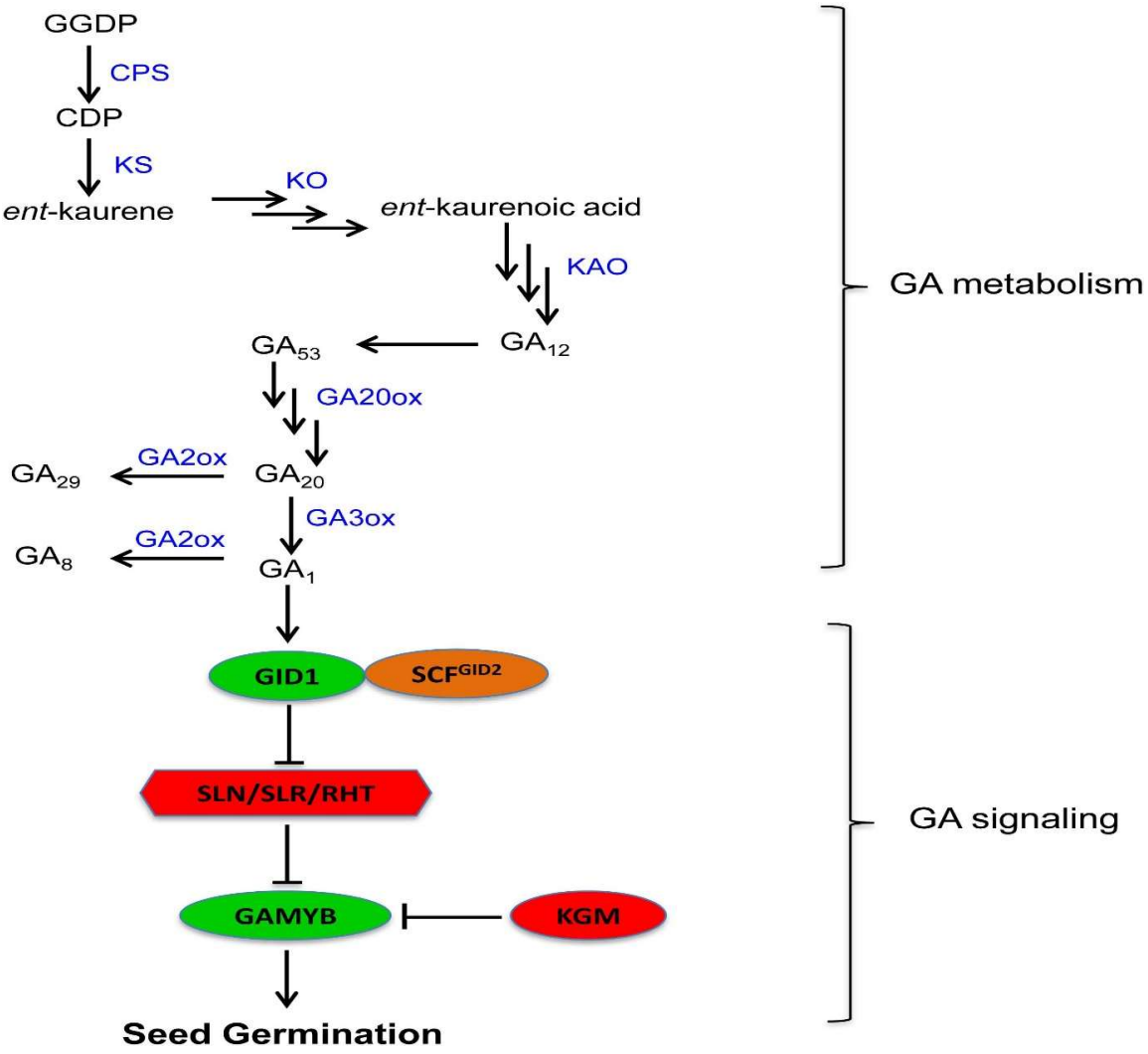


Figure 2. GA metabolism and signalling pathway in plants (Tuan *et al.* 2018).

2) Brassinosteriods

Brassinosteriods (BRs) are plant steroid hormones involved in stem elongation and leaf expansion that also enhance seed germination (Clouse and Sasse 1998). While BR biosynthesis and signalling mutants germinate well, they are more complex than wild-type to ABA inhibition of seed germination, signifying that these alterations decrease germination potential (Clouse and Sasse 1998; Sugimoto *et al.* 2010). In *Arabidopsis*, epibrassinolide (EBR) and brassinolide (BL) application disables non-germination of GA biosynthetic and *sleepy 1* (*sly1*) GA signalling mutants (Steber and McCourt 2001).

3) Ethylene

Ethylene promotes dormancy breaking in seeds via interactions with ABA signalling. Seed of *ethylene resistant* (*etr*) *1* receptor mutants and *ethylene insensitive* (*ein*) *2/enhanced response to aba* (*era*) *3* mutants exhibit increased dormancy correlated with increased sensitivity to ABA in seed germination and increased ABA synthesis (Beaudoin *et al.* 2000; Ghassemian *et al.* 2000; Chiwocha *et al.* 2005).

4) Nitric Oxide, Nitrate, and Active Oxygen Species

Many nitrogen-containing compounds, including NO gas, nitrite (NO_2^-), nitrate (NO_3^-), nitrogen dioxide, ammonium, azide, and cyanide, promote dormancy release and seed germination in many species, possibly as a means of sensing soil N availability (Bethke *et al.* 2018). Even nitrate provided during seed development via the maternal plant leads to lower dormancy (Alboresi *et al.* 2005). The nitrogen-containing compounds may promote seed germination via effects on metabolism, oxidation state and by causing vacuolation and cell wall weakening (Bethke *et al.* 2007; Bethke *et al.* 2018). NO is also reported to induce germination in the absence of far red light (Beligni and Lamattina 2000). Nitrate and dormancy-relieving conditions induce the expression of genes that encode enzymes in the pentose phosphate pathway, which may provide essential nutrients for germination (Finch-Savage *et al.* 2007).

1.4.4 Seed dormancy in *Lolium rigidum*

Primary seed dormancy in *L. rigidum* helps in prolonged seed survival, seed bank persistence and staggered seedling emergence. It also helps in preventing an untimely seed germination after summer rainfall events (Steadman *et al.* 2003b). Summer rainfall is often sufficient to promote germination, but germination that occurs in response to the summer rainfall is usually fatal and doesn't ensure seedling survival (Steadman *et al.* 2003a). Dormancy is gradually lost during the hot dry weather conditions and a majority of emergence occurs in late autumn (Peltzer and Matson 2002), but cohorts can continue to emerge throughout winter (McGowan 1970), enabling plants to escape early-season weed control. Factors within the seed that allow protracted establishment of *L. rigidum* seeds remains unclear and is worthy of investigation. The gradual decline in seed dormancy due to hot weather is referred to as after-ripening, which is common in winter annuals (Baskin and Baskin 1998). The rate of loss of dormancy during the after-ripening period is dependent upon temperature. The higher the temperature, faster is the dormancy loss in *L. rigidum* seeds (Steadman *et al.* 2004a).

It has been shown that hydrated seeds of *L. rigidum* become sensitised to light, making them conditionally dormant and these seeds germinate when exposed to light (Steadman *et al.* 2004b). Steadman *et al.* (2004a) conducted a field and laboratory experiment to determine the impact of hydrated storage (dark stratification) on dormancy loss in 22 *L. rigidum* populations. They observed an increased germination rate (95%) in the seeds that were exposed to light. This study concluded that dark stratification has a significant impact on increasing the light sensitivity of dormant *L. rigidum* seeds.

A shallow cultivation at the time of the first rainfall of the growing season stimulates weed seedling emergence, permitting the use of a non-selective herbicide or a second cultivation for weed control prior to crop seeding (Pratley 2000). The emergence of *L. rigidum* is usually improved by the use of double cultivation although methodology and results vary

(Gill 1996; Medd *et al.* 1996). Since light exposure has a stimulatory effect upon *L. rigidum* germination, understanding the conditions that enhance dark stratification would enable optimizing the timing of cultivation to increase weed seedling emergence and maximize the soil seed bank depletion (Steadman *et al.* 2004a).

1.4.5 Seed dormancy in *Bromus diandrus*

Gill and Blacklow (1985) showed the presence of variation in the duration of seed dormancy among different populations of *B. diandrus* collected across Australia, even when grown at a common location. Furthermore, they observed that seed storage conditions had no impact on the rate of dormancy release. Based on these findings they concluded that variation in seed dormancy amongst different populations was under genetic control and provided evidence that Australian populations of *B. diandrus* had adapted to the local climate.

Cheam (1986) observed that seed germination of *B. diandrus* was higher in complete darkness as compared to light. From their study it was concluded that an alternating temperature of 20/10 ℃ with 24h darkness was ideal for maximum germination of *B. diandrus*. In a later study, they also showed that seed germination in dormant seeds of *B. diandrus* was stimulated by chilling (cold stratification), which enables some of the seed bank to emerge later under cooler winter conditions and escape non-selective herbicides used for weed control before sowing crops (Cheam 1988). Studies from Spain (Del Monte and Dorado 2011) have also highlighted the photosensitivity mechanism in *B. diandrus*, with seeds displaying decreased germination under light conditions and requiring burial/darkness to initiate germination. In contrast, Harradine (1986) reported a slight stimulatory impact of light on the germination of *B. diandrus*.

Kleemann and Gill (2006) reported that *B. diandrus* populations from South Australia showed much longer dormancy than reported previously. Treatment of seeds with gibberellic acid (0.001 M GA3) significantly increased germination, which indicated high seed viability

but deep dormancy (under hormonal control in the embryo). In another study by Kleemann and Gill (2013), three *B. diandrus* populations were collected from cropping fields and adjacent fence lines to determine variation in seed dormancy. The populations collected from the fence lines were less dormant and germinated at 3 months after maturity, while the populations collected from within the cropping fields showed <3% germination at 8 months after maturity. The longer dormancy and delayed seedling emergence in field populations indicated selection for higher seed dormancy in this species by management practices used for crop production. Delayed seedling emergence would allow many plants to establish after the application of pre-sowing weed management tactics and lead to increased weed infestations in crops. It is also possible that selection for higher seed dormancy could lead to greater persistence of the seed bank to the next year.

1.5 Research aims and objectives

Information on seed ecology and biology is critical for understanding the adaptation of weeds to different environmental factors and management practices, which could aid in designing better integrated weed control strategies. The presence of genetic variation for seed dormancy within *L. rigidum* and *B. diandrus* populations would explain why some populations have rapidly adapted to management practices such as delayed crop sowing or higher frequency of crop production and lower frequency of pasture phases. Therefore the aim of our study was to enhance understanding of within population variation in seed dormancy in *L. rigidum and B. diandrus*. Hence, our studies focussed on answering the following questions:

1. Is the variation in seed dormancy within these weed populations under genetic control or heritable (low *vs* high dormancy cohorts or intra-population variation)?

2. Are the differences in seed dormancy between low and high dormancy cohorts associated with the expression of *GA20ox* and *ABA1* genes during the after-ripening period?
3. Does moisture stress during reproductive development influence the level of seed dormancy in *L. rigidum* and *B. diandrus*?

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Chapter 2: Selection for seed dormancy within *Bromus diandrus* Roth populations alters ABA1 and GA20ox gene expression

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Selection for seed dormancy within *Bromus diandrus* populations alters *ABA1* and *GA20ox* gene expression

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ABSTRACT

Context. *Bromus diandrus* Roth is a serious weed of cereal-based cropping systems in South Australia. Its adaptation to local climate and production systems, prolific seed production and evolution of herbicide resistance have made this weed difficult to manage. **Aims.** Studies were undertaken to determine genetic variation in seed dormancy within *B. diandrus* populations and its association with genes involved with gibberellic acid and abscisic acid synthesis. **Methodology.** Seeds from five *B. diandrus* populations were sampled during 2017 from commercial fields and planted during 2018 in pots. Protracted seedling emergence in these populations allowed selection of low and high dormancy cohorts. Seeds produced by these cohorts from each population were evaluated for variation in seed dormancy in the following year. The process of cohort selection and seed dormancy evaluation was repeated. The mechanism regulating seed dormancy was investigated in 2020 by assessing seeds of low and high dormancy cohorts concurrently for dormancy and for expression of *ABA1* and *GA20ox* genes, using quantitative real-time PCR. **Results.** There was at least a two-fold difference in seed dormancy among populations collected from different farms. Low and high dormancy cohorts within each population maintained consistent differences in seed dormancy in both years of assessment, suggesting genetic control over this trait. Differences in seed dormancy between low and high dormancy cohorts were significantly correlated with *ABA1* and *GA20ox* gene expression. **Conclusions.** Large differences in seed dormancy exist between individuals in *B. diandrus* populations. The study has provided evidence of genetic variation for seed dormancy within *B. diandrus* populations, which was associated with *ABA1* and *GA20ox* gene expression. **Implications.** Presence of genetic variation for seed dormancy could play an important role in adaptation to escape pre-sowing weed-control tactics, meaning that *B. diandrus* could become an even greater problem in field crops grown in this region.

Keywords: brome grass, cohorts, genetic variation, high dormancy, low dormancy, qPCR, seedling emergence, South Australia.

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Introduction

Bromus diandrus, known as brome grass or ripgput brome, is a grass weed commonly found in wheat-growing regions of South Australia characterised by cool wet winters and hot dry summers (Kleemann and Gill 2006). The weed is highly competitive with crops and has evolved resistance to herbicides that inhibit acetyl CoA carboxylase (ACCase), acetolactate synthase (ALS) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Reitano and Watts 2006; Owen *et al.* 2015*b*). In a farmer survey conducted by Llewellyn *et al.* (2016), *B. diandrus* was ranked the fourth most problematic weed of Australian cereal-based cropping systems. Characteristics such as causing yield loss in cereal crops, setting of a large number of seeds (600–3000 seeds/plant), staggered germination, providing an alternative host for pathogens, contamination of grains, and herbicide resistance make *B. diandrus* one of the most problematic weeds of South Australian crops (Reitano and Watts 2006). It has been suggested that the wide geographic distribution of *B. diandrus* might be due to its high genetic diversity (Gill and Blacklow 1985). Its seeds germinate

readily under cold moist conditions, usually occurring in late autumn and early winter. However, some populations show protracted germination throughout the growing season. Presence of primary seed dormancy helps *B. diandrus* plants to escape knockdown herbicides and infest cereal crops (Reitano and Watts 2006).

Seed dormancy is an adaptive trait imposed during the final phases of embryo development, and it prevents seed germination from preceding the completion of seed maturation (Baskin and Baskin 1998). The transition between dormancy and germination represents a critical stage in the life cycle of higher plants and is an important ecological and commercial trait (Rodríguez-Gacio *et al.* 2009). The continuity of seed dormancy after maturity is variable among different plant species. However, in several species where dormancy is maintained, it allows formation of a soil seedbank that can remain viable for many years and avoidance of environmental conditions that do not favour seedling emergence (Gubler *et al.* 2008). Seed dormancy and germination are induced and regulated by environmental factors such as light, moisture, temperature and nutrients, and genetic factors such as the concentrations of abscisic acid (ABA) and gibberellic acid (GA) (Finkelstein *et al.* 2008). ABA and GA are plant hormones considered vital for the regulation of seed dormancy and germination (Tuan *et al.* 2018).

Previous studies have shown that ABA is responsible for dormancy induction (Feurtado and Kermode 2007). An increase in ABA during seed maturation is correlated with the beginning of seed dormancy and results in inhibition of germination (Nambara *et al.* 2010). On the other hand, GA is known to stimulate seed germination by inducing hydrolytic enzymes, which weaken the endosperm and result in the development of the embryo (Qasem 2019). Biosynthesis of GA is regulated by GA20-oxidase (*GA20ox*) and GA3-oxidase (*GA3ox*) genes (Tuan *et al.* 2018). Previous studies of the mechanism of seed dormancy and germination in cereals such as wheat (Jacobsen *et al.* 2013; Son *et al.* 2016; Tuan *et al.* 2018), maize (White *et al.* 2000), barley (Jacobsen *et al.* 2002) and rice (Kim *et al.* 2009; Liu *et al.* 2014), weeds such as *Euphorbia esula* (Chao *et al.* 2014), and *Arabidopsis* species (Finkelstein *et al.* 2002, 2008) identified *ABA1*, *GA20ox* and *GA3ox* genes as being important for determination of seed dormancy.

