Physiological and Molecular Mechanisms of Dormancy and Germination in Grass Weed Seeds

Thomas Edward Holloway

Thesis submitted for the degree of

Doctor of Philosophy in Biological Sciences

Royal Holloway, University of London

October 2019

for Lena

Born 18th October 2017

Declaration of Authorship

I, Thomas Edward Holloway, hereby declare that this Thesis and the work presented therein is entirely my own. Where I have presented or consulted the work of others, this is always clearly stated.

Signed:

Date:

Abstract

Agricultural weeds are a serious threat to sustainable intensification. Reduction of the 'crop protection toolbox' due to herbicide resistance and a changing regulatory environment necessitates the development of alternative weed control strategies. Seed dormancy is a key trait determining weed emergence within crop fields however there is a fundamental lack knowledge on the mechanisms regulating seed dormancy in weeds, particularly at the molecular level. This Thesis aims to investigate the mechanisms of dormancy and germination in a panel of economically important grass (Family Poaceae) weeds in order to gain an insight into the diversity of mechanisms regulating germination in weed species. An ecophysiological characterisation of dormancy in five grass weed species demonstrates the role of dormancy in regulating germination responses to changing temperatures. Screening of a target-catalogue compound library identifies novel dormancy breaking responses across species to identify targets for manipulating weed emergence in the field and demonstrates diversity in the regulation of germination across grass species. Advances in next-generation sequencing technologies and analysis of publically available transcriptome datasets are employed to investigate the mechanisms of weed seed responses to temperature. Based on this analysis, dormancy-specific cell wall remodelling processes are identified leading to the hypothesis that cell wall strengthening is involved in dormancy enforcement. Biomechanical methods are employed to demonstrate that the coleorhiza, a tissue surrounding the embryonic radicle, becomes reinforced in dormant grass seeds. Model species and grass weeds are contrasted and examples of the conservation, divergence and convergence of mechanisms are discussed. The findings from this Thesis will support improved weed emergence models and provide targets for weed management at the level of the seed, as well as contributing to a broader understanding of the diversity in the regulation of germination across species.

Acknowledgements

The last four years have been the most enriching time in my life thanks to the support and input from my supervisors, colleagues, friends and family. Firstly I want to thank Dr. David Stock (Syngenta Ltd.), who was instrumental in initiating the collaboration that included this project and motivated my decision to apply for the PhD. David passed away during the project but his enthusiasm and kindness will be fondly remembered.

I am grateful to Professor Gerhard Leubner-Metzger and Dr. Kazumi Nakabayashi who are influential in my development as an independent researcher. Thank you Gerhard for your constant support and encouragement, and for allowing me the freedom to follow my own interests and ideas. I am very thankful to Kazumi for late night discussions and my practical laboratory training, but also for teaching me to think critically and work accurately. I will remember Kazumi's example for the rest of my career.

I want to express great gratitude and admiration to Syngenta who I have found to be an organisation that is committed to collaboration and the development of young researchers. Too many Syngenta colleagues have supported this project to list them all here. I am particularly thankful those who made my placement at Jealott's Hill enjoyable and engaging. Anne Seville took over from David as my industrial supervisor and has gone above and beyond with her contribution to this project. I am grateful to Anne for many things, but mostly for making me feel that my work is valuable to Syngenta. I also wish to thank Syngenta colleagues in North Carolina for their bioinformatics contribution that went further than what I thought possible for such a challenging species.

I feel very lucky to have been a part of The Seed Biology Group of Prof. Leubner, which has been much more than a group of colleagues over the course of my PhD. I specifically want to thank my fellow PhD students Edd, Giles, Matt and Waheed for making me laugh and keeping me sane. Thank you to Jake for teaching me everything I know about bioinformatics and for putting up with annoying questions. I wish all the best to James and Tina for their future together and good luck to Michi for his new career. Thanks also to friends outside of academic that remind me that there is a life outside of seeds.

I am grateful to my parents their love, support and understanding during my studies. You taught me to be questioning and to follow my own path. Thank you for picking me up when things went wrong and for believing in me.

Durante mis estudios también me he convertido en parte de otra familia que me ha brindado más apoyo del que esperaba. Gracias a mi suegro, que es una persona muy honesta, paciente y, a menudo, la voz de la razón en tiempos estresantes. A mi suegra que es una persona muy amorosa y que me ha apoyado durante todo mi doctorado, especialmente mientras escribía en Asturias. Gracias también a Tati-MJ, cuando vienes de visita me haces reír tanto que olvido el estrés de mi trabajo. Gracias también a Victoria y a todos los demás por hacerme sentir parte de vuestra familia.

Finally I want to say thank you to Marta, my friend, co-author and wife, who deserves her own acknowledgements chapter. Although this thesis is dedicated to our daughter, who was born during the RNA extractions for the transcriptome, she knows that without her this thesis would never have been completed. If you meet someone who you can spend all day with and still miss when you're away then you're very lucky person. Thank you for all your support, especially during the difficult last months writing this thesis.

Thank you all.

Table of Contents

List of Figures

(Manuscripts not included)

Chapter 8. Materials and Methods

Chapter 9. Appendix

List of Tables

(Manuscripts not included)

Abbreviations

Abbreviation	Full Form
% v/v	Percentage concentration as volume per volume
% w/v	Percentage concentration as weight per volume
% w/w	Percentage of mass
(+)-PAO4	Propenyl-abscisic acid with an O-butyl chain
1°/2°…	Primary/secondary …
13-LOX	*13-lipoxygenase*
1-MCP	1-methylcyclopropene
2ODD	*2-oxoglutarate–dependent dioxygenase*
530_{EX}	Excitation at a wavelength of 530 nm
590_{EM}	Emission filter at 590 nm
5-mC	5-methylcytosine
AA1	Abscisic acid agonist 1
AAO	*Abscisic aldehyde oxidase*
ABA	Abscisic acid
ABA8'OH	*Abscisic acid 8'hydroxylase*
ABF	*ABSCISIC ACID RESPONSE ELEMENT BINDING FACTOR*
ABI3/5	*ABSCISIC ACID INSENSITIVE 3/5*
ABRE	Abscisic acid responsive element (promoter *cis* element)
ACC	1-amino-cyclopropane-1-carboxylic acid
ACCase	*Acetyl CoA carboxylase*
ACO	*1-amino-cyclopropane-1-carboxylic acid oxidase*
ACX	*Acyl CoA oxidase*
AdoMet	S-adenosyl-methionine
AHDB	Agricultural and Horticultural Development Board
AI	Active ingredient
AIL1	*AP2-LIKE ETHYLENE-RESPONSIVE TRANSCRIPTION*
ALS	Acetolactate synthase
ANOVA	Analysis of variance
AOC	*Allene oxide cyclase*
AOS	*Allene oxide synthase*
APC	Anaphase promoting complex
AR	After-ripened
$AR_{100\%}$	Incubation time required to generate a fully after-ripened seed batch
$AR_{50\%}$	Incubation time required to generate a half-maximally after-ripened seed batch
AR_x	After-ripened for 'x' number of days

AS	Adaxial scale
AS_6	3′-hexasulfanyl abscisic acid
AS_n	3′-alkylsulfanyl abscisic acid
ATP	Adenosine triphosphate
AUC	Area under the curve
AVG	2-aminoethoxyvinyl glycene
aW	Water activity
BBSRC	Biotechnology and Biological Sciences Research Council
BLAST	Basic local alignment search tool
bp	Nucleotide base pairs
BSA	Bovine serum albumin
BUSCO	Benchmarking universal single-copy orthologs
BWA	Burrows-Wheeler aligner
C.I.	Confidence interval
CABI	Centre for Agriculture and Bioscience International
CBTC	Methyl 6-chloro-3*H*-1,2,3-benzodithiazole-4-carboxylate 2-oxide
CCD	*Carotenoid cleavage dioxygenase*
CD	Conditionally dormant
CDK	*Cyclin-dependant kinase*
cDNA	Complementary DNA
CE	Coleorhiza emergence
COI1	*CORONATINE INSENSITIVE 1*
CPS	*ent-copalyl diphosphate synthase*
CPT	Coleoptile
CRTISO	*Carotene isomerase*
CRZ	Coleorhiza
CS	Caryopsis
CTAB	Hexadecyltrimethylammonium bromide
CTR1	*CONSTITUTIVE TRIPLE RESPONSE 1*
Cvi	*Arabidopsis thaliana* Cape Verde Islands ecotype
CWRP	Cell wall remodelling protein
D	Dormant
D8/14/27/53	*DWARF 8/14/27/53*
DAD1	*DEFECTIVE IN ANTHER DEHISCENSE 1*
DAD2	*DECREASED APICAL DOMINANCE 2* (D14 ortholog, *Petunia hybrida*)
DAPI	4′,6-diamidino-2-phenylindole
DEG	Differentially expressed gene
DELLA	Components of the gibberellin signalling pathway characteristically containing a '-DELLA-' motif

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOG1	*DELAY OF GERMINATION 1*
DS	Diaspore (dispersal unit)
DTT	Dithiothreitol
EB	Epiblast
EBF	*ETHYLENE INSENSITIVE BINDING F-BOX*
EC	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
EIL	*ETHYLENE INSENSITIVE-3-LIKE*
EIN2/5	*ETHYLENE INSENSITIVE 2/5*
ELISA	Enzyme-linked immunosorbent assay
END	Endosperm
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EREBP	*ETHYLENE RESPONSE ELEMENT BINDING PROTEIN*
ERF	*ETHYLENE RESPONSE FACTOR*
ERH	Equilibrium relative humidity
ETR2	*ETHYLENE RECEPTOR 2*
EXPB	β-expansin
FDR	False discovery rate
FH	Freshly harvested
F_{max}	Maximum force
FPKM	Fragments per kilobase of transcript per million mapped reads
g ha-1/kg ha-1	Grams/kilograms per hectare
GA	Bioactive gibberellins
$GA_1/GA_3/GA_4/GA_7$	Gibberellin A1/A3/A4/A7 (the bioactive gibberellins)
GA13ox	*Gibberellin 13-oxidase*
GA20ox	*Gibberellin 20-oxidase*
GA3ox	*Gibberellin 3-oxidase*
GA4	*GIBBERELLIN DEFICIENT 4 (ga3-oxidase 1)*
GA5	*GIBBERELLIN DEFICIENT 5 (ga20-oxidase 1)*
GAI	*GIBBERELLIN INSENSITIVE* (Arabidopsis DELLA protein)
GGPP	Geranylgeranyl diphosphate
GID1	*GIBBERELLIN INSENSITIVE DWARF 1*
GIMP	GNU image manipulation program
gMAX	Maximum germination percentage
GO	Gene ontology
GUS	B-glucuronidase reporter system
HA	Aryl-C_1N hydroxamic acid
HEMA	2-hydroxyethyl methyl acrylate

HPPD	4-hydroxyphenyl-pyruvate
HR	Herbicide resistance
HYDB	B-Ring carotene hydroxylase
ICN	Illinois Climate Network
IPS	InterProScan (Blast2GO feature)
IWM	Integrated weed management
JA	Jasmonic acid
JA-Ile	Jasmonoyl isoleucine
JAR	*Jasmonoyl isoleucine conjugate synthase*
JAZ	*JASMONATE ZIM DOMAIN PROTEIN*
JIN	*JASMONATE INSENSITIVE*
KAI2	*KARRIKIN INSENSITIVE 2*
KAO	*ent-kaurenoic acid oxidase*
KAT	*L-3-ketoacyl-coa-thiolase*
KO	*ent-Kaurene oxidase*
KS	*ent-kaurene synthase*
MAX1/3/4	*MORE AXILLARY GROWTH 1/3/4*
MD	Morphological dormancy
MIDAS	Met Office Integrated Data Archive System
MOA	Mode of action
MPE	Micropylar endosperm
mRNA	Messenger ribonucleic acid
MUSCLE	Multiple sequence comparison by log-expectation
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	2,5-norbornadiene
NCBI	National Center for Biotechnology Information
NCED	*9-cis-epoxycarotenoid dioxygenase*
NCGR	National Centre for Genome Resources
ND	Non-dormant
NGS	Next-generation sequencing
NJ	Neighbour joining (phylogenetic methodology)
ns	Not statistically significant at a given alpha value
NSP	N-substituted phthalimides
NV	Non-vernalised
OP	Operculum
OPC-8	3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid
OPDA	*cis*-(+)-12-oxophytodienoic acid
OPDA-Ile	Isoleucine conjugate of cis-(+)-12-oxophytodienoic acid
OPR	*OPDA reductase*
OSR	Oil Seed Rape (*Brassica napus*)

P	Probability statistic
P450	*Cytochrome P450 monooxygenases*
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Physiological dormancy
PDS	*Phytoene destaurase*
PE	Paired-end (sequencing reads)
PF	Puncture force
PHS	Pre-harvest sprouting
PHX	Prohexadione calcium
pI	Isoelectric point
PML	Plumule
PP2Cs	*Type 2C protein phosphatases*
PSII	Photosystem II
PSY	*Phytoene synthase*
PVP	Polyvinylpyrrolidone
PY	Physical dormancy
PYL	*PYRABACTIN RESISTANT-LIKE*
PYR	*PYRABACTIN RESISTANT*
Q.S.	*Quantum satis* (adjusting to a required volume)
Qsd1	QTL seed dormancy 1 (Barley)
QTL	Quantitative trait locus
RAD	Radicle
RAP23/24	*RELATED TO AP2 3/4*
RC	Root cap
RCAR	*REGULATORY COMPONENTS OF ABA RECEPTOR*
rcbL	*Large subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase*
RE	Radicle emergence
RGA	*REPRESSOR OF GA1-3* (encodes an *Arabidopsis thaliana* DELLA protein)
RHT	*REDUCED HEIGHT 1* (encodes a *Triticum aestivum* DELLA protein)
RIL	Recombinant inbred line
RIN	RNA integrity number
RLE	Relative level of expression
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTE	*REVERSION-TO-ETHYLENE-INSENSITIVITY*
RuBisCO	*Ribulose-1,5-bisphosphate carboxylase/oxygenase*

SA	Summer annual
SCF	*SKP CULLIN F-BOX*
SDR	*Short-chain dehydrogenase/reductase*
SDS	Sodium dodecyl sulphate
SE	Standard error
SEM	Shoot emergence
SHT	Shoot
SLN1	*SLENDER 1* (encodes an *Hordeum vulgare* DELLA protein)
SLR1	*SLENDER RICE 1* (encodes an *Oryza sativa* DELLA protein)
SnRK2	*SNF1-RELATED PROTEIN KINASE*
SOD	*Superoxide dismutase*
T+P	Testa and pericarp
T_{50}^{-1}	The inverse of the time taken to reach half maximal germination
T_b	Base (minimum) temperature for germination
T_c	Ceiling (maximum) temperature for germination
TCO	*trans*-cyclooctane
TMB	Trimethylbutenolide
T_o	Optimum temperature for germination
TPS	*Terpene synthase*
TTC	2,3,5-triphenyltetrazolium chloride
V	Vernalised
WA	Winter annual
WSB	Agricultural soil weed seedbank
XEH	Xyloglucan endohydrolase (enzyme activity)
XET	Xyloglucan endotransglycosylase (enzyme activity)
XLLG	Xyloglucan nonasacharide
XLLG-SR	Sulforhodamine-conjugated xyloglucan nonasacharide
XTH	*Xyloglucan endotransglycosylase/hydrolase*
XXXG	Xyloglucan heptasacharide
ZDS	*Z-carotene desaturase*
ZEP	*Zeaxanthin epoxidase*
ZISO	*Z-carotene isomerase*

1. General Introduction

1.1 The importance of weeds in agriculture

1.1.1 What is a weed?

Weeds are plants that have co-evolved with humans to in order to take advantage of the niches created by agriculture (Baker, 1991). These weeds typically evolved from species specialising in disturbed habits, for example on the migratory routes of grazing animals, and share similar traits that contribute to their 'weediness' existing in a state of constant adaptation to changing agronomic practises (Neve *et al.* 2009). Weeds are typically self-compatible and highly fecund, adaptable to different environmental conditions through phenotypic plasticity and produce seeds that are able to survive for long periods of time in the soil (Baker, 1974). Another key component of weediness is the ability to effectively compete with crops for resources such as light, water and nutrients. The effect of weed competition on later crop establishment and yield, as well as the reproductive success of weeds, is determined by the weed-crop interaction during early crop establishment (Zimdahl, 1988). Therefore the ability of weeds to synchronise their emergence with crop establishment is a key weediness trait.

Understanding the economic impact of weed competition with crops is complicated by the ubiquitousness of weeds in agricultural systems – since it is almost impossible to eradicate them from a field it is hard to compare a weedy and weed free-field. However it is possible compare the theoretical 'attainable yield', or maximum yield achievable in a certain context, to the yields achieved when no efforts are made to control weeds. For example, in wheat there is a 50% potential global yield loss when pests and weeds are uncontrolled, that drops to an actual yield loss 29% due to the use of crop protection products. Actual percentage losses vary considerably in different regions

depending primarily on technology uptake. For example a 35% increase in yield could be gained from eradicating pests in wheat in Central Africa and Southeast Asia, whereas only a 14% increase in yield could be gained by eradicating all wheat pests in Northern Europe (Oerke, 2006). The importance of weeds in agricultural systems can be gauged by the amount farmers spend trying to control weeds. In 2012 global pesticide expenditure totalled approximately $56 billion at the farm level, herbicides accounting for 45% of this cost to farmers (Atwood & Paisley-Jones, 2017).

1.1.2 Loss of the crop protection toolbox

For centuries farmers have relied on a combination of mechanical control and crop rotation to control weeds, however there was a shift in the middle of the 20th century towards the use of chemical weed control (Zimdahl, 2018). Herbicides became prevalent due to their effectiveness and ease of application and the development of transgenic crops with tolerance to popular herbicides reduced the need for more traditional rotational cropping practises. This increased dependence on herbicides is causing concern about the environmental and public health effects of some herbicides (Römer *et al.*, 2019). The regulatory environment has responded by taking older chemistry off the market whilst creating more barriers to the registration of new herbicides (Chauvel *et al.*, 2012; NFU, 2015). Herbicides that are in development must also compete with established herbicides for which herbicide-tolerant crops have been developed. Consequently no new herbicide mode of actions (MOAs) have been commercialised in the last 30 years (Duke, 2012; Davis & Frisvold, 2017).

This reliance on a limited range of herbicide chemistries has contributed to increasing levels of herbicide resistance (Fig. 1.1) in weed populations globally (Peterson *et al.*, 2018). Herbicide resistance (HR) is an example of the evolutionary capacity of agricultural weeds, where under a continual selective pressure against a specific

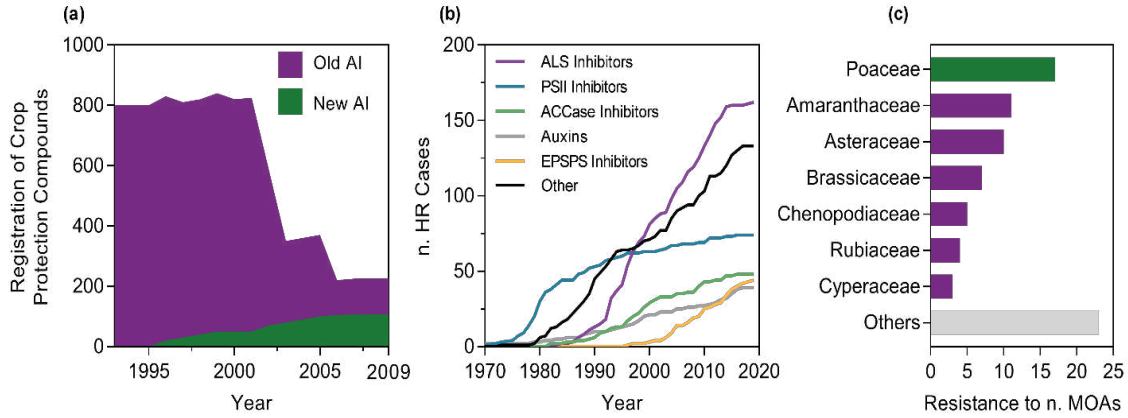


Fig. 1.1. Loss of the 'crop protection toolbox'. (a) The number of crop protection products registered in the UK with those registered from 1995 onwards shaded in green. Redrawn from NFU (2009). **(b)** Levels of reported herbicide resistance cases across individual species for different herbicide modes of action. **(c)** The number of resistances to different herbicide modes of action across weed families. **(a,b).** Data from Heap (2019).

target, resistance develops either by changes in the active site of the target protein or through other metabolic processes involved in herbicide detoxification or biokinetics (Délye *et al.*, 2015). Concomitant with the decline in the release of new herbicides, resistance has developed to all available MOAs and in many weed species resistance has evolved to multiple herbicide MOA within individual populations (Heap, 2019). For example, some populations of *Lolium rigidum* have developed resistance to 7 independent MOAs, making it difficult to find registered herbicides to control weeds (Owen *et al.*, 2014). Amongst herbicide resistant weeds, the grasses (family Poaceae) have developed resistance to the greatest number of herbicide MOAs (Heap, 2019).

The combined effects of reduced herbicide discovery, pesticide regulation, HR and a loss to traditional weed control techniques in high-input arable farming has led to a reduction in the ‘crop protection toolbox’ - the tools which farmers have to control weeds. This situation is causing a shift in weed science away from herbicide chemistry-driven research towards developing a better understanding of weed biology in order to develop more integrated approaches to weed management (Westwood *et al.*, 2018).

Integrated weed management (IWM) is an agronomic concept that takes a holistic approach to weed control based on the synergistic effects of different weed control practises such as tillage, cultivar competitiveness, biological control and herbicide applications (Swanton & Weise, 1991). Whilst there has been increasing interest in IWM in recent years, possibly as a result of the reduction in the crop protection toolbox, IWM practises remain difficult to implement in the field (Harker & O'Donovan, 2013). This is perhaps because IWM practises are dependent on an understanding of the effects of different agronomic practises on weed biology. There is diversity in weed populations across climactic regions, cropping systems, between fields and even between different areas of a single field (Bhowmik, 1997). Consequently IWM strategies have to be tailored to specifically for a particular context on-farm, a practise that is typically not economically viable for field crops.

1.2 Seedbank dynamics determine weed emergence patterns

1.2.1 Weed seedbank dynamics explain emergence patterns

One component of IWM involves understanding and manipulating the emergence patterns of weeds (Dyer, 1995). The concept of the 'soil weed seedbank' (WSB) describes the pool of ungerminated weeds seed stored in the soil that have the capacity to germinate and emerge as weeds. Weed populations are regulated by the dynamics of the WSB (Buhler *et al.*, 1997). This has particular relevance to HR weeds, where at any point many different HR genotypes may be present in the WSB and emerge across seasons, making the HR profiles of weeds present in a single season difficult to predict (Schwartz-Lazaro & Copes, 2019). Agronomic practises can have different effects on the WSB that can be exploited to manage the timing and extent weed emergence. For the example of tillage, shallow cultivation in combination with

fallowing (leaving land unplanted) can be used to stimulate the emergence of weeds at a convenient time during the cropping cycle. These weeds can then be controlled with herbicides or soil inversion before planting the crop (Dyer, 1995). Conversely such tillage practises can also bring deeply buried weed seeds to the surface of the WSB, stimulating the emergence of unexpected species that were buried in previous seasons (Zimdahl, 2018). An understanding of how WSB dynamics regulate weed emergence patterns is therefore particularly important both for predicting and controlling weed emergence.

1.2.2 Inputs and outputs to the weed seedbank

The vast majority of weeds seeds in the WSB come from annual, rather than perennial weeds and therefore the input of seeds to the WSB is dependent on the number of weeds that flower and produce seed within a season. Successful weeds are typically highly fecund, producing hundreds of seeds per plant where only the complete lifecycle of a single offspring is required to maintain the population (Baker, 1974). Consequently WSBs in fields with moderate infestations can have more than 1 million viable weed seeds m^{-2} (1 seed mm^{-2}) buried at different depths (Baskin & Baskin, 2006). These seeds can have either of two fates: to germinate and attempt to survive in competition with other weeds and the crop or to remain ungerminated and gradually lose the ability to emerge as the processes of seed ageing result in irreparable damage and eventual exhaustion of the seed along with seed predation and pathogenesis (Long *et al.*, 2014).

These two fates are controlled by the inherent seed properties of dormancy and longevity respectively. Seed longevity is a property of seeds that allows them to persist long-term in the WSB. This phenomenon is well exemplified by the long term seed burial experiment initiated by Dr. William James Beal in 1879, where seed samples have been taken every 5 years until the present day. This experiment has

demonstrated that, whereas some species persist for only a few years, many seeds are able to germinate after over 120 years of burial and produce normal seedlings (Telewski & Zeevaart, 2002). From an evolutionary perspective, this property of seeds allows plants to disperse their seeds through time as well as space as a bet-hedging strategy that spreads the risk of reproductive failure where the threat of plant mortality is high (Cohen, 1966). This is the typical situation for agricultural weeds, where cultivation adds an element of unpredictability and weed populations are subjected to mass mortality events such as those caused by tillage and herbicide applications. Consequently seed longevity is an adaptive trait that is considered to contribute strongly to the 'weediness' of agricultural weeds (Baker, 1991).

The inherent property of seeds that creates the need for longevity is dormancy. There are many definitions of seed dormancy (Hilhorst, 1995; Li & Foley, 1997; Finch-Savage & Leubner-Metzger, 2006; Bewley *et al.*, 2013) that have a common argument: seed dormancy is any block on the germination of a seed under otherwise favourable conditions for germination. In the context of agricultural weeds, seed dormancy is a restraint on germination of weeds that prevents their emergence until their chances of contributing their offspring to the WSB are greatest. Seed dormancy also allows plants to transfer information about previous environmental conditions across multiple generations in order to optimise germination timing in changing environments (Auge *et al.*, 2017a,b; Vayda *et al.*, 2018). This trans-generational control of the depth of dormancy can be the result of environmental factors perceived by the developing embryo ('maternal zygotic effects') or from factors that are perceived earlier by the vegetative mother plant ('true zygotic effects') that are transduced to the embryo through epigenetic mechanisms (Penfield & MacGregor, 2017).

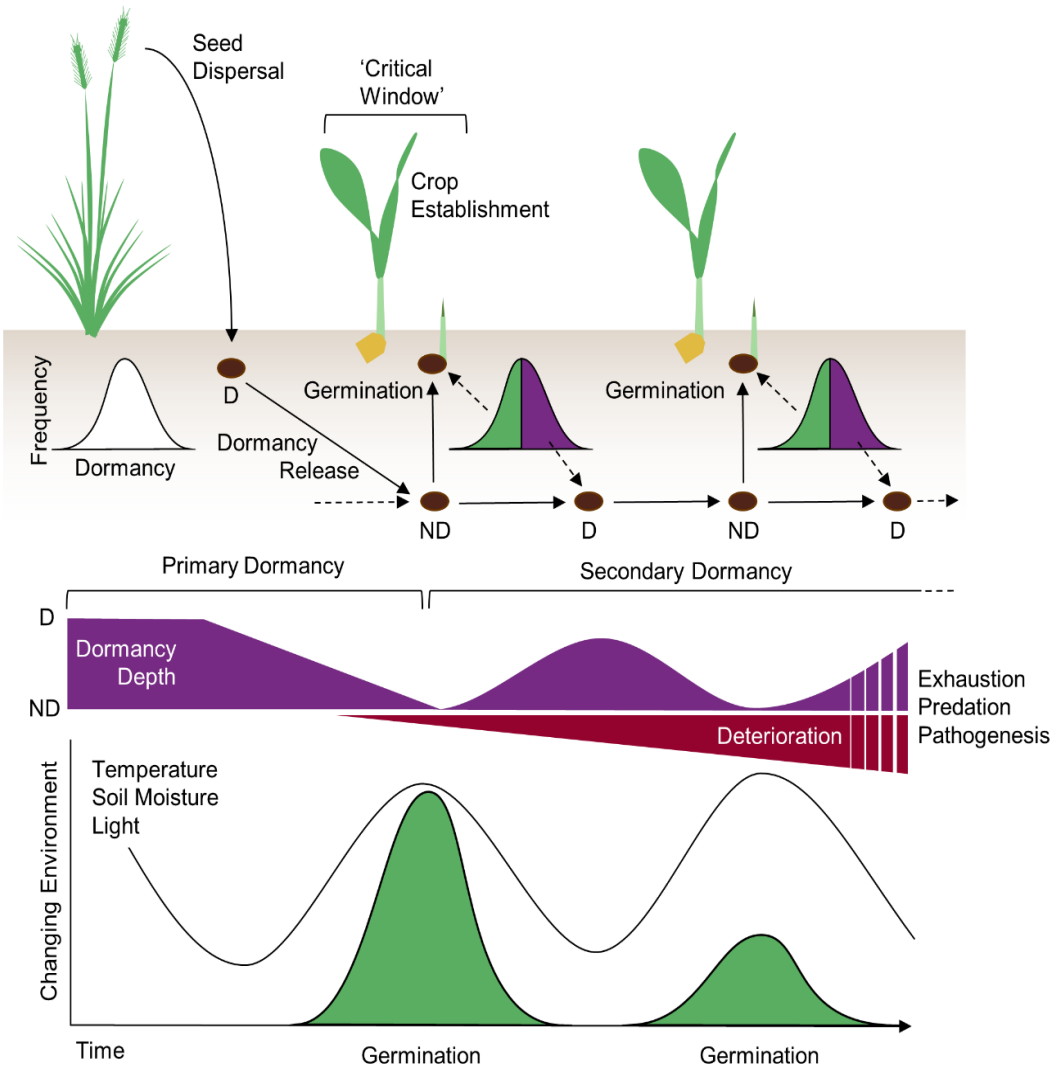


Fig. 1.2. Seed dormancy in an agricultural context. Weed seeds are typically dispersed with primary dormancy. As this dormancy is released a fraction of the population germinates and the remaining fraction may enter secondary dormancy. As dormancy levels cycle with environmental conditions the fraction of the population with less dormancy germinate. This way weeds can emerge during the 'critical window' for crop competition. If seeds remain for long periods in the weed seedbank they accumulate damage that may eventually lead to ageing and exhaustion.

1.2.3 Ecology of seed dormancy

From an ecological perspective, physiological seed dormancy is classified into two types depending on the timing when dormancy is imposed. Primary dormancy is induced before dispersal during seed maturation and limits the range of conditions under which the seed can germinate (Baskin & Baskin, 2014). Seeds with primary dormancy are typically dispersed into conditions that prevent their germination. In

temperate environments where there are seasonal changes in environmental conditions, as the seasons progress there is eventually an overlap between the range of temperatures permitted for germination by primary dormancy and the current environmental conditions during which time the seed germinates (Fig. 1.2) (Finch-Savage & Leubner-Metzger, 2006). For winter annuals, seeds are typically dispersed in the summer when soil moisture is too low to permit germination. In this 'dry state' seeds may undergo a process of 'after-ripening' whereby dormancy is gradually lost, permitting germination when water becomes available if other environmental conditions are favourable (Bewley *et al.*, 2013).