After autumn rainfall events, *B. diandrus* seedlings tend to emerge quickly, but these early cohorts can be easily managed by farmers with pre-sowing application of herbicides (Reitano and Watts 2006). The most problematic cohorts to control are those that emerge with, or slightly later than, the crop. These cohorts of *B. diandrus* tend to compete aggressively with crops and set large numbers of seeds, which contribute to the soil seedbank for the future (Kleemann and Gill 2013). Although early and late weed cohorts have been observed in many weed species including *B. diandrus*, the genetic basis of regulation of their behaviour remains unclear. Why do some weed seedlings within a population emerge later than others? Based on this knowledge gap, we designed studies to determine the level of variation in seed dormancy within different field-collected populations of *B. diandrus* and whether this seed dormancy is a heritable trait. Moreover, we considered the role of expression of genes considered important in regulating seed dormancy in weed species. Therefore, our studies focused on answer the following questions:

1. Are there differences in seed dormancy within *B. diandrus* populations present in the same field?
2. If so, is the variation in seed dormancy within these weed populations under genetic control or heritable (low vs high dormancy cohorts)?
3. Are the differences in seed dormancy between low and high dormancy cohorts associated with the expression of *GA20ox* and *ABA1* genes?

Materials and methods

Seed collection

Mature seeds of five populations of *B. diandrus* were collected from wheat crops on farmer fields on different farms in the medium–high rainfall crop production zone in South Australia during November 2017 (Table 1). Long-term annual rainfall at these collection sites ranges from 362 mm to 525 mm. The seeds of *B. diandrus* were oven dried at 40°C and then cleaned and placed in brown paper bags, and stored in a laboratory at 20°C/10°C day/night temperature until used in the experiments (April–November 2018).

Seedling emergence pattern of *B. diandrus* populations

This experiment was conducted during April–October in 2018, 2019 and 2020, in pots (33 cm by 28 cm by 5 cm) placed outdoors under natural environmental conditions at Waite Campus, The University of Adelaide. This period is locally referred to as the growing season, when frequent rainfall events occur and crops are grown in the field. Seeds of each of the five *B. diandrus* populations were placed in

Table 1. Latitude and longitude and long-term average annual rainfall for the farms in South Australia from which *B. diandrus* seeds were collected in 2017.

Town	Latitude and longitude	Average annual rainfall (mm)
Riverton	34.16°S, 138.75°E	525.6
Mallala	34.44°S, 138.51°E	402.3
Malinong	35.38°S, 139.56°E	379.2
Wynarka	35.20°S, 139.59°E	372.4
Smithville	35.21°S, 140.36°E	362.5

pots (three replicates of 50 seeds per pot) containing standard cocoa peat potting mix produced by mixing 540 L cocoa peat, 220 L water, and 60 L sand before steaming for 1 h (Handreck and Black 2010; Boutsalis *et al.* 2012). The experiment was laid out in randomised complete block design and the seeds were covered with a thin layer of potting mix soil to ensure complete darkness, because germination in this species can be inhibited by exposure to light (Del Monte and Dorado 2011; Kleemann and Gill 2013).

The pots were hand-watered if no rain was received for three consecutive days. Each pot received a uniform amount of water of ~1.25 L and all pots experienced similar moisture and temperature conditions. From each population, the early cohorts, which emerged 7–21 days after sowing, were carefully uprooted and transplanted into larger pots (25 cm deep, 18 cm diameter) for seed production and collection after maturity. The seedlings emerging midseason were also uprooted but discarded. The late cohorts, which emerged close to the end of the growing season at 54–70 days after sowing, were also uprooted and transplanted into pots. The experiment was terminated when seedling emergence ceased in spring. Plants of these early and late cohorts of *B. diandrus* were the source of low (LD) and high (HD) seed dormancy populations. The pattern of seed dormancy and seedling emergence in these LD and HD cohorts of *B. diandrus* was assessed in the next growing season (2019).

Additional pots (50 seeds per pot) of unselected Mallala and Riverton populations were used to produce fresh bulk seed for selection of LD and HD cohorts in 2019 (Fig. 1). Additionally, a population of *B. diandrus* was collected from the gardens of Waite campus and grown in separate pots. The garden population was used as a control to compare the dormancy pattern of LD and HD cohorts from cropped lands with that of a population collected from a non-cropped area. The selection of LD and HD cohorts and experimental evaluation over 3 years of study are displayed in Fig. 1.

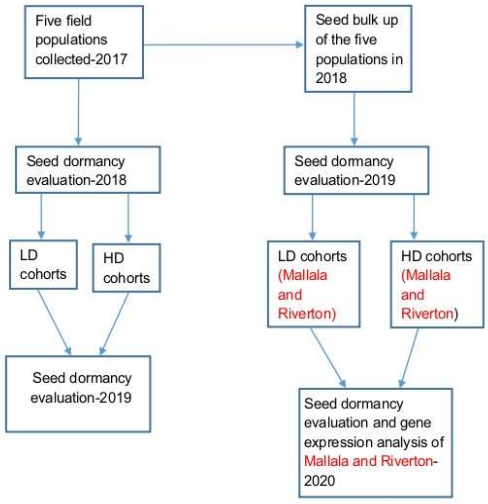


Fig. 1. Selection of populations for dormancy analysis.

Expression of genes associated with seed dormancy in *B. diandrus*

Selection of candidate and housekeeping genes

Because *ABA1* and *GA20ox* genes are involved in the initial steps of biosynthesis pathways of these plant hormones, they are known to have a significant impact on the expression of seed dormancy induction and release (Arora *et al.* 2003; Tuan *et al.* 2018). After careful review of the literature, we focused on the expression of the genes *ABA1* and *GA20ox* in *B. diandrus* and its relationship with changes in seed dormancy during the after-ripening period. In order to avoid bias in quantitative real-time PCR (qPCR) analysis, an internal control gene (i.e. a housekeeping gene) is used to normalise the expression data (Yuan *et al.* 2012). We initially tested our cDNA with *Actin*, *GADPH*, *NADH*, *Tubulin* and *ELF*. The most stable gene expression was observed with *Tubulin* and *ELF*, which were therefore selected as internal control genes for the qPCR studies.

Selection of populations

The LD and HD cohorts of the two most contrasting *B. diandrus* populations (Riverton and Mallala) in 2018 and 2019 were selected for studies on the expression of target genes (*ABA1* and *GA20ox*) shown to be important in regulating seed dormancy. The garden population of *B. diandrus* was also used in these studies for direct comparison with field populations from the crop production zone. Seeds from three individual plants from the LD and HD cohorts of Riverton and Mallala populations and seeds from three individuals of the garden population were harvested in November 2020, oven-dried, cleaned and stored in brown paper bags maintained at 20°C/10°C. Although there were large differences in the timing of seedling emergence of LD and HD cohorts, their plants reached maturity at the same time. Therefore, environmental conditions during seed maturation of LD and HD plants is unlikely to have played an important role in the expression of seed dormancy. These seeds were investigated to determine changes in seed dormancy and gene expression during the after-ripening period, at 30, 60, 90 and 120 days after maturity.

qPCR analysis for gene expression

The expression of housekeeping genes and *ABA1* and *GA20ox* was investigated by qPCR analysis at four time points (30, 60, 90 and 120 days after maturity) during dormancy maintenance and release. RNA was extracted from the three individual plants from the garden population

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and the LD and HD cohorts of the Riverton and Mallala field populations at these four time points. Three replicates of 10 seeds for LD and HD cohorts were used at each time point. The seeds were imbibed for 24 h at 20°C/10°C in Petri dishes wrapped in aluminium foil to ensure complete darkness. After incubation for 24 h, the imbibed seeds were removed from the Petri dish and immediately placed in liquid nitrogen to avoid degradation of RNA. The seeds were then ground to fine powder using a ball mill (MM301; Retch, Haan, Germany) before RNA extraction using the RNeasy Mini Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Integrity of RNA was evaluated via gel electrophoresis. Multiple cDNA synthesis reactions from each RNA sample were performed using a Tetro cDNA Synthesis Kit (Bioline, London, UK), and the cDNA was pooled to ensure enough cDNA from a single source for all qPCR analyses. Gene expression for seed dormancy was analysed via qPCR, using similar methods to those described by Malone *et al.* (2016) and Adu-Yeboah *et al.* (2020). A SensiMix (Bioline/Meridian Bioscience) assay with Dual-Labeled BHQ FRET Probes (Bioresearch Technologies, Petaluma, CA, USA) was used. Primers and probes for the candidate genes *ABA1* and *GA20ox* and control genes *Tubulin* and *ELF* were designed using RealTimeDesign qPCR assay design software (Bioresearch Technologies) (Table 2). Each qPCR reaction of 10 μL contained 5 μL SensiMix, 0.4 μL target and control gene F and R primers, 0.1 μL target and control gene probe, 1.5 μL H_2O and 2.5 μL cDNA. Reactions were run on a Rotor-Gene 6000 real-time thermal cycler (Qiagen) with the following parameters: 3 min at 95°C, followed by 45 cycles of 15 s at 95°C and 16 s at 60°C. Primer efficiencies were calculated and found to range from >96% to <99%. For all cDNA samples, both genes of interest, *ABA1* and *GA20ox*, were multiplexed with each control gene, *Tubulin* and *ELF* in separate reactions, and the data for each sample were normalised against both housekeeping genes.

Seed dormancy test

Seeds from the Riverton, Mallala and the garden populations were also assessed for dormancy status. At 30, 60, 90 and 120 days after maturity, seeds of these subpopulations were placed on two layers of Whatman No. 1 filter paper in 15-cm-diameter Petri dishes. Each Petri dish had 25 seeds, with three replicates of each population. Each Petri dish was given 5 mL distilled water, sealed with Parafilm to avoid moisture loss, and then covered with aluminium foil to ensure total darkness. The dishes were placed in an incubator set at 20°C/10°C, which was shown by Gill and Blacklow (1985) and Kleemann and Gill (2006) to be the optimum temperature for the germination of *B. diandrus*. Petri dishes were opened after 14 days and assessed for germination.

Statistical analyses

Seedling emergence pattern of *B. diandrus* populations

Seedling emergence data were converted to cumulative emergence percentage and then fitted to a logistic function in Prism 9 (GraphPad Software, San Diego, CA, USA) to estimate the time taken to reach 50% emergence (t_{50}), which was used as the indicator of seed dormancy. The fitted model was:

$$Y = 100/(1 + 10^{(\log t_{50} - X) \times b}) \quad (1)$$

where Y is the cumulative germination percentage, X is the time or days, t_{50} is germination rate, and b is the hillslope, which describes the steepness of the curves. Because there was no cohort × season interaction in the emergence pattern of LD and HD cohorts within each population, the results from 2019 to 2020 were combined and used to fit the logistic model in Prism 9. Root mean square error (RMSE) and standard error values were calculated to test the goodness of fit for the logistic model.

Gene expression analysis

The candidate gene runs for qPCR were compared with both housekeeping genes (*ELF* and *Tubulin*) to normalise the data. The average of the C_T values of the two control genes was taken, and a modified version of the ΔC_T ($2 = {}^{-\Delta C_T}$) method was used to analyse data from the qPCR (Livak and Schmittgen 2001; Gaines *et al.* 2010). The unpaired *t*-test

Table 2. Primers and probes for housekeeping and candidate genes used in qPCR.

Primers/probes for housekeeping genes		Primers/probes for candidate genes	
Primer/probe name	Sequence (5′–3′)	Primer/probe name	Sequence (5′–3′)
ELF-F	TGAGGCTGGTATCTCCAAGG	*Bd* GA20ox-F	GGCGGTACAAGAGCTGCTTG
ELF-R	CACACCAAGAGTGAAAGCAAGAAG	*Bd* GA20ox-R	CCACCACCGTTGATCCATCTC
ELF probe	ATGGCCAGACCCGTGAGCACG+TET	*Bd* GA20ox probe	ACCGGGCCGTGGTCAACAGC+FAM
Tubulin-F	ACATGGCCTGCTGTCTCAT	*Bd* ABA1-F	AGGTGGCTGCATGGCTATTGAG
Tubulin-R	GGTGCGCTTGGTCTTGATG	*Bd* ABA1-R	CCACGGGAGTTCTAGACTTGAC
Tubulin probe	TACCGTGGTGATGTTGTGCCCA+TET	*Bd* ABA1 probe	TGGTTAACCAACTGGCTGTAGAGCTTG+FAM

D

was performed in Prism 9 to assess differences in gene expression between LD and HD cohorts at each time point.

Correlation analysis between seed germination (%) from the Petri dish experiment and expression of *ABA1* and *GA20ox* was performed in Prism 9.

Results

Seedling emergence pattern of *B. diandrus* populations

There were significant differences in seedling emergence pattern among *B. diandrus* populations collected in South Australia. During 2018, the shortest time to reach 50% emergence (t_{50}) was observed in the Riverton population (10.3 days), followed by Wynarka (17.7 days) and Smithville (19.4 days) (Table 3). By contrast, t_{50} was much greater in Malinong (26.8 days) and Mallala (28.5 days) populations. High R^2 (0.91–0.96) and low RMSE indicates that the logistic model fitted well to the observed seedling emergence pattern (Table 3).

Seedling emergence ceased around late June to mid-July, which was up to 80 days after sowing. There was no clear relationship between annual rainfall at the site of collection and seed dormancy in *B. diandrus* populations. However, the population from the highest rainfall site (Riverton) had the lowest seed dormancy, whereas the population from the medium rainfall zone (Mallala) had the highest seed dormancy.

Seedling emergence pattern of seeds of LD and HD cohorts of Riverton and Mallala populations was investigated in 2019 and 2020. Regression analysis of pooled data for the two experimental runs showed significant differences in emergence pattern between LD and HD cohorts in both populations (Table 4). For example, seeds of the LD cohort of Riverton had a t_{50} of 23.5 ± 0.77 days, whereas the HD cohort of the same population had a t_{50} of 36.0 ± 0.97 days. Similarly, the LD cohort of the Mallala population had a t_{50} of 33.7 ± 1.27 days, compared with 45.7 ± 0.81 days for the HD cohort. By contrast, the garden population selected from a non-agricultural habitat had a t_{50} of only 8.7 ± 0.42 days.

Table 3. Estimated parameters of the logistic function (Eqn 1) fitted to cumulative emergence at different times for the five populations of *B. diandrus* in 2018.

Population	t_{50} (days)	*b*	R^2	RMSE
Riverton	10.3 (1.02)	3.2 (0.48)	0.91	10.9
Wynarka	17.7 (0.75)	3.0 (0.31)	0.95	7.9
Smithville	19.4 (0.92)	3.3 (0.44)	0.93	9.8
Malinong	26.8 (1.00)	3.6 (0.41)	0.94	9.5
Mallala	28.5 (0.75)	5.4 (0.66)	0.96	8.5

t_{50}, time taken to reach 50% of total cumulative emergence; *b*, slope of the fitted curve; RMSE, root mean square error. Values in parentheses are standard errors.

Table 4. Estimated parameters of the logistic function (Eqn 1) fitted to cumulative emergence at different times for the garden population and LD and HD cohorts of two populations of *B. diandrus* with pooled data from 2019 and 2020.

Population	t_{50} (days)	*b*	R^2	RMSE
Riverton-LD	23.5 (0.77)	2.8 (0.23)	0.92	9.8
Riverton-HD	36.0 (0.97)	3.9 (0.39)	0.90	11.9
Mallala-LD	33.7 (1.27)	3.0 (0.33)	0.86	14.0
Mallala-HD	45.7 (0.81)	8.1 (1.03)	0.90	12.8
Garden	8.7 (0.42)	1.84 (0.13)	0.95	6.9

t_{50}, time to reach 50% of total cumulative emergence; *b*, slope; RMSE, root mean square error. Values in parentheses are standard errors.

The logistic function (variable slope) fitted well to the seedling emergence pattern of LD and HD cohorts, as indicated by the R^2 and RMSE values of the fitted model (Table 4). Seeds of the HD cohort of the two populations consistently showed a longer lag phase at the start of the growing season than the seeds of the LD cohort. By contrast, the garden population was non-dormant and showed virtually no lag phase at the start, completing 100% of final emergence within 56 days after sowing (Fig. 2). There were significant ($P < 0.05$) differences in t_{50} between LD and HD cohorts within each population (Table. 4).

Gene expression analysis

Expression of *GA20ox* and *ABA1* genes in the LD and HD cohorts

Expression of *GA20ox* and *ABA1* in seeds produced by LD and HD cohorts showed significant differences during the after-ripening period (Fig. 3). At 30 days after seed maturity, *GA20ox* gene expression was relatively low in both LD and HD cohorts. At this early post-maturity stage, there was a significant difference in *GA20ox* gene expression between LD and HD cohorts of Mallala (104% and 85%) (Fig. 3). Over time, the expression of *GA20ox* increased, and at 90 days after maturity, the LD cohorts of both populations had higher gene expression (128% and 130%) of *GA20ox* than the HD cohorts (117% and 119%) (Fig. 3). There was a gradual increase in the expression of *GA20ox* in both LD and HD cohorts at 30, 60 and 90 days after maturity; however, at 120 days after maturity, the expression of *GA20ox* declined. In the Petri dish study where seed germination and expression of *GA20ox* were investigated concurrently, there was a significant positive correlation between *GA20ox* expression and seed germination (Fig. 4).

In contrast to *GA20ox*, *ABA1* gene expression showed a significant decline over time. At 30 days after maturity, the LD and HD cohorts of Mallala had a higher *ABA1* gene expression (98% and 117%) than Riverton. From 30 to

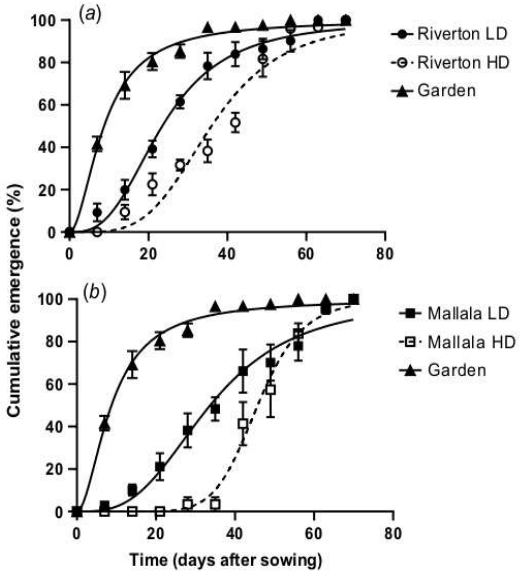


Fig. 2. Cumulative emergence of seeds of LD and HD cohorts of two field populations of *B. diandrus*, (*a*) Riverton and (*b*) Mallala, and a garden population, fitted with the logistic function. Bars indicate the standard error.

60 days after maturity, *ABA1* expression increased in both LD and HD cohorts of Riverton and then declined over time (Fig. 3). At 60 days after maturity, *ABA1* expression in Riverton was significantly higher in seeds of the HD (129%) than LD (114%) cohorts, whereas a decline in the *ABA1* activity in the LD and HD cohorts of Mallala (113 and 117%) was observed. At 120 days after maturity, *ABA1* expression levels of HD cohorts of Riverton and Mallala (95% and 103%) had also dropped significantly, correlating with the loss of seed dormancy (Figs 3, 4). The general trajectory of *ABA1* gene expression showed a steady decline during the after-ripening period, but overall, expression remained higher in seeds of the HD than the LD cohort. Riverton seeds showed an early increase in *ABA1* gene expression from 30 to 60 days (from 107% to 129%), followed by a sharp decline from 90 to 120 days after maturity (from 121% to 95%) (Fig. 3). There was significant negative correlation between *ABA1* expression and seed germination (Fig. 4).

Discussion

From the results of this 3-year study, it is evident that there is considerable variation in seedling emergence among and within the populations of *B. diandrus* collected from single fields on different farms in South Australia (Table 3). Most of this variation was associated with the population from Riverton, which had much lower seed dormancy than the other populations. This variation in seedling emergence among the populations reflects differences in seed dormancy, likely due to differences in management practices used as well as environmental factors (Gill 1996; Owen *et al.* 2015*a*). Our results are consistent with the findings of Gill and Blacklow (1985), who selected *B. diandrus* populations from 14 sites across southern Australia and grew them at a common location in Western Australia. The pattern of loss of seed dormancy varied among the populations, from which Gill and Blacklow (1985) concluded that seed dormancy in these populations was under genetic control. In our study, the differences in seed dormancy between early and late cohorts of *B. diandrus* populations from the same field clearly showed the presence of genetic variation for seed dormancy.

Previous research has shown a strong interaction between genotype and environment in the determination of primary seed dormancy (Tan *et al.* 2006; Goggin *et al.* 2010), and it has been suggested that the genetic component is likely to be mediated by several different genes (Goggin *et al.* 2010). Chao *et al.* (2014) conducted qPCR studies to investigate dormancy patterns in buds and seeds of *Euphorbia escuela*, and found downregulation of *ABA1* gene expression between paradormant buds and seeds imbibed for 21 days and endodormant buds and seeds imbibed for 1 day. Owing to the constant expression of the *ABA1* gene in dormant buds and seeds, Chao *et al.* (2014) concluded that the downregulation of *ABA1* could specify that ABA synthesis was lower in paradormant buds and seeds than endodormant buds and seeds. By contrast, our studies showed significant differences in the expression of *ABA1* and *GA20ox* between LD and HD cohorts of *B. diandrus*, and the expression of these genes changed over time as seeds became non-dormant. Freshly harvested dormant seeds showed a higher expression of *ABA1* and a lower expression of *GA20ox* than seeds at 2–3 months after maturity.

A balance between the synthesis and catabolism of ABA and GA is of pivotal importance for controlling the equilibrium between seed dormancy and germination (Gutierrez *et al.* 2007). The stability of ABA/GA is determined by the antagonistic control of ABA and GA on each other through their reciprocal regulation of the transcription of their metabolic genes (Seo *et al.* 2006; Toh *et al.* 2008). Environmental signals control the equilibrium between ABA and GA by altering the expression of metabolic enzymes, and also positive and negative regulators of both hormones, many of which are response-regulated, that increase or diminish the dormancy levels (Finkelstein *et al.* 2008). There were significant differences in the expression of *ABA1* in the LD and HD cohorts, which correlated well with seed dormancy in these cohorts. There was a steady decline in *ABA1* gene expression with time in LD and HD cohorts of all the populations, but the overall expression of

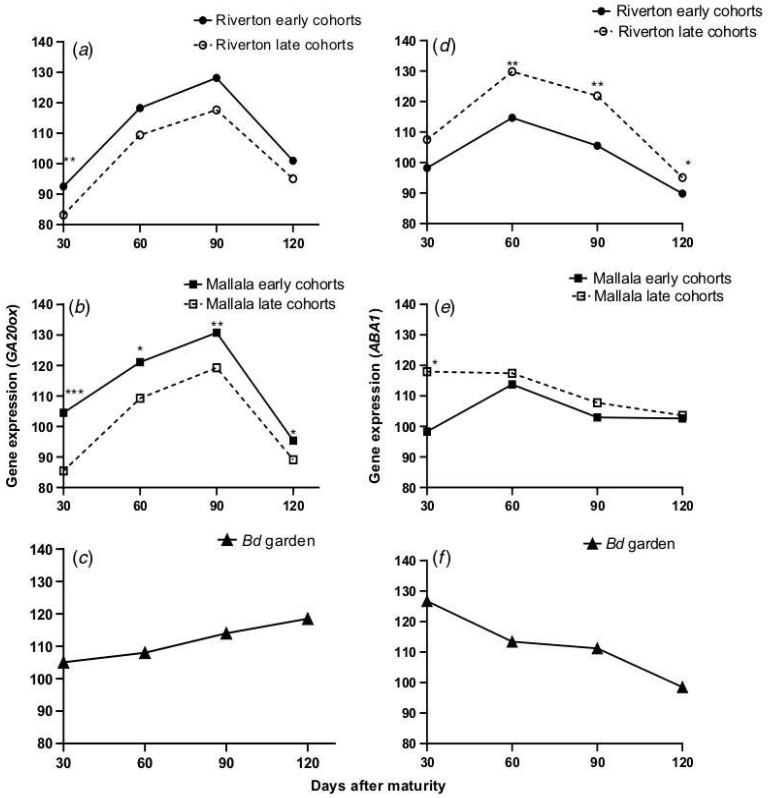


Fig. 3. Relative gene expression of (*a*–*c*) *GA20ox* and (*d*–*f*) *ABA1* (*ELF* and *Tubulin* were the control genes) of the seeds of LD and HD cohorts of (*a*, *d*) Riverton and (*b*, e) Mallala, and (*c*, *f*) the garden population at four time points after seed maturity. Results of the unpaired two-sample *t*-tests are also shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

this gene tended to be higher in the HD than the LD cohorts (Fig. 3). Although *GA20ox* expression increased over time, the LD cohorts maintained higher *GA20ox* gene expression than the HD cohorts. Because the differences in *ABA1* and *GA20ox* expression between the seeds of LD and HD cohorts persisted even at 9 months after maturity, they may also contribute to the long-term dormancy of *B. diandrus* seeds and, hence, persistence of the seedbank.

In previous studies, *B. diandrus* and *Bromus rigidus* populations collected from the fence line were less dormant than those collected from the adjacent cropping field (Kleemann and Gill 2006, 2013). Based on these results, it was speculated that seed dormancy in populations of these two species is likely to be under genetic control. Along the fence lines, weed populations are likely to evolve in response to competitive interactions that would favour early-emerging, low dormancy cohorts. By contrast, weed populations within cropping fields evolve in response to management practices such as tillage and pre-sowing herbicide applications, which are likely to favour later emerging cohorts (avoidance). In another self-pollinating species, *Bromus tectorum*, the progeny of six different populations grown in the greenhouse showed large differences in germination behaviour that indicated the presence of a strong genetic mechanism involved in the regulation of seed dormancy (Meyer and Allen 1999). In our study, cohorts (subpopulations) of *B. diandrus* populations were created by separating the LD and HD cohorts through growing them at a common location. These cohorts maintained significant differences in dormancy as reflected by seedling emergence pattern in 2019 and 2020. There was at least a two-fold difference in the t_{50} of seedling emergence between the LD and HD cohorts. It can be argued that seeds

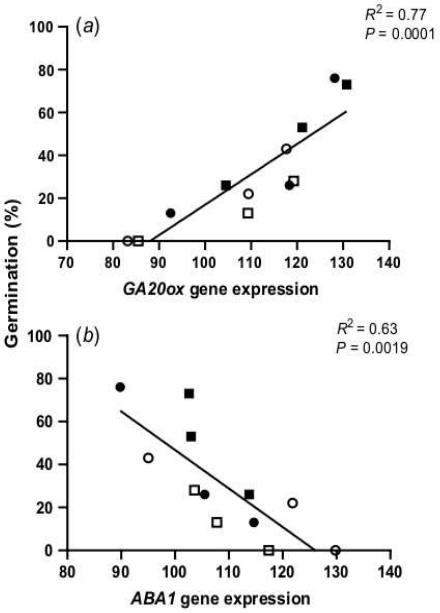


Fig. 4. Correlation analysis between seed germination and expression of (*a*) *GA20ox* and (*b*) *ABA1* genes. Riverton LD (●) and HD (○) cohorts and Mallala LD (■) and HD (□) cohorts.

of LD cohorts have a lower dormancy; hence, their seedlings emerged earlier than seedlings of HD cohorts. This variation in seedling emergence pattern between cohorts grown at the same site strongly supports our hypothesis that seed dormancy of *B. diandrus* is under genetic control and these field populations contain individuals with contrasting levels of seed dormancy. These results also highlight the key finding that seed dormancy is a heritable trait in field-collected populations of *B. diandrus*. Individuals within *B. diandrus* populations also possess variable expression of genes regulating seed dormancy. From a weed-management perspective, these individuals with variable levels of seed dormancy (polymorphism) will allow *B. diandrus* populations to adapt rapidly to changes in management practices such as sowing time of crops and use of pre-sowing herbicides. Conversely, change in land use to more frequent or longer pasture phases could shift the balance towards lower seed dormancy in weed populations.

Conclusion

Previous studies of seed dormancy in *B. diandrus* have not investigated the role of specific genes responsible for controlling the trait. The findings from our research have enhanced understanding of genes involved in regulating seed dormancy, their expression over time, and how loss of dormancy is associated with changes in the expression of *ABA1* and *GA20ox* genes. The LD cohorts of *B. diandrus* populations had significantly greater expression of *GA20ox,* leading to earlier dormancy release, than the HD cohorts. This study also provides information about the presence of considerable genetic variation for seed dormancy within field populations of *B. diandrus*; this variation could enable the weed to adapt rapidly to escape pre-sowing weed-control tactics. Our research has also revealed large differences in seed dormancy among populations, likely associated with differences in management practices used for weed control.

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Data availability. The data that support this study are available in the article in the form of figures and tables.

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Chapter 3: Genetic control of seed dormancy in *Lolium rigidum* and its association with *GA20ox* and *ABA1* expression

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Contribution to the Paper	Planned the study, Conducted all experiments, analysed and interpreted data, wrote 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.		
Signature		Date	09-06-2022

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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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 molecular work, helped in data analysis and interpretation and edited the manuscript.		
Signature		Date	9/6/22

Name of Co-Author	Christopher Preston		
Contribution to the Paper	Supervised development of research, data analysis and interpretation of result and edited the manuscript.		
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Contribution to the Paper	Supervised development of research, data analysis and interpretation of result and edited the manuscript.		
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Genetic control of seed dormancy in *Lolium rigidum* and its association with *GA20ox* and *ABA1* expression

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ABSTRACT

Context. *Lolium rigidum* Gaudin (annual ryegrass) has remained the most problematic weed of crop production in Australia for more than 20 years. There is some evidence that this weed species can rapidly adapt to management practices including delayed crop sowing. **Aims.** Studies were undertaken to determine genetic variation for seed dormancy within *L. rigidum* populations and its association with genes involved with gibberellic acid and abscisic acid synthesis. **Methodology.** Populations of *L. rigidum* were grown in pots to select low and high dormancy cohorts. Seeds produced by these cohorts from each population were assessed for variation in seed dormancy. Seeds of high and low dormancy cohorts were concurrently assessed for seed dormancy and expression of *LrABA1* and *LrGA20ox* genes, using quantitative real-time PCR. **Results.** Presence of differences greater than two-fold in seed dormancy between populations from the same farm indicated *in situ* selection for seed dormancy, most likely in response to management. Low and high dormancy cohorts of all populations maintained clear differences in seed dormancy in both years of assessment. Differences in seed dormancy between low and high dormancy cohorts were significantly correlated with *LrABA1* and *LrGA20ox* gene expression. **Conclusions.** This investigation has provided clear evidence of the presence of genetic variation for seed dormancy within *L. rigidum* populations. **Implications.** The presence of genetic variation for seed dormancy in *L. rigidum* populations will allow this weed to adapt rapidly to changes in weed management practices such as delayed sowing of crops.

Keywords: emergence, genes expression, high dormancy, *Lolium rigidum*, low dormancy, *LrABA1*, *LrGA20ox*, variation.

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Introduction

Seed dormancy has been defined as 'one of the least understood phenomena in seed biology' (Finch-Savage and Leubner-Metzger 2006), and remains confusing despite recent advances. This confusion reflects the likelihood that dormancy is not a single phenomenon, but a condition with many contributing causes. Dormancy is the inability of mature, viable seeds to germinate under conditions that would otherwise be ideal for germination. For a better understanding of seed dormancy and germination, it is vital to differentiate between dormancy (which is induced during seed maturation on the mother plant), and germination. Both are different physiological states, yet they depend on one another. Germination occurs only after dormancy release, and dormancy release can only be seen upon the start of germination (Bentsink *et al.* 2018). In genetic terminology, dormancy is epistatic to germination.