In summer annuals, seeds are dispersed during the colder months, when soil moisture is not a limiting factor. There is often a requirement for these imbibed seeds to experience cold temperatures ('cold-stratification') in order for dormancy to be released. For example in seeds of the common summer annual maize weed *Echinochloa crus-galli* (barnyard grass) primary dormant seeds do not respond to after-ripening (even after 400 days) yet 1-2 weeks of cold stratification is sufficient to break dormancy (Honek *et al.*, 1999). On the other hand, many winter annual weeds such as *Ambrosia artemisiifolia* (common ragweed) respond to after-ripening (Baskin & Baskin, 1985) and may require periods of warm stratification to break dormancy (Baskin & Baskin, 2014).

Secondary dormancy occurs post-dispersal, often when seeds fail to germinate within the first season and encounter prolonged conditions unfavourable to germination. For example secondary dormancy can be induced in the model species *Arabidopsis thaliana* by imbibition at high temperature (Donohue *et al.*, 2007) or by prolonged incubation under temperatures below the minimum permissive temperature for germination (Penfield & Springthorpe, 2012). Other factors such as moisture content, light and the chemical or gaseous environment of the WSB can also influence the entrance and exit from secondary dormancy (reviewed in Baskin & Baskin, 2014).

Whilst there is a tendency to consider primary and secondary dormancy as two distinct processes, in nature dormancy exists in a continuum across time (Baskin & Baskin, 1985) and the maternal environment as well as the primary dormancy levels of seeds influences the subsequent depth of secondary dormancy (Auge *et al.*, 2015). In the laboratory, dormancy types can be distinguished by transferal of seeds between different fixed imbibitional environments, however in the field dormancy levels 'cycle' depending on environmental conditions (Baskin & Baskin, 2014).

1.3 Seed dormancy mechanisms

Seed dormancy has been extensively studied both at ecological and molecular levels yet dormancy remains 'one of the least understood phenomena in the field of seed biology', partly due to a lack of interaction between these two disciplines (Finch-Savage & Leubner-Metzger, 2006). Whilst ecological studies have investigated dormancy cycling in a wide range of species (reviewed in Baskin & Baskin, 2014), molecular studies of dormancy have focused almost entirely on the model species *A. thaliana* and *Hordeum vulgare* (barley) that have typically have only a shallow dormancy (Cohn, 1996). Understanding of the mechanisms underlying seed dormancy came initially from forward genetics approaches using mutants with abnormal dormancy phenotypes and then by mapping of quantitative trait loci (QTL) and more recently through omics-technologies and reverse genetics approaches (Nakabayashi *et al.*, 2017).

1.3.1 Genetic regulation of physiological seed dormancy in model species

Primary seed dormancy is imposed during the seed maturation phase when the seed develops the ability to germinate and survive desiccation (Bewley *et al.*, 2013). During maturation the interaction between four key transcription factors, ABSCISIC ACID

INSENSITIVE 3 (ABI3), FUSCA 3 (FUS3) and LEAFY COTYLEDON 1 & 2 (LEC1 & 2), regulate both seed maturation and the acquisition of dormancy. Disruption of these genes leads to aberrant seed development including severely reduced seed dormancy (reviewed by Holdsworth *et al.*, 2008). Further identification of mutants with reduced dormancy phenotypes in both *Zea mays* (maize) and Arabidopsis identified a central role for abscisic acid (ABA) metabolism in seed dormancy. In maize, a number of *viviparous* mutants that germinated whilst still on the cob were identified (McCarty, 1995) that were later found to have disrupted genes with roles in ABA biosynthesis. One of these genes was V*iviparous14* (*vp14*) that encodes 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key rate limiting ABA biosynthesis enzyme (Schwartz *et al.*, 1997). Additional ABA biosynthesis mutants in Arabidopsis, such as *aba-deficient 1* defective in zeaxanthin epoxidase, the enzyme responsible for the first step in ABA biosynthesis, also have a reduced dormancy phenotype (Koornneef *et al.*, 1982). Mutants of ABA catabolism such as *cyp707a2*, encoding an ABA 8'hydroxylase, exhibit an increased dormancy phenotype (Okamoto *et al.*, 2006).

Confirming the finding that ABA is an important positive regulator of dormancy, mutants of genes involving ABA signalling components also show reduced dormancy phenotypes. During ABA signalling, ABA binds to PYR/PYL/RCAR receptor proteins that then form a complex with Type 2C Protein Phosphatases (PP2Cs) releasing the repression of SNF1-related protein kinases (SnRK2s) on downstream regulators of ABA responsive genes (reviewed by Nambara *et al.*, 2010). Mutants of both PP2Cs (e.g. *ABA insensitive 1 & 2*) and SnRK2s again result in altered dormancy phenotypes (Koornneef *et al.*, 1984; Fujii *et al.*, 2007).

Classical mutant screening approaches have also identified negative regulators of seed dormancy (Nakabayashi *et al.*, 2017). In species with a non-deep physiological dormancy, exogenous bioactive gibberellin (GA) application promotes germination (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006). A number of

mutants that show a reduced apical dominance and repressed germination have been identified in crop and model species (reviewed by Ross, 1994) and have later been characterised as having disrupted GA biosynthesis or signalling. In Arabidopsis, the mutants *ga1* through *ga5,* that have with reduced gibberellin accumulation and germination, has been identified as having disrupted in the GA biosynthesis genes. Of these genes, *GA4* (G*ibberellin 3-oxidase 1*) and *GA5* (*Gibberellin 20-oxidase 1*) have been identified as the most important genes regulating the accumulation of gibberellins during germination (Yamaguchi, 2008). Mutants with aberrant gibberellin signalling, such as the *gibberellin insensitive dwarf 1* (*gid1*) mutant of Arabidopsis, barley and rice, that were found to be deficient in the GA receptor (Ueguchi-Tanaka *et al.*, 2005) have an increased dormancy phenotype that is not restored by exogenous GA application in Arabidopsis *gid1a gid1c* double knockouts (Voegele *et al.*, 2011). Negative regulators of gibberellin activity have been identified in genes encoding members of the RGA/GAI family of proteins that repress GA responses and are negatively regulated by the GA-GID1 complex. Disruption of these genes in Arabidopsis (*rga* & *gai*), barley (*sln1*), maize (*d8*), rice (*slr1*) and wheat (*rht*) result in diverse plant height phenotypes (Peng *et al.,* 1999; Olszewski *et al.*, 2002).

The antagonistic interaction of ABA and GA (Fig. 1.3) has been widely researched and is considered the core of the hormonal regulation of dormancy and the transition to germination (Finch-Savage & Leubner-Metzger, 2006). However additional hormonal signalling pathways have been shown to influence dormancy through their interaction with ABA and GA signalling (reviewed by Linkies & Leubner-Metzger, 2012). Ethylene is a gaseous hormone produced by the methionine cycle (Yang & Hoffman, 1984) that can revert the effect of exogenous ABA application when its precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) is applied to *Lepidium sativum* (garden cress) seeds (Linkies *et al.*, 2009).

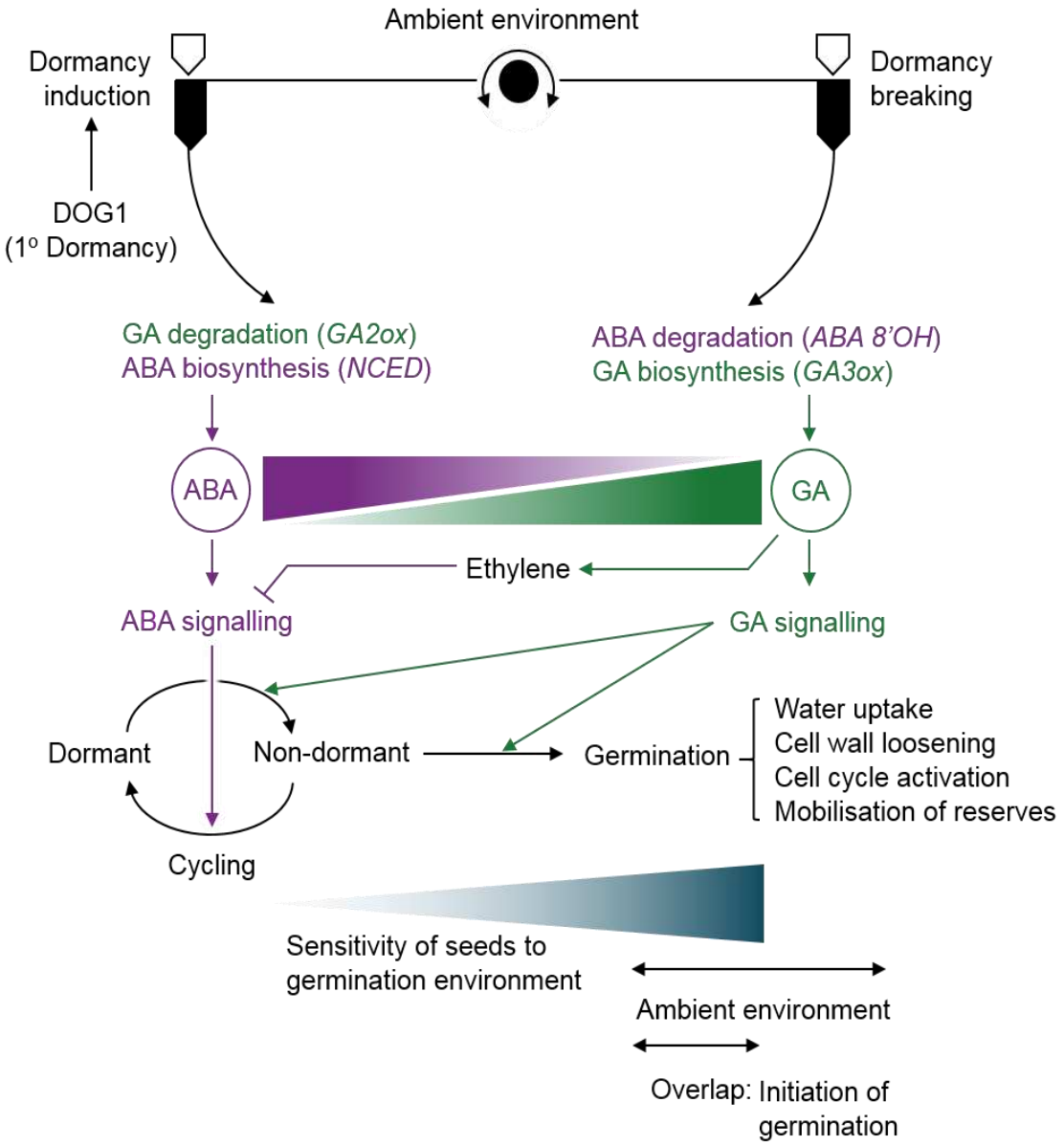


Fig. 1.3. Hormonal regulation of seed dormancy in Arabidopsis. Model for the regulation of dormancy and germination by ABA, GA and ethylene. Ambient environmental factors affect the balance between ABA and GA. In the dormant state ABA synthesis and signaling dominate, and in the non-dormant state GA signaling and synthesis induce the transition to germination. Cross-talk between ABA and GA may take place via ethylene synthesis and signaling. The ABA:GA balance cycles according to environmental conditions, and when there is an overlap between the temperatures required for germination as set by the ABA:GA balance and the ambient environment the germination process is induced. This model is based on microarray data from Cadman *et al.* (2006) and is modified from a figure in Finch-savage & Leubner-Metzger (2006) to include information from Linkies & Leubner-Metzger (2011) and Graeber *et al.* (2012).

Ethylene signalling mutants have differing sensitivities to exogenous ABA application. For example the ethylene receptor mutant *etr2* is hypersensitive to ABA and has a

greater cold stratification requirement to break primary dormancy (Beaudoin *et al.*, 2007). Forward genetics approaches have also identified a role for jasmonates (JA), hormones generally associated with defence and stress responses, in seed dormancy. For example the JA-insensitive mutants *coi1-16* and *jin4* are ABA-hypersensitive (reviewed by Linkies & Leubner-Metzger, 2012).

Despite the effectiveness of the forward genetics approach to identify the core hormonal regulation of dormancy, this approach is limited when investigating gene families where there is a high level of redundancy (Nakabayashi *et al.*, 2017). This short-coming in seed dormancy research was overcome using recombinant inbred lines (RILs) of parents with contrasting dormancy phenotypes through the analysis of quantitative trait loci (QTLs). Numerous seed dormancy QTL studies have been performed in Arabidopsis, barley and rice identifying both conserved and unique QTLs. In Arabidopsis the first major QTL to be cloned was the *DELAY OF GERMINATION 1* (*DOG1*) gene where a loss of function in *dog1* leads to a complete lack of seed dormancy (Bentsink *et al.*, 2006) and DOG1 protein levels typically correlate with levels of dormancy across different maternal environmental conditions (Nakabayashi *et al.*, 2012). In monocots, QTL analysis for dormancy and the pre-harvest sprouting (PHS) trait has identified more than 150 QTLs, most of which have an unknown molecular function (reviewed in Li *et al.*, 2019). For example, one QTL that has been identified as a major determinant of reduced seed dormancy in barley is *Qsd1* that encodes an alanine aminotransferase which controls seed dormancy through an unknown mechanism (Sato *et al.*, 2016).

More recently '-omics' approaches have been employed to understand the downstream genes regulated by specific environmental signals, hormones and mutations. Microarray-based studies in Arabidopsis have identified a pool of transcripts specific to the dry state that have an influence on the dormancy of imbibed seeds (Nakabayashi *et al.*, 2005; Nelson *et al.*, 2017). There is an overrepresentation of genes controlled by

ABA responsive elements (ABREs) in the dry seed transcriptome and the expression of many dry seed transcripts is affected in *abi5,* an ABA-insensitive mutant (Nakabayashi *et al.*, 2005). The role of gibberellins during germination has been investigated through exogenous GA application to a GA biosynthesis mutant, identifying GA-responsive genes associated with progression of the cell cycle, cell wall remodelling and the regulation of other hormones (Ogawa *et al.*, 2003). Microarray technology has also been used to compare primary dormancy, after-ripening and cycling secondary dormancy, identifying many of similarities in the ABA:GA dynamics of dormancy states, but also some transcripts specific to either dormancy type (Cadman *et al.*, 2006). The ongoing development of next-generation sequencing (NGS) technologies, such as RNAseq for global transcriptome analysis, have also permitted the study of the molecular mechanisms of dormancy and germination in non-model species such as *Suaeda spp.* (seepweed) (Wang *et al.*, 2017; Xu *et al.*, 2017), *Aethionema arabicum* (stonecress) (Wilhelmsson *et al.*, 2019), *Cunninghamia lanceolata* (chinese fir) (Cao *et al.*, 2016) and the parasitic weed *Phelipanche aegyptiaca* (Bao *et al.*, 2017) but to date no NGS experiments have been performed in non-model monocot seeds. These studies confirm the conserved role of ABA and GA signalling processes across diverse species, but also identify species specific processes indicating that there are diverse mechanisms interacting with the core ABA:GA system to regulate germination across different species.

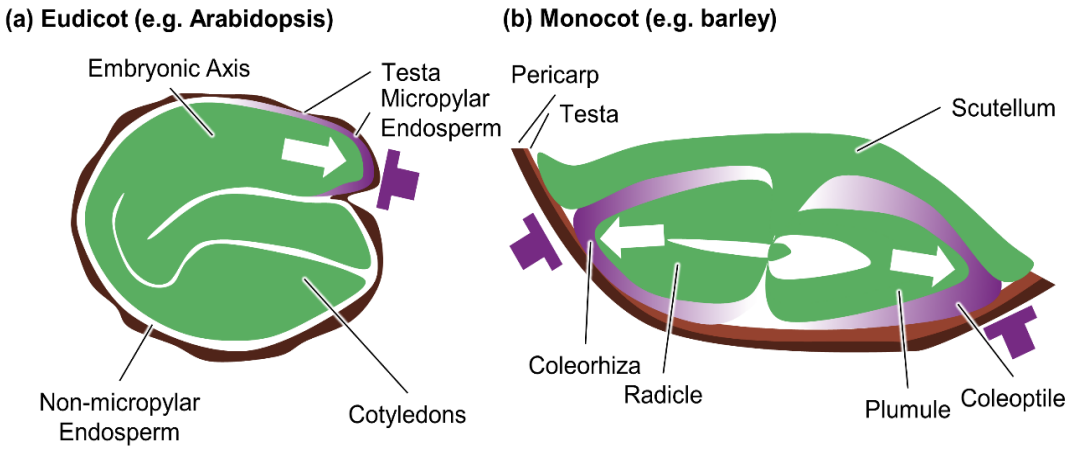


Fig. 1.4. Comparative morphology of eudicot and monocot embryos. (a) Schematic of a eudicot seed showing the embryo with two cotyledons surrounded by endosperm and testa. **(b)** Schematic of a grass embryo showing embryonic root (radicle) and shoot (plumule) surrounded by the coleorhiza and coleoptile respectively all contained within the pericarp and testa. Arrows represent growth potential and caps represent barriers potentially restraining this growth.

1.3.2 The role of different organs

The majority of studies on the regulation of germination and dormancy through hormonal and transcriptomic mechanisms comes from work sampling whole undissected seeds (Graeber *et al.*, 2010). However, seeds are structurally complex and the different embryonic structures within the seed provide different contributions to seed dormancy (Linkies *et al.*, 2010). In terms of its morphology, a seed can be broken down into two functional compartments: the embryo that expands in response to environmentally-mediated hormonal signals, and the covering layers that restrain the growth of the embryo up to a threshold point, above which germination occurs (Steinbrecher & Leubner-Metzger, 2017). The endosperm in Arabidopsis is a living tissue that actively produces ABA in response to environmental conditions that can prevent the growth of excised embryos (Lee *et al.*, 2010, 2012). The part of this endosperm that surrounds the radicle is the micropylar endosperm (MPE), which is a key tissue that regulates the expansion of the embryonic axis through physical restraint (Fig. 1.4a). The force required to puncture this tissue, and hence complete germination,

is changed in response to cold stratification and hormone application (reviewed by Steinbrecher & Leubner-Metzger, 2017).

Monocotyledonous seeds lack an MPE (Fig. 1.4b) that has such an important role in Arabidopsis seed dormancy and germination. Instead, the embryonic axis (formed from the embryonic root 'radicula' and embryonic shoot 'plumule') is surrounded by non-vascularised embryonic tissues called the coleorhiza and coleoptile (Fig. 1.4b), which expand during germination and are thought to have a role in protecting the growing embryo during the germination process (Sargent & Osborne, 1980; Sargent *et al.*, 1981; Debaene-Gill *et al.*, 1994). However, it has been suggested that the coleorhiza of monocots and the MPE of dicots are functionally related tissues, and that the coleorhiza may have an important role in regulating the dormancy in grasses (Millar *et al.*, 2006). In germinating barley grains ABA 8'hydroxylase accumulates specifically in the coleorhiza indicating an important tissue-specific division of labour in hormone metabolism (Millar *et al.*, 2006). Quantification of ABA in dormant and after-ripened barley embryos showed that ABA is present at high levels in the coleorhiza and that this level is reduced after 24 hours imbibition in AR coleorhiza but remains high in dormant coleorhizae (Barrero *et al.*, 2009). Tissue specific microarrays also identified coleorhiza-specific differentially expressed genes (DEGs) involved in ABA catabolism, cell wall modification and jasmonate metabolism (Barrero *et al.*, 2009). Mannanases, that are thought to have a role in cell wall loosening, have a strong localisation signal in the coleorhiza, indicating a requirement for cell wall loosening that is also seen in the MPE (González-Calle *et al.*, 2015).

Weakening of the MPE is a prerequisite for the completion germination of many eudicot species (Steinbrecher & Leubner-Metzger, 2017). This weakening is directed by environmentally-mediated hormonal mechanisms involving reactive oxygen species (ROS) (Müller *et al.*, 2009). Ethylene and GA increase the weakening effect (Müller *et al.*, 2006; Müller *et al.*, 2009) through the increased production of cell wall remodelling

proteins (CWRPs) such as expansins, glucanases and mannanases that loosen the polysaccharide bonds within the MPE cell walls (Chen & Bradford, 2000; Chen *et al.*, 2002; Leubner-Metzger, 2003; Martínez-Andújar *et al.*, 2012). Biomechanical techniques have been applied to confirm that regulation of CWRPs results in a decrease in the force required to puncture the MPE in a number of crop and model species (reviewed by Steinbrecher & Leubner-Metzger, 2017) such as in Arabidopsis, *L. sativum* (garden cress), *Lactuca sativa* (lettuce) and *Solanum lycopersicum* (tomato), yet these techniques have yet to be applied to the coleorhiza of grasses.

1.4 Manipulation of seed dormancy as a target for weed management

1.4.1 Current practices in the management for the weed seedbank

Seed dormancy is a highly adaptive trait in agricultural weeds that determines their timing of emergence and consequently their competitiveness and detrimental effects on crops. Prior to knowledge of the mechanisms of dormancy in seeds, seed dormancy has been used as a target for the management of weeds (Dyer, 1995). Agronomic practices can have a substantial effect on the number of viable seeds in the WSB (Clements *et al.*, 1996). For example conventional tillage practices can be used to bury viable weed seeds and prevent their germination within a particular season, whereas in conservation tillage practices seed return to the WSB is typically much greater (Forcella & Lindstrom, 1988) with seeds remaining in the top centimeter of the soil profile (Yenish *et al.*, 1992). The time of day during which tillage takes place has also been used to control weed emergence. Where tillage occurs at night, emergence can be minimised since seeds with a light requirement to break dormancy do not germinate (Wesson & Wareing, 1969) and strong artificial irradiance during tillage promotes weed emergence (Scopel *et al.*, 1994). However an awareness of the environmental impacts of conventional tillage has led to a shift towards conservation tillage systems in which

weed pressure is typically higher and accordingly there is a greater dependence on herbicides (reviewed by Busari *et al.*, 2015). This shift is necessitates the development of alternative WSB control approaches that less dependent on soil disturbance (Dyer, 1995).

1.4.2 Management of the weed seedbank using chemicals.

Whilst most chemical weed control practices target processes in seedlings to adult plants, weeds typically spend the majority of their lifecycle as dormant seeds in the WSB, invulnerable to herbicides (Papenfus *et al.*, 2015). An alternative to the use of tillage for the management of the WSB could be to use chemicals to manipulate the emergence patterns of weeds. This approach would have a number of important advantages. Stimulating the emergence of weeds before the planting of the crop would allow weeds to be controlled with an herbicide, or by alternative means for HR weeds, without the concern of herbicide tolerance (safety/selectivity) in the crop. Alternatively weeds could be forced to germinate during periods that would be unfavorable for their further growth, causing seedling mortality or 'suicidal germination'. If germination stimulants were combined with pre-emergence herbicides ('Flush and Kill'), synchronous weed emergence immediately after application would cause weed seedlings to receive the greatest herbicide dose before herbicide concentrations are reduced by degradation. Conversely, germination inhibitors could be used to uncouple the synchronised emergence of weeds with crops, preventing weeds from emerging during the critical 'competition window' where the effects of weed competition on crop yield are greatest. This system would also be likely to be compatible with conservation tillage practices, since in these systems weed seeds typically inhabit the uppermost layer of soil where they would easily be targeted by germination stimulants.

This approach has been demonstrated on a small scale with parasitic *Striga* and *Orobanche spp.* weeds of maize, sorghum and millet in Africa and Asia (reviewed by Zwanenburg *et al.*, 2016). Parasitic weeds are entirely dependent on their host-plants, and hence the germination in these plants is initiated only through perception of low concentrations of the root exudates from their host plants such as strigolactones (Cook *et al.*, 1966, 1972; Brooks *et al.*, 1985). Initially it was discovered that these parasitic weed seeds responded to germination stimulants, such as ethylene, that could be pumped under pressure into the soil and stimulate the germination of *Striga* but not *Orobanche spp.* (Eplee, 1975). Germination in the absence of a nearby host-plant caused the death of the seedling and hence this technique was an effective and specific *Striga spp*. control method. However application in the field was technically challenging and cost-limiting at larger scales (Parker, 1991). The development of easily synthesisable synthetic strigolactone analogs, such as Nijmegen-1 (Thuring *et al.*, 1997), made chemical control of the parasitic WSB more feasible at very low doses (<7 g ha^{-1}) with very promising field trial results (Zwanenburg *et al.*, 2016). However the factors that limit the widespread use and commercialisation of 'suicidal germination' are agronomic rather than biological. Strigolactone analogs typically have low soil mobility and stability and therefore application timing was critical to avoid drought periods when germination could not occur (Babiker & Hamdoun, 1982; Babiker *et al.*, 1987, 1988). Much of the continuing work on 'suicidal germination' of parasitic weeds is now focused on developing strigolactone analogs and mimics with greater stability against hydrolysis in the soil (Zwanenburg *et al.*, 2016).

Whilst research into chemical manipulation of the parasitic WSB has progressed substantially, nearly to the point of commercialisation, research into the chemical control of the non-parasitic WSB is limited to just a handful of papers. This may be in part due to a lack of the specific and potent chemistry that exists for non-parasitic weeds. In contrast to the quantities of strigolactone analogs applied to induce suicidal

germination (<10 g ha^{-1}), some GA mimics and ABA biosynthesis inhibitors need to be applied at 3.5 kg ha^{-1} and 367 g ha^{-1} respectively to see a stimulatory effect in a narrow range of species (Donald & Tanaka, 1993; Goggin & Powles, 2014). Therefore if germination stimulants are to become successful WSB management tools then more potent germination stimulants with activity against a broader range of weed species will be required.

A key limitation to the development of these 'Flush and Kill' strategies is the lack of knowledge regarding the mechanisms of dormancy and germination in agricultural weeds, particularly at the molecular level (Dyer, 1995). Across the literature on germination stimulants in weeds, there is also a lack of standardisation in the methods employed as well as the origin and dormancy status of the seeds used. Results even between comparisons of the effect of individual compounds in a single species across different experiments are often confounding. The same challenge applies also to attempts to model weed emergence, where the data used to parameterise emergence models often vary considerably due to differences between seed batches. This thesis attempts to transfer knowledge from model species to the study of seed dormancy in a panel agricultural grass weeds. A physiological characterisation of the dormancy in these species is conducted in order to define conditions for further experiments. The contribution of different embryonic organs to grass seed dormancy is also investigated to identify the biomechanical processes involved in the germination of grasses. Developments in NGS technologies are employed to develop an understanding of the molecular mechanisms of dormancy and germination a grass weed in order to identify potential targets for WSB management. A small compound library is screened against this panel of weed species to identify MOAs with potential for WSB germination stimulants.

1.4.3 Selection of a panel of grass weed species with diverse life history traits

To investigate dormancy and germination in grass weeds a selection of economically important weed species with a variety of growth habits from different agronomic contexts was made (Fig. 1.5 and 1.6). In general grass weeds that had the greatest economic impact due to HR were selected. Weeds have a high degree of phenotypic plasticity (Grundy, 2003) that, along with the globalisation of agriculture, has allowed many weeds to become ubiquitous around the world. This phenotypic plasticity makes the lifecycles of weeds difficult to define since within a single species many growth forms and lifecycle habits may be present depending on the environment in which they grow.

***Alopecurus myosuroides* Huds. (blackgrass)**

Alopecurus myosuroides is a facultative winter annual what has a major peak of emergence in autumn and a minor emergence in spring, and is a successful weed of winter and spring cereals in Europe (Fig. 1.5a) (Clarke *et al.*, 2015). *Alopecurus myosuroides* has been described has the most destructive agricultural weed in Europe (Lutman *et al.*, 2013) due to its high fecundity and competitive ability in winter cereals (Maréchal & Henriet, 2012; Moss, 2017). *Alopecurus myosuroides* has complex herbicide resistance traits, with many populations in Europe having multiple resistance to up to five herbicide MOAs (Heap, 2019). The increased use of conservation tillage as well as a long term trend for earlier autumn sowing has favored the increase in *A. myosuroides* populations across Europe (Moss, 2017). In this work we use *A. myosuroides* as a 'model' to investigate the molecular mechanisms of dormancy and germination since it has a diploid genome, facilitating *de novo* transcriptome assembly (Aude *et al.*, 2015).

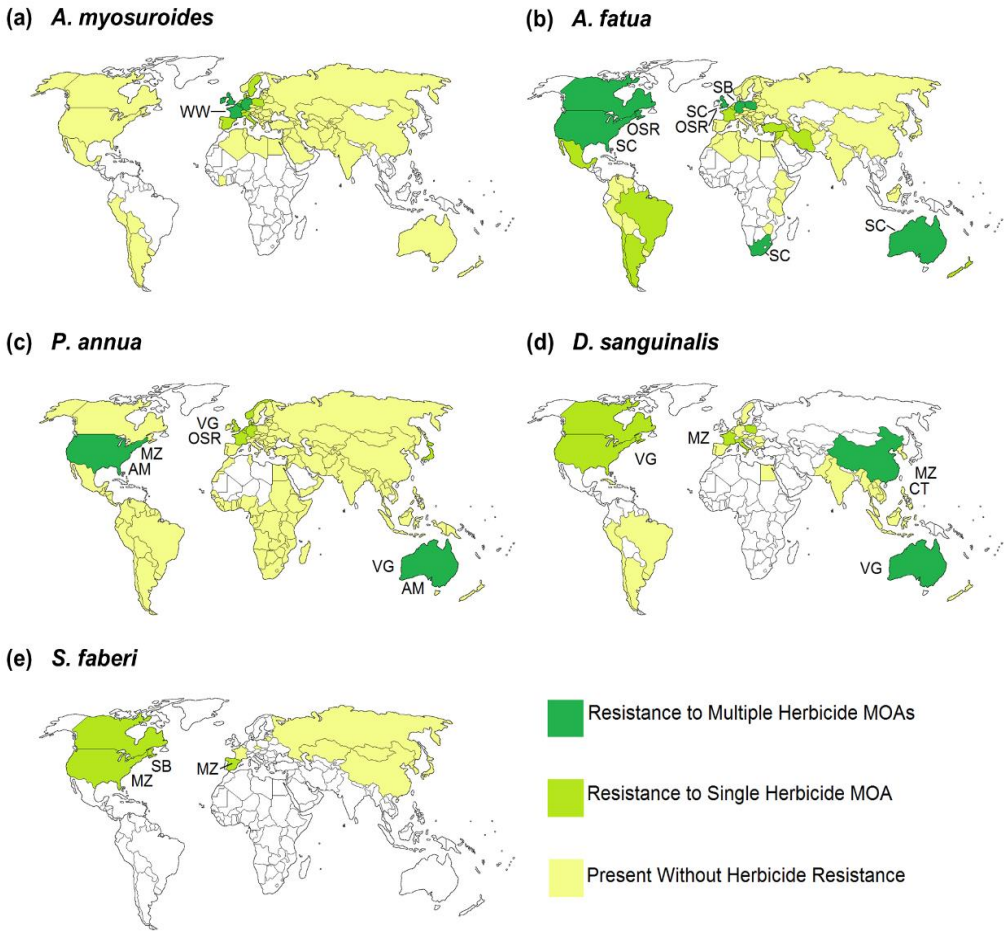


Fig. 1.5. Global patterns of herbicide resistance in selected grass weeds. Global distribution maps of weed species based on political boundaries showing affected crops. The Intensity of green colour reflects non-resistant presence of the weed, resistance to a single herbicide mode of action (MOA) or resistance to multiple herbicide MOAs. Letters represent affected crops: AM, amenity; CT, cotton; MZ, maize; OSR, oil seed rape; SC, spring cereals; SB, sugar beet; VG, vegetables; WW, winter wheat. Data were obtained from Heap (2019) and the CABI Invasive Species Compendium (CABI 2019).