In weeds such as *Lolium rigidum* Gaud. (annual ryegrass), seed dormancy is an adaptive trait that enables seed germination to coincide with favourable environmental conditions (Qasem 2019). Seed dormancy and germination are induced and regulated by environmental factors such as light, moisture, temperature and nutrients, and genetic factors such as the concentration of abscisic acid (ABA) and gibberellic acid (GA) (Finkelstein *et al.* 2008). Studies have shown that ABA is responsible for dormancy induction (Finch-Savage and Leubner-Metzger 2006; Feurtado and Kermode 2007).

An increase in ABA during seed maturation is correlated with the beginning of seed dormancy and it results in inhibition of germination (Finkelstein *et al.* 2008). GA is known to stimulate seed germination by inducing hydrolytic enzymes, which weaken the endosperm and result in the development of the embryo (Qasem 2019).

Among the different plant hormones, ABA and GA are considered vital for the regulation of seed dormancy and germination (Tuan *et al.* 2018). The level of ABA contained in the mature seeds is responsible for determining seed dormancy (Finkelstein *et al.* 2008; Nambara *et al.* 2010). In mature seeds and buds, the biosynthesis of ABA is regulated by the genes *ABA1, NCED3* and *ABA2* (Arora *et al.* 2003; Ruttink *et al.* 2007; Wang *et al.* 2016). The biosynthesis of GA, which releases seed dormancy and helps in seed germination, is regulated by *GA20-oxidase* (*GA20ox*) and *GA3-oxidase* (*GA3ox*) (Tuan *et al.* 2018). Previous studies of the mechanism of seed dormancy and germination in cereals such as wheat (Jacobsen *et al.* 2013; Son *et al.* 2016; Tuan *et al.* 2018), maize (White *et al.* 2000), barley (Jacobsen *et al.* 2002) and rice (Kim *et al.* 2009; Liu *et al.* 2014), in weeds such as *Euphorbia esula* (Chao *et al.* 2014), and in *Arabidopsis* species (Finkelstein *et al.* 2002, 2008) identified *ABA1, GA20ox* and *GA3ox* as being important for determination of seed dormancy. Hence, in this study, we also focus on the expression of the genes *ABA1* and *GA20ox* in regulating seed dormancy in field populations of *L. rigidum*.

In Australia, *L. rigidum* produces seeds in spring, which tend to be dormant in summer. The primary seed dormancy in *L. rigidum* is controlled by temperature, which ensures seedling emergence occurs in late autumn and winter (Peltzer and Matson 2002), thereby avoiding fatal seed germination after summer rainfall events in southern Australia (Steadman *et al.* 2003, 2004). The presence of seed dormancy in *L. rigidum* seeds leads to staggered seedling emergence throughout the growing season, making this weed extremely difficult to control (Ellery *et al.* 2003; Goggin *et al.* 2011).

Environmental conditions during seed maturation can influence the level of seed dormancy (Milberg and Andersson 1994; Andersson and Milberg 1998; Steadman *et al.* 2004). *Lolium rigidum* is a cross-pollinated species that is widely distributed across climatic zones in southern and Western Australia. This wide geographic distribution has resulted in selection for varying levels of seed dormancy; for example, seeds collected from high-rainfall zones tend to be less dormant than those collected from dry areas (Peltzer and Matson 2002). The primary dormancy in *L. rigidum* appears to be under the influence of environmental and genetic factors (Goggin *et al.* 2010).

In research so far, there has been greater focus on demonstrating differences in seed dormancy between weed populations growing in contrasting environmental conditions, whereas less effort has gone into understanding variation in seed dormancy within weed populations, including *L. rigidum*, and its potential role in adaptation to management tactics. Environmental conditions in the Australian agricultural landscape tend to be highly variable, and it is important to understand how this variability impacts on the expression of seed dormancy in important weed species.

Information on seed ecology and biology is a prerequisite for understanding the adaptation of weeds to different environmental factors, which could aid in designing better integrated weed-control strategies. Presence of genetic variation for seed dormancy within *L. rigidum* populations would explain why some populations have rapidly adapted to management practices such as delayed crop sowing. Therefore, the aim of our study was to improve understanding of the dormancy behaviour of *L. rigidum* seeds, which in turn will help to provide better understanding of weed management. Hence, our studies focused on answering the following questions:

(1) Do *L. rigidum* populations present on South Australian cropping farms show variation in seed dormancy?
(2) Is the variation in seed dormancy within these weed populations under genetic control (low vs high dormancy cohorts)?
(3) Are the differences in seed dormancy between low and high dormancy cohorts associated with the expression of *GA20ox* and *ABA1* genes?

Materials and methods

Seed collection

Mature seeds of seven different populations of *L. rigidum* were collected from wheat fields on three farms in the medium- and high-rainfall areas in South Australia during November 2017 (Table 1). Seeds were collected from farms near Hilltown (HT1 and HT2), Paskeville (PSK1 and PSK2) and Roseworthy Agricultural College (RAC1, RAC2 and RAC3). Dry spikes of *L. rigidum* were hand-threshed to extract seeds, cleaned and stored in a laboratory at 20°C until used in the experiments (April–November 2018).

All sampling locations had quite similar temperature conditions, but Hilltown had considerably higher annual rainfall (542.5 mm) than Paskeville (388.5 mm) and Roseworthy (434.8 mm).

Seedling emergence pattern of different *L. rigidum* populations

In this study, seed emergence pattern was used as the indicator of seed dormancy. The experiment was conducted from April to November for 3 years (2018, 2019 and 2020) in pots placed outdoors under natural environmental conditions at The University of Adelaide, Waite Campus.

Table 1. Long-term maximum and minimum temperatures and rainfall at the three locations from which *L. rigidum* seeds were collected in 2017.

	Temperature (°C)						Rainfall (mm)		
	Hilltown		Paskeville		Roseworthy		Hilltown	Paskeville	Roseworthy
	Mean max.	Mean min.	Mean max.	Mean min.	Mean max.	Mean min.			
January	30.4	14.9	32.0	15.6	31.9	14.9	18.4	15.4	17.0
February	29.6	14.8	30.6	15.1	31.1	14.7	18.4	18.0	19.6
March	26.2	12.2	28.2	13.5	28.3	12.8	16.1	17.4	16.9
April	21.8	9.1	24.1	10.4	24.2	10.5	33.9	30.9	30.9
May	17.1	6.5	19.4	8.3	19.7	8.6	48.8	45.0	37.9
June	13.5	4.8	16.1	5.8	16.2	6.5	57.9	51.6	45.2
July	13.0	4.0	15.6	5.3	15.5	5.7	63.8	48.2	44.4
August	14.2	4.3	17.0	4.8	16.5	5.2	58.7	46.9	45.7
September	17.4	6.1	20.4	6.2	19.9	6.4	50.2	40.2	45.0
October	21.2	7.9	24.2	8.4	23.8	7.7	41.0	34.1	32.0
November	25.1	10.7	27.6	11.3	27.7	10.7	30.7	20.7	25.0
December	27.7	12.7	29.5	13.5	29.7	12.7	21.3	19.5	25.1
Annual	21/9		23/10		22/10		542.5	388.5	434.8

In southern Australia, April is the onset of autumn when weather conditions are cooler and rainfall events increase in frequency (Table 1). The study was undertaken during the autumn–spring part of the growing season, which receives frequent rainfall events when cereal crops are grown in this region.

The experiment was laid out in randomised complete block design with three replicates of each population. Seeds of *L. rigidum* were placed in the pots (33 cm by 28 cm by 5 cm; 50 seeds per pot), which contained standard cocoa peat potting mix produced by mixing 540 L cocoa peat, 220 L water and 60 L sand before steaming for 1 h (Handreck *et al.* 2002; Boutsalis *et al.* 2012). The seeds were covered with a thin layer of potting mix soil to ensure complete darkness and watered. In order to avoid water stress, the pots were hand-watered if no rain was received for three consecutive days. Each tray received ~1.25 L water and all the trays and pots experienced similar moisture and temperature conditions.

Seedlings were counted and removed from pots on a weekly basis. Plants that emerged during the first 14 days after sowing were considered low dormancy (LD) cohorts (early emerging). The LD cohorts (five plants/replicate) from each population were carefully uprooted and transplanted into larger pots (25 cm deep, 18 cm diameter) for seed production. The seedlings emerging midseason were also uprooted but discarded. Similarly, the high dormancy (HD) cohorts (i.e. late emerging) that emerged 60–70 days after sowing were uprooted and transplanted into separate pots. The pots containing the LD and HD cohorts from all populations were separately covered with pollen sleeves to prevent cross-pollination between the cohorts or populations. Although plants of LD cohorts established 6 weeks earlier than HD cohorts, all plants reached maturity within 1 week of each other.

Seeds collected from these LD and HD cohorts were used to assess the pattern of dormancy and seedling emergence in 2019. Because seed dormancy is transient in nature, plants from unselected or bulk seeds of three populations (RAC3, HT2 and PSK1) were grown in pots in 2019 to select LD and HD cohorts for seed dormancy evaluation in 2020. The method used to separate the LD and HD cohorts in 2019 was identical to that described above for the previous growing season. Fig. 1 represents the method of producing LD and HD populations over the 3 years.

Association between seed dormancy and target gene expression of LD and HD cohorts

Based on the differences in seed dormancy between LD and HD cohorts, three contrasting populations were selected for the expression of target genes (*LrABA1* and *LrGA20ox*) known to regulate seed dormancy. Seeds from three individual plants from the LD and HD cohorts of HT2, PSK1 and RAC3 populations were oven-dried, cleaned and stored in paper bags in a laboratory maintained at 20°C/10°C. The LD and HD cohorts of these three populations were investigated at 30, 60 and 90 days after maturity (DAM) to determine changes in seed dormancy and gene expression during the after-ripening period.

Seed dormancy assessment

At 30, 60 and 90 DAM, seeds of the selected populations and cohorts were placed on two layers of Whatman No. 1 filter paper in 15-cm-diameter Petri dishes. Each Petri dish

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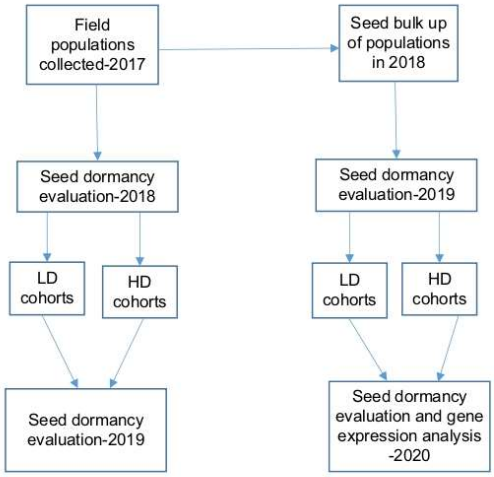


Fig. 1. Selection of populations for seed dormancy analysis.

had 25 seeds with three replicates of each population × cohort. Distilled water (5 mL) was added to seeds in each Petri dish and then sealed with Parafilm to avoid moisture loss. Petri dishes were placed in an incubator set at 20°C/10°C with a photoperiod of 12 h. Petri dishes were opened after 14 days and assessed for germination.

Quantitative real-time PCR (qPCR) analysis for gene expression

At 30, 60 and 90 DAM, RNA was extracted from seeds of LD and HD cohorts. Seeds of the three selected *L. rigidum* populations were imbibed for 24 h at 20°C/10°C with a photoperiod of 12 h. There were three replicates of 25 seeds per individual plant used in this study. After 24 h, the imbibed seeds were removed from the Petri dish and immediately placed in liquid nitrogen to avoid any degradation of RNA. Seeds were then ground to fine powder with a Retsch Ball Mill (MM301; Retch, Haan, Germany). RNA extraction was performed by using the RNeasy Mini Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was evaluated via gel electrophoresis. The Tetro cDNA Synthesis Kit (Bioline, London, UK) was used to synthesise multiple cDNAs from each RNA replicate, and the cDNA reactions of each RNA replicate were pooled separately and frozen at −20°C for use in qPCR.

Partial gene sequences of *LrABA1* and *LrGA20ox* were amplified by using the primers described in Table 2. Sequence comparison using the NCBI Basic Local Alignment Search Tool (BLASTn) (Altschul *et al*. 1990; NCBI Resource Coordinators 2016) confirmed the identity of the genes, with the *LrABA1* fragment showing 93.8% homology to *Triticum dicoccoides ABA1* (accession no. XM_037629902.1) and *LrGA20ox* showing 98.5% homology to *Lolium perenne GA20ox* (accession no. AY014281.1). Similarly, partial gene sequence of *Lr-αTubulin* and *LrELF* were amplified by using primers described in Malone *et al.* (2016). Sequence comparison using NCBI BLASTn (Altschul *et al.* 1990; NCBI Resource Coordinators 2016) showed 98% homology to *Triticum aestivum Tubulin* (accession no. AK451994.1) and 93% homology to *Hordeum vulgare Elongation factor* (*ELF*) (accession no. XM-045125981.1), respectively. These sequences were used to design primers and probes for qPCR (Table 2), using RealTimeDesign qPCR assay design software (Biosearch Technologies, London, UK).

Gene expression for seed dormancy was analysed via qPCR, using similar methods to those described by Malone *et al.* (2016) and Adu-Yeboah *et al.* (2020). A SensiMix (Bioline/Meridian Bioscience, London, UK) assay with Dual-Labelled BHQ FRET probes (Biosearch Technologies) was used. cDNA templates were amplified in a 10-μL reaction mixture containing 5 μL SensiMix, 0.4 μL each target and control primer, 0.1 μL each target and control probe, 2 μL H_2O and 2 μL cDNA. Reactions were run on a Rotor-Gene 6000 real-time thermal cycler (Qiagen) with the following

Table 2. Primers and probes for housekeeping and candidate genes used in qPCR.

Primer/probe name	Sequence (5′–3′)	Primer/probe name	Sequence (5′–3′)
Primers for *LrABAta1* amplification		Primers for *LrGA20ox* amplification	
ABA-1F	AAATGCAGTGGTATGCATTTCACRAGG	GA20-1F	GACATCCCGYCGCAGTTCATCTG
ABA-1R	GCCTTRTCAGAAAGCCGGCAACT	GA20-1R	CGGTAGTGCTTCTGCGTGAASTC
Primers/probes for housekeeping genes		Primers/probes for candidate genes	
ELF-F	TGAGGCTGGTATCTCCAAGG	GA20ox-F	GCACTACCGGCGCTTCTT
ELF-R	CACACCAAGAGTGAAAGCAAGAAG	GA20ox-R	CAGGGCGGGTAGTAGTTGA
ELF Probe	ATGGCCAGACCCGTGAGCACG+TET	GA20ox probe	CGAGGGCAACGAATCCATAATGCGG+FAM
Tubulin-F	ACATGGCCTGCTGTCTCAT	ABA1-F	AGGTGGCTGCATGGCTATTGAG
Tubulin-R	GGTGCGCTTGGTCTTGATG	ABA1-R	CCACGGGAGTTCTAGACTTGAC
Tubulin probe	TACCGTGGTGATGTTGTGCCCA+TET	ABA1 probe	TGGTTAACCAACTGGCTGTAGAGCTTG+FAM

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parameters: 3 min at 95°C, followed by 45 cycles of 15 s at 95°C and 16 s at 60°C. Primer efficiencies were calculated and found to range from >96% to <99%. Each qPCR reaction containing the cDNA from LD and HD cohorts of the three populations was run with a gene of interest (*LrABA1* and *LrGA20ox*) and two control genes (*Lr-αTubulin* and *LrELF*). The cDNA from HT2, PSK1 and RAC3 (LD and HD cohorts) was tested with the primers and probes for housekeeping genes and candidate genes (Table 2).

Statistical analyses

Seedling emergence pattern of different *L. rigidum* populations

Seedling emergence data were converted to cumulative emergence percentage and then fitted to a logistic function to estimate the time to 50% of emergence (t_{50}), which was used as the indicator of seed dormancy. All statistical analyses were performed with the software GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).The fitted model used was:

$$Y = 100/(1 + 10^{(\log t_{50} - X)b}) \quad (1)$$

where Y is the percentage of cumulative germination, X is the time (days), germination rate (t_{50}) is the time required for the germination of half of the total germinated seeds, and b is the hill slope, which describes the steepness of the curves.

An analysis of variance was performed to determine the season × cohort interaction for the 2 years of seed dormancy assessments. The P-values for the cohort × year interaction for 2019 and 2020 data for HT2, PSK1 and RAC3 were 0.63, 0.98 and 0.46 (non-significant, $P > 0.05$), respectively. Therefore, seedling emergence data for each cohort for the 2 years were pooled and used to fit the logistic function. Root-mean-square error (RMSE) and standard error (s.e.) values were calculated to test the goodness of fit for the logistic model.

Gene expression analysis

For gene expression analysis, the candidate gene runs for qPCR were compared with both housekeeping genes (*LrELF* and *Lr-αTubulin*) to normalise the data. The average of the C_T values of two control genes was used and a modified version of the ΔC_T ($2 = ^{-\Delta C_T}$) method was used to analyse data from the qPCR (Livak and Schmittgen 2001; Gaines *et al*. 2010). An unpaired *t*-test was performed for gene expression between LD and HD cohorts at each time point. Data for seed germination (%) of different populations x cohorts and *LrGA20ox* and *LrABA1* gene expression at 30, 60 and 90 DAM was used to undertake a correlation analysis.

Results

Seedling emergence pattern of different *L. rigidum* populations

In 2018 the shortest time to reach 50% of final seasonal emergence (t_{50}) was observed in RAC1 (11.2 days), followed by HT1 (13.4 days) and RAC2 (17.6 days) (Table 3), whereas t_{50} was two–three-fold greater in HT2 (23.7 days), PSK1 (33.8 days), PSK2 (28.8 days) and RAC3 (26.9 days). There were significant differences in emergence pattern between populations collected from the same farm. For example, HT2 had a t_{50} of 23.7 days, compared with 13.4 days for HT1. Similarly, t_{50} for RAC3 (26.9 days) was more than two-fold greater than RAC1 (11.2 days).

There were significant differences between LD and HD cohorts with regard to t_{50} for all populations (Table 4). There was at least a two-fold difference in the t_{50} of seedling emergence between LD and HD cohorts. Lower seed dormancy in LD cohorts was reflected in earlier seedling emergence than the HD cohorts; for example, seeds of the

Table 3. Estimated parameters of the logistic function (Eqn 1) fitted to the cumulative emergence at different times for the seven populations of *L. rigidum* in 2018.

Population	t_{50} (days)	*b*	R^2	RMSE
HT1	13.4 (0.54)	2.799 (0.24)	0.96	6.274
HT2	23.7 (0.79)	1.952 (0.11)	0.97	5.675
PSK1	33.8 (1.28)	2.434 (0.21)	0.93	8.728
PSK2	28.8 (0.79)	3.865 (0.34)	0.96	7.545
RAC1	11.2 (0.48)	2.093 (0.14)	0.96	5.375
RAC2	17.6 (2.47)	1.431 (0.25)	0.73	16.89
RAC3	26.9 (1.61)	1.729 (0.17)	0.89	10.06

t_{50}, time taken to reach 50% of total cumulative emergence; *b*, slope of the fitted curve; RMSE, root-mean-square error. Values in parentheses are standard errors.

Table 4. Estimated parameters of logistic function (Eqn 1) fitted to the cumulative emergence at different times for LD and HD cohorts of the three populations of *L. rigidum* with the pooled data from 2019 and 2020 evaluations.

Population	t_{50} (days)	*b*	R^2	RMSE
HT2-LD	19.8 (1.18)	1.82 (0.16)	0.76	12.61
HT2-HD	42.5 (1.40)	3.03 (0.29)	0.67	13.62
PSK1-LD	16.3 (1.10)	1.69 (0.16)	0.92	12.50
PSK1-HD	36.9 (1.39)	2.49 (0.23)	0.89	13.07
RAC3-LD	28.5 (1.23)	2.29 (0.20)	0.90	12.56
RAC3-HD	46.5 (1.58)	2.93 (0.31)	0.64	14.51

t_{50}, time taken to reach 50% of total cumulative emergence; *b*, slope; RMSE, root-mean-square error. Values in parentheses are standard errors.

LD cohort of RAC3 required 28.5 days to reach t_{50}, compared with 46.5 days for the HD cohort of the same population.

The logistic function (variable slope) fitted well to the seedling emergence pattern of LD and HD cohorts, as seen by the R^2 values of the fitted model (Table 4), which varied from 0.64 to 0.92 ($P < 0.001$). The RMSE (%) also indicated a good agreement between predicted and observed seedling emergence. Seeds of the HD cohort of the three populations consistently showed a longer lag phase at the start of the growing season than seeds of the LD cohort (Fig. 2). There were significant ($P < 0.05$) differences in t_{50} between the LD and HD cohorts of each population (Table 4). From the results of this study, it is evident that there is a considerable variation in seedling emergence between and within populations of *L. rigidum*.

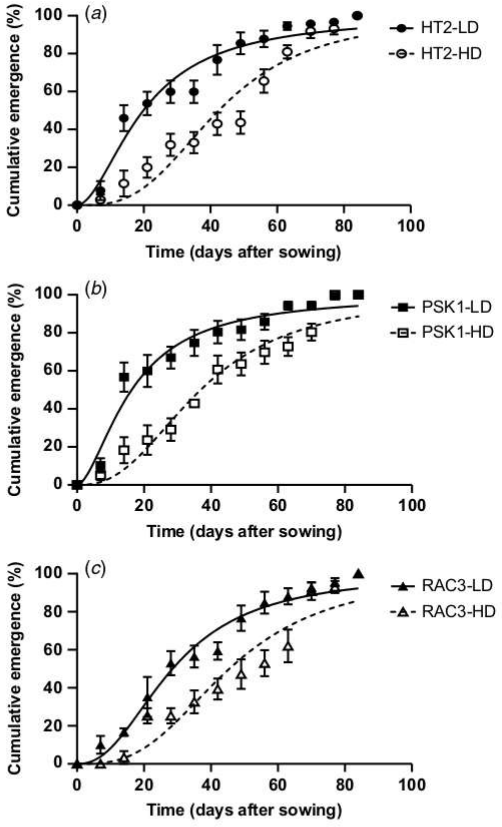


Fig. 2. Cumulative emergence of seedlings of LD and HD cohorts of three *L. rigidum* populations fitted with the logistic function. Parameters of the fitted model are shown in Table 4. Capped bars indicate standard error of the mean (±s.e.).

Gene expression analysis

Expression of *LrGA20ox* and *LrABA1* genes in LD and HD cohorts

Expression of *LrGA20ox* and *LrABA1* in seeds produced by LD and HD cohorts showed significant differences over time (Figs 3 and 4). At 30 DAM, there was no significant difference in *LrGA20ox* gene expression between LD and HD cohorts of RAC3 (87% and 88%) and HT2 (82% and 81%) (Fig. 3), which were both highly dormant and had a lower early-season germination. However, seeds produced by the LD cohorts of PSK1 had significantly higher *LrGA20ox* gene expression (94%) than seeds from its HD cohorts (83%) (Fig. 3). The expression of *LrGA20ox* in seeds increased during after-ripening, and at 90 DAM, the LD cohorts of all three populations had significantly higher (119%, 110% and 107%) *LrGA20ox* gene expression than the HD cohorts (98%, 85% and 89%) (Fig. 3). The increase in *LrGA20ox* expression showed a positive correlation with seed germination in Petri dishes (Fig. 5). These results also correlate well with data from pot studies, where maximum seedling emergence was observed at ~84 DAM, when *LrGA20ox* expression in seeds was at its highest level (Fig. 2).

In contrast to *LrGA20ox*, *LrABA1* gene expression showed a significant decline during the after-ripening period. The LD and HD cohorts of all populations had higher *LrABA1* gene expression at 30 DAM, which declined over time (Fig. 4). At 60 DAM, the *LrABA1* expression level in RAC3 was significantly higher in seeds of HD cohorts (115%) compared to the LD cohorts (95%), while a decline in the *LrABA1* activity in the LD and HD cohorts of HT2 and PSK1 was observed. At 90 DAM, the *LrABA1* expression levels of HD cohorts of HT2, PSK1 and RAC3 (93%, 93% and 95%) showed a sharp decline. The general trajectory of *LrABA1* gene expression was a steady decline after maturity (after-ripening period), but overall expression remained higher in the HD cohorts than the LD cohorts (Fig. 5). Surprisingly, RAC3 seeds showed an early increase in *LrABA1* gene expression from 30 to 60 DAM (from 103% to 115%) followed by a sharp decline from 60 to 90 DAM (from 115% to 95%) (Fig. 4). There was a significant negative correlation between *LrABA1* gene expression and seed germination (Fig. 5).

Discussion

This study showed significant differences in seed dormancy between populations of *L. rigidum* collected from the same farm in South Australia. These differences were particularly evident in populations from Roseworthy and Hilltown, where there were two-fold differences in seed dormancy (t_{50}) between populations from the same farm. Although HT1 and HT2 come from a site with much higher annual rainfall, they possessed similar seed dormancy to populations collected from two farms in the medium-rainfall zone. However,

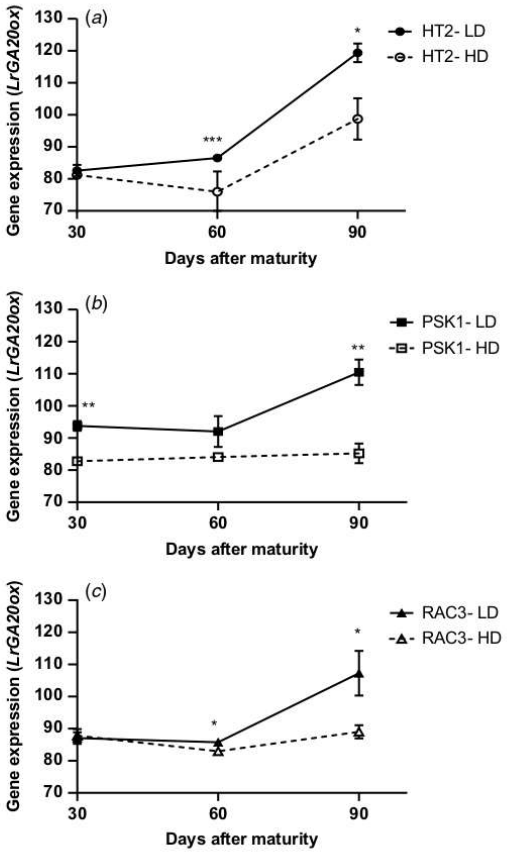


Fig. 3. Relative *LrGA20ox* gene expression analysis (with *Lr-αTubulin* and *LrELF* as control genes) of the seeds of LD and HD cohorts of RAC3, PSK1 and HT2 populations at 30, 60 and 90 days after seed maturity. Results of the unpaired two sample *t*-tests are also shown: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; capped bars indicate standard error.

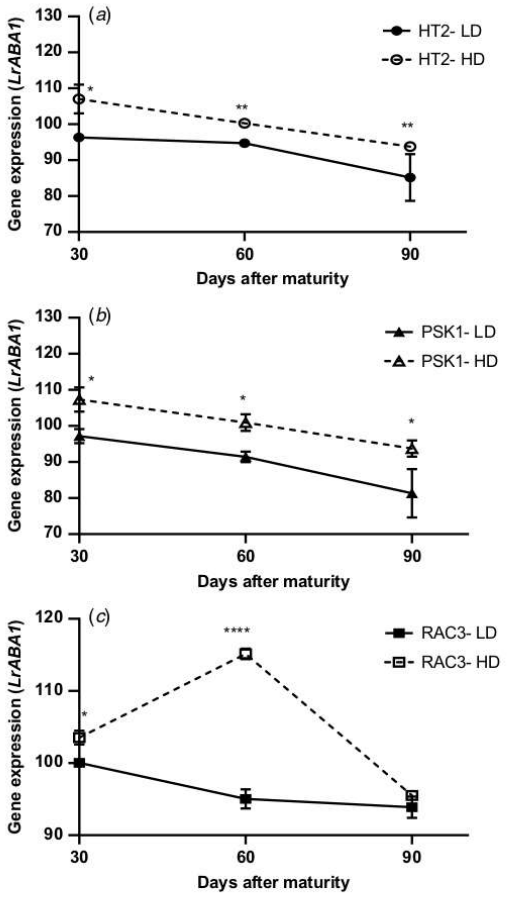


Fig. 4. Relative *LrABA1* gene expression analysis (with *Lr-αTubulin* and *LrELF* as control genes) of the seeds of LD and HD cohorts of RAC3, PSK1 and HT2 populations at 30, 60 and 90 days after seed maturity. Results of the unpaired two sample *t*-tests are also shown: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; capped bars indicate standard error.

differences in seed dormancy were smaller between two populations from Paskeville (Table 3). This variation in seed dormancy between populations from a single farm is likely due to differences in selection pressure imposed by the management practices (such as relative frequency of crop and pasture production in the field or time of crop sowing) used in different fields on the same farm (Gill 1996; Owen *et al*. 2015). It is quite likely that pasture phases will favour low dormancy, whereas crop production tactics are likely to select for genotypes with high dormancy. This initial investigation provides some evidence for the presence of genetic variation for seed dormancy between populations of *L. rigidum* in South Australia.

Seedling emergence in the investigated *L. rigidum* populations occurred for more than 2 months, which offered an opportunity to select the LD and HD cohorts of plants. Seeds produced by these cohorts also displayed clear differences in seed dormancy in studies undertaken in the subsequent year (Fig. 2). For example, LD seeds from Paskeville (PSK1) required only 16 days to reach 50% of annual emergence (t_{50}), compared with 37 days for seeds of the HD cohorts (late cohorts) from the same farm. Large differences in seed dormancy between LD and HD cohorts were also observed in populations from Hilltown and Roseworthy. Although plants of LD cohorts established up

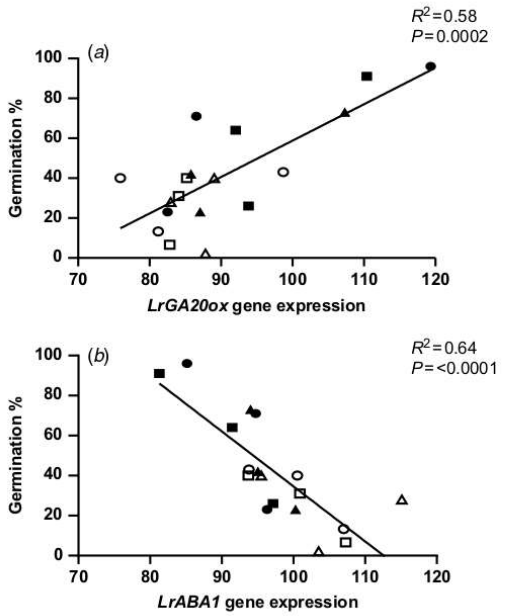


Fig. 5. Correlation analysis between seed germination and expression of *LrGA20ox* and *LrABA1*. HT2 LD (●) and HD (○) cohorts, PSK1 LD (■) and HD (□) cohorts, and RAC3 LD (▲) and HD (Δ) cohorts.

to 4–6 weeks earlier than the HD cohorts, all plants matured within 1 week of each other. Therefore, differences in seed dormancy observed between LD and HD cohorts are unlikely to be associated with environmental conditions during seed maturation and are instead likely to be related to genes regulating seed dormancy. Ellery *et al.* (2003) showed that differences in seed dormancy between *L. rigidum* populations persisted even after they had been grown at a common location. Therefore, seed dormancy in *L. rigidum* is likely to be under genetic control, which would enable populations to adapt to escape pre-sowing weed-control tactics (Ghersa *et al.* 1994; Steadman *et al.* 2003; Gundel *et al.* 2008).

Indirect evidence for genetic control of seed dormancy in the self-pollinated species *Bromus diandrus* and *B. rigidus* has been reported. Populations of these species collected from the fence line were less dormant than those collected from the adjacent cropping field (Kleemann and Gill 2006, 2013). Given that the fence-line and in-crop populations experienced the same climatic conditions, the authors concluded that seed dormancy in these two species is likely to be under genetic control and in response to management practices used for weed control. In another self-pollinating species *B. tectorum*, the progeny of six different populations grown in the greenhouse showed large differences in germination behaviour, which indicated the presence of a genetic mechanism involved in the regulation of seed dormancy (Meyer and Allen 1999).