Avena fatua L. (common wild oat)

Avena fatua is a native of the Central Asia and is considered to one of the oldest weeds of cultivation (Paterson & Jones, 1977) that has now become naturalised across the globe. More recently *A. fatua* has become a problematic weed of winter and spring cereals in the USA, Canada and the UK (Fig. 1.5b) (CABI, 2019). *Avena fatua* is another facultative winter annual and typically has a minor emergence peak in autumn and the major peak in spring and hence is a problem in spring cereals (Clarke et al. 2015). Like *A. myosuroides, A. fatua* has complex herbicide resistance traits with

populations in the USA and Canada having resistance to up to four herbicide MOAs (Heap, 2019). Before the genomic era, *A. fatua* was used as a model species to study dormancy in grasses (Simpson, 1990) however research into the molecular mechanisms of dormancy in this species is limited by its very large hexaploid genome (Stewart *et al.*, 2009). In this work *A. fatua* is used to understand the roles of different tissues during germination since its large embryo size facilitates dissection and tissue preparation.

***Poa annua* (UK: annual meadowgrass, USA: annual bluegrass)**

Poa annua is a highly cosmopolitan tetraploid weed that is found across the globe including in the Arctic and Antarctic (Fig. 1.5c) and is present in both annual and perennial biotypes (CABI, 2019). Individual *P. annua* plants are low growing and have a poor competitive ability against many crops, however their lifecycle can be completed within 6 weeks and they continue to flower and produce seed year round except in cold winter conditions and so *P. annua* populations can grow exponentially within a season if not controlled (Clarke *et al.*, 2015). Herbicide resistance in *P. annua* typically occurs on golf courses, where some populations have multiple resistance to five herbicide MOAs (Heap, 2019).

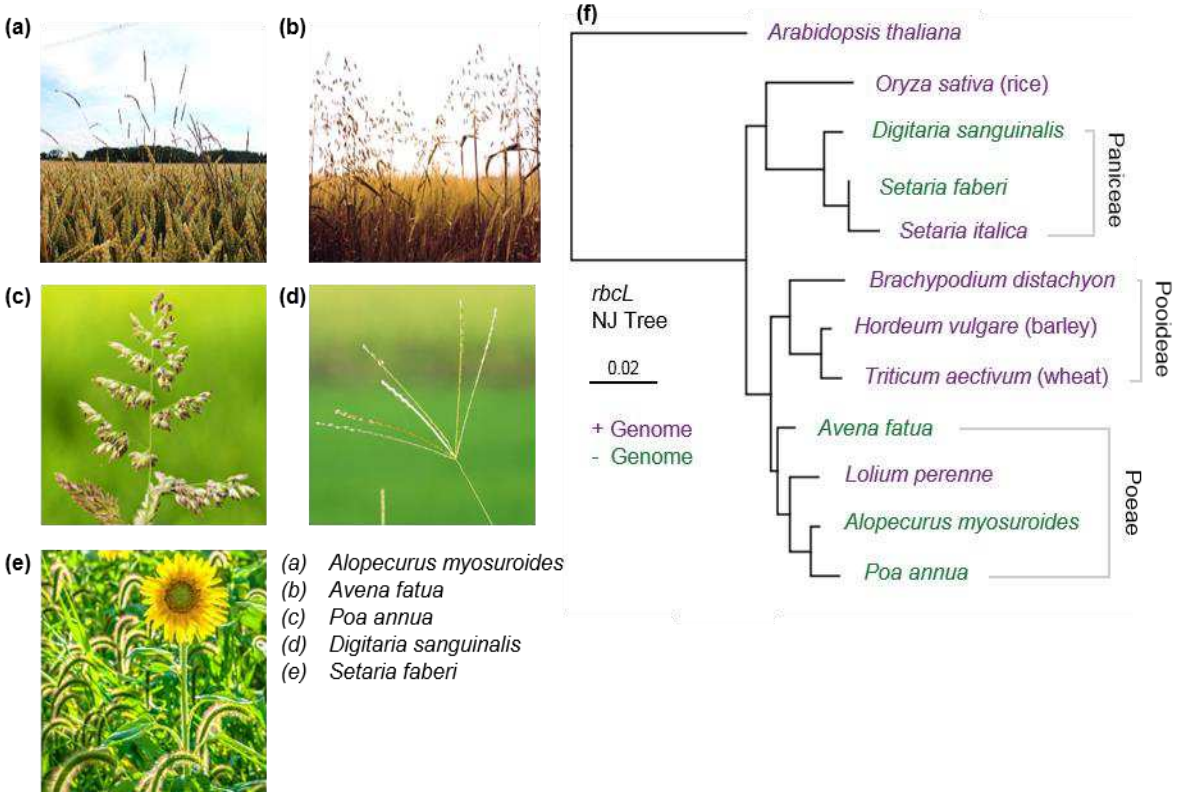


Fig. 1.6. Photographs and phylogenetic tree of selected grass weed species. **(a-e)** Photographs of grass weeds **(a)** *Alopecurus myosuroides* at seed dispersal in a wheat field, UK. **(b)** *Avena fatua* at seed dispersal in a wheat field, UK. **(c)** *Poa annua* inflorescence at flowering. **(d)** *Digitaria sanguinalis* inflorescence at flowering. **(e)** *Setaria faberi* during seed maturation in a sunflower crop, USA. Image credits: **(a)** taken by the author, **(b-e)** purchased from shutterstock.com. **(f)** Neighbor joining (NJ) tree of a MUSCLE alignment of the genomic sequence of the large subunit of RuBisCO (*rbcL*). Species with an available genome are shown in purple, and with no genome in green. Scale bar represents 0.02 substitutions per base.

Digitaria sanguinalis (large crabgrass, hairy crabgrass)

Digitaria sanguinalis is a highly competitive weed in Southern Europe and the USA (Fig. 1.5d) as a weed of maize, beets and vegetable crops that can return up to 150,000 seeds to the weed seed bank from a single plant (CABI, 2019). *D. sanguinalis* is a summer annual and emergence typically occurs during the summer months and flowering and seed dispersal occurs during the autumn and winter months (Cardina *et al.*, 2007). The oldest cases of HR in *D. sanguinalis* occur in vegetables in Australia, however more recently resistance has developed to a variety of MOAs in vegetables in the USA and Canada (Heap, 2019).

***Setaria faberi* (giant foxtail, Japanese bristlegrass)**

Setaria faberi is a native of China however it has been introduced in the USA, where it is one of the most important weeds of the maize belt and has become the subject of many studies modelling weed emergence (CABI, 2019). Like *D. sanguinalis, S. faberi* has a summer annual habit emerging in spring and setting seed during late summer and autumn (Fausey *et al.*, 1997). There are many reports of herbicide resistance in *S. faberi* to acetolactate synthase (ALS) inhibitors in the USA in maize crops (Heap, 2019).

1.5 Aims and objectives

1.5.1 Overall objective of the project

The development of chemical control strategies for the WSB are limited by a lack of understanding of the physiological and molecular mechanisms of dormancy and germination in weeds. This work aims to investigate these mechanisms under controlled conditions in a range of grass weed species with diverse life history traits in order to identify targets that could be exploited for WSB management.

1.5.2 Specific aims of the project

Aim 1: Develop an understanding of the role of imbibitional temperature on the germination of grass weed seeds with defined levels of dormancy and investigate how these patterns of germination can be explained by patterns of gene expression.

> **Objective 1a:** Develop a quantitative measure of the effect of after-ripening on dormancy release.
>
> **Objective 1b:** Perform germination kinetics using seed batches with defined dormancy levels across a range of temperatures to identify how dormancy influences the temperature window permissive to germination.
>
> **Objective 1c:** Investigate the molecular mechanisms involved in the interaction between seed dormancy, after-ripening and imbibitional temperatures in a selected species using transcriptomic approaches.

Aim 2: Using information and experimental conditions obtained from Aim 1, select and test putative germination stimulants on seed batches with defined dormancy levels.

Objective 2a: Screen diverse compounds against a selected weed species and compare the response to effective compounds across the species.

Objective 2b: Compare the response to effective compounds across seed batches with differing dormancy levels to understand the degree to which dormancy determines compound efficacy.

Objective 2c: Compare the response to effective compounds across seed batches with differing genotypes to understand the degree to which batch effects influence compound responses.

Aim 3: Investigate the hypothesis that the coleorhiza and MPE share a common function in the physical restraint of the radicle during dormancy and germination.

Objective 3a: Optimise and employ the biomechanics approaches developed for the MPE to the coleorhiza to determine if the MPE and coleorhiza share a conserved function in the mechanical restraint of radicle expansion.

Objective 3b: Identify cell wall remodeling processes that may be involved in the regulation of coleorhiza biomechanical properties by analysing publically available transcriptome datasets.

Objective 3c: Develop biochemical approaches to investigate the dynamics of identified cell wall remodeling process in distinct embryonic tissues.

1.6 Structure of the thesis

This thesis is composed of both experimental chapters in a monograph format as well as research papers that have been prepared for submission to peer-reviewed journals. Chapter 2 provides an ecophysiological characterisation of dormancy in different species and gives a description of seed batches as well as experimental conditions for further experiments. Chapter 3 describes the results from the screening of germination stimulants across different grass weed species with defined dormancy levels. Chapter 4 contains a manuscript prepared for submission to New Phytologist (Online ISSN: 1469-8137) describing the results from an RNAseq experiment in *A. myosuroides* to understand the changes in gene expression associated with dormancy enforcement and release at different temperatures aiming identify potential targets for germination stimulants. Chapter 5 contains a manuscript prepared for submission to Nature Plants (Online ISSN: 2055-0278) describing the biomechanical role of the coleorhiza in the regulation of germination in *A. fatua* and how this can be explained by the dynamics of cell wall remodeling enzymes. Chapter 6 contains a manuscript prepared for submission to Seed Science Research (Online ISSN: 1475-2735) describing how vernalisation of *A. myosuroides* plants affects the seed dormancy of their progeny. In Chapter 7 the results from both monograph and manuscript chapters are discussed as well as the future perspectives for research into germination stimulants in weeds. A detailed description of the methods employed in both monograph and manuscript chapters is provided in Chapter 8. An appendix for all Chapters is provided in Chapter 9.

1.7 References

Atwood D, Paisley-Jones C. **2017**. *EPA Report: Pesticide Industry Sales and Usage: 2008-2012 Market Estimates*. Washington D.C.

Aude J, Gardin C, Gouzy J, Carrère S, Délye C. **2015**. ALOMYbase, a resource to investigate non-target-site-based resistance to herbicides inhibiting acetolactate-synthase (ALS) in the major grass weed *Alopecurus myosuroides* (black-grass). *BMC Genomics* **16**: 1–22.

Auge GA, Blair LK, Burghardt LT, Coughlan J, Edwards B, Leverett LD, Donohue K. **2015**. Secondary dormancy dynamics depends on primary dormancy status in *Arabidopsis thaliana*. *Seed Science Research* **25**: 230–246.

Auge GA, Blair LK, Neville H, Donohue K. **2017a**. Maternal vernalization and vernalization-pathway genes influence progeny seed germination. *New Phytologist* **216**: 388-400.

Auge GA, Leverett LD, Edwards BR, Donohue K. **2017b**. Adjusting phenotypes via within- and across-generational plasticity. *New Phytologist* **216**: 343–349.

Babiker AGT, Hamdoun AM. **1982**. Factors affecting the activity of GR7 in stimulating germination of *Striga hermonthica* (Del.) Benth. *Weed Research* **22**: 111–115.

Babiker AGT, Hamdoun AM, Rudwan A, Mansi NG, Faki HH. **1987**. Influence of soil moisture on activity and persistence of the strigol analogue GR24. *Weed Research* **27**: 173–178.

Babiker AGT, Ibrahim NE, Edwards WG. **1988**. Persistence of GR7 and Striga germination stimulant(s) from *Euphorbia aegyptiaca* Boiss. in soils and in solutions. *Weed Research* **28**: 1–6.

Baker HG. **1974**. The Evolution of Weeds. *Annual Review of Ecology and Systematics* **5**: 1–24.

Baker HG. **1991**. The continuing evolution of weeds. *Economic Botany* **45**: 445–449.

Bao YZ, Yao ZQ, Cao XL, Peng JF, Xu Y, Chen MX, Zhao SF. **2017**. Transcriptome analysis of *Phelipanche aegyptiaca* seed germination mechanisms stimulated by fluridone, TIS108, and GR24. *PLoS ONE* **12**: 1–19.

Barrero JM, Talbot MJ, White RG, Jacobsen J V, Gubler F. **2009**. Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant physiology* **150**: 1006–1021.

Baskin JM, Baskin CC. **1985**. The annual dormancy cycle in buried weed seeds: a continuum. *BioScience* **35**: 492–498.

Baskin JM, Baskin CC. **2004**. A classification system for seed dormancy. *Seed Science Research* **14**: 1–16.

Baskin JM, Baskin CC. 2006. The natural history of soil seed banks of arable land. *Weed Science* **54**, 549-557.

Baskin CC, Baskin JM. **2014**. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. Oxford: Academic Press.

Beaudoin N, Serizet C, Gosti F, Giraudat J. **2007**. Interactions between abscisic acid and ethylene signaling cascades. *The Plant Cell* **12**: 1103-1115.

Bentsink L, Jowett J, Hanhart CJ, Koornneef M. **2006**. Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in Arabidopsis. *PNAS* **103**: 17042–17047.

Bewley J, Bradford K, Hilhorst H, Nonogaki H. **2013**. *Seeds - Physiology of Development, Germination and Dormancy*. London: Springer.

Bhowmik P. **1997**. Weed biology: importance to weed management. *Weed Science* **45**: 329–336.

Brooks DW, Bevinakatti HS, Powell DR. **1985**. The absolute structure of (+)-strigol. *Journal of Organic Chemistry* **50**: 3779–3781.

Buhler DD, Hartzler RG, Forcella F. **1997**. Weed seed bank dynamics. *Journal of Crop Production* **1:1**, 145-168.

Busari MA, Kukal SS, Kaur A, Bhatt R, Dulazi AA. **2015**. Conservation tillage impacts on soil, crop and the environment. *International Soil and Water Conservation Research* **3**: 119–129.

CABI. **2019**. Invasive Species Compendium. URL: www.cabi.org/isc/ (Last Accessed 01.10.2019)

Cadman CSC, Toorop PE, Hilhorst HWM, Finch-Savage WE. **2006**. Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant Journal* **46**: 805–822.

Cao D, Xu H, Zhao Y, Deng X, Liu Y, Soppe WJJ, Lin J. **2016**. Transcriptome and degradome sequencing reveals dormancy mechanisms of *Cunninghamia lanceolata* seeds . *Plant Physiology* **172**: 2347–2362.

Cardina J, Herms CP, Herms DA, Forcella F. **2007**. Evaluating phenological indicators for predicting giant foxtail (*Setaria faberi*) emergence. *Weed Science* **55**: 455–464.

Chauvel B, Guillemin J, Gasquez J, Gauvrit C. **2012**. History of chemical weeding from 1944 to 2011 in France: Changes and evolution of herbicide molecules. *Crop Protection* **42**: 320–326.

Chen F, Bradford KJ. **2000**. Expression of an expansin is associated with endosperm weakening during tomato seed germination. *Plant Physiology* **124**: 1265–1274.

Chen F, Nonogaki H, Bradford KJ. **2002**. A gibberellin-regulated xyloglucan endotransglycosylase gene is expressed in the endosperm cap during tomato seed

germination. *Journal of Experimental Botany* **53**: 215–223.

Clarke J, Ginsburg D, Clare K, Tonguc L. **2015**. *The Encyclopaedia of Arable Weeds*. Kenilworth, UK: AHDB.

Clements DR, Benott DL, Muephy D, Swanton CJ. **1996**. Tillage effects on weed seed return and seedbank composition. *Weed Science* **44**: 314–322.

Cohen D. **1966**. Optimizing reproduction in a randomly varying environment. *Journal of Theoretical Biology* **12**: 119–129.

Cohn MA. **1996**. Operational and philosophical decisions in seed dormancy research. *Seed Science Research* **6**: 147–154.

Cook C, Whichard L, Turner B, Wall M. **1966**. Germination of witchweed (*Striga lutea* Lour.): isolation and properties of a potent stimulant. *Science* **154**: 4–6.

Cook CE, Whichard LP, Wall ME, Egley GH, Coggon P, Luhan PA, Mcphail AT. **1972**. Germination stimulants. ii. the structure of strigol: A potent seed germination stimulant for witchweed (*Striga lutea* Lour.). *Journal of the American Chemical Society* **94**: 6198–6199.

Davis AS, Frisvold GB. **2017**. Are herbicides a once in a century method of weed control? *Pest Management Science* **73**: 2209–2220.

Debaene-Gill SB, Allen PS, Gardner JS. **1994**. Morphology of the perennial ryegrass (*Lolium perenne*; Poaceae) coleorhiza and emerging radicle with continuous or discontinuous hydration. *American journal of botany* **81**: 739–744.

Délye C, Duhoux A, Pernin F, Riggins CW, Tranel PJ. **2015**. Molecular Mechanisms of Herbicide Resistance. *Weed Science* **63**: 91–115.

Donald WW, Tanaka FS. **1993**. The germination stimulant AC94377 reduces seed survival of wild mustard (*Sinapis arvensis*). *Weed Science* **41**: 185–193.

Donohue K, Heschel MS, Chiang GCK, Butler CM, Barua D. **2007**. Phytochrome mediates germination responses to multiple seasonal cues. *Plant, Cell and Environment* **30**: 202–212.

Duke SO. **2012**. Why have no new herbicide modes of action appeared in recent years? *Pest Management Science* **68**: 505–512.

Dyer WE. **1995**. Exploiting weed seed dormancy and germination requirements through agronomic practices. *Weed Science* **43**: 498–503.

Eplee RE. **1975**. Ethylene: a witchweed seed germination stimulant. *Weed Science* **23**: 433–436.

Fausey JC, Renner KA, 1997. Germination, emergence, and growth of giant foxtail (*Setaria faberi*) and fall panicum (*Panicum dichotomiflorum*). *Weed Science* **45**: 423–425.

Finch-Savage WE, Leubner-Metzger G. **2006**. Seed dormancy and the control of germination. *New Phytologist* **171**: 501–523.

Forcella F, Lindstrom MJ. **1988**. Weed seed populations in ridge and conventional tillage. *Weed Science* **36**: 500–503.

Fujii H, Verslues PE, Zhu JK. **2007**. Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. *Plant Cell* **19**: 485–494.

Goggin DE, Powles SB. **2014**. Fluridone: A combination germination stimulant and herbicide for problem fields? *Pest Management Science* **70**: 1418–1424.

González-Calle V, Barrero-Sicilia C, Carbonero P, Iglesias-Fernández R. **2015**. Mannans and endo-β-mannanases (MAN) in *Brachypodium distachyon*: Expression profiling and possible role of the *BdMAN* genes during coleorhiza-limited seed germination. *Journal of Experimental Botany* **66**: 3753–3764.

Graeber K, Linkies A, Müller K, Wunchova A, Rott A, Leubner-Metzger G. **2010**. Cross-species approaches to seed dormancy and germination: Conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae *DOG1* genes. *Plant Molecular Biology* **73**: 67–87.

Grundy AC. **2003**. Predicting weed emergence: A review of approaches and future challenges. *Weed Research* **43**: 1–11.

Harker KN, O'Donovan JT. **2013**. Recent weed control, weed management, and integrated weed management. *Weed Technology* **27**: 1–11.

Heap I. **2019**. International Survey of Herbicide Resistance. URL: www.weedscience.org (Last Accessed 02.10.2019.)

Hilhorst HWM. **1995**. A critical update on seed dormancy. I. Primary dormancy. *Seed Science Research* **5**: 61–73.

Holdsworth MJ, Bentsink L, Soppe WJJ. **2008**. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179**: 33–54.

Honek A, Martinkova Z, Jarosik V. **1999**. Annual cycles of germinability and differences between primary and secondary dormancy in buried seeds of *Echinochloa crus-galli*. *Weed Research* **39**: 69–79.

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM. **1982**. The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) heynh. *Theoretical and Applied Genetics* **61**: 385–393.

Koornneef M, Reuling G, Karssen CM. **1984**. The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiologia Plantarum* **61**: 377–383.

Lee KP, Piskurewicz U, Turečková V, Carat S, Chappuis R, Strnad M, Fankhauser C, Lopez-Molina L. **2012**. Spatially and genetically distinct control of seed germination by phytochromes A and B. *Genes and Development* **26**: 1984–1996.

Lee KP, Piskurewicz U, Turečková V, Strnad M, Lopez-Molina L. **2010**. A seed coat bedding assay shows that RGL2-dependent release of abscisic acid by the endosperm controls embryo growth in Arabidopsis dormant seeds. *PNAS* **107**: 19108–19113.

Leubner-Metzger G. **2003**. Functions and regulation of β-1,3-glucanases during seed germination, dormancy release and after-ripening. *Seed Science Research* **13**: 17–34.

Li B, Foley ME. **1997**. Genetic and molecular control of seed dormancy. *Trends in Plant Science* **2**: 384–389.

Li C, Nonogaki H, Barrero J (Eds.). **2019**. *Seed Dormancy, Germination and Pre-Harvest Sprouting*. Lausanne, Switzerland: Frontiers Media.

Linkies A, Graeber K, Knight C, Leubner-Metzger G. **2010**. The evolution of seeds. *New Phytologist* **186**: 817–831.

Linkies A, Leubner-Metzger G. **2012**. Beyond gibberellins and abscisic acid: How ethylene and jasmonates control seed germination. *Plant Cell Reports* **31**: 253–270.

Linkies A, Mu K, Morris K, Turec V, Strnad M, Lynn JR, Finch-savage WE. **2009**. Ethylene interacts with abscisic acid to regulate endosperm rupture during germination : a comparative approach using *Lepidium sativum* and *Arabidopsis thaliana*. *The Plant Cell* **21**: 3803–3822.

Long RL, Gorecki MJ, Renton M, Scott JK, Colville L, Goggin DE, Commander LE, Westcott DA, Cherry H, Finch-Savage WE. **2014**. The ecophysiology of seed persistence: A mechanistic view of the journey to germination or demise. *Biological Reviews* **90**: 31-59.

Lutman PJW, Moss SR, Cook S, Welham SJ. **2013**. A review of the effects of crop

agronomy on the management of *Alopecurus myosuroides*. *Weed Research* **53**: 299–313.

Maréchal P, Henriet F. **2012**. Ecological review of black-grass (*Alopecurus myosuroides* Huds.) propagation abilities in relationship with herbicide resistance. *Biotechnology, Agronomy, Society and Environment* **16**: 103–113.

Martínez-Andújar C, Pluskota WE, Bassel GW, Asahina M, Pupel P, Nguyen TT, Takeda-Kamiya N, Toubiana D, Bai B, Gõrecki RJ, *et al.* 2012. Mechanisms of hormonal regulation of endosperm cap-specific gene expression in tomato seeds. *Plant Journal* **71**: 575–586.

McCarty DR. **1995**. Genetic control and integration of maturation and germination pathways in seed development. *Annual reviews in Plant Physiology* **46**: 71–93.

Millar AA, Jacobsen J V., Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F. **2006**. Seed dormancy and ABA metabolism in Arabidopsis and barley: The role of ABA 8'-hydroxylase. *Plant Journal* **45**: 942–954.

Moss S. **2017**. Black-grass (*Alopecurus myosuroides*): Why has this weed become such a problem in western europe and what are the solutions? *Outlooks on Pest Management*.

Muller K, Linkies A, Vreeburg RAM, Fry SC, Krieger-Liszkay A, Leubner-Metzger G. **2009**. *In vivo* cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. *Plant Physiology* **150**: 1855–1865.

Müller K, Tintelnot S, Leubner-Metzger G. **2006**. Endosperm-limited Brassicaceae seed germination: Abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant and Cell Physiology* **47**: 864–877.

Nakabayashi K, Bartsch M, Xiang Y, Miatton E, Pellengahr S, Yano R, Seo M,

Soppe WJJ. **2012**. The time required for dormancy release in Arabidopsis is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. *Plant Cell* **24**: 2826–2838.

Nakabayashi K, Graeber K, Leubner-Metzger G. **2017**. Genetics of dormancy. *Encyclopedia of Applied Plant Sciences* **1**: 504–508.

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E. **2005**. Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: Epigenetic and genetic regulation of transcription in seed. *Plant Journal* **41**: 697–709.

Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, Kamiya Y. **2010**. Abscisic acid and the control of seed dormancy and germination. *Seed Science Research* **20**: 55–67.

Nelson SK, Ariizumi T, Steber CM. **2017**. Biology in the dry seed: transcriptome changes associated with dry seed dormancy and dormancy loss in the arabidopsis GA-insensitive *sleepy1-2* mutant. *Frontiers in Plant Science* **8**: 1–21.

Neve P, Vila-Aiub M, Roux, F. 2009. Evolutionary-thinking in agricultural weed management. *New Phytologist* **184(4)**: 783-793.

NFU. **2015**. *The impact of losing plant protection products on UK food and plant production*. Stoneleigh, Warwickshire, UK.

Oerke EC. **2006**. Crop losses to pests. *Journal of Agricultural Science* **144**: 31–43.

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S. **2003**. Gibberellin biosynthesis and response during Arabidopsis seed germination. *The Plant Cell* **15**: 1591–1604.

Okamoto M, Kuwahara A, Seo M, Kushiro T, Asami T, Hirai N. **2006**. *CYP707A1* and *CYP707A2*, which encode abscisic acid 8'-hydroxylases, are indispensable for proper control of seed dormancy and germination in Arabidopsis. *Plant Physiology* **141**:

97–107.

Olszewski N, Sun TP, Gubler F. **2002**. Gibberellin signaling: Biosynthesis, catabolism, and response pathways. *Plant Cell* **14**: 61–80.

Owen MJ, Martinez NJ, Powles SB. **2014**. Multiple herbicide-resistant *Lolium rigidum* (annual ryegrass) now dominates across the Western Australian grain belt. *Weed Research* **54**: 314–324.

Papenfus HB, Kulkarni MG, Posta M, Finnie JF, Van Staden J. **2015**. Smoke-isolated trimethylbutenolide inhibits seed germination of different weed species by reducing amylase activity. *Weed Science* **63**: 312–320.

Parker C. **1991**. Protection of crops against parasitic weeds. *Crop Protection* **10**: 6–22.

Paterson JG, Jones DP. **1977**. Wild oats in world agriculture. *The Journal of Applied Ecology* **14**: 994-995.

Penfield S, MacGregor DR. **2017**. Effects of environmental variation during seed production on seed dormancy and germination. *Journal of Experimental Botany* **68**: 1–7.

Penfield S, Springthorpe V. **2012**. Understanding chilling responses in Arabidopsis seeds and their contribution to life history. *Philosophical Transactions of the Royal Society* **367**: 291–297.

Peng *et al.* 1999. 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400**: 256-261.

Peterson MA, Collavo A, Ovejero R, Shivrain V, Walsh MJ. **2018**. The challenge of herbicide resistance around the world: a current summary. *Pest Management Science* **74**: 2246–2259.

Römer U, Schaak H, Mußhoff O. **2019**. Pre-Print: The perception of crop protection: Explicit vs. implicit association of the public and in agriculture. *Journal of Environmental*

Psychology.

Ross JJ. **1994**. Recent advances in the study of gibberellin mutants. *Plant Growth Regulation* **15**: 193–206.

Sargent JA, Mandi S Sen, Osborne DJ. **1981**. The loss of desiccation tolerance during germination: An ultrastructural and biochemical approach. *Protoplasma* **105**: 225–239.

Sargent JA, Osborne DJ. **1980**. A comparative study of the fine structure of coleorhiza and root cels during the early hours of germination of rye embryos. *Protoplasma* **104**: 91–103.

Sato K, Yamane M, Yamaji N, Kanamori H, Tagiri A, Schwerdt JG, Fincher GB, Matsumoto T, Takeda K, Komatsuda T. **2016**. Alanine aminotransferase controls seed dormancy in barley. *Nature Communications* **7**: 1-9.

Schwartz-Lazaro LM, Copes JT. **2019**. A review of the soil seedbank from a weed scientists perspective. *Agronomy* **9**: 1–13.

Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR. **1997**. Specific oxidative cleavage of carotenoids by VP14 of maize. *Science* **276**: 1872–1874.

Scopel AL, Ballare CL, Radosevich SR. **1994**. Photostimulation of seed germination during soil tillage. *New Phytologist* **126**: 145–152.

Simpson G. **1990**. *Seed Dormancy in Grasses*. Cambridge: Cambridge University Press.

Steinbrecher T, Leubner-Metzger G. **2017**. The biomechanics of seed germination. *Journal of Experimental Botany* **68**: 765–783.

Stewart CN, Tranel PJ, Horvath DP, Anderson J V., Rieseberg LH, Westwood JH, Mallory-Smith CA, Zapiola ML, Dlugosch KM. **2009**. Evolution of weediness and invasiveness: Charting the course for weed genomics. *Weed Science* **57**: 451–462.

Swanton CJ, Weise SF. **1991**. Integrated weed management: The rationale and approach. *Weed Technology* **5**: 657–663.

Telewski FW, Zeevaart JAD. **2002**. The 120-yr period for Dr. Beal's seed viability experiment. *American Journal of Botany* **89**: 1285–1288.

Thuring JWJF, Bitter HH, De Kok MM, Nefkens GHL, Van Riel AMDA, Zwanenburg B. **1997**. N-phthaloylglycine-derived strigol analogues. Influence of the D-ring on seed germination activity of the parasitic weeds *Striga hermonthica* and *Orobanche crenata*. *Journal of Agricultural and Food Chemistry* **45**: 2284–2290.

Ueguchi-Tanaka M, Ashikari M, Nakajima M, Itoh H, Katoh E, Kobayashi M, Chow TY, Hsing YIC, Kitano H, Yamaguchi I, *et al.* 2005. *GIBBERELLIN INSENSITIVE DWARF1* encodes a soluble receptor for gibberellin. *Nature* **437**: 693–698.

Vayda K, Donohue K, Auge GA. **2018**. Within- and trans-generational plasticity: Seed germination responses to light quantity and quality. *AoB PLANTS* **10**: 1-17.