Previous research by Goggin *et al.* (2010) on *L. rigidum* showed that seed dormancy was correlated with differences in stress protein expression and peroxidase activity. However, their research did not identify expression of any specific genes associated with seed dormancy. In our studies, we showed significant differences in the expression of *LrABA1* and *LrGA20ox* genes between LD and HD cohorts of *L. rigidum*, and the expression of these genes changed during the after-ripening period. Freshly harvested seeds showed a much higher expression of *LrABA1* and a lower expression of *LrGA20ox*. There were significant differences in the expression of *LrABA1* in the LD and HD cohorts, which correlated well with seed dormancy in these cohorts. There was a steady decline in *LrABA1* gene expression with time in LD and HD cohorts of all the populations, but the overall expression of this gene tended to be higher in the HD than the LD cohorts (Figs 4 and 5). Although *LrGA20ox* expression increased over time, the LD cohorts maintained higher *LrGA20ox* gene expression than the HD cohorts. Because the differences in *LrABA1* and *LrGA20ox* expression between the seeds of LD and HD cohorts persisted even 9 months after maturity, it may also contribute to a longer seedbank persistence of *L. rigidum*. Higher expression of *LrGA20ox* and a decline in the expression of *LrABA1* is consistent with the classic model in which the state of seed dormancy is correlated with ABA/GA ratio (White *et al.* 2000). Seo *et al.* (2006) hypothesised that ABA acts negatively to GA, which means that once ABA levels fall below a threshold level, GA increases and hence germination begins.

In contrast to the evidence available on the effect of environmental factors on seed dormancy, the genetic basis of seed dormancy in *L. rigidum* has not been well characterised (Goggin *et al.* 2010). Previous seed dormancy studies in *L. rigidum* have not attempted to investigate its genetic basis within or between the populations (e.g. Ellery *et al.* 2003; or Owen *et al.* 2015). In our study, cohorts (subpopulations) of *L. rigidum* populations were created by separating them into LD and HD and growing them at a common location. These cohorts maintained significant differences in dormancy as reflected by seedling emergence in 2019 and 2020 (Table 4 and Fig. 2). Seeds of these cohorts also showed significant differences in the expression of *LrABA1* and *LrGA20ox* genes, correlated with loss of seed dormancy. The results of this investigation strongly support our hypothesis that seed dormancy of *L. rigidum* is under genetic control, and that the different cohorts of the same field populations contain contrasting levels of seed dormancy.

Conclusion and implications

Previous studies of seed dormancy in *L. rigidum* have not investigated the role of specific genes responsible for controlling seed dormancy. The findings from our research have enhanced the understanding of genes involved in regulating seed dormancy, their expression over time, and how loss of dormancy is associated with changes in the expression of *LrABA1* and *LrGA20ox* genes. LD cohorts of *L. rigidum* populations had significantly greater expression of *LrGA20ox* leading to earlier dormancy release than the HD cohorts. Future studies could also focus on the contribution of other genes (such as *ABA2*, *GA2-ox* or *GA3-ox*) to the regulation of seed dormancy.

Field populations also show considerable genetic variation for seed dormancy between cohorts, which can be selected with selection pressure, and hence could enable this weed to adapt rapidly to escape pre-sowing weed-control tactics. Our research has also shown large differences in seed dormancy between *L. rigidum* populations from the same farm, which is likely to be associated with selection pressure imposed by different management practices used for weed control. Farmers with high dormancy populations of *L. rigidum* are likely to experience less effective pre-sowing weed control and will need to integrate other management tactics to minimise weed competition and prevent the build-up of the seedbank.

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Data availability. The data that support this study are available in the article in the form of figures and tables.

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Chapter 4: Impact of water stress during reproductive development on seed dormancy in *Bromus diandrus* Roth and *Lolium rigidum* Gaudin

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Contribution to the Paper	Planned the study, Conducted all experiments, analysed and interpreted data, wrote 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.		
Signature		Date	09-06-2022

Co-Author Contributions

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Context: *Bromus diandrus* Roth and *Lolium rigidum* Gaudin are regarded as the most problematic weeds of cereal based cropping systems of Australian agriculture. Adaptation to environmental stresses, protracted seed germination after sowing of crops and herbicide resistance have made these weeds serious threats to crop production.

Aims: Studies were undertaken to determine the impact of moisture stress during reproductive development on the extent of seed dormancy and the expression of genes involved with GA and ABA synthesis in *B. diandrus* and *L. rigidum* populations.

Methodology: A pot study was undertaken at two locations, with two populations each of *B. diandrus* and *L. rigidum*. Water stress was applied from either GS31 or GS60 stage until seed maturation, along with a well-watered treatment (control). Seeds of stressed *vs* well-watered treatments were concurrently assessed for seed dormancy and the expression of *ABA1* and *GA20ox* genes using qPCR.

Results: The seeds from GS31 treatment, where plants experienced the longest water stress, were most dormant in both the weed species. Water stress treatments altered the expression of the *GA20ox* gene in particular, which was correlated with the level of dormancy in seeds of *B. diandrus* and *L. rigidum*.

Conclusions: This investigation has provided clear evidence of the impact of water stress on seed dormancy and on the expression of genes involved in regulating seed dormancy in *B. diandrus* and *L. rigidum*.

Implications: As spring rainfall in the Australian agricultural landscape tends to be highly variable, weeds are likely to experience variable levels of water stress during reproductive development, which in turn is likely to influence seed dormancy, weed seedling emergence and effectiveness of pre-sowing weed management next season.

Keywords

Water stress, seed germination, dormancy, *ABA1*, *GA20ox*, genetic variation, crop-weed competition.

Introduction

Seed dormancy plays a key role in survival of weed seeds in the soil (Ghersa *et al.* 1997). For managing weed populations, seed dormancy is problematic because it is associated with the development of persistent seed banks. Seed dormancy also causes difficulties in predicting when weed seedlings are likely to emerge and can reduce the effectiveness of weed control measures (Ghersa and Holt 1995). Both genetic factors and the maturation environment experienced by the parent plants play important roles in setting the level of seed dormancy (Fenner *et al.* 2005). In particular, the maturation environment experienced by the parent plant has been shown to play a fundamental role in determining seed dormancy in weeds (Steadman *et al*. 2003; 2004). Several environmental factors, such as photoperiod, water availability, temperature, light, and nutrient supply, are known to influence seed quality, including seed size, dormancy, and germinability (Fenner 1991; Hilhorst and Toorop 1997; Baskin and Baskin 1998; Gutterman 2000). Among these water availability is an important limiting factor, which effects plant growth and reproductive development and often prevents the plants from reaching the maximum growth potential in rain-fed environments (Patterson 1995; Jaleel *et al.* 2009). Inadequate soil moisture affects crop-weed competition significantly and generally weeds dominate under stress due to greater plasticity as compared to the crops (Chauhan and Mahajan 2014).

Spring rainfall in southern Australia can be quite variable, and this variation in rainfall pattern and increased aridity consistent with a warming climate, has the potential to alter weed distribution and their impact on crop production (Ramesh *et al*. 2017). Therefore, it is important to understand how water stress experienced by weeds during seed development will influence the expression of seed dormancy in important weeds. Low spring rainfall during the reproductive phase of weeds could also have an impact on the quality of weed seeds, in

particular their dormancy status. Sharif-Zadeh and Murdoch (2000) showed that seed dormancy of *Cenchrus ciliaris* L. increased when water stress was present during seed maturation. Whereas, water stress applied at the onset of flowering until completion of flowering resulted in a significant reduction in plant biomass, seed production and quick dormancy release in wild radish (Eslami *et al*. 2010).

Previous studies have shown that early maturing seeds have higher levels of seed dormancy as compared to the late maturing seeds (Barton 1962; Frost 1971; Pourrat and Jacques 1975; Ceccato *et al.* 2015). This variation is partially facilitated by maternal control of seed coat properties (Kendall *et al*. 2011; MacGregor *et al*. 2015). For example, variations in the seed coat thickness have been reported in the genus *Chenopodium* (Ceccato *et al*. 2015). However in *Arabidopsis*, the maternal environment is known to modify the contents of abscisic acid (ABA) and gibberellic acid (GA) in mature seeds, which affects physiological behaviour of the embryo (Footitt *et al*. 2011). Among the different plant hormones, ABA and GA are considered vital for the regulation of seed dormancy and germination (Tuan *et al*. 2018). In mature seeds and buds, the biosynthesis of ABA has been shown to be regulated by *ABA1, NCED3* and *ABA2* genes (Arora *et al*. 2003; Ruttink *et al*. 2007; Wang *et al.* 2016). While GA releases seed dormancy and helps in seed germination, its biosynthesis is regulated by GA20-oxidase (*GA20ox*) and GA3-oxidase (*GA3ox*) genes (Tuan *et al*. 2018). As *ABA*1 and *GA20ox* genes are involved in the initial steps of biosynthesis pathways of these plant hormones, they are known to have a significant impact on the expression of seed dormancy induction and release (Arora *et al*. 2003; Tuan *et al.* 2018).

Bromus diandrus Roth is a winter annual weed commonly found in agricultural areas characterized by cool wet winters and hot dry summers (Kon and Blacklow 1988) and is a serious weed of crops in South Australia (Kleemann and Gill 2006). *Lolium rigidum* Gaudin is regarded as the most problematic weed prevalent in grain growing regions of South Australia

(Kloot 1983). Both of these weed species can be highly competitive with crops, including wheat, and can also be contaminants of harvested grains (Gill and Blacklow 1985; Gill *et al*. 1987; Gill 1996; Steadman *et al*. 2004a; Llewellyn *et al.* 2016). The life cycle of both weeds is similar to many annual grass species in southern Australia. They germinate in autumn-winter, grow rapidly during spring and set seeds in summer (Steadman *et al.* 2003; Kleemann and Gill 2006). Upon maturation seeds are mostly dormant, which prevents fatal germination during infrequent rainfall events that occur during summer in Australia (Steadman *et al*. 2004a).

Lolium rigidum has high genetic variability (Owen *et al.* 2007), which can lead to large variations in germination responses of populations. Previous studies by Steadman *et al* (2004b) on *L. rigidum* confirmed that seed dormancy is affected by moisture and temperature stress; seeds produced under cool temperatures and moisture stress were quick to lose dormancy as compared to the seeds collected from well-watered plants. None of the previous studies examined the impact of moisture stress on seed dormancy variation and expression of genes responsible for controlling this variation in *B. diandrus* and *L. rigidum*. At present it is unclear what effect water stress during reproductive development will have on seed dormancy in *B. diandrus* and *L. rigidum*. Therefore, studies were undertaken with the following objectives:

1. determine the influence of moisture stress during reproductive development on seed dormancy in *L. rigidum* and *B. diandrus*; and
2. determine the effect of water stress on the expression of important genes (ABA1 and GA20ox) known to be associated with seed dormancy during the after-ripening period.

Materials and methods

Seed collection

Mature seeds of two populations each of *Lolium rigidum* and *Bromus diandrus* were collected in December 2019 from crop fields in South Australia. The *L. rigidum* populations (RAC1 and RAC3) were collected from a single farm at Roseworthy and *B. diandrus* populations were collected from two different farms located near the towns of Mallala and Riverton. The collected seeds were oven dried, cleaned and stored in a laboratory until used in the experiments (April to November 2020).

Effect of water stress during reproductive development on seed dormancy

This experiment was laid out in completely randomized design and replicated at two locations, Roseworthy campus (R) and the Waite campus of The University of Adelaide (W) during April to November 2020. These two experimental sites are 50 km apart. The experiment was replicated at these two sites to assess the stability of the effect of water stress treatments on seed dormancy. Weed plants were exposed to water stress by ceasing irrigation at the GS31 (1st node) and GS60 stage (flowering). The control treatment, in which plants were irrigated regularly to prevent water stress, was also included to compare the effects of water stress. Water stress treatments were replicated 3 times at each location.

The control pots were maintained at the field capacity throughout the experiment whereas soil water content of water stress treatments was allowed to decline to 25% of the field capacity (FC) based on regular assessment of pot weight. Thereafter, pots in the water stress treatments were maintained close to 25% FC by regular watering. The field capacity was determined using the method described by Steadman *et al*., (2004b). Briefly, the pots containing dry potting mixture were weighed and then watered until water started to drain at the bottom of the pots. Pots were then allowed to drain for 6 hours and then reweighed to determine FC. Seeds of *L.*

rigidum and *B. diandrus* populations (3 replicates/population and stress treatment) were placed in pots (8 litre capacity with 250mm diameter) containing a potting mix (Handreck *et al.* 2002; Boutsalis *et al.* 2012). When seedlings reached the two leaf stage, plant density was reduced to five plants/pot for *L. rigidum* and three plants/pot for *B. diandrus*. The pots were kept outdoors under natural environmental conditions and well-watered until the start of water stress treatments. When the plants reached the GS31 (1st node stage), all the pots were moved into the glasshouse to avoid rainfall and ensure consistency of temperature and light conditions for all the treatments. Water content for the first stress treatment (GS31) was allowed to decline to 25% of the FC and then maintained at this level until maturity. The other pots were irrigated regularly and maintained close to the FC until the plants reached GS60 (flowering stage) and the second water stress treatment was imposed. Plants allocated to water stress treatments were weighed twice a week and the soil moisture raised to 25% of the FC. Throughout the experiment, pots of the no stress treatment (control) were well-watered and maintained close to the FC.

1) Seed production and seed mass

Harvest maturity was defined as the point at which no green colour remained in the stems and seeds. At maturity, seeds of each stress treatment from the two study sites were collected and placed in separate paper bags. The seeds were oven dried at 40℃ and then cleaned and stored in a laboratory at standard temperature conditions of 20/10 ℃ until used for seed dormancy and gene expression studies (December 2020 to June 2021). After cleaning, seeds were counted to determine seed production and samples of 100-seeds were weighed to determine seed mass.

2) Seed dormancy behaviour

Seeds of different water stress treatments were assessed for seed dormancy (germinability) every 2 months from December 2020 to June 2021. Seeds (25) were placed in 15cm diameter Petri dishes lined with Whatman No 1 filter paper; there were 3 replicates of each treatment. The filter paper was moistened with 5mL of distilled water and the 25 seeds spread over the surface. The Petri dishes were placed in an incubator set at 20/10 ℃, with 12h photoperiod for *L. rigidum*. As germination in *B. diandrus* seeds can be inhibited by exposure to light, Petri dishes containing its seeds were covered with aluminium foil to ensure complete darkness. Seed germination was assessed after 14 days and the experiment was terminated.

Expression of target genes associated with seed dormancy

After a careful review of the literature, we focussed on the expression of the genes *ABA1* and *GA20ox* in seeds of *B. diandrus* and *L. rigidum* and its relationship with changes in seed dormancy during the after-ripening period.

At the same time as the investigation of seed dormancy in water stress treatments, weed seeds were also used to extract the RNA and determine the expression of target genes (ABA1 and GA20ox). For each treatment there were three replicates of 25 seeds/population of *L. rigidum* and 15 seeds/population of *B. diandru*s tested at 30, 90 and 150 days after maturity to determine changes in the seed dormancy and gene expression levels during the after-ripening period. Quantitative polymerase chain reaction (qPCR) analysis of the expression of housekeeping genes (*αTubulin* and *ELF*) and target genes (*ABA1* and *GA20ox*) was conducted at each assessment to determine the pattern of candidate gene expression during dormancy maintenance and release.

RNA extraction and cDNA synthesis from seeds

The seeds imbibed for 24 hours at 20/10 ℃ were removed from the Petri dishes and immediately placed in liquid nitrogen to avoid any degradation of RNA. The seeds were then ground to a fine powder using a Retsch Ball Mill (MM301, RETSCH, Artisan Technology Group). RNA extraction was performed using the RNeasy mini extraction kit (Qiagen) according to the manufacturer's instructions. RNA integrity was evaluated via gel electrophoresis. Using the Tetro cDNA synthesis kit (Bioline), multiple cDNA synthesis reactions from each RNA sample were performed and the cDNA was pooled to ensure enough cDNA from a single source was available for the qPCR analysis.

Gene expression for seed dormancy was analysed via quantitative PCR (qPCR) using similar methods to that described by Malone *et al.* (2016) and Adu-Yeboah *et al.* (2020). A Sensimix (Bioline, Meridian Bioscience) assay using Dual-Labelled BHQ FRET probes (Bioresearch Technologies, Petaluma, CA, USA) was used. Primers and probes for the candidate genes *ABA1* and *GA20ox* and control genes *αTubulin* and *ELF* were designed using Real Time Design qPCR assay design software (Bioresearch Technologies). cDNA templates were amplified in a 10µL reaction mixture containing 5µL of Sensimix (Bioline, Meridian Bioscience), 0.4µL of each target and control primers, and 0.1µL of each target and control probe, 1.5µL of H_2O and 2.5µL of cDNA. Reactions were run on a Rotor Gene 6000 real-time thermal cycler with the following parameters: 3 min at 95℃, followed by 45 cycles of 15s at 95 ℃ and 16s at 60 ℃. Primer efficiencies were calculated and found to range from > 96% to < 99%. Each qPCR reaction containing the cDNA from the control (no stress), water stress at the GS31 and GS60 stage from both the populations of *L. rigidum* and *B. diandrus* was run with a gene of interest (*ABA1* and *GA20ox*) and 2 control genes (*αTubulin* and *ELF*). This protocol was repeated with seeds at 90 and 120 days after maturity (DAM).

Statistical analysis

All statistical analysis was performed with the software GraphPad Prism version 9 (GraphPad Software, San Diego, CA). An analysis of variance (ANOVA) was performed on the data of seed germination in different water stress treatments at the two sites to determine the effect of population x site and population x stress x site. The *P* values of population x site were 0.77 and 0.83 for *B. diandrus* and *L. rigidum*, respectively. The *P* values for population x stress x site were 0.084 (*B. diandrus*) and 0.483 (*L. rigidum*). Since the *P* values for site were non-significant ($P > 0.05$), the germination data for the two sites were pooled.

For gene expression analysis, the candidate gene runs for qPCR were compared with both housekeeping genes (*αTubulin* and *ELF*) to normalize the data. The average of the C_T values of 2 control genes was used and a modified version of the ΔC_T ($2 = {}^{-\Delta CT}$) method was used to analyse data from the quantitative PCR (Livak and Schmittgen 2001; Gaines *et al.* 2010). The calculated CT values were then used to run ANOVA. A correlation analysis between the gene expression levels and seed germination (%) of both the weed species was performed to determine the relationship between seed germination and gene expression levels during the after-ripening periods.

Results

Impact of water stress on seed production and seed mass

In both *L. rigidum* and *B. diandrus*, the number of seeds produced and the weight of 100 seeds was similar at both the locations (Table 1 and 2). However, there was a significant effect of water stress treatments on seed production. As expected, plants growing without water stress (control) produced more seeds and seeds of greater mass (Table 1 and 2). Conversely, plants exposed to water stress at GS31, which experienced stress for the longest period, had the lowest seed production.

Table 1. The effect of water stress treatments on *B. diandrus* seed production

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Table 2. The effect of water stress treatments on *L. rigidum* seed production

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Effect of water stress treatments on seed dormancy

The water stress treatments had a significant impact on seed dormancy in *B. diandrus* populations. Plants grown in well-watered conditions produced seeds with a lower level of dormancy as compared to plants that experienced water stress at the earliest stage of development (GS31). At 30 days after maturity (DAM), some germination was observed in the control (20%) and water stress imposed at the flowering stage (11%). However, there was no seed germination observed in plants exposed to water stress at GS31 (Fig. 1). During the after-ripening period, there was a gradual increase in seed germination in the control as well as water stress treatments. Significant differences were observed in seed germination between water

stress and control treatments until 90 DAM. However, at 210 DAM, seed germination in both the water stressed and control treatments was similar in both populations of *B. diandrus* (Fig. 1).

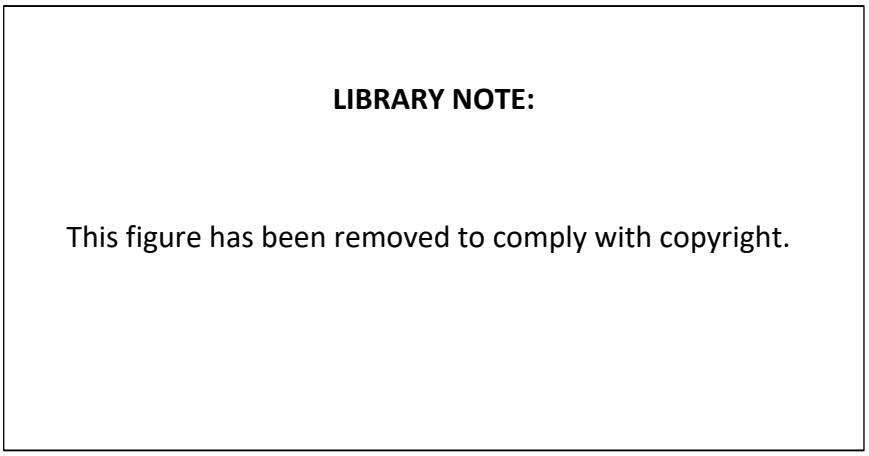


Fig.1. Germination pattern of two *B. diandrus* populations under water stress and no stress treatments. The error bar show $\pm$ standard error, and the symbols show significant differences at $p<0.05$ (*) or $p<0.01$ (**).

L. rigidum populations also showed significant differences in seed germination pattern in response to water stress treatments (Fig. 2). The seeds from the no stress treatment were less dormant and started germination at 30 DAM (Fig. 2). Water stress imposed at GS60 had little effect on seed dormancy in comparison to GS31, where the seeds of plants exposed to water stress were most dormant and took longer to start germination (Fig. 2). For instance, at 90 DAM for GS31 only 8% and 22% seed germination was observed in RAC1 and RAC3, compared to approximately 50% germination in the no stress treatment of both the populations, respectively (Fig 2). Significant differences in germination of different water stress treatments during the after ripening period indicates strong impact of water stress on seed dormancy of *L. rigidum* and *B. diandrus* populations.

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Fig 2. Germination pattern of two *L. rigidum* populations under water stress and no stress treatments. The error bar show ± standard error, and the symbols show significant differences at $p<0.05$ (*) or $p<0.01$ (**).

ABA1 gene expression

There were significant differences in the expression levels of *ABA1* between the water stress treatments in Mallala and Riverton populations of *B. diandrus*. During the after-ripening period, the *ABA1* gene expression levels in no stress and water stress at GS60 declined in both *B. diandrus* populations. The differences in the *ABA1* expression levels among the treatments were more noticeable at 90 DAM. Higher *ABA1* expression levels were observed for Mallala and Riverton in the no stress treatments (113%, 104%) as compared to GS31 (85%, 74.5%) and GS60 (96%, 0%) (Fig 3a, b). The expression of *ABA1* gene declined at 150 DAM as compared to earlier assessments at 30 and 90 DAM. At 150 DAM no detectable expression was observed in the water stress at GS31 and GS60 treatments of Riverton, nor the GS31 treatment of Mallala. The differences in the expression levels of GS31 and GS60 were non-significant.

The analysis of *ABA1* gene expression in *L. rigidum* populations showed significant differences between the seeds produced by plants exposed to water stress at the GS31, GS60 and no stress treatments. The *ABA1* expression levels were lower for the no stress treatments of RAC1 and RAC3 compared to the water stress applied at GS31 stage (Fig. 3c and d).The decline in *ABA1* expression levels from 30 to 150 DAM was more noticeable in all the treatments of RAC3 as compared to RAC1.

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Fig. 3. Relative *ABA1* gene expression analysis (*αTubulin* and *ELF* were the control genes) of the seeds of stress *vs* control treatments of two *B. diandrus* (a, b) and two *L. rigidum* (c, d) populations at 30, 90 and 150 Days after maturity (DAM). The symbols show significant differences at $p<0.05$ (*), or $p<0.01$ (**) or $p<0.001$ (***).

GA20ox gene expression

In contrast to *ABA1, GA20ox* gene expression showed a significant increase during the after-ripening period. All the treatments of both populations of *B. diandrus* had a lower *GA20ox* expression at 30 DAM, but there was an increase over time in all treatments at 150 DAM (Fig. 4a, b). For example at 30 DAM, *GA20ox* gene expression for Mallala was 107% for no stress treatment while at 150 DAM the *GA20ox* expression had increased to 132% (Fig. 4a). Similar trends showing increase in *GA20ox* gene expression levels over time were observed in the Riverton population as well, where *GA20ox* expression increased significantly between 30 and 150 DAM (160%) (Fig. 4b). Consistent with the Mallala population, *GA20ox* expression levels were higher for the seeds of well-watered plants than water stress at the GS31 stage in Riverton *B. diandrus*, especially at 150 DAM.

During the after-ripening period in *L. rigidum* populations, *GA20ox* gene expression increased while *ABA1* gene expression declined. Significantly higher *GA20ox* gene expression levels were observed in the no stress (control) treatment, as compared to the water stress at GS31 (Fig. 4c and d). At 30 DAM, RAC1 and RAC3 had lower *GA20ox* expression for all the stress treatments followed by a gradual increase in the gene expression over time (Fig. 4c and d). At 150 DAM a similar trend of increase in *GA20ox* expression levels during the after-ripening period was observed in all the stress treatments of RAC3 (Fig. 4d). The correlation analysis between seed germination and *GA20ox* gene expression showed a significant positive relationship for all water stress treatments in both populations of *B. diandrus* and *L. rigidum* (Fig. 5). However a negative correlation between *ABA1* expression levels and seed dormancy was observed for all the water stress treatments of both the populations of *B. diandrus* (R^2= 0.29) and *L. rigidum* (R^2= 0.34).

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Fig. 4. Relative *GA20ox* gene expression (*αTubulin* and *ELF* were the control genes) analysis of the seeds of stress *vs* control treatments of two *B. diandrus* (a, b) and two *L. rigidum* (c, d) populations at 30, 90 and 150 Days after maturity (DAM). The symbols show significant differences at $p<0.05$ (*), or $p<0.01$ (**) or $p<0.001$ (***).

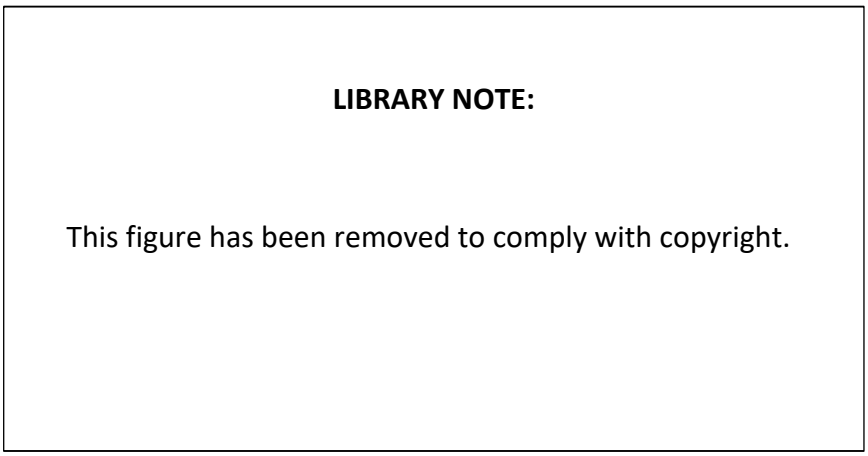


Fig. 5. Correlation analysis between seed germination and the expression of *GA20ox* in (a) two *B. diandrus* and (b) two *L. rigidum* populations. The symbols represent GS31 (●○) GS60 (▲△) and control (■□) treatments of both the *B. diandrus* and *L. rigidum* populations, respectively.

Discussion

Studies on climate change have shown that the increasing temperatures in Australia associated with global warming will result in reduced rainfall events and more frequent droughts (DEE, 2017). In South Australia, the crop growing areas already experience variable climatic conditions especially in spring where spring rainfall is highly variable. The ecological consequences of this climatic variability in weed seed dormancy are unclear. Weed population dynamics are greatly influenced by seed production and the success of weed management systems is highly dependent on preventing replenishment of the soil seed bank over time (Norris 2003). However, there have been limited investigations of the effect of moisture stress on weed seed dormancy and the genetic regulation of seed dormancy in response to water stress remains unclear.

In this study, water stress during reproductive development had a significant effect on the fecundity and seed mass of *B. diandrus* and *L. rigidum* populations. Plants of water stress treatments produced fewer seeds, as well as seeds of lower mass compared to the well-watered plants (Tables 1 and 2). Similar findings were reported by Eslami *et al.* (2010), where the plants of wild radish (*Raphanus raphanistrum*) exposed to different levels of water stress showed 9-85% reduction in the number of seeds/plant as compared to the non-stressed plants. The ability of *L. rigidum* and *B. diandrus* to only show modest reduction in seed production under reduced soil moisture availability (25% FC) shows that both of these weed species can survive and reproduce under dry climatic conditions often experienced in spring in Australia.

Analysis of seed dormancy through germination assessments showed that seeds produced in no water stress treatments lost seed dormancy more quickly than plants exposed to water stress from the GS31 (Fig. 1 and 2). Plants exposed to water stress from GS60 onwards produced seeds with similar seed dormancy as the control in *B. diandrus*. Even though water

stress applied at GS60 had a significant effect on seed mass of *B. diandrus* populations compared to the no stress (control), this late stress did not have a significant effect on seed dormancy. In contrast, water stress applied at GS31 not only reduced seed production and seed mass, it also produced seeds with higher dormancy than no stress and late stress (GS60). However in *L. rigidum*, plants that experienced water stress at GS60 showed a significant reduction in seed mass and intermediate level of seed dormancy between the control and water stress at GS31. Importantly, both water stress treatments had greater seed dormancy in *L. rigidum* than the no stress treatment. According to Sharif-Zadeh and Murdoch (2000), water stress imposed on plants of *Cenchrus ciliaris* at anthesis and caryopses maturation stages increased dormancy in *C. ciliaris* seeds. In their study, seeds exposed to water stress treatments had 14 to 19% germination as compared to 67% for the control (no water stress) treatment. From these results they concluded that greater dormancy in seed produced by water stressed plants in *C. ciliaris* might be associated with reduced water permeability of glumes in response to water stress. According to Sharif-Zadeh and Murdoch (2000), reduced water permeability in seeds of water stressed plants may not only protect seeds from drought stress it also imposes dormancy, which is itself an important survival mechanism.

In our study, earlier onset of water stress after the 1st node stage applied to *B. diandrus* and *L. rigidum* plants resulted in the production of fewer but more dormant seeds. In contrast, seeds produced by plants exposed to water stress later from flowering onward had lower levels of dormancy and germinated earlier than seeds from plants exposed to stress after the 1st node stage. These results differ from the findings of Steadman *et al.* (2004b), where *L. rigidum* seeds produced under water stress in atypical cool conditions for spring had lower dormancy as compared to the seeds grown under warm and well-watered conditions. In our study, pots were maintained in the greenhouse under typical warm spring temperature conditions during reproductive development, similar to what would be experienced in the field. These differences

in ambient temperature conditions during water stress may explain the disparity in response to water stress between our study and Steadman *et al.* (2004b).

Some of the differences in the influence of water stress on weed seed dormancy between our research and published literature may be related to the methodology used to impose water stress. In our study, water stress was imposed by cessation of watering at a pre-determined growth stage and pots were rewatered up to 25% of the field capacity. It is highly likely that plants of *B. diandrus* and *L. rigidum* would have experienced severe water stress after several days of the end of irrigation. However in a study on *Avena fatua* by Peters (1982), plants were exposed to water stress after panicle emergence, and the pots were only watered when the flag leaf started showing signs of wilting. Similarly, a study on *Sorghum halepense* subjected plants to cycles of drought by withholding water for 5 days and then rewatering the plants again (Benech-Arnold *et al*. 1992). In contrast, water stress to *Bromus tectorum* plants was imposed by changing polyethylene glycol (PEG) solutions, where the onset of water stress would have been instantaneous (Thill *et al.* 1979). Given the disparity in the methodology used to expose weed plants to water stress, it understandable to see the differences in its effect on seed dormancy.