Voegele A, Linkies A, Müller K, Leubner-Metzger G. **2011**. Members of the gibberellin receptor gene family *GID1* (*GIBBERELLIN INSENSITIVE DWARF1*) play distinct roles during *Lepidium sativum* and *Arabidopsis thaliana* seed germination. *Journal of Experimental Botany* **62**: 5131–5147.

Wang L, Wang HL, Yin L, Tian CY. **2017**. Transcriptome assembly in *Suaeda aralocaspica* to reveal the distinct temporal gene/miRNA alterations between the dimorphic seeds during germination. *BMC Genomics* **18**: 1–21.

Wesson G, Wareing PF. **1969**. The induction of light sensitivity in weed seeds by burial. *Journal of Experimental Botany* **20**: 414–425.

Westwood JH, Charudattan R, Duke SO, Fennimore SA, Marrone P, Slaughter DC, Swanton C, Zollinger R. **2018**. Weed management in 2050: Perspectives on the future of weed science. *Weed Science* **66**: 275–285.

Wilhelmsson PKI, Chandler JO, Fernandez-Pozo N, Graeber K, Ullrich KK, Arshad W, Khan S, Hofberger JA, Buchta K, Edger PP, *et al.* 2019. Usability of reference-free transcriptome assemblies for detection of differential expression: A case study on *Aethionema arabicum* dimorphic seeds. *BMC Genomics* **20**: 1-19.

Xu Y, Zhao Y, Duan H, Sui N, Yuan F, Song J. **2017**. Transcriptomic profiling of genes in matured dimorphic seeds of euhalophyte *Suaeda salsa*. *BMC Genomics* **18**: 1–14.

Yamaguchi S. **2008**. Gibberellin metabolism and its regulation. *Annual Review of Plant Biology* **59**: 225–251.

Yang SF, Hoffman NE. **1984**. Ethylene biosynthesis and its regulation in higher plants. *Annual Reviews in Plant Physiology* **35**: 165–175.

Yenish JP, Doll JD, Buhler DD. **1992**. Effects of tillage on vertical distribution and viability of weed seed in soil. *Weed Science* **40**: 429–433.

Zimdahl R. **1988**. The concept and application of the critical weed-free period. In: Altieri MA, Liebman M, eds. Weed Management in Agroecosystems: Ecological Approaches. Florida: CRC Press, 145–155.

Zimdahl R. **2018**. *Fundamentals of Weed Science*. Colorado: Academic Press.

Zwanenburg B, Mwakaboko AS, Kannan C. **2016**. Suicidal germination for parasitic weed control. *Pest management science* **72**: 2016–202

2. An Ecophysiological Approach to Understanding Grass Weed Seed Dormancy and Germination

2.1 Abstract

Germination represents the most important 'decision' in the lifecycle of agricultural weeds. By synchronising their emergence with crop establishment, annual weeds take advantage of a 'competitive window' to maximise their fitness. Dormant weed seeds perceive seasonal changes in environmental conditions in order to determine the optimal timing for germination. In this chapter the relationship between imbibitional temperature and seed dormancy is investigated. Controlled after-ripening is used as a method to generate seed batches with defined dormancy levels. Dormant grass weed seeds are limited in the range of temperatures in which they germinate. As dormancy is released by controlled after-ripening, this range of temperatures permissive to germination widens. In winter annual weeds (e.g. *Alopecurus myosuroides* and *Avena fatua*) dormancy restricts germination to cooler temperatures and the loss of dormancy permits germination at warmer temperatures. In summer annuals (e.g. *Digitaria sanguinalis* and *Setaria faberi*) dormancy performs the opposite role, limiting germination to warmer temperatures and dormancy release permits germination at cooler temperatures. Data for the response of dormant seed batches to a gradient of temperatures is used to calculate quantitative parameters ('cardinal temperatures') to describe this effect. When compared to example soil temperature and emergence data, these cardinal temperatures predicted seasonal emergence patterns in some species, demonstrating the applicability of this approach for informing weed emergence models. Finally, this chapter provides a physiological basis for further work investigating the mechanisms of dormancy in grass weed seeds.

2.2 Introduction

2.2.1 Ecology and classification of weed seed dormancy

Seed dormancy is the single most important factor determining weed emergence in the field (Grundy, 2003). This dormancy can be imposed by physical restraint of the growth of the embryo by thick covering layers (physical dormancy, PY), by the presence of an immature embryo that has to develop before germination can occur (morphological dormancy, MD) or be regulated through interaction with the environment (physiological dormancy, PD) with dormancy in different species having different contributions from each of these components (Baskin & Baskin, 2004). Whilst PY and MD dormancy types are essentially set at the point of seed dispersal, PD seeds exist in a 'dormancy continuum' where dormancy level cycles across the season depending on environmental conditions (Baskin & Baskin, 1985). Much of our understanding of this dormancy cycling comes from the study of the changes in the germinability of weed seeds, that typically show PD, exhumed from arable fields across the year (Baskin & Baskin, 1985, 2014). Temperature plays a fundamental role in the regulation of dormancy cycling since treatments that release or enforce dormancy widen or restrict the range of temperatures under-which dormant seeds will germinate in many PD species (Vegis, 1963, 1964, Baskin & Baskin, 1985, 2014).

There are many systems for the classification of seed dormancy (reviewed in Baskin & Baskin, 2014) however for the purposes of this chapter the system proposed by Baskin & Baskin (2004) is used because it provides a robust description of the role of temperature in the regulation of PD (Fig. 2.1). This system divides PD into three types depending on the strength of treatment required to break dormancy. Seeds with 'deep' PD to not respond to gibberellin application and require long periods of cold stratification to germinate whereas in seeds with 'non-deep' PD gibberellin application and periods of dry storage (after-ripening) promote germination. In an 'intermediate'

type seeds respond when a combination of both of these treatments are applied (Baskin & Baskin, 2004). Agricultural weed seeds typically fall into the 'non-deep PD' category, potentially allowing them to respond rapidly to changes in environmental conditions (Baskin & Baskin, 2014).

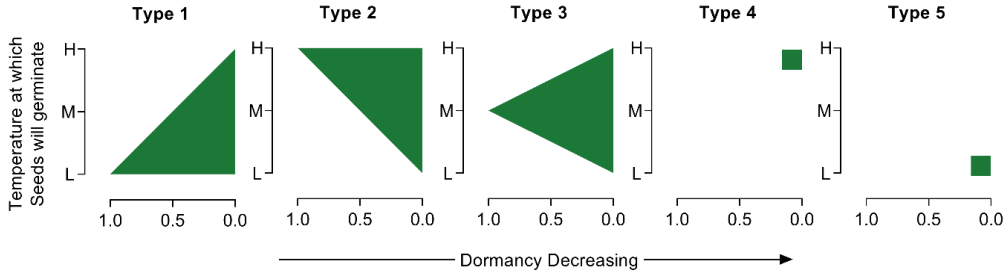


Fig. 2.1. Types of non-deep physiological dormancy (PD). At high dormancy levels germination is restricted to a narrow range of temperatures, and as dormancy is released the range of temperatures under which a non-deep PD seed will germinate widens. Types 1-3 non-deep PD show a continuum of change in temperature ranges, where as in types 4 & 5 dormancy release results in germination only at specific high (H) or low (L) temperatures. Figure modified from Baskin & Baskin (2004).

During the loss of dormancy in 'non-deep' PD seeds, the range of temperatures under which a seed population will germinate increases until there is an overlap between this 'temperature window' and the ambient conditions in the soil seedbank, at which point germination occurs (Finch-Savage & Leubner-Metzger, 2006). In climates characterised by a seasonal variation in temperature, the direction in which this temperature window opens typically reflects the lifecycle of the plant (Baskin & Baskin, 2014). For example in obligate winter annual ecotypes of Arabidopsis that emerge in autumn, dormancy of exhumed seeds is released by cold temperatures (Baskin & Baskin, 1983) whereas comparable studies on summer annual weed species, such as *Chenopodium album*, show a germination preference for warm temperatures in the dormant state (Baskin & Baskin, 1987). Through the interaction of dormancy and environmental signals such as temperature, weed seeds determine the optimal timing

Table 2.1. Summary of weed lifecycle, dormancy and germination preferences. Life Cycle: WA, winter annual; SA, summer annual. Dormancy (based on classification system from Baskin & Baskin (2004): CD, conditionally dormant; ND, non-dormant; PD, physiological dormancy; PY, physical dormancy. Light: L, light; D, dark; where inequalities represent preference for either condition. Temperature range: T_b, base temperature; T_o, optimum temperature; T_c, ceiling (maximum) temperature.

Species	Life Cycle	Dormancy	Light	Temperature Range (°C) T_b	T_o	T_c	References
Alopecurus myosuroides	WA[1,2]	PD[4] PY[3,4] CD[1] ND[1]	L>D[4]	3[3] 0[4]	26[3] 23[4]	 32[4]	[1](Kazinczi & Hunyadi 1992) [2](Wellington & Hitchins, 1966) [3](Colbach *et al.*, 2002b,a) [4](Holloway, 2016)
Avena fatua	WA[a]	PD[1,3] ND[b] PY[b]	L>D[2]	 4[4]	16[3] 12[4] 22[a]	 24[4] 30[a]	[1](Fennimore *et al.*, 1998) [2](Boyd & Van Acker, 2004) [3](Myers, 1997) [4](Sawhney, 1989)
Digitaria sanguinalis	SA[1]	CD[1] ND[b] PY[2] PD[3,4]	L>D[b]		35[b] 30[2]		[1](Masin *et al.*, 2006) [2](Gallart *et al.*, 2008) [3](Miller *et al.*, 1965) [4](Gianfagna, 1951)
Poa annua	WA[b]	PD[1]	L[2]	<5[1]	10[1]	25[1]	[1](Standifer & Wilson, 1988) [2](Ohadi *et al.*, 2010)
Setaria faberi	SA[2]	PD[b,4]	-	<7[1] <0[3]	20[1] 24[3]	>30[1]	[1](Fausey & Renner, 1997) [2](Dekker, 2003) [3](Leon *et al.*, 2004) [4](Stanway, 2016)

[a](Simpson, 1990), [b](Baskin & Baskin, 2014)

for germination in order to maximize their chances of seedling survival and gain a competitive advantage over the crop (Zimdahl, 1988, 2018; Grundy, 2003).

2.2.2 Dormancy can predict weed emergence patterns

An understanding of the interaction between seed dormancy and temperature is therefore important for agronomists attempting to model weed emergence in the field. An ability to predict both the level of seed dormancy and weed emergence patterns have important applications in optimising the timing of weed management strategies

such as tillage and herbicide application (Dyer, 1995). Classical weed emergence models rely on empirical measurements of emergence in the field in comparison to meteorological data (e.g. Roberts & Feast, 1970; Stoller & Wax, 1973; Roberts & Potter, 1980). However data collection for these methods is highly resource intensive and so these models are largely descriptive. More recent emergence models consist of sub-models that integrate separate elements contributing to emergence; such as dormancy, germination and pre-emergence growth, to give quantitative emergence predictions (e.g. Vleeshouwers & Kropff, 2000). These models are parameterised using data generated under laboratory conditions and validated against field emergence patterns. A key limitation of this approach is the quality of input parameters that are typically derived from the literature. In many cases, predictions about seed dormancy are made based on data generated on seed batches with unknown dormancy levels across a limited number of conditions due to a lack of available data (Colbach *et al.*, 2006a,b).

Within the study species in this thesis, there is disparity across the literature with respect to the type of dormancy expressed by different species (Table 2.1). There is also considerable variation in the cardinal temperatures (Bradford, 2002) permissive to germination (T_b, base temperature; T_o, optimum temperature; T_c, ceiling maximum temperature) that are used to parameterise emergence models (Table 2.1). Whilst it is likely that genotype plays a role in these parameters, differences in dormancy levels between batches is likely to be a predominant factor determining these differences due to role of dormancy in determining the temperature window permissive to germination. A better understanding of the physiological responses of weed seeds to temperature will also support research into both the molecular mechanisms of weed seed dormancy and germination stimulants by providing appropriate incubation temperatures for compound screening and molecular analysis.

2.2.3 Using after-ripening to generate seed batches with defined dormancy levels

In order to develop an understanding of how dormancy influences the temperature responses of weed seeds it would be advantageous to be able to compare the changes in temperatures permissive to germination in seed batches as dormancy levels are reduced. Controlled after-ripening is a useful method to achieve this because the dormancy of large seed batches can be released in a controlled and predictable manner and batches can be stored for further analysis. Under natural conditions, after-ripening occurs in seeds of winter annuals that disperse their seeds during the summer when soil moisture is too low to support germination (Bewley *et al.*, 2013). Under this dry condition (<20% seed moisture content) it is generally accepted that enzyme-dependant processes including transcription and translation are inactive (Bewley *et al.*, 2013). It has therefore been proposed that after-ripening occurs though spontaneous biochemical modifications involving reactive oxygen species (ROS), such as oxidation and carbonylation, to the pool of stored mRNA and protein present in dry seeds after dispersal (Bailly *et al.*, 2008; El-Maarouf-Bouteau & Bailly, 2008; Bazin *et al.*, 2011b,a; Nelson *et al.*, 2017).

The rate of after-ripening in model species is primarily dependent on temperature and seed moisture content (Bazin *et al.*, 2011a), where after-ripening occurs more rapidly at intermediate moisture contents and is inhibited at moisture contents greater than 70% (w/w) (Bewley *et al.*, 2013). Conversely the rate of after-ripening can be greatly reduced when seeds are stored dry at freezing temperatures, allowing for the preservation of dormancy levels of seed batches generated by controlled after-ripening. This approach permits the production of seed batches with defined dormancy levels, where the same batch can reliably be used for multiple experiments over time.

2.2.4 Aims and objectives

In this chapter, the relationship between primary dormancy and temperature is investigated across five grass weed species with differing life history traits. We aim to generate robust temperature responses that can better inform weed emergence models and further analysis of the mechanisms of dormancy in weed species. Controlled after-ripening is explored as a method to generate weed seed batches with defined dormancy levels. The responses of these seed batches to imbibition across a temperature gradient demonstrates the role of dormancy in setting the permissive temperatures for germination in grass weeds. Calculated cardinal temperatures (T_b, T_o & T_c) for weed seed batches with differing levels of dormancy are compared to example soil temperature and weed emergence data to demonstrate the usefulness of these parameters in predicting patterns of weed emergence in the field.

2.3 Results & Discussion

2.3.1 Morphophysiological characterisation of the germination process in grass weeds

The dispersal unit (diaspore) of the species investigated consists of a single caryopsis (grain) held within maternally derived tissues such as glumes (lemma or palea), in contrast to grains of free-threshing cereal crops such as wheat or rye, that lack these structures at dispersal (Hubbard, 1968). These covering layers provide multiple barriers to water and oxygen that can enforce dormancy through reducing uptake into the caryopsis (Simpson, 1990). Additionally they have a role in seed persistence through the release of anti-microbial compounds (Raviv *et al.*, 2018) and can aid the dispersal of diaspores (Peart, 1984). In the five weed species tested the caryopsis was retained within these covering layers during the germination process.

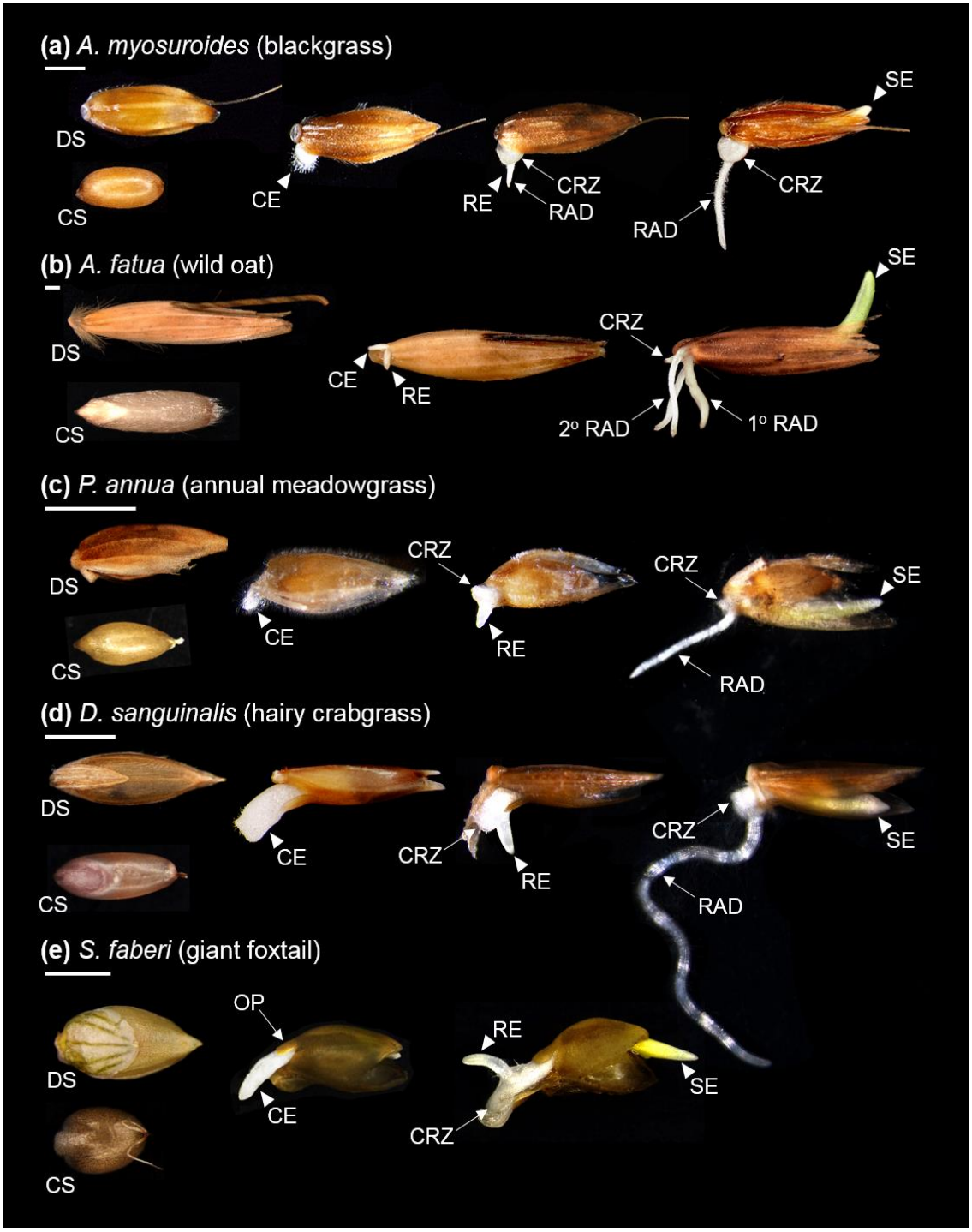


Fig. 2.2. Visible events during the germination of grass weed seeds. CE, coleorhiza emergence; CRZ, coleorhiza; CS, caryopsis; DS, diaspore; OP, operculum; RAD, radicle (1º, primary radicle; 2º secondary radicles); RE, radicle emergence; SE; shoot emergence. Scale bar represents 2 mm. Additional seedling images in Appendix Fig. 9.6.

In order to be able to quantitatively compare the germination of different grass weed seeds a standard measure for the initiation and completion of germination was required. There was much diversity in the morphology of germinating grasses, particularly in the relative size of the coleorhiza; an embryonic tissue that covers the radicle. For example the coleorhiza of *A. fatua* (Fig. 2.2b) was small when compared with the length of the caryopsis (1/10 of the length) however in other species, such as *D. sanguinalis* (Fig. 2.2d), the coleorhiza expanded to nearly the length of the caryopsis. In *D. sanguinalis* and *S. faberi* an operculum (analogous to a flap or lid) was present on the lemma that allowed the large coleorhiza to emerge from this ridged structure (Fig. 2.2e).

Irrespective of this morphological diversity, the emergence of the coleorhiza from the margins of the lemma and palea was the first visible sign that germination was occurring across all the grass weeds tested therefore coleorhiza emergence was chosen to distinguish between germinated and non-germinated diaspores across all the experiments in this chapter. Coleorhiza emergence was typically followed by the rupture of the coleorhiza by a single primary radicle, with the exception of *A. fatua*, where secondary radicles ruptured the lateral sides of the coleorhiza. Radicle emergence was typically followed by the emergence of the shoot (plumule surrounded by the coleoptile) with the exception of *S. faberi* (Fig. 2.2e), where radicle and shoot emergence occurred simultaneously.

In order to investigate the roles of different tissues during the germination process we chose to work with *A. fatua* due to its large caryopsis size (Fig. 2.2b) that facilitates dissection, microscopy and tissue preparation for further analysis. A tissue embedding protocol was optimised to address the problem of poor infiltration of embedding media into the starchy caryopses (Chapter 8.3). After-ripened *A. fatua* caryopses were imbibed for varying lengths of time and embedded, sectioned and stained according to this protocol. After 24 hours of imbibition, the cells of the embryo have become fully

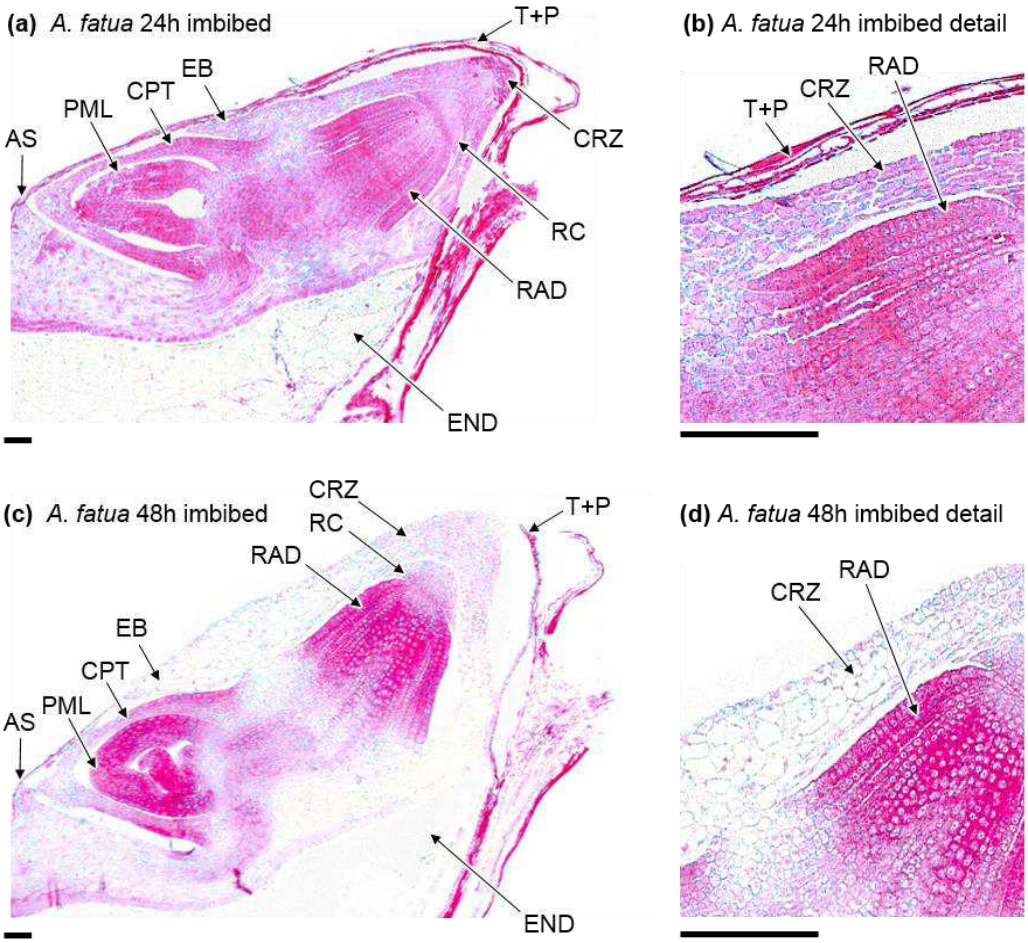


Fig. 2.3. Changes in embryo morphology during early germination of *A. fatua*. Micrographs of after-ripened *A. fatua* embryos at 24 (a,b) or 48 (c,d) hours after imbibition. Micrographs show 5 µm lateral sections stained with safranin and methylene blue to stain nuclei blue and cell walls pink. Scale bar represents 200 µm. AS, adaxial scale; CPT, coleoptile; CRZ, coleorhiza; EB, epiblast; END, endosperm; PML, plumule; RAD, radicle; RC, root cap; T+P, testa and pericarp.

hydrated and surrounded by the testa and pericarp (Fig. 2.3a,b). After 48 hours of imbibition, a localised expansion of cells in the lateral sides of the coleorhiza push the densely cellularised tip of the coleorhiza out, rupturing the testa and pericarp.

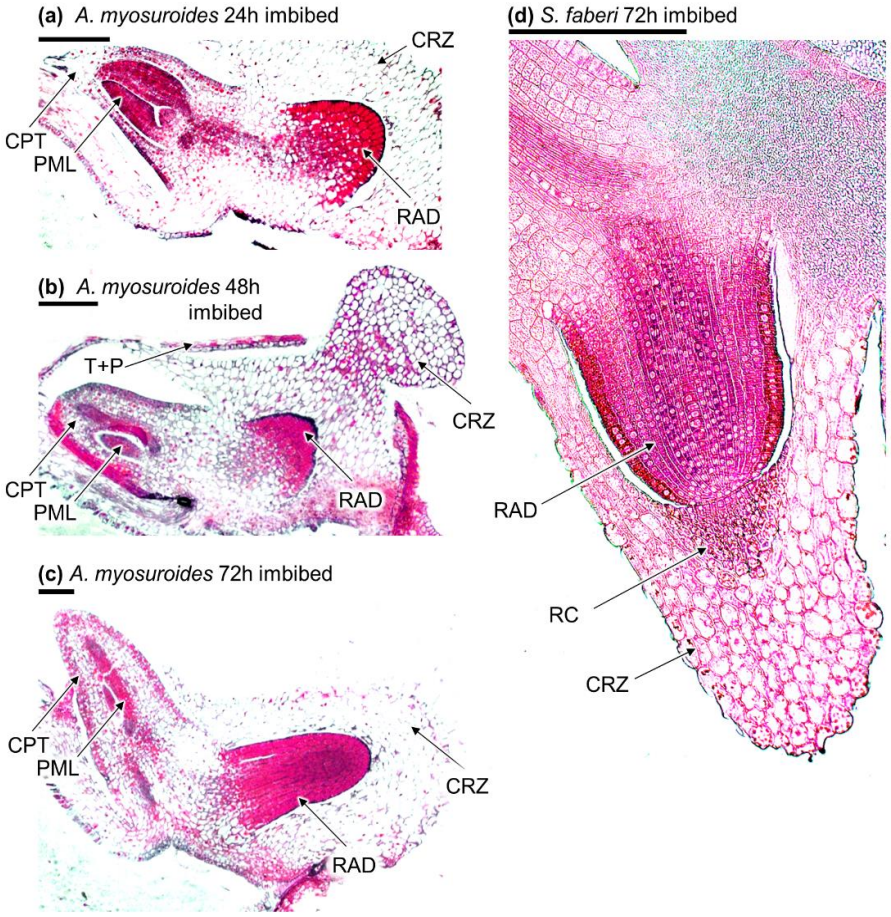


Fig. 2.4. Changes in embryo morphology during the germination of *A. myosuroides* and *S. faberi*. Micrographs of after-ripened *A. myosuroides* (a-c) *and S. faberi* (d) embryos at 24 (a) or 48 (b) and 72 (c,d) hours after imbibition. Micrographs show 5 µm lateral sections stained with safranin and methylene blue to stain nuclei blue and cell walls pink. Scale bar represents 200 µm. CPT, coleoptile; CRZ, coleorhiza; PML, plumule; RAD, radicle; RC, root cap; T+P, testa and pericarp.

This expansion of the coleorhiza occurs before any significant expansion of the cells of the radicle indicating that coleorhiza expansion is not occurring due to tension from the radicle but instead is an independent process that represents the first morphological change in the embryo during germination. Similar experiments were conducted with *A. myosuroides, D. sanguinalis* and *S. faberi* (Fig. 2.4, *D. sanguinalis* not shown) that demonstrated this dramatic expansion of coleorhiza cells during early germination. For example, in *A. myosuroides* the coleorhiza expands to form an outpouching which the radicle then expands into (Fig 2.4b,c) and in *S. faberi* coleorhiza cells expand dramatically and become highly vacuolated (Fig. 2.4d). Taken together these results

demonstrate that germination in grass weeds is a process, rather than a single event, involving different steps and distinct tissues that may be independently regulated.

2.3.2 Generation of seed batches with defined dormancy levels

The seed material used in this chapter, and through-out the rest of this thesis, come from a number of sources including collection from the field, propagation under controlled environment conditions and from an industrial seedbank (Table 2.2). In order to investigate primary dormancy, it is necessary to harvest seeds at the point of maturity as they are dispersed from the mother plant, before dormancy levels become reduced due to uncontrolled after-ripening. To preserve this initial dormancy state, freshly harvested (FH) seed batches were subjected to a seed storage protocol (Chapter 8.1) involving drying the seed batch down to less than 10% moisture contents and freezing the batches in hermetically sealed containers containing a desiccant.

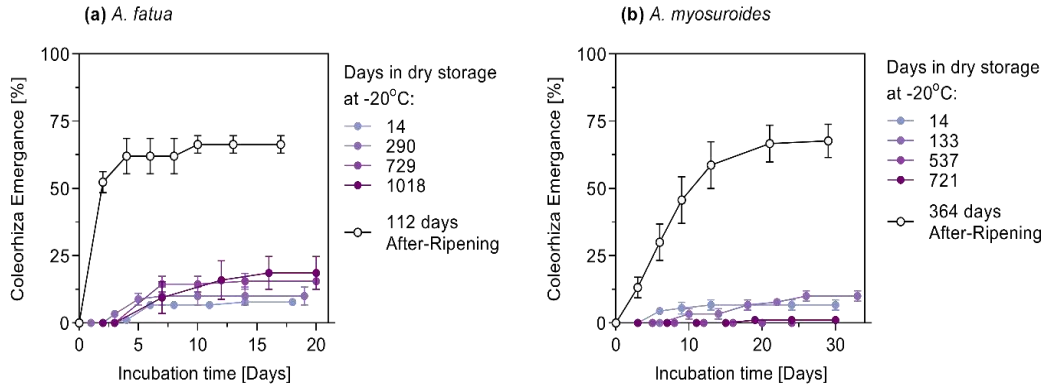


Fig. 2.5. Storage of seeds at -20°C preserves dormancy. The effect of different periods of dry frozen storage on cumulative coleorhiza emergence (germination) of (a) *A. fatua* LH840-D and (b) *A. myosuroides* LH170-D seeds in comparison to fully after-ripened seed (*A. fatua* LH840-AR_{112}, *A. myosuroides* LH170-AR_{364}) from the same batch. Individual lines represent a comparison of experiments conducted at different times under the same conditions (20°C under constant light). Error bars show standard error of the mean.