Most of the previous studies on the impact of water stress on weed seed dormancy have not investigated mechanisms associated with seed dormancy. In our studies, seeds of water stressed plants of *B. diandrus* and *L. rigidum* were not only assessed for loss of seed dormancy during the after-ripening period but also for changes in the expression of two important genes known to regulate seed dormancy. ABA is a plant hormone well known to be linked with dormancy induction (Bentsink *et al.* 2018). Kanno *et al*. (2010) verified that ABA was synthesized in both maternal and zygotic tissues during seed development, and maternal ABA can be translocated to the embryos and induce primary seed dormancy. GA is the other major plant hormone involved in the regulation of seed dormancy and germination (Finch-Savage

and Leubner-Metzger, 2006). The level of biologically active GA in plant tissues is determined by the balance between its biosynthesis and inactivation (Yamaguchi, 2008). The biosynthesis of GA is regulated mainly by reactions catalysed by GA 20-oxidase (*GA20ox*) and GA 3-oxidase (*GA3ox*), while its inactivation is controlled primarily by GA 2-oxidase (*GA2ox*). In general ABA and GA play antagonistic roles in regulating seed dormancy (Wang *et al.* 2016).

In our studies the qPCR analysis of seeds of water stress treatments showed higher *ABA1* levels soon after seed maturity (30 DAM) followed by a general decline as seed became non-dormant. The clearest effect of water stress on *ABA1* was observed in RAC3 population of *L. rigidum*, which showed significantly lower *ABA1* expression in no stress treatment than the two water stress treatments (Fig. 3d). In *B. diandrus* populations and RAC1 *L. rigidum*, there was no clear difference in *ABA1* expression between water stress treatments. Over the after-ripening period, there was a decline in *ABA1* expression and a sharp increase in *GA20ox* gene expression levels in all the treatments (Fig. 3 and 4). The large observed increase in *GA20ox* gene expression levels during the after-ripening period suggests variation in seed dormancy between the water stress treatments was strongly linked with *GA20ox* expression. The increase in *GA20ox* expression levels from 30 DAM to 150 DAM suggests a contribution of this gene to the loss of seed dormancy during the after-ripening period. This was reflected in a significant positive correlation between *GA20ox* expression and seed germination in *B. diandrus* and *L. rigidum* (Fig. 5). Even though there was a negative association between *ABA1* expression and loss of seed dormancy, the relationship was not as strong as for *GA20ox*. The results of this study showed that *GA20ox* expression plays a significant role in the regulation of seed dormancy in these two grass weed species. When seeds of these weed species developed without water stress, they showed higher expression levels of *GA20ox* than those that experienced water stress during reproductive development.

Conclusion

Soil moisture conditions in which plants of *B. diandrus* and *L. rigidum* undergo reproductive development and mature had a strong influence on the levels of seed dormancy in these two important weed species. Water stress treatments also had a significant effect on the expression of *ABA1* and *GA20ox* genes, which are known to be involved in the regulation of seed dormancy. The results of this study showed much greater differences in *GA20ox* between water stress treatments than *ABA1*.

As expected, water stressed plants produced fewer seeds than well-watered plants, but had elevated levels of seed dormancy. As spring rainfall in Australian agricultural regions can be highly erratic, weeds are likely to not only produce different amount of seed between seasons, but it is also likely that the seeds produced will differ in their dormancy status. Since the seeds of *B. diandrus* and *L. rigidum* collected from plants that experienced water stress at GS31 stage were the most dormant, it is likely dry conditions during the reproductive stage will result in replenishment of the seedbank with weed seeds of greater dormancy status. Therefore, growing season rainfall received during reproductive development of weeds in the previous season is likely to influence the timing of their establishment and effectiveness of pre-sowing weed control tactics in the next year.

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Chapter 5: General Discussion And Conclusion

5.1 General Discussion

Lolium rigidum and *Bromus diandrus* are winter annual weeds of cereal crops and pastures that have naturalised across southern and Western Australia. Both these weed species produce seed in summer, which remain dormant in the soil and replenish the soil seed bank. Some seeds lose their dormancy over summer and autumn and initial seedling emergence is often observed after rainfall events in late autumn and early winter. Previous studies reported staggered germination and varying levels of seed dormancy in the populations of *L. rigidum* and *B. diandrus* collected from the same localities, which points to the presence of genetic variation in seed dormancy with these populations. Some of the previous research on *L. rigidum* and *B. diandrus* has provided indirect evidence for presence of genetic variation in seed dormancy (Gill and Blacklow 1985; Steadman *et al.* 2003a; Steadman *et al.* 2003b; Steadman *et al.* 2004a; Steadman *et al.* 2004b; Chauhan *et al.* 2006; Kleemann and Gill 2006; Goggin *et al.* 2011; Kleemann and Gill 2013). However, the genes involved in controlling seed dormancy have not been identified and changes in their expression during the after-ripening period have not been investigated.

5.1.1 Variation in seed dormancy in *Lolium rigidum* populations

In this study, seeds of seven different *L. rigidum* populations were collected from crop fields in South Australia in the summer of 2017. In April 2018 (autumn), a pot experiment was conducted to investigate seed emergence patterns in these populations. There were large differences in the pattern of seedling emergence between these populations. The time taken to reach 50% of the cumulative emergence (t_{50}) was estimated by fitting a logistic function and

used as the indicator of seed dormancy. The t_{50} for 3 populations from Roseworthy farm of the University of Adelaide ranged from 10 d to 27 d. Similarly, the t_{50} for two populations from Hilltown varied from 13 d for HT1 to 23 d for HT2. The presence of close to two-fold difference in t_{50} between *L. rigidum* populations from the same farm (i.e. same climate) provided initial evidence of genetic factors involved in regulating seed dormancy in this weed species. Seedling emergence in these populations continued for close to 2 months, which offered an opportunity to select early and late cohorts from within these populations. It was hypothesised that the early cohorts are likely to have arisen from a low dormancy (LD) fraction and conversely late cohorts would have come from a high dormancy (HD) fraction of the population. Plants of early and late cohorts were allowed to cross-pollinate with individuals of the same cohort by enclosing them in pollen-proof enclosures. This was vital as *L. rigidum* is an obligate out-crossing species. Fresh seeds of unselected populations were used to repeat the cycle of cohort selection.

Seeds collected from LD and HD cohorts were investigated for seed dormancy in 2019 and 2020. As expected, seeds from the LD cohort germinated earlier than the HD cohorts. Differences in seedling emergence between the LD and HD cohorts of the same population were consistent in the two selection experiments and indicated that seed dormancy in these populations is under genetic control. In previous studies, researchers have speculated about the presence of genetic variation of seed dormancy. For example, seeds collected from high rainfall zones tend to be less dormant compared to those collected from dry areas (Peltzer and Matson 2002). Others have stated that the presence of seed dormancy in *L. rigidum* seeds leads to staggered seedling emergence throughout the growing season, making this weed extremely difficult to control (Ellery *et al.* 2003; Goggin *et al.* 2011). Even though no evidence was provided, it has been suggested that the primary dormancy in *L. rigidum* appears to be under the influence of environmental and genetic factors (Goggin *et al.* 2010).

In order to investigate genetic regulation of seed dormancy, quantitative polymerase chain reaction (qPCR) technique was used on seeds of LD and HD cohorts. *ABA1* and *GA20ox* genes are known to play an important role in the biosynthesis of plant hormones ABA and GA. Both of these hormones are known to be important in regulating seed dormancy. Importantly, qPCR studies with seeds of the LD and HD cohorts of *L. rigidum* populations showed significant differences in the expression of *ABA1* and *GA20ox* genes during the after-ripening period. *ABA1* expression at 30 days after maturity was higher in HD cohorts as compared to the LD cohorts. A significant decline in the expression of *ABA1* was observed over time although it remained higher in HD cohorts at 90 days after maturity as well. Meanwhile, the expression of *GA20ox* was higher in the LD cohorts than the HD cohorts. The decline in *ABA1* expression and increase in *GA20ox* expression over time shows that the loss of seed dormancy is associated with changes in the expression of these important genes. This study provided a valuable insight into the genetic mechanism of seed dormancy variation within the populations of *L. rigidum*, which is likely to play an important role in enabling adaptation of these populations to different management practices.

5.1.2 Variation in seed dormancy in *Bromus diandrus* populations

Seed dormancy was assessed in five *B. diandrus* populations collected from fields in cropping areas of South Australia in 2017. There were significant differences in seedling emergence pattern between *B. diandrus* populations. The shortest time taken to reach 50% of final emergence (t_{50}) was observed in the Riverton population (10.3 d), followed by Wynarka (17.7 d) and Smithville (19.4 d). In contrast, t_{50} was much later in more dormant populations which came from Malinong (26.8 d), and Mallala (28.5 d). Despite the differences in t_{50}, seedling emergence in these populations continued up to 80 days after sowing (DAS). This protracted emergence pattern allowed selection of seedlings from low dormancy (LD) and high dormancy (HD) cohorts, as previously described for *L. rigidum*. Seeds produced by the LD and

HD cohorts were collected at maturity and investigated for seedling emergence patterns in 2019. A further selection of LD and HD cohorts was made from unselected seed of these populations in 2019 and tested in 2020. Both years of selection of early and late cohorts showed consistent behaviour in the pot studies. Seedlings of LD cohorts emerged earlier than the HD cohorts, reflecting differences in their seed dormancy. There was a 2-fold difference in the t_{50} of LD vs HD cohorts. Consistent differences in the timings of seedling emergence in the LD and HD cohorts of the same population indicated the presence of genetic control of seed dormancy. Our results help explain previous reports of differences in seed dormancy between weed populations even after they were grown at a common location. For example, Gill and Blacklow (1985) reported large differences in seed dormancy of *B. diandrus* populations collected from 14 sites in Western Australia even after they were grown in a common garden. From this observed variation in seed dormancy, they hypothesized that seed dormancy in *B. diandrus* is under genetic influence. Similarly, differences in dormancy levels of *B. diandrus* populations collected from fence-line and adjacent cropping field also pointed towards the genetic control of seed dormancy in this self-pollinated grass weed species (Kleemann and Gill 2006; 2013).

Previous research has shown the importance of *ABA1* and *GA20ox* genes in the expression of seed dormancy (Arora *et al.* 2003; Wang *et al.* 2016; Tuan *et al.* 2018). In our studies, qPCR was used to determine changes in *ABA1* and *GA20ox* gene expression between LD and HD cohorts of different populations. There were significant differences in the expression of *ABA1* in the LD and HD cohorts, which correlated well with seed dormancy in these cohorts. There was a steady decline in *ABA1* gene expression with time in LD and HD cohorts of all the populations, but the overall expression of this gene tended to be higher in the HD cohorts than in LD cohorts. While *GA20ox* expression increased over time as seeds became non-dormant, the LD cohorts maintained a higher *GA20ox* gene expression than the HD

cohorts. As the differences in *ABA1* and *GA20ox* expression between the seeds of LD and HD cohorts persisted even at 9 months after maturity, they may also contribute to the long-term dormancy of *B. diandrus* seeds and hence persistence of the seed bank.

5.1.3 Effect of water stress on seed dormancy

As water stress during reproductive development can be a frequent occurrence in Australia, it is important to understand the effect of this environmental stress on weed seed dormancy. Therefore, a pot experiment was conducted in 2021 to assess the effect of water stress during reproductive development on seed dormancy in two different populations each of *L. rigidum* and *B. diandrus*. Gene expression analysis was also performed to determine association between seed dormancy and *ABA1* and *GA20ox* expression in seeds collected from different water stress treatments. In this study, water stress during reproductive development had a significant effect on the fecundity and seed mass of *B. diandrus* and *L. rigidum* populations. Plants of water stress treatments produced fewer seeds, as well as seeds of lower mass, compared to the well-watered plants. Germination assessments through Petri dish experiments performed at 30, 90,150 and 210 days after maturity (DAM), showed that seeds produced in no water stress treatments lost seed dormancy more quickly than plants exposed to water stress at the GS31 (1st node stage). Seeds of plants where water stress was imposed at flowering (GS60) showed a lower response in terms of seed dormancy. It is feasible that cessation of watering at GS60 stage (flowering) may have allowed these plants to grow on stored soil water and reproduce unimpeded for several days before water stress became a limiting factor. Importantly, in *L. rigidum* both water stress treatments had greater seed dormancy than the no stress treatment, while in *B. diandrus* the seeds from the water stress at GS31 stage were more dormant as compared to the well-watered control and stress imposed at GS60. In field conditions, more dormant seeds produced in response to water stress could be

fewer in number but will reside in the seed bank for extended period of times and increase weed infestations in the future.

The qPCR analysis of seeds of water stress treatments showed higher *ABA1* levels soon after seed maturity (30 DAM) and a decline in *ABA1* gene expression levels at 90 and 150 DAM. However, there was no significant change in *ABA1* expression in response to water stress. Over the after-ripening period, there was a decline in *ABA1* and a sharp increase in *GA20ox* gene expression in all the treatments. A significantly higher *GA20ox* gene expression levels were observed in the no stress (control) treatment, as compared to the water stress at GS31. At 30 DAM, the populations had lower *GA20ox* expression for all the stress treatments followed by a gradual increase in the gene expression over time. At 150 DAM a similar trend of increase in *GA20ox* expression levels during the after ripening period was observed. An increase in *GA20ox* expression levels from 30 DAM to 150 DAM (80%-110%), suggests a contribution of this gene in the loss of seed dormancy during the after-ripening period. The correlation analysis between seed germination and *GA20ox* gene expression showed a positive relationship for all water stress treatments in both populations of *B. diandrus* and *L. rigidum***.**

5.2 Conclusions

This research project provides in-depth understanding of genetic variation in seed dormancy within the populations of *L. rigidum* and *B. diandrus*. From our research it became evident that seed dormancy in both these weed species is a heritable trait. Selection of low and high dormancy cohorts within these populations turned out to be an effective approach for investigating genetic variation in seed dormancy. The use of qPCR for investigating the expression of dormancy regulating genes (*ABA1 and GA20ox*) of the LD and HD cohorts provided valuable insights into the mechanism regulating seed dormancy. Previous studies of seed dormancy in these weed species have not investigated the role of specific genes

responsible for controlling seed dormancy. The findings from this research have enhanced understanding of the genes involved in regulating seed dormancy, their expression over time, and how loss of dormancy is associated with changes in the expression of *ABA1* and *GA20ox* genes. Presence of individuals with large variation in seeds dormancy within populations of *B. diandrus* and *L. rigidum* will enable them to rapidly adapt to changes in management practices such as delayed crop sowing. This research has also shown large differences in seed dormancy between *L. rigidum* populations from the same farm, which is likely to be associated with selection pressure imposed by different management practices used for weed control. Farmers with high dormancy populations of *L. rigidum* are likely to experience less effective pre-sowing weed control and will need to integrate other management tactics to minimise weed competition and prevent the build-up of the seedbank.

This study also established that water stress during reproductive development doesn't only affect seed production (fecundity), but also seed dormancy in these two weed species. Results of these studies indicate that seeds produced when water is non-limiting are less dormant than those produced when the parent plants were under water stress for an extended period during reproductive development. Weeds growing under water stress from floral initiation (GS31) will produce more dormant seeds, which may be more difficult to control before sowing crops the next season. To conclude, the findings reported here have improved understanding of the dormancy behaviour of *L. rigidum* and *B. diandrus* populations in South Australia and will provide guidance for designing integrated weed management programs.

5.2.1 Future implications

The studies conducted on the seed dormancy and germination behaviour of *L. rigidum* and *B. diandrus* populations have highlighted important aspects of genetic control of seed dormancy. In our studies, we focussed on the expression of *ABA1* and *GA20ox* as the key

genetic control of seed dormancy. Future studies on genes regulating seed dormancy in weeds may also include GA2-Oxidase and GA3-Oxidase, which are downstream from *GA20ox* investigated in this project.

The protracted seed germination observed in the populations of these weed species has implications for weed control. The early cohorts will be relatively easy to control with pre-sowing management tactics. However, late emerging cohorts will tend to escape pre-sowing management and can contribute to weed seed production especially in poorly competitive crops. Therefore, strategies to enhance crop vigour and weed suppressive ability combined with effective pre-emergent herbicides need to carefully considered and used in fields infested with high dormancy populations of these weed species. Future research should also investigate weed management tactics required to enhance weed suppression to minimise seed output of late (high dormancy) cohorts.

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