Table 2.2. Description of seed batches used in this thesis. Batch number refers to a naming system where AR_x shows the number of days of after-ripening where x is number of days. Shared genotype is represented by symbols where the parent line is identified by '♀'. For the sources of seeds: [a]seed batches provided by Syngenta Ltd. from the Seed Store at Jealott's Hill International Research Centre; [b]seed batches collected from arable fields by the author; [c]seed batches propagated by the author under controlled environment conditions. 'State' describes the dormancy of seed batches: D, dormant; ≈D unknown intermediate dormancy level; ND, not dormant; $AR_{x\%}$, after ripened at 50 % relative humidity at 20°C where x% represents a proportion of after-ripening relative to fully after ripened ($AR_{100\%}$); 'Aged' batches are those with low viability.

Species	Batch	Harvest Date	Source	State
Alopecurus myosuroides	LH841	Sept. 2013	UK[a]	ND
	LH100▲♀	May. 2015	Germany[a]	ND
	LH150	July. 2015	UK, Berkshire[b]	D
	LH128	Dec. 2018	UK, Berkshire[a]	ND
	LH216	2008	France[a]	≈D
	LH312	March 2018	UK, Berkshire[a]	ND
	LH170-D	June 2017	UK, Berkshire[b]	D
	LH170-AR_{120}	June 2017	UK, Berkshire[b]	$AR_{50\%}$
	LH170-AR_{364}	June 2017	UK, Berkshire[b]	$AR_{100\%}$
	LH192V-D▲	Sept. 2017	UK, RHUL[c]	D
	LH192V-AR_{196}▲	Sept. 2017	UK, RHUL[c]	$AR_{50\%}$
	LH192NV-D▲	Sept. 2017	UK, RHUL[c]	D
	LH192NV-AR_{196}▲	Sept. 2017	UK, RHUL[c]	$AR_{100\%}$
Avena fatua	LH401	Aug. 2013	UK, Aberdeenshire[a]	ND
	LH110	Aug. 2016	USA, Mississippi[a]	Aged
	LH125	Aug. 2018	UK, Nottinghamshire[a]	≈D
	LH937	Nov. 2014	USA[a]	ND
	LH840-D	Aug. 2016	UK, Hampshire[b]	D
	LH840-AR_{112}	Aug. 2016	UK, Hampshire[b]	$AR_{100\%}$
Digitaria sanguinalis	LH173■♀	Jan. 2016	USA[a]	ND
	LH181-D■	Nov. 2018	UK, RHUL[c]	ND
	LH181-AR_{140}■	Nov. 2018	UK, RHUL[c]	D
	LH181-AR_{196}■	Nov. 2018	UK, RHUL[c]	$AR_{100\%}$
Poa annua	LH101•♀	Jan. 2016	UK, Berkshire[a]	ND
	LH161-D	Oct. 2016	UK, Berkshire[a]	D
	LH161-AR_{110}	Oct. 2016	UK, Berkshire[a]	$AR_{100\%}$
	LH011V-D•	Nov. 2018	UK, RHUL[c]	D
Setaria faberi	LH905▼♀	July 2014	Serbia[a]	ND
	LH179-D▼	Sept. 2017	UK, RHUL[c]	D
	LH-179-AR_{280}▼	Sept. 2017	UK, RHUL[c]	$AR_{100\%}$

This method was effective in preserving the primary dormancy of all 5 species. In *A. fatua*, dry storage at -20°C gradually released dormancy in a dose-dependent manner so that after 1000 days of storage germination had increased from ~5% to ~15% when imbibed at 20°C under constant light (Fig. 2.5a). However this effect was not observed in other species, such as *A. myosuroides.*

In order to after-ripen seed batches in a defined and reproducible manner, saturated solutions of hygroscopic salts were used to generate defined relative humidities in sealed containers as described by Vertucci & Roos (1993). A dew point hygrometer was used to determine the equilibrium relative humidity (ERH) generated by these salt solutions at different concentrations and temperatures (Fig. 2.6a,b). Intermediate relative humidities that generate seed moisture contents between 20-40% are typically the most effective for after-ripening (Bewley *et al.*, 2013). On this basis, saturated solutions of $MgCl_2$ (32 %ERH at 20°C) and $Ca(NO_3)_2$ (53 %ERH at 20°C) were chosen.

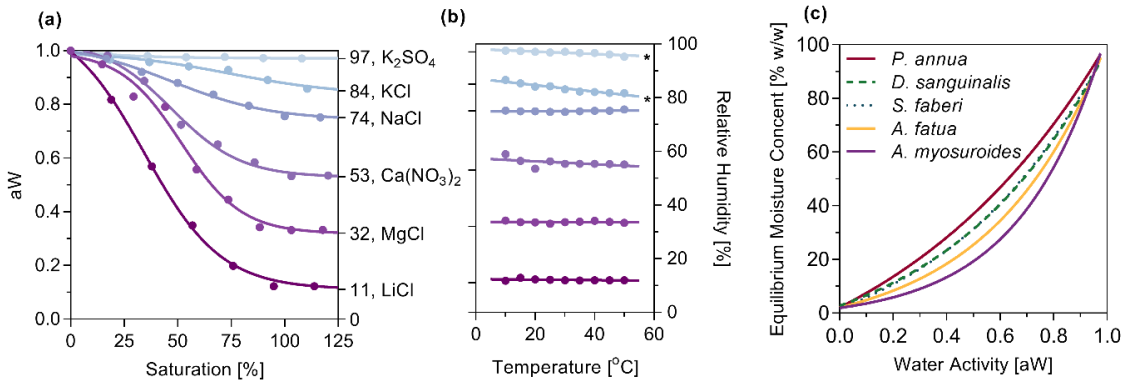


Fig. 2.6. Using salt solutions to generate seed batches with a known moisture content. (a) Concentration (% w/v) (relative to the saturation point) of different hygroscopic salts causes a predictable change in the equilibrium relative humidity (%ERH = 100 x aW) of the atmosphere above the solution. (b) The thermal stability of relative humidities generated by saturated solutions of different hygroscopic salts. An asterisk represents salt solutions where the linear regression slope is significantly non-zero ($p < 0.001$). (c) Sorption isotherm curves for diaspores from five grass weed species showing the relationship between the water activity (aW) of the storage condition and the moisture contents of the seeds (% w/w).

Table 2.3. The effect of seed storage at defined humidity on seed water content. Water activity of seeds of five species of grass weed seeds after one week stored above saturated solutions of $MgCl_2$ or $Ca(NO_3)_2$ measured using a dew point hygrometer. These values were used to interpolate the moisture contents (% w/w) of the seeds using a five parameter asymmetric sigmoidal curve (Gottschalk & Dunn, 2005) fitted to the sorption isotherms (Fig. 2.6c).

	Water activity (aW)		Interpolation of % Water Content		
Species	**$MgCl_2$**	**$Ca(NO_3)_2$**	**R^2**	**$MgCl_2$**	**$Ca(NO_3)_2$**
P. annua	0.339 ± 0.002	0.476 ± 0.011	0.9969	23.4	34.8
D. sanguinalis	0.332 ± 0.002	0.483 ± 0.009	0.9918	18.9	29.9
S. faberi	0.332 ± 0.017	0.484 ± 0.010	0.9945	18.6	30.0
A. fatua	0.332 ± 0.001	0.479 ± 0.007	0.9955	14.5	29.5
A. myosuroides	0.331 ± 0.004	0.482 ± 0.013	0.9982	10.3	18.1

It has been suggested that seed moisture contents, rather than water activity, is the key factor determining the rate of after-ripening (Bazin *et al.*, 2011a). The relationship between the ERH and moisture contents of any matrix can be described at a particular temperature using sorption isotherms that are typically non-linear and in seeds are dependent on the composition of seed storage organs such as differences in starch and oil contents (Weitbrecht *et al.*, 2011). In order to determine if there are differences in the moisture contents of seeds stored above defined ERHs that may affect the rate of after-ripening, sorption isotherms were constructed for five weed species (Fig. 2.6c). Interpolation of moisture contents from these curves for seeds stored above saturated solutions of $MgCl_2$ or $Ca(NO_3)_2$ identified large differences in moisture contents although the seeds had been equilibrated at defined ERHs (Table 2.3). For example, when stored above saturated $MgCl_2$, *P. annua* seeds achieved a moisture contents of 23.4% whereas *A. myosuroides* seeds only 10.3% moisture contents (Table 2.3).

If moisture content is a more reliable predictor of after-ripening rate than the ERH of the storage condition then we expect to see differences in the rate of after-ripening that reflect this dissimilarity. Generating 'after-ripening curves' showing the change in maximum cumulative germination (gMAX) over time in an after-ripening condition reveals differences in the after-ripening requirement of the grass weed seeds tested (Fig. 2.7). A four parameter logistic curve fitted to the after-ripening curves was used to

interpolate the half-maximal after-ripening time ($AR_{50\%}$) across the species stored at either 30% or 50% ERH. Values were not interpolated for *S. faberi* since the relationship between after-ripening and gMAX was ambiguous (Fig. 2.7f). Comparisons of the after-ripening rate across species demonstrated significant differences in $AR_{50\%}$ (One-way ANOVA, $F(6,14) = 47.72$, $p<0.0001$). The difference between the 30% and 50% ERH storage conditions was significant for *P. annua* (F-Test, $F(1,40) = 7.40$, $p=0.0096$) however it was not for the other species tested.

A. fatua was the species with the lowest $AR_{50\%}$ at both 30 and 50% ERH (Table 2.4) however did not have the highest moisture contents under these storage conditions. The species with the greatest difference in interpolated between the two storage humidities tested were *P. annua* and *A. fatua*, however a difference in $AR_{50\%}$ between these two conditions was only detected in *P. annua*. On the other hand, *A. myosuroides*, which achieved the lowest moisture contents under both humidity conditions (Table 2.3), also took the longest to reach $AR_{50\%}$ (Table 2.4). Taken together these results suggest that moisture content may play a role in determining the after-ripening requirements of grass weed seeds however other factors, such as the depth of dormancy, are also likely to play a role. It is clear from studies in Arabidopsis involving multiple ecotypes or mutants that after-ripening has a strong genetic component related to dormancy loci independent of the sorption isotherm properties of seeds (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2015; Auge *et al.*, 2017). Taken together the rate of after-ripening is likely determined by both the physical properties of seeds and their depth of dormancy. These results also demonstrate that after-ripening curves can be used to make comparisons between species when their sorption isotherm properties are equivalent, for example when comparing ecotypes of the same species, but not to make comparisons between species with different sorption properties.

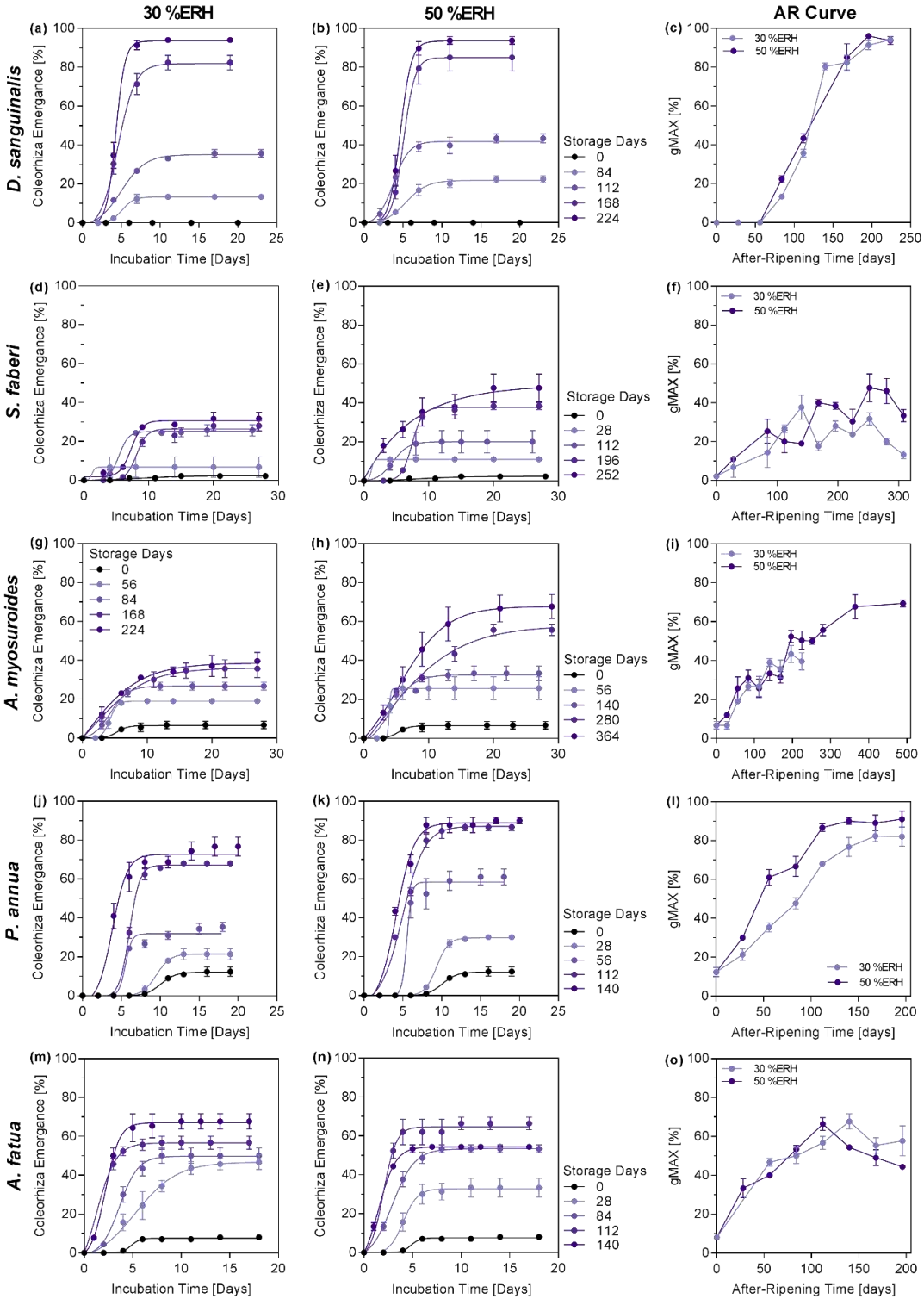


Fig. 2.7. After-ripening in five grass weed species. Representative germination curves showing coleorhiza emergence for *D. sanguinalis* LH181-D (a,b), *S. faberi* LH179-D (d,e), *A. myosuroides* LH170-D (g,h), *P. annua* LH161-D (j,k) and *A. fatua* LH840-D (m,n) stored at either 30% (a,d,g,j,m) or 50% equilibrium relative humidity (ERH) (b,e,h,k,n) for a variable number of days. (c,f,i,l,o) Plots showing the change in maximum germination (gMAX) as a result of after-ripening at 30 of 50 %ERH. Error bars show standard error of the mean for triplicates of >30 diaspores incubated at 20°C under constant light.

Table 2.4. Interpolated times to reach half maximal after-ripening ($AR_{50\%}$) across five grass weed species. A four parameter logistic curve fitted to the after-ripening curves in Fig. 2.4 was used to interpolate the half-maximal after-ripening time across the species stored at either 30% or 50% equilibrium relative humidity. *P* value shows significant differences in $AR_{50\%}$ between the two humidities. ±SE represents the standard error of the interpolated $AR_{50\%}$ value for three replicates.

	R^2		$AR_{50\%}$					$AR_{100\%}$	
Species	**30%**	**50%**	**30%**	**±SE**	**50%**	**±SE**	***p***	**30%**	**50%**
D. sanguinalis	0.9879	0.9867	116.8	1.959	117.1	4.628	ns	224	196
S. faberi	*0.4766*	*0.6454*	-	-	-	-	-	-	-
A. myosuroides	-	0.8472	-	-	168.5	3.291	ns	-	364
P. annua	0.9613	0.9581	88.89	10.69	52.76	5.742	0.096	168	140
A. fatua	0.8945	0.8389	37.92	11.73	30.24	7.192	ns	140	112

2.3.3 The effect of after-ripening on the germination temperature window

Having determined the rate at which different grass weed species after-ripen, batches with defined dormancy levels could be generated. For each species, with the exception *S. faberi* that had an unpredictable response to after-ripening (Fig. 2.7f), fully after-ripened batches ($AR_{100\%}$) were produced by incubating FH dormant seeds until no further increase maximum germination was observed. For *S. faberi*, a 280 day after-ripened batch was used. $AR_{50\%}$ batches were also produced for *A. myosuroides* and *D. sanguinalis* at 50% ERH (Table 2.2) as representatives of grass weeds with either winter or summer annual habits respectively. These seed batches were imbibed across a gradient of temperatures under constant light using thermogradient plates and cumulative germination was assessed by counting coleorhiza emergence over time.

Across all the species after-ripening had a strong effect on the temperature preferences for germination (Fig. 2.8). In the species with a winter annual habit (*A. myosuroides* & *A. fatua*) germination in the dormant state was restricted to colder temperatures and after-ripening caused an upward shift in their temperature preference for germination (Fig. 2.8a,b) as described by Baskin & Baskin (2004) as 'Type 1 non-deep PD'. Conversely in the two summer annuals germination was restricted across all

temperatures tested. In the case of *D. sanguinalis* this was a complete inhibition (Fig. 2.8d) and in *S. faberi* there was a small peak of germination under warm temperatures (Fig. 2.5e). After-ripening in these species caused a downward widening of temperatures permissive to germination typical of 'Type 2 non-deep PD'. The dormant *P. annua* batch initially tested had an unusual pattern of peaks across the temperatures (Fig. 2.8c) that did not fit with any previously described dormancy type. To check if this effect was consistent the batch was repropagated and the experiment repeated with the offspring from a single mother plant. Interestingly this batch followed a similar pattern as the initial seed batch. The explanation for this effect is not clear, however it is possible that heteroblasty in dormancy levels of seeds matured at different positions on the *P. annua* inflorescence may play a role, as reported in *S. faberi* (Dekker, 2003).

In order to provide a quantitative description of the effect of after-ripening on the temperature range under which grass weed seeds germinated linear regression was used to interpolate the cardinal temperatures (T_b, T_o and T_c) for seed germination following Bradford (2002). Typically for the interpolation of these values, the inverse of the time taken to reach half maximal germination (T_{50}^{-1}) is used as an input. However in the case of the response of dormant seeds to different temperatures, the differences we see between different temperatures come from a difference in maximum germination not from a difference in germination speed. Additionally, the time for each seed within a population to germinate within the species we tested did not always follow a normal distribution, breaking a key assumption of the T_{50}^{-1} approach. Instead the area under the cumulative germination curve (AUC) was chosen as the regression parameter because this parameter has a linear relationship with both maximum germination and T_{50} (Appendix 9.2).

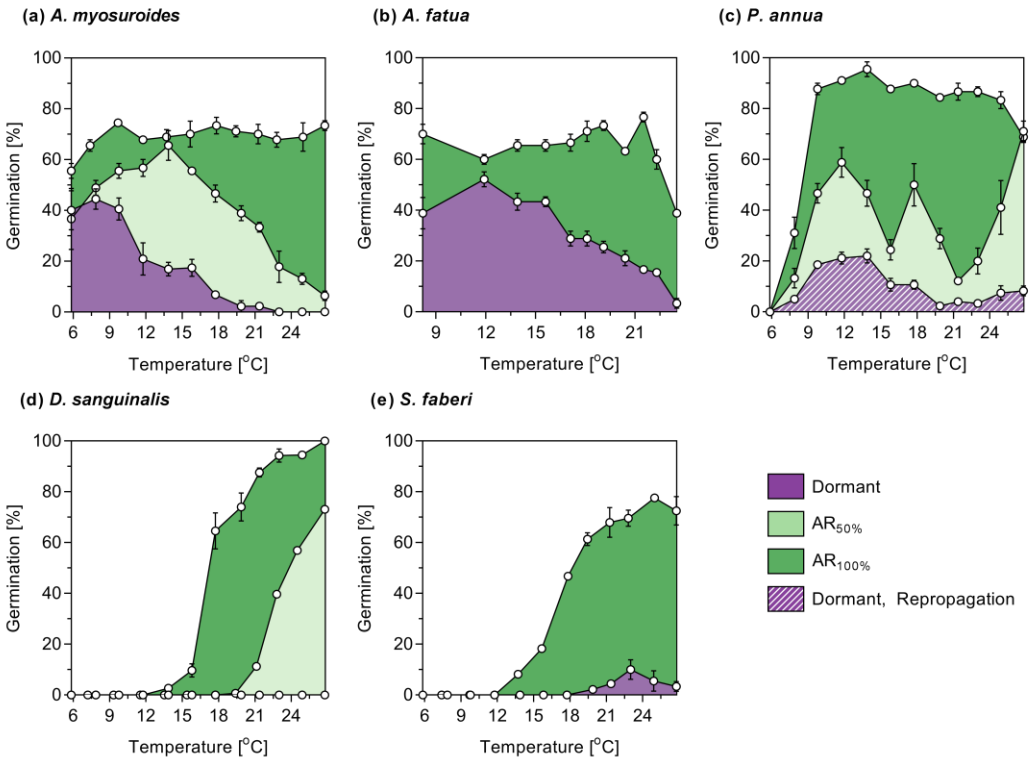


Fig. 2.8. The effect of imbibitional temperature on the germination of dormant and after-ripened grass weed seeds. Germination is recorded as the maximum cumulative percentage of germinated diaspores of (a) *A. myosuroides* LH170-D (purple), LH170-AR_{120} (light green) & LH170-AR_{364} (dark green); (b) *A. fatua* LH840-D (purple) & LH840-AR112 (dark green); (c) *P. annua* LH011-D (hashed purple), LH161-D (light green) & LH161-AR_{110} (dark green); (d) *D. sanguinalis* LH181-D (purple), LH181-AR_{110} (light green) & LH181-AR_{196} (dark green); (e) *S. faberi* LH179-D (purple) & LH179-AR_{280} (dark green). Error bars represent standard error of the means for triplicates of > 30 diaspores incubated under constant light. Germination curves are shown in Fig. 9.1.

Linear regression of the relationship between AUC and temperature was used to interpolate T_b and T_c and a segmental linear regression model was used to estimate the T_o. Regression plots for this analysis are shown in the appendix (9.3) and interpolated values for cardinal temperatures for different seed batches are shown in Table 2.5.

Table 2.5. Interpolated cardinal temperatures describing the responses of grass weed seeds with differing levels of dormancy to temperature. 'Range' shows the range of temperatures that were used for regression analysis. 'R^2' described the goodness-of-fit for the regression analysis, 'n' is the number of values used for regression analysis, 'value' shows the interpolated value of a particular parameter and '+/- C.I.' show the positive and negative values for the 95% confidence interval respectively.

Species	Batch	Parameter	Range (°C)	R^2	n	Value (°C)	+ C.I.	- C.I.
A.myosuroides	LH170-D	T_c	5-22	0.614	15	18.4	24.7	16.0
	LH170-D	T_o	5-20	0.781	13	6.8	8.0	4.5
	LH170-AR_{120}	T_c	14-27	0.822	22	30.0	32.6	28.2
	LH170-AR_{120}	T_b	4-16	0.716	18	-1.9	1.4	-8.5
	LH170-AR_{120}	T_o	4-28	0.822	36	13.5	14.6	12.3
	LH170-AR_{364}	T_b	4-15	0.637	15	-2.7	1.3	-
	LH170-AR_{364}	T_o	4-28	0.476	36	10.3	13.0	5.2
D. sanguinalis	$LHAR_{196}$	T_b	14-28	0.858	22	13.1	14.5	11.1
	LH-AR_{140}	T_b	19-28	0.919	15	19.5	20.2	18.7
S. faberi	LH-AR_{280}	T_b	11-28	0.917	27	11.8	12.7	10.5
P. annua	LH-161AR_{110}	T_b	4-16	0.924	18	5.0	5.8	3.8
	LH-161AR_{110}	T_o	4-28	0.942	36	14.8	17.5	13.7
A. fauta	LH840-D	T_c	12-24	0.803	33	27.1	29.2	25.6
	LH840-D	T_o	12-24	0.803	33	14.7	15.5	13.7
	LH840-AR_{110}	T_o	8-24	0.647	36	20.0	22.4	18.5
	LH840-AR_{110}	T_b	4-18	0.726	19	-2.1	1.8	-9.5

In order to understand if these extrapolated cardinal temperatures have any relation to emergence patterns in the field we obtained publically available soil temperature data for a site in the UK (Beaufort Park, Bracknell) and the USA (Springfield, Illinois) (ICN, 2019; MetOffice, 2019) and compared this to observations made on the emergence of these species in locations with similar climates (Fausey *et al.*, 1997; Davis *et al.*, 2003; Dekker, 2003; Cardina *et al.*, 2007, 2011; Liebman *et al.*, 2014; Clarke *et al.*, 2015; Taylor-Davies, 2017).

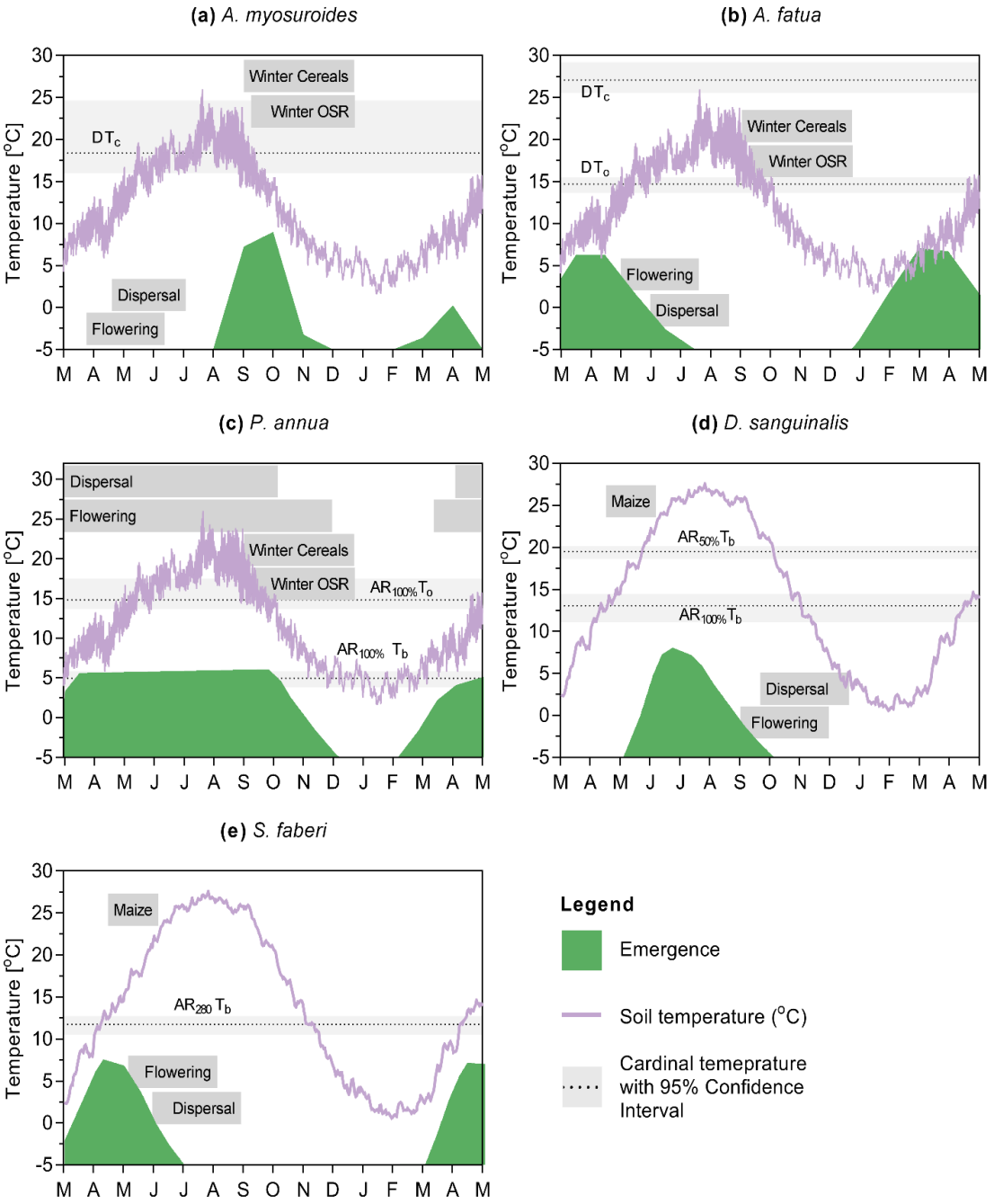


Fig. 2.9. Soil temperature and emergence patterns of grass weeds related to cardinal temepratures for germination. Weed seedling emergence was plotted across the year based on observations from Dekker (2003), Leibman *et al.* (2014), Fausey & Renner (1997), Cardina *et al.* (2007), Cardina *et al.* (2011), Clarke *et al.* (2015) and Taylos-Davis (2017). Soil temeprature data was obtained from (a-c) the Met Office MIDAS database showing a 5 year average for Beaufort Park, Bracknell (UK) at a 10 cm soil depth and (d,e) from The Illinois Climate Network database showing an 8 year average for exposed soil in Springfield, Illinois (USA) at a depth of four inches (10.16 cm). Cardinal temperatures for germination are plotted as dotted lines with the shaded area showing the 95% confidence interval.

Cardinal temperatures for each weed species (Table 2.5) were then plotted on these graphs to identify co-occurrence between seasonal changes in soil temperature and cardinal temperatures that reflect emergence patterns (Fig. 2.9). This approach is limited by the availability of weed emergence data and, since the emergence and soil temperature data come from different locations and times, quantitative conclusions cannot be drawn about the effect of seed dormancy and temperature on emergence patterns. It was not possible to interpolate a complete set of cardinal temperatures for all the batches due to the limited range of temperatures at which germination was assessed. For example, calculation of a robust T_b for dormant *A. myosuroides* and *A. fatua* was not possible since the colder temperatures tested were relatively optimal for germination (Fig. 2.8a,b). The opposite was the case for *D. sanguinalis* and *S. faberi* where the highest temperatures tested were optimal for germination hindering T_c estimation. In *P. annua* it was not possible to estimate T_o due to the complexity of the dormant seed germination response to different temperatures. Additionally, the seed batches tested do not come from the same locations and growth conditions as the soil temperatures and emergence data. However, despite these limitations, these data serve as a qualitative example of what emergence models attempt to achieve using similar parameters.

In some species the interpolated cardinal temperatures relate to key events during the weed lifecycle, and in other species not. For example, the winter annual *A. myosuroides*, seeds are dispersed from May-July at soil temperatures above T_c for the dormant state and as soil temperatures are reduced below T_c from August-November we see the major peak of emergence (Fig. 2.9a). The opposite pattern was observed in the summer annual *D. sanguinalis* where seeds were dispersed at soil temperatures below the T_b in the $AR_{50\%}$ state during October-January and as temperatures rise again to above T_b we see an emergence peak from June-September that ends when soil temperatures sink below the T_b again (Fig. 2.9d). However the same pattern was not

observed in the related summer annual species *S. faberi* (Fig. 2.9e). In *P. annua*, a facultative winter annual, emergence occurs year-round except during the winter months from December-February. This inhibition of emergence co-occurred with the reduction of soil temperatures below the T_b for the $AR_{100\%}$ state and as soil temperatures increased above T_b from February onwards, emergence in *P. annua* resumed. Taken together these observations demonstrate that cardinal temperatures estimated by germination assays under laboratory conditions do have the potential to be used to predict grass weed emergence patterns in the field in some species, however in other species there may be factors other than temperature that determine emergence patterns.

2.4 Conclusions

Primary seed dormancy limits the range of temperatures under which grass weed seeds germinate. Dormancy loss through controlled after-ripening widens this range of temperatures permitting germination under a broader range of environmental conditions. The rate of after-ripening depends in part on the sorption isotherm properties of seeds. This differential temperature response can be used to derive quantitative parameters that can be used as inputs to weed emergence models. The comparison of example weed emergence and soil temperature data with these parameters demonstrated that the interaction between temperature and dormancy can be used to predict seasonal grass weed emergence patterns in some species. The physiological description of the interaction between temperature, dormancy and germination will be used as the experimental basis for further research into the mechanisms of dormancy and germination in grass weed seeds.

2.5 References

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-de Vries H, Koornneef M. **2003**. Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genetics* **164**: 711–729.

Auge GA, Blair LK, Neville H, Donohue K. **2017**. Maternal vernalization and vernalization-pathway genes influence progeny seed germination. *New Phytologist*. **216**: 1-13.

Bailly C, El-Maarouf-Bouteau H, Corbineau F. **2008**. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *Comptes Rendus - Biologies* **331**: 806–814.

Baskin JM, Baskin CC. **1983**. Seasonal changes in the germination responses of buried seeds of *Arabidopsis thaliana* and ecological interpretation. *Botanical Gazzete* **144**: 540–543.

Baskin JM, Baskin CC. **1985**. The annual dormancy cycle in buried weed seeds: A continuum. *BioScience* **35**: 492–498.

Baskin JM, Baskin CC. **1987**. Temperature requirements for after-ripening in buried seeds of four summer annual weeds. *Weed Research* **27**: 385–389.

Baskin JM, Baskin CC. **2004**. A classification system for seed dormancy. *Seed Science Research* **14**: 1–16.

Baskin C, Baskin J. **2014**. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. Oxford: Academic Press.

Bazin J, Batlla D, Dussert S, El-Maarouf-Bouteau H, Bailly C. **2011a**. Role of relative humidity, temperature, and water status in dormancy alleviation of sunflower seeds during dry after-ripening. *Journal of Experimental Botany* **62**: 627–640.

Bazin J, Langlade N, Vincourt P, Arribat S, Balzergue S, El-Maarouf-Bouteau H, Bailly C. **2011b**. Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *The Plant Cell* **23**: 2196–2208.

Bentsink L, Jowett J, Hanhart CJ, Koornneef M. **2006**. Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in Arabidopsis. *PNAS* **103**: 17042–17047.

Bewley J, Bradford K, Hilhorst H, Nonogaki H. **2013**. *Seeds - Physiology of Development, Germination and Dormancy*. London: Springer.

Boyd N, Van Acker R. **2004**. Seed germination of common weed species as affected by oxygen concentration, light, and osmotic potential. *Weed Science* **52**: 589–596.

Bradford KJ. **2002**. Applications of hydrothermal time to quantifying and modeling seed germination and dormancy. *Weed Science* **50**: 248–260.

Cardina J, Herms CP, Herms DA. **2011**. Phenological indicators for emergence of large and smooth crabgrass (*Digitaria sanguinalis* and *D. ischaemum*). *Weed Technology* **25**: 141–150.

Cardina J, Herms CP, Herms DA, Forcella F. **2007**. Evaluating phenological indicators for predicting giant foxtail (*Setaria faberi*) emergence. *Weed Science* **55**: 455–464.

Clarke J, Ginsburg D, Clare K, Tonguc L. **2015**. *The Encyclopaedia of Arable Weeds*. AHDB.

Colbach N, Busset H, Yamada O, Dürr C, Caneill J. **2006a**. AlomySys: Modelling black-grass (*Alopecurus myosuroides* Huds.) germination and emergence, in interaction with seed characteristics, tillage and soil climate: II. Evaluation. *European Journal of Agronomy* **24**: 113–128.

Colbach N, Chauvel B, Durr C, Richard G. **2002a**. Effect of environmental conditions

on *Alopecurus myosuroides* germination. I. Effect of temperature and light. *Weed Research* **42**: 210–221.

Colbach N, Dürr C, Chauvel B, Richard G. **2002b**. Effect of environmental conditions on *Alopecurus myosuroides* germination. II. Effect of moisture conditions and storage length. *Weed Research* **42**: 222–230.

Colbach N, Durr C, Roger-Estrade J, Chauvel B, Caneill J. **2006b**. AlomySys: Modelling black-grass (*Alopecurus myosuroides* Huds.) germination and emergence, in interaction with seed characteristics, tillage and soil climate: I. Constructon. *European Journal of Agronomy* **24**: 113–128.

Davis A, Dixon P, Leibman M. **2003**. Cropping system effects on giant foxtail (*Setaria faberi*) demography : II. Retrospective perturbation analysis. *Weed Science* **51**: 930–939.

Dekker J. **2003**. The foxtail (Setaria) species-group. *Weed Science* **51**: 641–656.

Dyer WE. **1995**. Exploiting weed seed dormancy and germination requirements through agronomic practices. *Weed Science* **43**: 498–503.

El-Maarouf-Bouteau H, Bailly C. **2008**. Oxidative signaling in seed germination and dormancy. *Plant Signaling & Behavior* **3**: 175–182.

Fausey JC, Renner KA. **1997**. Germination, emergence, and growth of giant foxtail (*Setaria faberi*) and fall panicum. *Weed Science* **45**: 423–425.

Fennimore SA, Nyquist WE, Shaner GE, Myers SP, Foley ME. **1998**. Temperature response in wild oat (*Avena fatua* L.) generations segregating for seed dormancy. *Heredity* **81**: 674–682.

Finch-Savage WE, Leubner-Metzger G. **2006**. Seed dormancy and the control of germination. *New Phytologist* **171**: 501–523.

Gallart M, Verdú AMC, Mas MT. **2008**. Dormancy breaking in *Digitaria sanguinalis*

seeds: The role of the caryopsis covering structures. *Seed Science and Technology* **36**: 259–270.

Gianfagna AJ, Pridham AMS. 1951. Some aspects of dormancy and germination of crabgrass seed, *Digitaria sanguinalis* Scop. *Proceedings fo the American Society of Horticultural Science* **58**: 291-297.

Gottschalk PG, Dunn JR. **2005**. The five-parameter logistic: A characterization and comparison with the four-parameter logistic. *Analytical Biochemistry* **343**: 54–65.

Grundy AC. **2003**. Predicting weed emergence: A review of approaches and future challenges. *Weed Research* **43**: 1–11.

Holloway T. **2016**. Master's Thesis: Comprehensive physiological charachterisation of Alopecurus myosuroides Huds. germiantion as affected by karrikin 1 application. Department of Biological Sciences, Royal Holloway University of London.

Hubbard C. **1968**. *Grasses*. Bungay, Sussex: The Chaucer Press.

ICN. **2019**. Illinois Climate Network: Water and Atmospheric Resources Monitoring Program.

Kazinczi G, Hunyadi Keszthely (Hungary). Inst. for Plant Protection) K (Pannon U of AS. **1992**. A contribution to the germination biology of blackgrass (*Alopecurus myosuroides* Huds.). Mad. Fac. Landbouww. Univ. Gent. 57/3b, 1001-1048.

Leon RG, Knapp AD, Owen MDK. **2004**. Effect of temperature on the germination of common waterhemp (*Amaranthus tuberculatus*), giant foxtail (*Setaria faberi*), and velvetleaf (*Abutilon theophrasti*). *Weed Science* **52**: 67–73.

Liebman M, Miller ZJ, Williams CL, Westerman PR, Dixon PM, Heggenstaller A, Davis AS, Menalled FD, Sundberg DN. **2014**. Fates of *Setaria faberi* and *Abutilon theophrasti* seeds in three crop rotation systems. *Weed Research* **54**: 293–306.

Masin R, Zuin MC, Otto S, Zanin G. **2006**. Seed longevity and dormancy of four

summer annual grass weeds in turf. *Weed Research* **46**: 362–370.

Met Office. **2019**. *MIDAS Open: UK soil temperature data, v201901. Centre for Environmental Data Analysis*. URL: https://catalogue.ceda.ac.uk/uuid/245df050d57a500c183b88df509f5f5a (Last Accessed: 14.10.2019).

Miller PM, Ahrens JF, Stoddard EM. **1965**. Stimulation of crabgrass seed germination by 1,2-dibromo-3-chloropropane and ethylene dibromide. *Weeds* **13**: 13–14.

Myers S. **1997**. Developmental differences between germinating after-ripened and dormant excised *Avena fatua* L. embryos. *Annals of Botany* **79**: 19–23.

Nakabayashi K, Bartsch M, Ding J, Soppe WJJ. **2015**. Seed dormancy in Arabidopsis requires self-binding ability of DOG1 protein and the presence of multiple isoforms generated by alternative splicing. *PLoS Genetics* **11**: 1–20.

Nelson SK, Ariizumi T, Steber CM. **2017**. Biology in the dry seed: Transcriptome changes associated with dry seed dormancy and dormancy loss in the Arabidopsis GA-insensitive *sleepy1-2* mutant. *Frontiers in Plant Science* **8**: 1–21.

Ohadi S, Rahimian Mashhadi H, Tavakkol-Afshari R, Beheshtian Mesgaran M. **2010**. Modelling the effect of light intensity and duration of exposure on seed germination of *Phalaris minor* and *Poa annua*. *Weed Research* **50**: 209–217.

Peart MH. **1984**. The effects of morphology, orientation and position of grass diaspores on seedling survival. *Journal of Ecology* **72**: 437–453.

Raviv B, Godwin J, Granot G, Grafi G. **2018**. The dead can nurture: Novel insights into the function of dead organs enclosing embryos. *International Journal of Molecular Sciences* **19**.

Roberts HA, Feast PM. **1970**. Seasonal distribution of emergence in some annual weeds. *Experimental Horticulture*: 36–41.

Roberts HA, Potter ME. **1980**. Emergence patterns of weed seedlings in relation to cultivation and rainfall. *Weed Research* **20**: 377–386.

Sawhney R. **1989**. Temperature control of dormancy and germination in embryos isolated from seeds of dormant and nondormant lines of wild oats (*Avena fatua*) . *Canadian Journal of Botany* **67**: 128–134.

Simpson G. **1990**. *Seed Dormancy in Grasses*. Cambridge: Cambridge University Press.

Standifer LC, Wilson PW. **1988**. Dormancy studies in three populations of *Poa annua* L. seeds. *Weed Research* **28**: 359–363.

Stanway V. **2016**. Laboratory germination of giant foxtail (*Setaria faberi* Herrm.), at different stages of maturity. *Association of Official Seed Analysts and the Society of Commercial Seed Technologists* **61**: 85–90.

Stoller EW, Wax LM. **1973**. Periodicity of germination and emergence of some annual weeds. *Weed Science* **21**: 574–580.

Taylor-Davies B. **2017**. *Blackgrass: System BEN offers alternative solutions to resistance management*. Nuffield Scolar's Report. URL: https://www.nuffieldscholar.org/news/ben-taylor-davies-report-published/ (Last Accessed: 14.10.2019)

Vegis A. **1963**. Climatic control of germination, bud break, and dormancy. In: Evans LT., ed. Environmental control of plant growth. Oxford: Academic Press, 265–287.

Vegis A. **1964**. Dormancy in Higher Plants. **15**: 185–224.

Vertucci CW, Roos EE. **1993**. Theoretical basis of protocols for seed storage II. The influence of temperature on optimal moisture levels. *Seed Science Research* **3**: 201–213.

Vleeshouwers L, Kropff M. **2000**. Modelling field emergence patterns arable weeds.

New Phytologist **148**: 445–457.

Weitbrecht K, Müller K, Leubner-Metzger G. **2011**. First off the mark: Early seed germination. *Journal of Experimental Botany* **62**: 3289–3309.

Wellington PS, Hitchins S. **1966**. Seed doramncy and the winter annual habit in blackgrass (Alopecurus myosuroides Huds.). *Journal of the National Institute of Agricultural Botany* **10**: 628–643.

Zimdahl R. **1988**. The concept and application of the critical weed-free period. In: Altieri MA., Liebman M., eds. Weed Management in Agroecosystems: Ecological Approaches. Florida: CRC Press, 145–155.

Zimdahl R. **2018**. *Fundamentals of Weed Science*. Colorado: Academic Press.

3. Chemical Genetics of Grass Weed Seed Dormancy and Germination.

3.1 Abstract

Seed dormancy in weed seeds is a highly adaptive trait that contributes to weed competitiveness in the field. Germination stimulants provide the opportunity to manipulate the dormancy, and therefore emergence patterns, of weed seeds in the soil seedbank as a target for weed management. In this Chapter, the literature on germination stimulants is reviewed to identify compounds with potential for weed seedbank management. A target-catalogue compound library was developed with compounds that have known activity in model species as well as analogs with *in vitro* activity. These compounds were screened across five grass weed species (*A. fatua, A. myosuroides, D. sanguinalis, P. annua* and *S. faberi*) with differing life history traits. Additional screens were conducted on lead compounds to determine the effect of temperature, dormancy and seed batch-effects on the effectiveness of the germination stimulants. Smoke-derived compounds, carotenoid biosynthesis inhibitors and gibberellin receptor agonists were identified as promising germination stimulants in grass weeds. However, the effect of these lead compounds was often species- and dormancy-specific reflecting the complexity of dormancy mechanisms across the species. The factors influencing responses to germination stimulants are discussed along with the research needs for the development of more effective weed seed-specific germination stimulants.

3.2 Introduction

3.2.1 Chemical manipulation of seed persistence as a target for weed control

Seed dormancy a highly adaptive trait in determining the timing of emergence of agricultural weeds. Weeds have become adapted to high-input agricultural systems by synchronising their emergence with the cropping cycle to avoid control measures and maximize their competitiveness with the crop (Zimdahl, 2018). The effect of weeds on crop yield is typically determined by a 'competitive window' during the establishment of crops where weeds have their most damaging effects on crop growth (Roberts *et al.*, 1982). Effective weed management strategies therefore focus on creating 'weed-free' periods during this critical window (Zimdahl, 1988). An emerging approach to achieve this is through chemical manipulation of the WSB, either to cause premature germination of dormant weed seeds that can be removed using additional control methods, or through inhibition of weed seed germination to avoid weed emergence during this critical window (Dyer, 1995; Papenfus *et al.*, 2015). This concept has received some attention in the literature (Bond & Burch, 1990; Donald & Tanaka, 1993; Carmona & Murdoch, 1995; Long *et al.*, 2011; Goggin & Powles, 2014; Papenfus *et al.*, 2015) however the results are often contradictory within and across species. In this Chapter the literature on germination modifying compounds is reviewed and a series of compounds from a target-catalogue library are screened for dormancy breaking and germination stimulant effects across a panel of grass weed species.

3.2.2 Inorganic compounds targeting dormancy and germination

Non-organic nitrate-containing compounds, such as nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), cyanide (CN^-), azide (N_3^-) and nitrogen containing gasses such as nitrogen monoxide (NO) and dioxide (NO_2) promote the release from dormancy in a

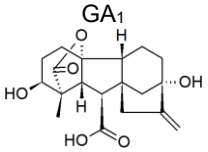
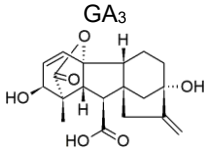
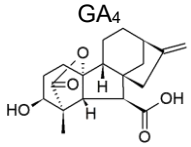
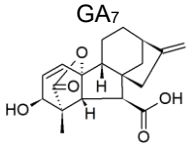
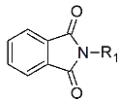
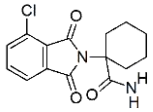
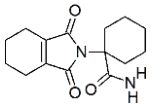
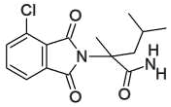
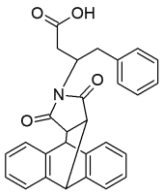
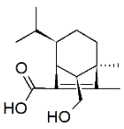
broad range of species (Bethke *et al.*, 2007). The mechanisms through which N-containing compounds stimulate germination is unknown although it has been proposed that responses to N-containing compounds may be an adaptation to germinate specifically on nitrogen rich soils (Pons, 1989). One hypothesis focuses on the potential role of NO_3^- and NO_2^- as electron acceptors in respiratory metabolism through oxidation of NADPH or by affecting electron transport allowing greater carbon flow through the pentose phosphate pathway, liberating additional metabolites required for early germination (Roberts, 1973). There is also some evidence to suggest that NO_3^- interacts with ABA metabolism to regulate germination in Arabidopsis (Matilla *et al.*, 2015). Cyanide and azide are respiratory inhibitors with a strong dormancy breaking effect, however the mechanism by which they stimulate germination is again unknown (Bethke *et al.*, 2007).

3.2.3 Compounds targeting gibberellin metabolism and signalling

Gibberellins are a family of tetracyclic diterpene endogenous phytohormones that have a well characterised role in the positive regulation of developmental processes such as seed germination, stem elongation, flowering and fruit development (Yamaguchi, 2008). Whilst there are 136 naturally occurring gibberellins in plants and fungi, approximately 100 of which are present in plants, only GA_1, GA_3, GA_4 and GA_7 (Table 3.1a) have a signalling activity (Yamaguchi, 2008). Gibberellin-deficient mutants of Arabidopsis (e.g. *ga1-3*) typically have reduced germinability that can be restored by the exogenous application of bioactive gibberellins (Kucera *et al.*, 2005). Gibberellin biosynthetic genes are also characteristically expressed during the germination process in Arabidopsis (Ogawa *et al.*, 2003). Gibberellins are perceived by the GIBBERELLIN INSENSITIVE DWARF 1 (GID1) receptor in Arabidopsis. Upon binding of bioactive GAs to the GID1 binding pocket, its conformation is changed to increase its affinity for DELLA proteins that inhibit the transcription of GA-responsive genes in the

absence of GA, leading to F-Box-mediated proteasomal degradation of DELLAs and transcription of GA-responsive genes (Hartweck, 2008).

Table 3.1. Gibberellin signalling agonists

Gibberellin Signalling Agonists		
(a) Gibberellic Acids GA$_1$  GA$_3$  GA$_4$  GA$_7$ 	Endogenous phytohormones[1,2] involved in germination[3] and organ elongation. Used for dormancy breaking in non-deep physiologically dormant species[4]. GA$_3$ is commonly used in horticulture and agriculture to break bud dormancy and stimulate germination respectively[5].	[1](Yamaguchi, 2008) [2](Hartweck, 2008) [3](Ogawa *et al.*, 2003) [4](Baskin & Baskin, 2014) [5](Rademacher, 2016)
(b) N-Substituted Phthalimides Generic  AC-94377  AC-99524  AC-92803 	Selective agonists of the gibberellin receptor *Gibberellin Insensitive Drawf 1* (GID1)[1,2]. Promotes the germination of GA biosynthesis mutants in Arabidopsis. AC94377 promotes germination in some weed species[3,4,5] and promotes emergence of *Sinapis arvensis* in the field at 3.4 kg ha^{-1} [5].	[1](Yalpani *et al.*, 1989) [2](Jiang *et al.*, 2017a) [3](Diehl & Walworth, 1977) [4](Bond & Burch, 1990) [5](Donald & Tanaka, 1993)
(c) Others 67D  Helminthosporic Acid 	67D is a putative GID1 agonist that promotes germination in Arabidopsis seeds with inhibited GA biosynthesis[1]. Helminthsporic acid is a GID1 agonist promotes germination in Arabidopsis seeds with inhibited GA biosynthesis[2].	[1](Jiang *et al.*, 2017b) [2](Miyazaki *et al.*, 2017)

Several non-endogenous GID1 agonists have been identified that show a germination stimulating effect. N-substituted phthalimides (NSPs), particularly those with an N-substituted cyclohexane moiety (Table 3.1b), have potent gibberellin-like germination stimulant properties (Diehl & Walworth, 1977). Like GAs, the NSP AC-94377 (Table 3.1b), is able to restore the germination of GA-deficient mutants (Jiang *et al.*, 2017a) and promotes the germination of a number of weed species in under field and laboratory conditions (Thomas, 1984; Gott & Thomas, 1986; Bond & Burch, 1990;

Donald & Tanaka, 1993). Interestingly in celery (*Apium gravolens* L.) AC-94377 and GA_{4+7} promote seed germination, however seedlings from GA treated seeds were abnormally elongated and etiolated. AC-94377 treated seeds showed normal seedling development, suggesting developmental specificity for AC-94377 activity (Gott & Thomas, 1986). Work with radiolabelled GA_4 and AC-94377 demonstrated that GAs and AC-94377 compete for the same binding site in cucumber (*Cucumis sativus* L.) hypocotyls (Yalpani *et al.*, 1989). After the discovery of the GID1 receptor in rice (*Oryza sativa* L.) (Ueguchi-Tanaka *et al.*, 2005), AC-94377 was identified as a specific GID1 agonist using a yeast-2-hybrid approach in Arabidopsis (Jiang *et al.*, 2017a).

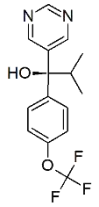
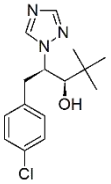
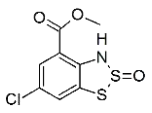
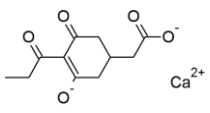
Two additional compounds with GA-like activity are 67D and Helminthosporic Acid (Table 3.1c). 67D was identified as part of a compound library screen against Arabidopsis seeds imbibed with a gibberellin biosynthesis inhibitor (paclobutrazol), and was shown both to bind to GID1 and compete for active site binding with GA_4 (Jiang *et al.*, 2017b). Helminthsporic acid, extracted from the pathogenic fungus *Helminthosporium sativum*, was found to remove the light requirement for germination in tobacco (*Nicotiana tabacum* L.) (Hashimoto & Tamusa, 1967) and later work identified that helminthsporic acid was a selective agonist of GID1 in Arabidopsis and rice (Miyazaki *et al.*, 2017).

In plants, gibberellin is synthesised from geranylgeranyl diphosphate (GGPP) by three classes of enzymes: terpene synthases (TPSs, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS)), cytochrome P450 monooxygenases (P450s, *ent*-Kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO)) and 2-oxoglutarate–dependent dioxygenases (2ODDs, GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox)) (Yamaguchi, 2008). Whilst GA3oxs are responsible for the last catalytic step of bioactive GA biosynthesis, another class of 2ODDs, GA 2-oxidases (GA2oxs), are responsible for gibberellin deactivation (Yamaguchi, 2008). Mutations in TPSs and

P450s genes cause severe dwarfing phenotypes in Arabidopsis (Koornneef & van der Veen, 1980) and germination of these mutants depends on exogenous application of GAs (Kucera *et al.*, 2005).

A range of GA biosynthesis inhibitors, such as flurprimidol and paclobutrazol (Table 3.2a), have been developed for the crop production market due to their applicability for preventing cereal lodging and reducing the need to mow turf grass (i.e. inhibition of stem elongation) (Rademacher, 2016). Similar reduced germination phenotypes are observed when gibberellin biosynthesis is inhibited using inhibitors of KO. For example flurprimidol and paclobutrazol have been found to inhibit germination in both cress (*Lepidium sativum* L.) and Arabidopsis (Debeaujon & Koornneef, 2000; Müller *et al.*, 2006).

Table 3.2. Inhibition of gibberellin synthesis and catabolism

Inhibition of Gibberellin Metabolism			
(a) Inhibition of *ent*-kaurene oxidase			
Flurprimidol 	Paclobutrazol 	Flurprimidol and pacloburtazol are inhibitors of *ent*-Kaurene Oxidase (KO) that inhibit the accumulation of bioactive GAs[1]. They are commonly used in plant production to reduce stem elongation[2].	[1](Rademacher, 2000) [2](Rademacher, 2016)
(b) Inhibition of GA oxidases			
CBTC 	Prohexadione-Ca (PHX) 	CBTC is a specific inhibitor of *Gibberellin 2-Oxidase* (GA2ox) that promotes germination and elongation in Arabidopsis by inhibiting GA catabolism[1]. PHX is a non-specific inhibitor of both GA2ox and *Gibberellin 3-Oxidase* (GA3ox), inhibiting rice shoot elongation at low concentrations and promoting at high concentrations[2].	[1](Otani *et al.*, 2010) [2](Nakayama *et al.*, 1990b)

Inhibitors of 2ODDs also have an effect on germination kinetics (Table 3.2b). Prohexadione calcium (PHX), an inhibitor of 2ODDs (GA2/3/20oxs), decreases bioactive GA biosynthesis (Nakayama *et al.*, 1990a) and consequently inhibits germination in Arabidopsis (Otani *et al.*, 2010). A more specific inhibitor of GA2oxs, CBTC, was identified through screening against a recombinant Arabidopsis GA2ox2, causing an accumulation of labelled GA_4 *in vitro* (Otani *et al.*, 2010). CBTC was found to slightly increase germination rate and seedling stem elongation in Arabidopsis, suggesting CBTC inhibits GA2ox with a high degree of specificity (Otani *et al.*, 2010).

3.2.4 Compounds targeting abscisic acid metabolism and signalling

Abscisic acid is a phytohormone with a key role in enforcing dormancy and regulating germination. Mutants in ABA signalling components, such as the well characterised *viviparous-1* (*vp1*) mutant in maize (*Zea mays*), germinate even before seed dispersal (McCarty *et al.*, 1991). Transgenic lines overexpressing ABA biosynthetic genes, such as *9*-cis-*epoxycarotenoid dioxygenases* (*NCEDs*) typically have enhanced dormancy (Thompson *et al.*, 2000) and inhibitors of ABA biosynthesis release seed dormancy (Grappin *et al.*, 2000; Ali-Rachedi *et al.*, 2004). ABA also has a role in the thermoinhibition of Arabidopsis germination at high temperatures (Toh *et al.*, 2008). During ABA signalling, ABA forms a complex with PYR/PYL/RCAR family of proteins (ABA receptors) and type 2C protein phosphatase (PP2Cs) causing the phosphorylation of downstream targets that otherwise inhibit the expression ABA-responsive genes (Raghavendra *et al.*, 2010). The PYL agonist pyrabactin (Table 3.3a), was found to inhibit germination in Arabidopsis via the PYRABACTIN RESISTANCE 1 (PYR1) ABA receptor, inducing highly correlated patterns of gene expression with exogenous ABA in the seed and less correlated expression in the seedling, indicating a level of developmental specificity for the ABA signalling machinery and pyrabactin (Park *et al.*, 2009). A number of PYL antagonists have been

developed to inhibit ABA signalling. The elucidation of crystal structures for the Arabidopsis PYR1 dimer and its interaction with ABA (Nishimura *et al.*, 2009; Santiago *et al.*, 2009) facilitated the development of PYL antagonists by rational design (Hayashi & Kinoshita, 2014).

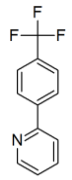
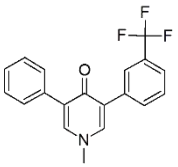
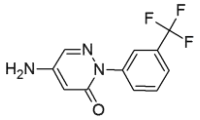
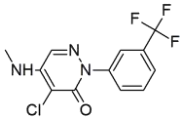
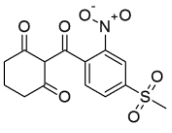
Takeuchi *et al.*, (2014) found that ABA analogs with long 3' alkyl chains were able to block PYL-PP2C interaction by steric hindrance. A six-carbon alkyl substituted ABA (Table 3.3b, AS_6) was found to be the most effective promoter of germination when Arabidopsis seeds were imbibed in ABA (Takeuchi *et al.*, 2014). This finding led the way for the development of additional 3' and 4'-modified ABA analogs with antagonistic activity against PYLs. (+)-PAO4, a propenyl-ABA with an *O*-butyl chain, (Table 3.3b) is a slightly more potent antagonist in Arabidopsis and a significantly more potent

Table 3.3. ABA signalling agonists and antagonists

ABA Signalling Agonists and Antagonists			
(a) PYL receptor Agonists			
(+)-Abscisic acid	Pyrabactin	Endogenous hormone typically associated with dormancy and the inhibition of germination. High in the dry state[1]. Pyrabactin is a *Pyrabactin Resistance 1* (PYL1) ABA receptor agonist specific to seeds in Arabidopsis with some agonistic activity to PYL2. Inhibits germination in Arabidopsis[2].	[1]Nambara *et al.*, (2010) [2](Park *et al.*, 2009)
(b) PYL Antagonists			
3′-alkylsulfanyl ABA (AS_n)	AS_6	Modified ABA, S-hexyl chain blocks protein phosphatase 2C (PP2C) binding by steric hindrance. AS_6 recovers ABA-induced germination inhibition in Arabidopsis[1].	[1](Takeuchi *et al.*, 2014)
AA1	(+)-PAO4	(+)-PAO4 is a modified ABA, S-hexyl chain blocks PP2C binding by steric hindrance. (+)-PAO4 recovers ABA-induced germination inhibition in Arabidopsis, having a stronger effect in *Latuca sativa*[1]. AA1 recovers ABA-induced germination inhibition in Arabidopsis. Broadly interacts across many PYL receptors[2].	[1](Takeuchi *et al.*, 2015) [2](Ye *et al.*, 2017)

antagonist in lettuce (Takeuchi *et al.*, 2015). More recently, 4'-modified ABAs, such as 4′-*O*-phenylpropynyl ABA analogs, have been developed showing promising improvements in antagonistic activity over AS_6 in Arabidopsis seed germination (Takeuchi *et al.,* 2018) Aside from rational design, PYL antagonists such as ABA Antagonist1 (AA1) have been developed using chemical screens that have a lower physiological activity against ABA-inhibited seed germination in Arabidopsis, however are antagonistic to a broader range of PYLs (Ye *et al.*, 2017).

Table 3.4. Carotenoid Biosynthesis Inhibitors.

Carotenoid Biosynthesis Inhibitors			
Bleaching Herbicides			
Trifluoromethyl-phenylpyridones 	Fluridone 	Bleaching herbicide. Phytoene detasurase (PDS) inhibitor[1] that inhibits ABA systhesis by reducing precursor accumulation[2]. Releases thermos-inhibition in *L. sativa*[3] and Arabidopsis[4]. Breaks dormancy in *Lolium ridgium* in the field at 367 g ha^{-1} [5].	[1](Bartels & Watson, 1978) [2](Gamble & Mullett, 1986) [3](Yoshioka *et al.*, 1998) [4](Toh *et al.*, 2008) [5](Goggin & Powles, 2014)
Trifluoromethylphenyl aminopyridazones 	Norflurazone 	Bleaching herbicide and PDS inhibitor[1]. Inhibits ABA biosynthesis and overcomes light requirement for germination in *L. sativa*[2]. Breaks dormancy in Arabidopsis[3].	[1](Bartels & Watson, 1978) [2](Roth-Bejerano *et al.*, 1999) [3](Debeaujon *et al.*, 2000)
Dioxocyclohexanecarbaldehydes 	Mesotrione 	Bleaching herbicide inhibiting 4-hydroxyphenyl- pyruvate (HPPD) inhibiting plastiquinone synthesis resulting in a decrease in carotenoids[1].	[1](Mitchell *et al.*, 2001)

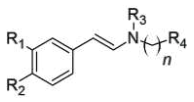
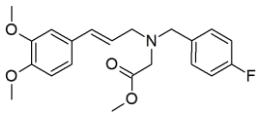
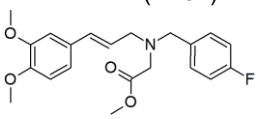
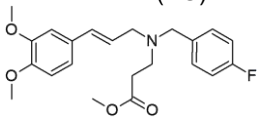
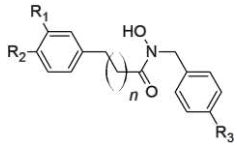
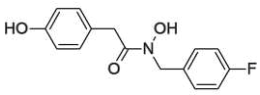
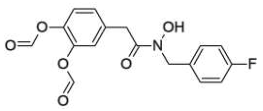
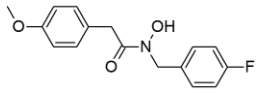
Inhibition of ABA biosynthesis is also a well described target for germination stimulants. Many of the components of ABA biosynthesis were discovered in carotenoid

biosynthesis mutants that had an albino phenotype and viviparous germination (McCarty, 1995). The *Phytoene Desaturase* (*PDS*) mutant *pds3*, that acts upstream of carotenoid biosynthesis converting phytoene to ζ-carotene, shows reduced carotenoid and ABA content and interestingly also impairs gibberellin biosynthesis (Qin *et al.*, 2007). A large number of PDS inhibitors, such as fluridone and norflurazone (Table 3.4) have been commercialised as bleaching herbicides for weed control (Bartels & Watson, 1978).

The downstream inhibition of ABA biosynthesis that occurs due to carotenoid biosynthesis inhibition (Gamble & Mullett, 1986) makes PDS inhibitors useful promoters of germination under conditions where ABA biosynthesis is limiting germination, for example during thermoinhibition at high temperatures (Yoshioka *et al.*, 1998; Toh *et al.*, 2008). It has also been suggested that PDS inhibitors are useful tools for WSB management due to their combined germination stimulant and herbicidal activities (Goggin & Powles, 2014). An additional group of bleaching herbicides that inhibit 4-hydroxyphenyl-pyruvate dioxygenase (HPPD) such as Mesotrione (Table 4), also inhibit carotenoid biosynthesis through inhibition of plastiquinone production that is required as a cofactor for PDS activity (Mitchell *et al.*, 2001) and have untested potential as germination stimulants.

Acting downstream of carotenoids in ABA biosynthesis are the carotenoid cleavage dioxygenases (CCDs), such as 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) that cleave the *cis*-isomers of violaxanthin and neoxanthin to produce xanthoxin (Nambara & Marion-Poll, 2005). NCEDs are a key rate limiting step for ABA production in seeds (Nambara *et al.*, 2010), are highly expressed during thermoinhibition in Arabidopsis (Toh *et al.*, 2008) and overexpression of tomato NCEDs generates a dormant phenotype (Thompson *et al.*, 2000). Inhibition of NCED activity is therefore an

Table 3.5. Inhibitors of NCED activity.

Inhibition of NCED activity			
(a) Phenylethylamines			
Generic 	Abamine 	Competitive inhibitor of NCED[1] that decreases ABA accumulation and enhances germination in *Lepidium sativum*[1], Arabidopsis C24[2] and barley[3].	[1](Han *et al.*, 2004) [2](Li *et al.*, 2012) [3](Yoshida & Asami, 2006)
Abamine (SY97) 	Abamine (SG) 	An abamine derivative having a greater inhibitory effect on ABA accumulation in Arabidopsis seedlings than SY97[1].	[1](Kitahata *et al.*, 2006)
(b) Hydroxamic Acids			
Generic 	D2 	Inhibitors of carotenoid cleavage dioxygenases (CCDs) including NCEDs[1,3]. Aryl-C_2N hydroxamic acids (including D1-7) are strong CCD inhibitors[1,3]. D4 releases *L.sativa* thermoinhibition[2] and D4 and D7 induces germination in tomato NCED overexpression line[2]. D4 reduces ABA accumulation in tomato seeds[2]. D2 and D4 have a weak inhibitory effect on *Z. mays* NCEDs[4].	[1](Sergeant *et al.*, 2009) [2](Awan *et al.*, 2017) [3](Harrison, 2014) [4](Chandler, 2015)
D7 	D4 		

interesting target for dormancy breaking compounds through inhibition of ABA accumulation. CCDs are also involved in a number of other plant metabolic processes, such as strigolactone biosynthesis, therefore the selectivity of inhibitors towards NCEDs is an important factor determining their applicability as germination stimulants (Villedieu-Percheron *et al.*, 2014).

Two classes of CCD inhibitors with specificity to NCEDs are phenylethylamines and hydroxamic acids (Table 5). Abamine (SY97) (ABA biosynthesis inhibitor with an amine moiety) is a phenylethylamine NCED inhibitor that acts as a competitive inhibitor for the

NCED catalytic site that was shown to reduce ABA contents in leaves and promote radicle growth in germinating cress seeds (Han *et al.*, 2004). SY97 was also shown to improve germination in barley (*Hordeum vulgare* L.) and in the Arabidopsis C24 ecotype (Yoshida & Asami, 2006; Li *et al.*, 2012). Structure-activity studies with abamine generated a number of structures with improved NCED inhibition activity such as abamineSG that has greater inhibitory effect on ABA accumulation in Arabidopsis seedlings than SY97 (Kitahata *et al.*, 2006).

Inhibitors with greater specificity for CCDs involved in strigolactone biosynthesis have also been developed based on the abamine structure (Kitahata *et al.*, 2011). More recently, structures similar to abamine, but with a hydroxamic acid linker between the substituted phenyl rings, have been described as potent CCD inhibitors (Sergeant *et al.*, 2009). In a comparison between tomato CCD1 and NCED1, aryl-C_1N hydroxamic acids (HAs), named F1-4, are strong inhibitors of CCD1 and not NCED1, aryl-C_2N HAs (named D1-7) and aryl-C_3N HAs (D8-13) all inhibit CCD1 and show varying degrees of NCED inhibition (Sergeant *et al.*, 2009). D4 releases thermoinhibition in lettuce while D4 and D7 induce germination in tomato *NCED* overexpression lines that otherwise have a dormant phenotype through increased ABA accumulation (Awan *et al.*, 2017).

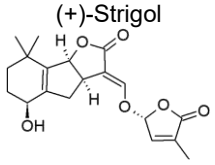
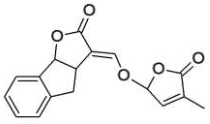
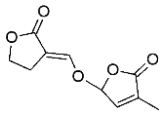
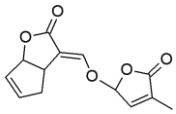
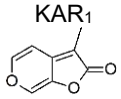
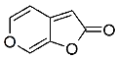
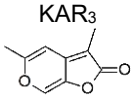
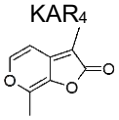
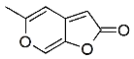
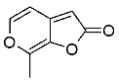
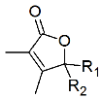
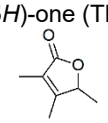
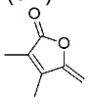
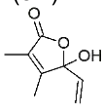
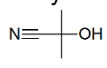
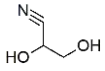
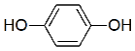
3.2.5 Smoke-derived compounds and strigolactones

Fire plays an important role in plant community succession by providing a recolonisation opportunity for plants from the removal of the leaf canopy and changes in soil chemistry. Consequently wildfires are often followed by flushes of germination and plant establishment (Brown & Smith, 2000). This observation led to the discovery that smoke was able to break dormancy and stimulate germination in some species (de

Lange & Boucher, 1990) and that some species even require fire cues for the completion of their lifecycle (Flematti *et al.*, 2013). The active substances produced by burning cellulose are extractable in water to produce 'smoke-water' (van Staden *et al.*, 1995), a complex mixture of compounds that has been found to stimulate germination in an estimated 1200 plant species (Flematti *et al.*, 2013; Jefferson *et al.*, 2014). Interestingly, many species that respond to smoke as a germination cue do not inhabit fire-prone environments indicating that their response to smoke is not an adaptation to a specific environment but rather occurs through a conserved mechanism. However in general, plants whose germination responds to smoke tend to be annuals with dormant and persistent seeds that prefer disturbed habitats (Mojzes *et al.*, 2015). Smoke applications are now commonly used in restoration ecology and in horticulture to improve germination and seedling establishment (Kulkarni *et al.*, 2011).

Bio-assay guided fractionation of smoke-water identified a potent germination stimulator, karrikin 1 (KAR_1) that stimulated germination of some fire-responsive species in the sub-nanomolar range (Flematti *et al.*, 2004a,b, 2005). Karrikins have structural similarity with the strigolactone family of phytohormones, both having butenolide and unsaturated ester moieties leading to the assumption that karrikins function through strigolactone signalling. This hypothesis was supported by evidence that strigolactone responsive parasitic *Striga* and *Orobanche spp.* respond to both low concentrations of KAR_1 and the synthetic strigolactone GR-24 (Daws *et al.*, 2008). However some smoke-responsive species do not respond to strigolactone application leading to the idea that strigolactones act through related but not identical signalling pathways (Chiwocha *et al.*, 2009; Nelson *et al.*, 2009). This observation was confirmed by the identification of the karrikin insensitive mutant *kai2* that is insensitive to KAR_1 but retains sensitivity to strigolactones (Waters et al., 2012a, b). In Arabidopsis,

Table 3.6. Strigolactones and smoke-derived compounds

Strigolactones & Smoke-Derived Compounds		
(a) Strigalactones (+)-Strigol  GR-24  GR5  GR7 	Strigol initiates germination in Striga sp. Parasitic weeds[1] and strigol analogs promote germination in some weed species[2]. GR24 overcomes thermoinhibition of germination in Arabidopsis[3].	[1](Cook *et al.*, 1966) [2](Pepperman & Bradow, 2016) [3](Toh *et al.*, 2012)
Smoke-Derived Compounds		
(b) Karrikinolides KAR_1  KAR_2  KAR_3  KAR_4  KAR_5  KAR_6 	Smoke is a well-known stimulator of germination. A bio-assay guided fractionation of smoke identified KAR_1 as a germination stimulant for *L. sativa*[1]. KAR_1 is the most abundant in smokewater, however structure activity analysis[2,3] demonstrated that KAR_2 is more potent in Arabidopsis[4]. Karrikiolides are perceived by KAI2, and share similar signalling components to strigolactone signalling however the physiological response of karrikin and GR24 treatments are distinct[5].	[1](Flematti *et al.*, 2004b) [2](Flematti *et al.*, 2007) [3](Flematti *et al.*, 2010) [4](Nelson *et al.*, 2009a) [5](Morffy *et al.*, 2016)
(c) Trimethylbutenolides Generic  3,4,5-trimethylfuran-2(5*H*)-one (TMB)  3,4-dimethyl-5-methylidenefuran-2(5*H*)-one  5-ethenyl-5-hydroxy-3,4-demethylfuran-2(5*H*)-one 	Another bioassay driven fraction of smoke identified TMB as a potent germination inhibitor[1] of *L. sativa*. A number of active analogs have been identified[2]. It has been proposed that KAR_1 and TMB do not compete for the same active site[3]. TMB also inhibits germination in a number of weed species[4].	[1](Light *et al.*, 2010) [2](Pošta *et al.*, 2013) [3](Soós *et al.*, 2012) [4](Papenfus *et al.*, 2015)
(d) Cyanohydrins Generic  Acetone cyanohydrin  Mandelonitrile  Glyceronitrile  Hydroquinone 	Cyanohydrins are cyanogenic compounds present both endogenously in plants and in smoke that can cause dormancy release and stimulate germination indirectly through cyanide evolution[1]. Smoke derived compound that stimulates germination in *L. sativa* at low concentrations[2].	[1](Flematti *et al.*, 2013) [2](Kamran *et al.*, 2017)

strigolactones are perceived by a KAI2 ortholog D14 and a hydrolase DAD2 that most likely degrades strigolactones during perception (Hamiaux *et al.*, 2012).

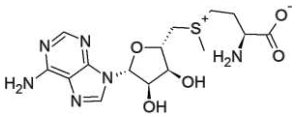
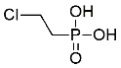
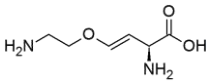
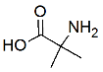
Additional smoke-fractionation experiments identified fractions with germination inhibitory properties (van Staden *et al.*, 2004). The structure of this inhibitory molecule was later identified as a racemic compound 2,3,4-trimethylbut-2-enolide (Trimethylbutenolide, TMB, Table 3.6c) (Light *et al.*, 2010). TMB inhibits the germination of lettuce as well as a number of weed species (Papenfus *et al.*, 2015) and a range of synthesised analogs also have germination inhibitory activity (Pošta *et al.*, 2013). Physiological and transcriptomic analysis of lettuce treated with KAR_1 and TMB indicated that these compounds do not compete for the same binding site (Soós *et al.*, 2012).

After the purification of active compounds from smoke it was noted that a number of smoke-responsive species were not responsive to KAR_1 (Flematti *et al.*, 2013). The application of additional bioassay-driven fractionations of smoke on these species identified cyanohydrins, such as glyceronitrile, (Table 3.6d) as potent germination stimulants that were less species specific (Flematti *et al.*, 2011). Cyanohydrins spontaneously hydrolyse to form cyanide, a known germination stimulant already discussed in section 3.2.2 of this Chapter.

3.2.6 Compounds targeting ethylene and metabolism and signalling

Much of the research on seed dormancy and germination has focused on the antagonistic relationship of GA and ABA. However other phytohormones such as ethylene also have an important role in the regulation of germination (Linkies &

Table 3.7. Ethylene agonists and biosynthesis inhibitors.

Ethylene Agonists and Biosynthesis Inhibitors			
(a) Ethylene Precursors			
S-adenosylmethionine (SAM) 	1-aminocyclopropane-1-carboxylic acid (ACC) 	SAM and ACC are the direct precursors of ethylene[1]. Inhibition of germination by ABA can be reverted by ACC application in *Lepidium sativum*[2].	[1](Rzewuski & Sauter, 2008) [2](Graeber *et al.*, 2010)
(b) Ethylene Signalling Molecules			
Ethylene 	Ethephon 	Ethylene is a gaseous phytohormone that stimulates germination through interaction with ABA signalling pathways[1,2,3]. Ethylene may also have some role in dormancy breaking[4]. Ethephon, a commercial plant growth regulator, is metabolised in plants to produce ethylene[5] and stimulates germination in a number of weed species[6].	[1](Linkies *et al.*, 2009) [2](Beaudoin *et al.*, 2007) [3](Ghassemian *et al.*, 2000) [4](Matilla-Vázquez, 2008) [5](Calvo *et al.*, 2004) [6](Carmona & Murdoch, 1995)
(c) Ethylene Biosynthesis Inhibitors			
2-aminoethoxyvinyl glycene (AVG) 	Aminoisobutyric Acid (AIB) 	There are many ethylene signalling and biosynthesis inhibitors due to their commercial applicability in delaying fruit ripening[1]. AVG[2] is an ACS inhibitor, and AIB inhibits ACC[3].	[1](Schaller & Binder, 2017) [2](Yu & Yang, 1979) [3](Sato & Esashi, 1980)
(d) Ethylene Signaling Antagonists			
trans-cyclooctene (TCO) 	1-methylcyclopropene (1-MCP) 	TCO[4], 1-MCP[5] and NBD[6] are gaseous ethylene receptor antagonists.	[4](Sisler *et al.*, 1990) [5](Sisler & Blankenship, 1998) [6](Sisler *et al.*, 1986)
2,5-norbornadiene (NBD) 		Inhibition of ethylene biosynthesis and signalling typically inhibits germination[7].	[7](Corbineau *et al.*, 2014)

Leubner-Metzger, 2012). Ethylene (Table 3.7b) is a gaseous hormone, produced as a by-product the methionine cycle (Yang & Hoffman, 1984), which interestingly inhibits growth of seedlings but promotes seed germination. During germination ethylene interacts with ABA and GA signalling pathways (Ghassemian *et al.*, 2000; Matilla &

Matilla-Vázquez, 2008; Linkies *et al.*, 2009). In Arabidopsis, mutants of the ethylene receptor *etr2* are hypersensitive to ABA application during germination (Beaudoin *et al.*, 2007). The inhibitory effect of exogenous ABA application on cress germination can also be reverted by the application of 1-amino-cyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene (Graeber *et al.*, 2010). Ethephon (Table 3.7b), a commercial plant growth regulator and promoter of fruit ripening (EPA, 1995) that is metabolised in plants to produce ethylene (Calvo *et al.*, 2004), stimulates the germination of a number of weed species (Carmona & Murdoch, 1995). Due to the commercial importance of controlling fruit ripening a number of ethylene biosynthesis and signalling inhibitors have been developed. Two key ethylene biosynthesis genes upregulated during germination and fruit ripening are ACC synthase (ACS) and ACC oxidase (ACO) (Linkies & Leubner-Metzger, 2012).

Application of 2-aminoethoxyvinyl glycene (AVG, an ACS inhibitor) or aminoisobutyric acid (AIB, an ACC inhibitor) inhibit germination in a number of species (Corbineau *et al.*, 2014). Inhibition of ethylene signalling has also been exploited by commercial gaseous ethylene receptor (ETR) antagonists such as *trans*-cyclooctane (TCO) (Sisler *et al.*, 1990), 1-methylcyclopropene (1-MCP) (Sisler & Blankenship, 1998) and 2,5-norbornadiene (NBD) (Sisler *et al.*, 1986) (Table 7c). ETR antagonists such as NBD inhibit germination in cress; an effect that is partially restored by ACC application (Linkies *et al.*, 2009).

3.2.7 Aims and objectives

Having produced an up-to-date summary of compounds that have germination stimulatory or inhibitory effects on model and crop species the effectiveness of a selection of these compounds in breaking the primary dormancy in grass weed seeds was tested. Working mostly with *A. myosuroides* the aim was to identify compounds that have a dormancy breaking activity. The project then aims to identify how conserved these dormancy breaking responses are by screening compounds across four additional weed species with differing life history traits (*A. fatua, D. sanguinalis, P, annua* and *S. faberi*). An additional aim is to identify how batch effects, such as dormancy level and after-ripening affect the responses of grass weed seeds to lead compounds by using both batches with defined dormancy levels (dormant, $AR_{50\%}$ and $AR_{100\%}$) and differing genotypes. By identifying dormancy breaking compounds the aim is to gain insight into the mechanisms regulating dormancy in these species.

3.3 Results

3.3.1 Compounds targeting gibberellin metabolism and signalling

Exogenous application of gibberellins is a standard method used to break non-deep physiological dormancy (Baskin & Baskin, 2014). Application of GA_3 and GA_{4+7} to *A. myosuroides* (LH170) had only a small dormancy breaking effect at the highest concentrations (50 & 100 µM) that was greater for GA_{4+7} (Fig. 3.1a,b). After-ripening this batch for 120 days moderately increased sensitivity to exogenous GA application (Fig. 1d,e). This effect of gibberellin application appears minimal, when compared to Arabidopsis ecotypes with impaired GA biosynthesis and completely inhibited germination, that require only 1 µM GA_{4+7} to reach complete germination within the population (Debeaujon & Koornneef, 2000).

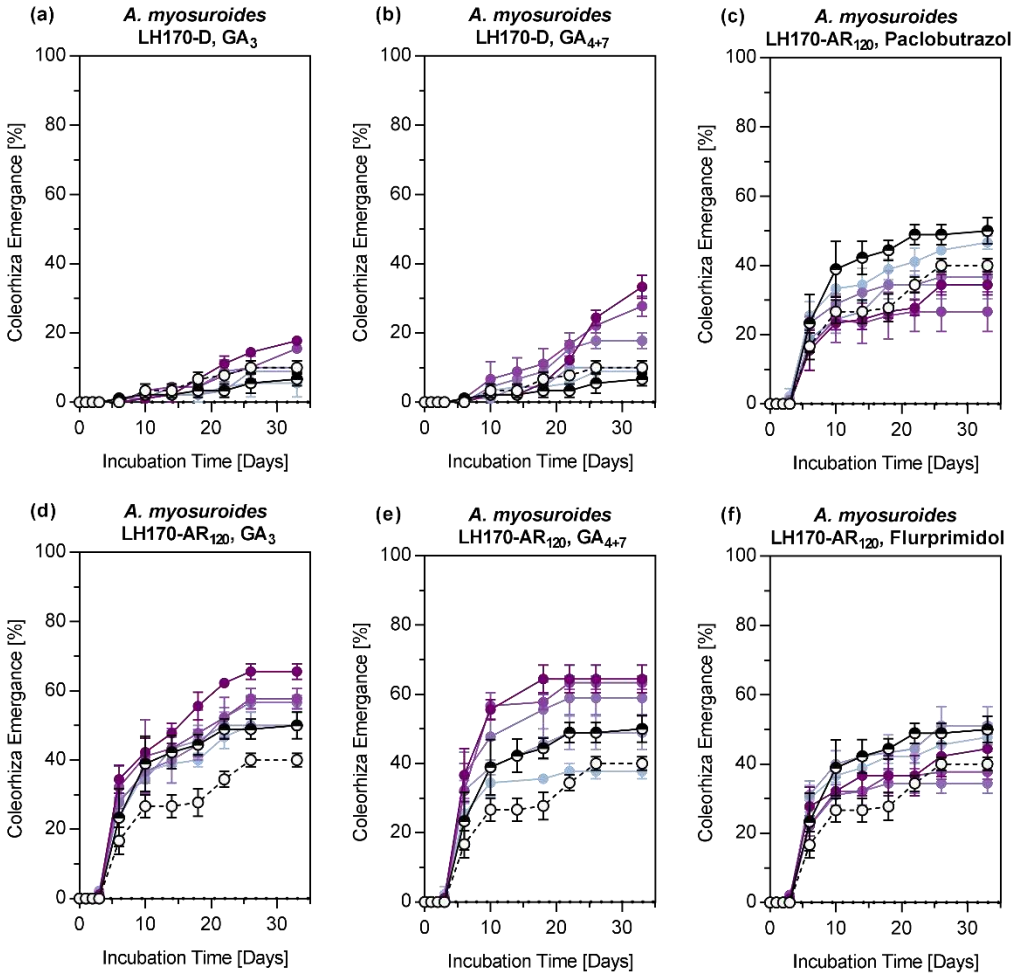


Fig. 3.1. The effect of GA and GA biosynthesis inhibitors on *A. myosuroides* germination. The effect of endogenous gibberellins, GA_3 (a,d) and GA_{4+7} (b,e) on the germination of dormant (a,b) and 120 day after ripened LH170. (c,f) The effect of gibberellin biosynthesis inhibitors paclobutrazol (c) and flurprimidol (f) on the germination of 120 day after-ripened LH170. (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 20°C under constant light.

Application of the KO inhibitors paclobutrazol and flurprimidol show a tendency towards inhibition at the highest concentrations which again seems minimal compared to the complete inhibition achieved in Arabidopsis by 1 µM paclobutrazol (Debeaujon & Koornneef, 2000). Taken together these results suggest that *A. myosuroides* germination is not dependant on *de novo ent*-kaurenoic acid biosynthesis or that paclobutrazol and flurprimidol are ineffective inhibitions of *A. myosuroides* KO. Interestingly, despite the ineffectiveness of GA_3 and GA_{4+7} at breaking *A. myosuroides*

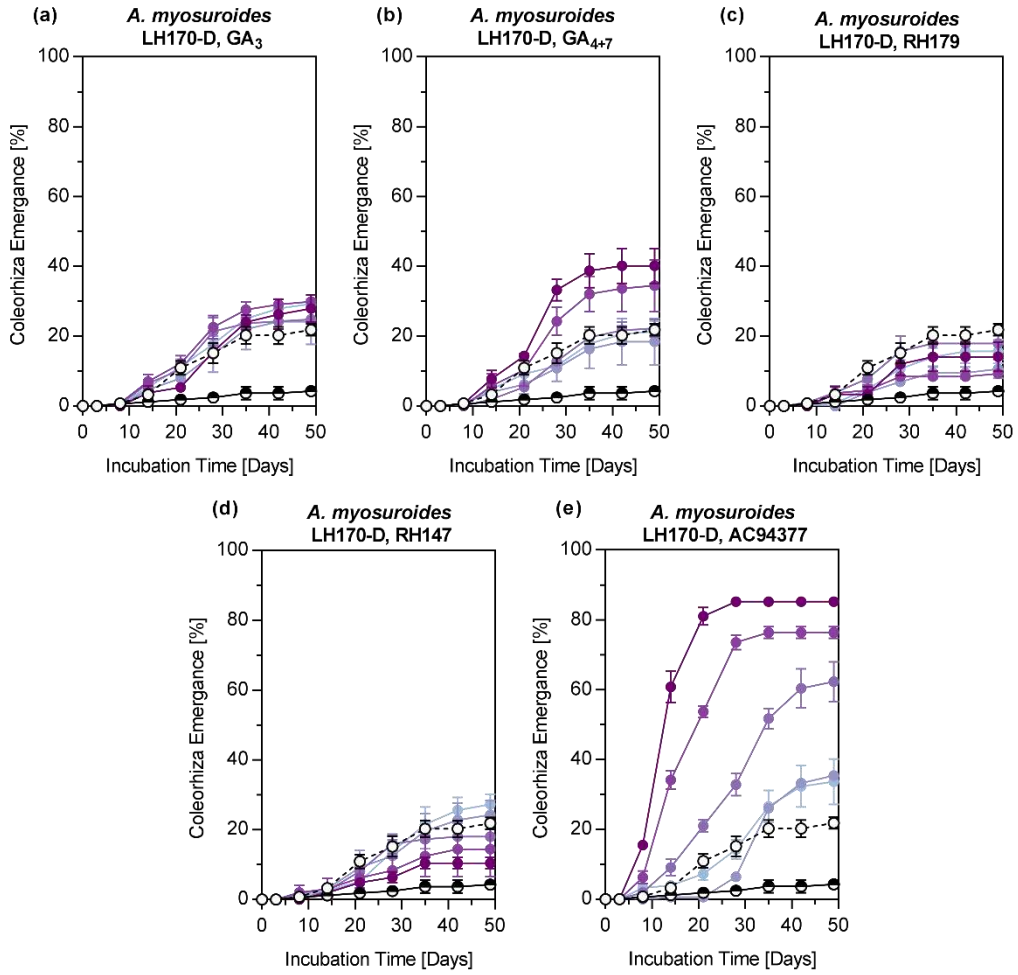


Fig. 3.2. The effect of GAs and phthalimide lactone analogs on dormant *A. myosuroides* germination. The effect of endogenous gibberellins, GA_3 (a) and GA_{4+7} (b) and N-substituted phthalimide lactone analogs (c-e) including AC94377 (e) on the germination of dormant *A. myosuroides* LH170. (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light.

dormancy, the N-substituted phthalimide lactone AC-94377 had a strong dormancy breaking effect achieving double the maximum germination reached by GA_{4+7} application (Fig. 3.2e). Other phthalimide lactone derivatives tested (structures not shown) had no significant dormancy breaking effect (Fig. 3.2c,d). AC-94377 is a GID1 agonist with high specificity in Arabidopsis, so this result suggests that the gibberellin signalling machinery is present in dormant *A. myosuroides* seeds that not respond

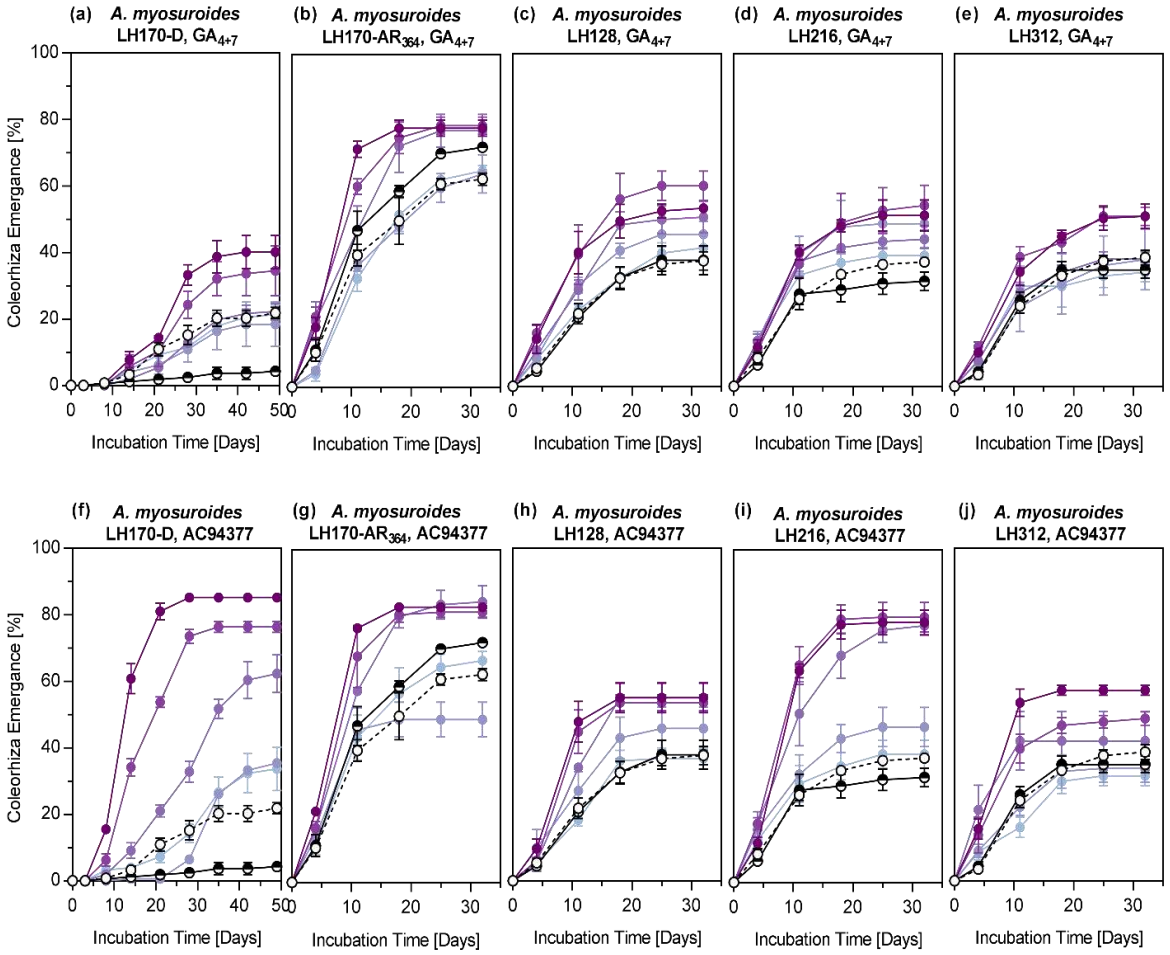


Fig. 3.3. Comparative effects of GA_{4+7} and AC-94377 across different *A. myosuroides* batches. (-○-, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light.

strongly to GA_3 or GA_{4+7} or that the effect is achieved through a different pathway. Additionally, inhibitors of GA2-oxidase has no dormancy breaking effect in *A. myosuroides* (Appendix Fig. 9.5) indicating that degradation of bioactive GAs was unlikely to be mechanism of dormancy enforcement.

Comparisons of the germination stimulant effect of AC-94377 and GA_{4+7} were performed using a 364 day after-ripened *A. myosuroides* batch (LH170-AR_{364}) and three additional *A. myosuroides* batches with intermediate dormancy levels. Whilst in

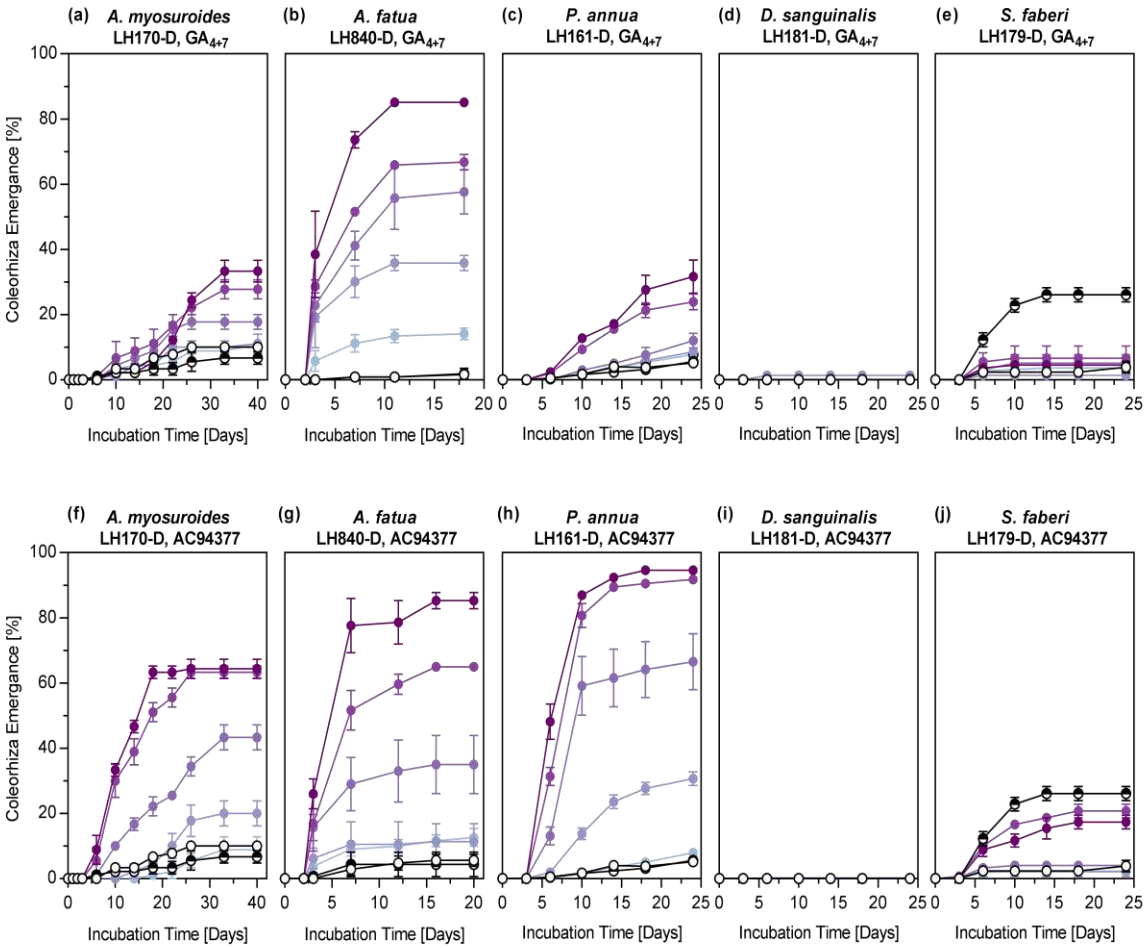


Fig. 3.4. Comparative effect of GA_{4+7} and AC-94377 across dormant batches of five grass weed species. (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 20°C under constant light.

the dormant batch (LH170-D) AC-93477 had a considerably stronger effect than GA_{4+7}, this difference in effect was lost after 364 days of after-ripening (Fig. 3.3g). The

additional seed batches responded to GA_{4+7} and AC-94377 similarly (Fig. 3.1c,e,h,j) with the exception of LH216 that responded more strongly to AC94377 (Fig. 3.3d,i). These results demonstrate that the relative effect of AC-94377, compared to GA_{4+7}, is comparatively dormancy-specific in *A. myosuroides*.

A comparison of the effects of GA_{4+7} and AC-94377 across five grass weed species showed that the species tested had very different sensitivities to both compounds (Fig.

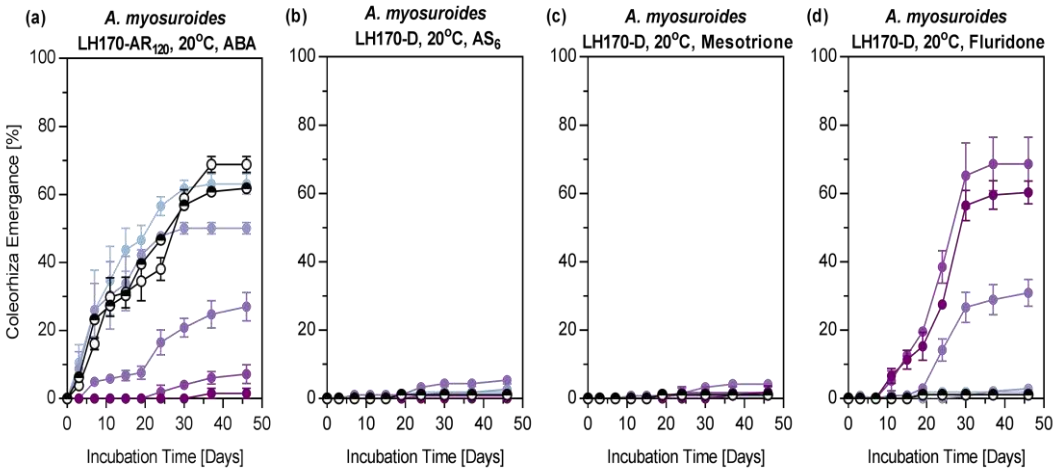


Fig. 3.5. **Effect of AS6, fluridone and mesotrione on dormant *A. myosuroides* germination**. (a) ABA inhibits germination in 120 day after ripened A. myosuroides (LH170-AR120). (b-d) The effect of AS6 (b) fluridone (c) and mesotrione (d) on the germination of dormant *A. myosuroides* (LH170-D). . (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 20°C under constant light.

3.4). In general species that responded to GA_{4+7} (*A. myosuroides, A. fatua* and *P. annua*) also responded to AC-94377 (Fig. 3.3a-c,f-h) and species that did not respond to GA_{4+7} also did not respond to AC-94377 (*D. sanguinalis* and *S. faberi*) (Fig. 3d,e,l,j). However for those species that did respond to AC94377, the sensitivity to GA_{4+7} did not determine the response to AC-94377. For example, *P. annua* had low sensitivity to GA_{4+7} compared to *A. fatua* yet responded strongly to AC-94377 whereas *A. fatua* was more sensitive to GA_{4+7} than AC-94377.

3.3.2 Compounds targeting abscisic acid metabolism and signalling

Abscisic acid inhibits the germination of after-ripened *A. myosuroides* at concentrations above 1 µM (Fig. 3.5a). The PYL antagonist AS_6 did not break dormancy at any of the concentrations tested indicating either that at the concentrations tested it does not

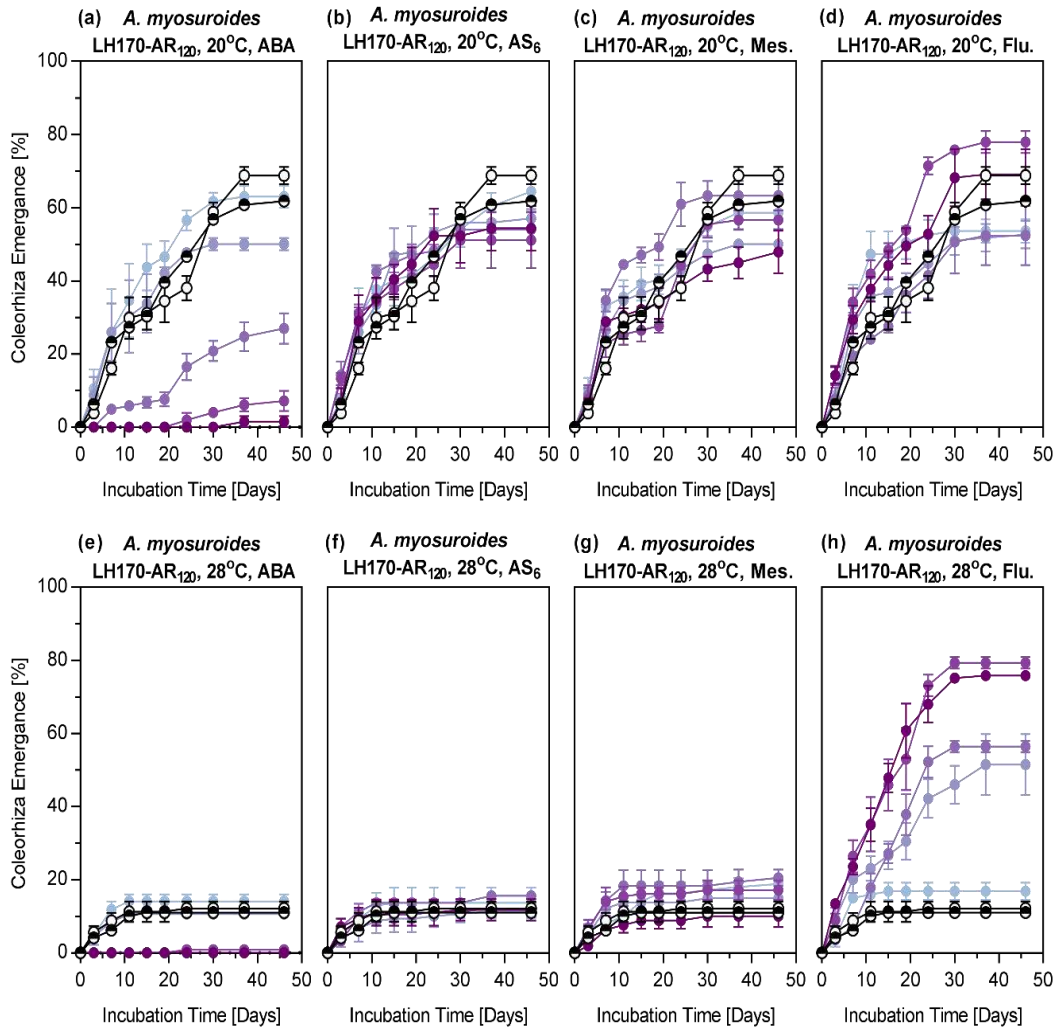


Fig. 3.6. Effect of ABA, AS_6, mesotrione and fluridone on thermoinhibited *A. myosuroides*. 120 day after-ripened A. myosuroides (LH170-AR_{120}) incubated at either 20°C (a-d) or 28°C (e-h) in constant light with ABA (a,e), AS6 (b,f), mesotrione (c,g) or fluridone (d,h). (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds.

inhibit the formation of *A. myosuroides* PYL-PP2C complexes or that ABA signalling does not have an important role in the enforcement of germination (Fig. 3.5b). The

PDS inhibiting beaching herbicide fluridone was an effective dormancy breaking treatment at concentrations above 10 µM (Fig. 3.5d) however the HPPD inhibitor mesotrione was not an effective dormancy breaking treatment (Fig. 3.5c). Whilst HPPD

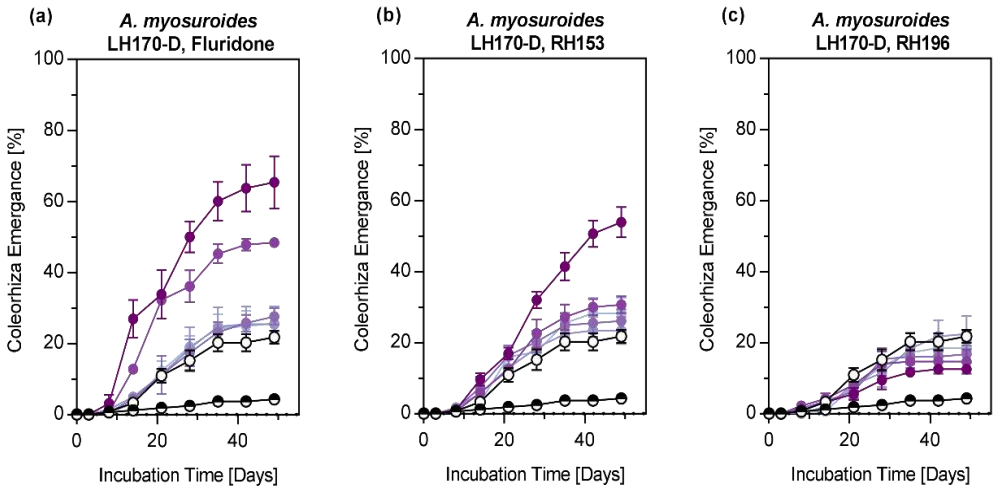


Fig. 3.7. Dormancy breaking effects of fluridone analogs on *A. myosuroides*. The effect of fluridone (a) and two fluridone analogs RH153 (b) and RH196 (c) on dormant *A. myosuroides* (LH170-D). (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light.

plastiquinone biosynthesis (Mitchell *et al.*, 2001), seedlings treated with up to 100 µM did not have symptomatic bleaching (data not shown) indicating some degree of resistance to this herbicide in the *A. myosuroides* batch used.

PDS inhibitors are able to release the thermodormancy induced by high temperature incubation in Arabidopsis and lettuce by reducing the accumulation of ABA (Yoshioka *et al.*, 1998; Toh *et al.*, 2008). In 120 day after-ripened *A. myosuroides* (LH170-AR_{120}) 28°C is an inhibitory temperature for germination and application of ABA greater than 1µM completely inhibit germination at this temperature (Fig. 3.6e). AS_6 did not appreciably affect germination at the relatively optimal temperature of 20°C (Fig. 3.6b) and failed to release thermoinhibition (Fig. 3.6f). Mesotrione had a tendency to inhibit germination at high concentrations in the after-ripened batch at 20°C (Fig. 3.6c) and did not release thermodormancy (Fig. 3.6g). Fluridone had a small stimulatory effect on

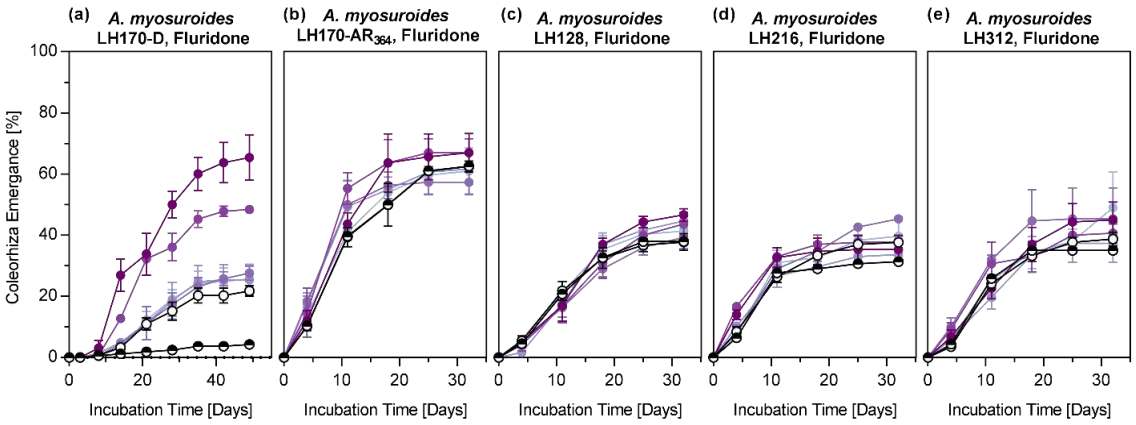


Fig. 3.8. Effect of fluridone across *A. myosuroides* batches. Fluridone effect on *A. myosuroides* batches with known dormancy level: dormant (a, LH170-D) fully after ripened (b, LH170-AR_{364}) and batches with intermediate dormancy levels LH128 (c), LH216 (d) and LH312 (e). (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light.

germination on after-ripened at 20°C (Fig. 3.6d) and was an effective thermodormancy releasing treatment (Fig. 3.6h) indicating that *de novo* biosynthesis of ABA is involved in the inhibition of germination at high temperatures in *A. myosuroides*. The observation that fluridone releases thermo- and primary dormancy (Fig. 3.5d) and AS_6 does not release either dormancy, suggests that ABA biosynthesis is involved in both processes and AS_6 is an ineffective inhibitor of ABA signalling in *A. myosuroides* at the concentrations tested. Dose-responses of two additional fluridone analogs (RH153 and RH196) demonstrated that fluridone had the strongest dormancy breaking effect in *A. myosuroides* above 10 µM (Fig. 3.7a). Whilst RH196 had no dormancy breaking effect (Fig. 3.7c), RH153 had a dormancy breaking effect that was 10-fold lower than fluridone (Fig. 3.7b).

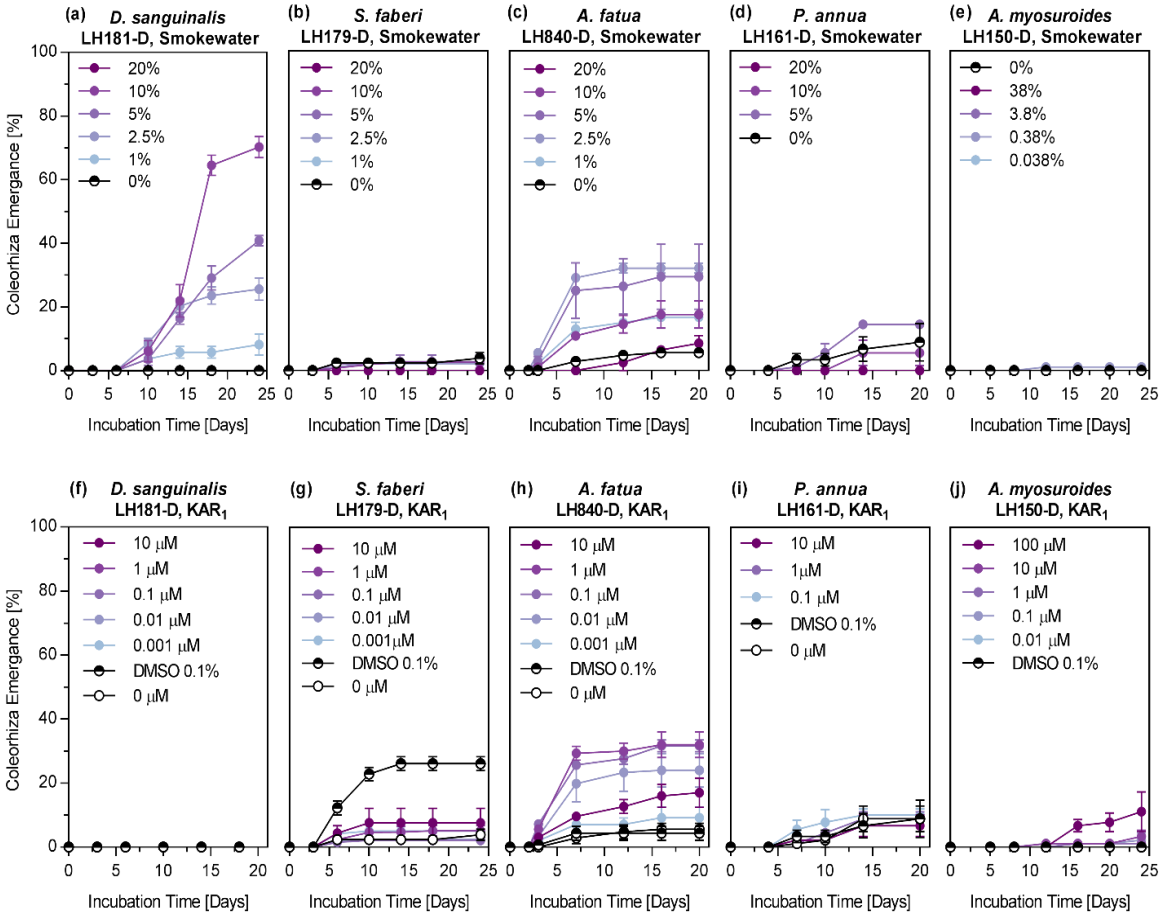


Fig. 3.9. Effect of smoke-water and KAR_1 on dormant grass weed seeds. Effect of smoke-water (Regen2000 Smokemaster™) (a-e) and KAR_1 (f-j) on dormant *D. sanguinalis* LH181-D (a,f), *S. faberi* LH179-D (b,g) *A. fatua* LH840-D (c,h), *P. annua* LH161-D (d,i) and *A. myosuroides* LH150-D (e,j). Dilutions of smoke-water are shown as a percentage dilution of volume per volume (%v/v). Error bars show standard error of the mean for triplicates of >30 seeds, incubated at 20°C under constant light.

A comparison of the effect of fluridone across *A. myosuroides* seed batches demonstrated that the stimulatory effect of fluridone was only present in the fully dormant seed batch (Fig. 3.8a). After-ripening this batch for 364 days ($LH170\text{-}AR_{364}$) minimised the stimulatory effect of fluridone (Fig. 3.8b). Fluridone did not stimulate the germination of the additional three *A. myosuroides* batches that had intermediate levels of dormancy, where as other compounds such as AC-94377 did under the same

conditions (Fig. 3.3). This suggests that *de novo* ABA biosynthesis is only a significant limitation on germination in batches where the dormancy level is high.

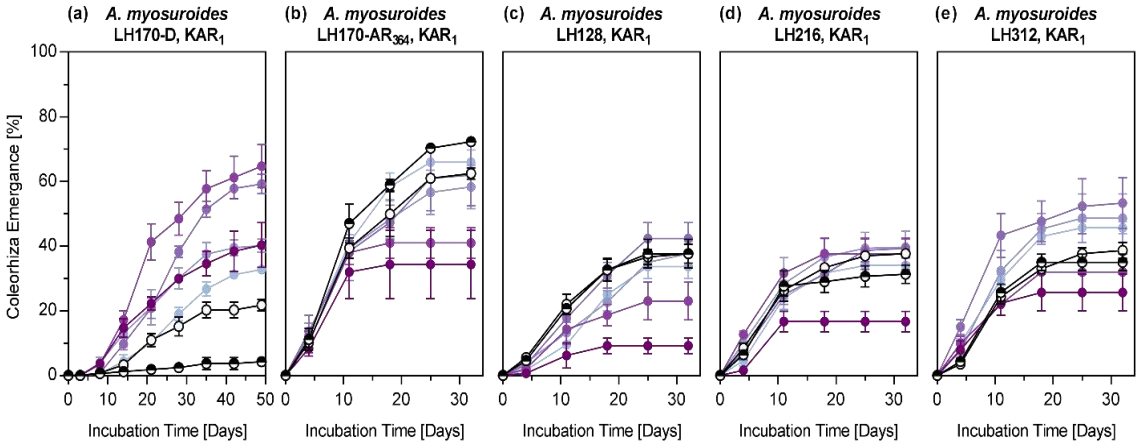


Fig. 3.10. The effect of KAR_1 across different *A. myosuroides* batches. KAR_1 effect on *A. myosuroides* batches with known dormancy level: dormant (a, LH170-D) fully after ripened (b, LH170-AR_{364}) and batches with intermediate dormancy levels LH128 (c), LH216 (d) and LH312 (e). (○, H_2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light.

3.3.3 The effect of smoke-derived compounds on grass weed seed germination

Smoke-water is a commonly used treatment to improve seed germination in horticulture and conservation (Kulkarni *et al.*, 2011) due to the ability of smoke to stimulate the germination of many species that do not inhibit fire prone habitats (Mojzes *et al.*, 2015). Research into the effects of smoke on germination in a variety of species is highly prolific (Jefferson *et al.*, 2014) yet the effect of smoke on many species remains ambiguous. For the example of *A. myosuroides,* some authors report that KAR_1 and smoke-water inhibit germination (Daws *et al.*, 2007) and others report that germination is promoted by smoke-water (Adkins & Peters, 2001). The same pattern has been observed in a number of agricultural weeds in the literature, including *A. fatua*

(Adkins & Peters, 2001; Kępczyński *et al.*, 2006; Daws *et al.*, 2007; Stevens *et al.*, 2007; Cembrowska-Lech & Kępczyński, 2017).

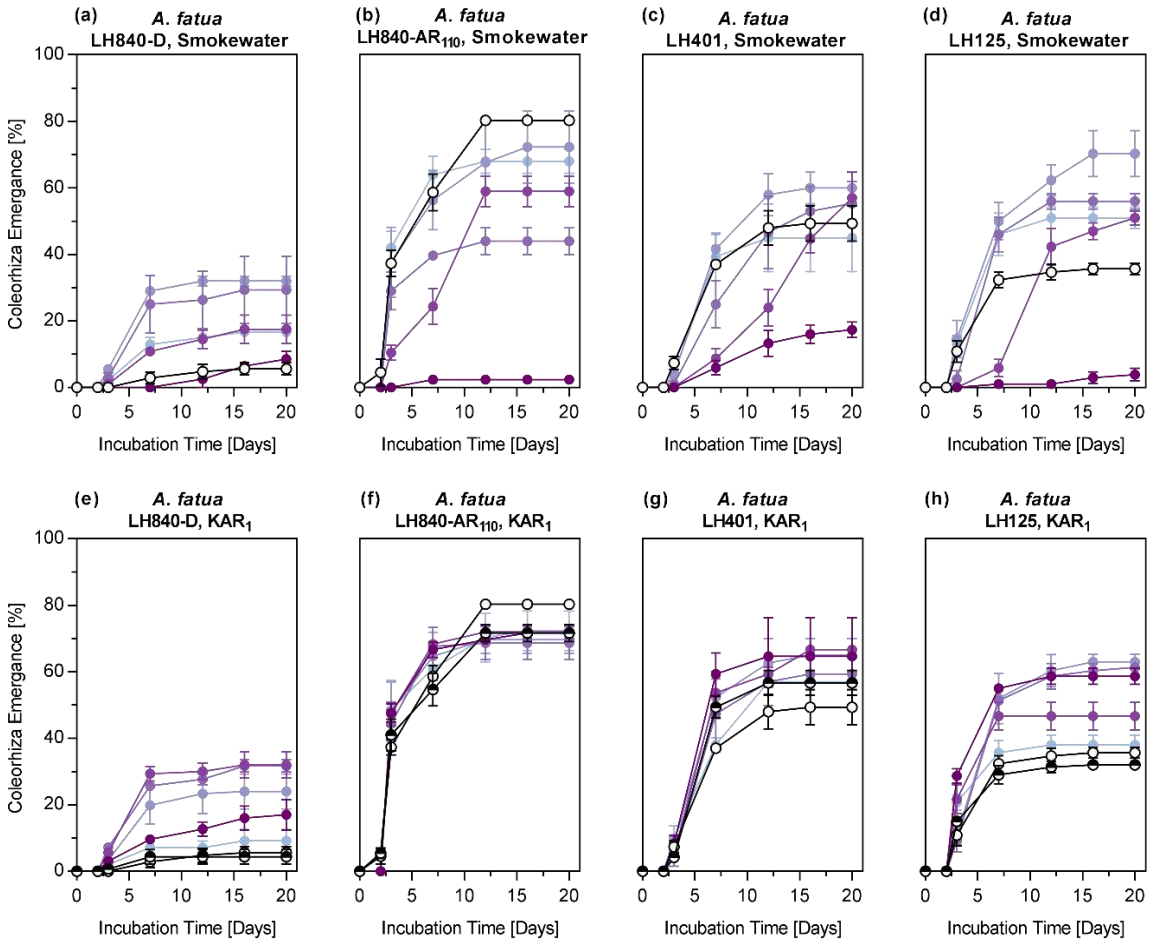


Fig. 3.11. Effect of smoke-water and KAR_1 on different *A. fatua* batches. The effect of smoke-water (Regen2000 Smokemaster™) (a-d) and KAR_1 (e-h) on (a,e) fully dormant (LH840-D), (b,f) fully after-ripened ($LH840\text{-}AR_{110}$) batches. (c-h) the effect of smoke-water and KAR_1 on batches with intermediate dormancy levels. Dilutions of smoke-water are shown as a percentage dilution (%v/v): ○, control; ●, 1%; ●, 2.5%; ●, 5%; ●, 10%; ●, 20%. KAR_1 was applied by molar concentration: ○, H_2O; half-shaded circle, 0.01% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM. Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 20°C under constant light.

To compare the germination stimulant effects of smoke-water and KAR_1, dilutions of a commercial smoke-water formulation "Regen2000 Smokemaster™" (Grayson, Tecnica Pty Ltd., Victoria, Australia) commonly used for horticultural applications was applied to

seeds in the germination medium. The responses of dormant seeds to smoke-water and KAR_1 were highly variable across the species (Fig. 9). *Digitaria sanguinalis* and *A. fatua* (Fig. 3.9a,c) both responded to smoke-water and *A. myosuroides*, *P. annua* and *S. faberi* did not (Fig. 3.9b,d,e). Higher concentrations of smoke-water (>10% v/v) were less effective or inhibitory. The response of *A. fatua* to KAR_1 was similar to the effect achieved by smoke-water application (Fig. 3.9h). The species that did not respond to smoke-water also did not respond to KAR_1 even when applied at high concentrations (>10 µM) (Fig. 3.9g,I,j). In *S. faberi*, KAR_1 was inhibitory to germination when compared to the solvent (DMSO) control (Fig. 3.9g). Interestingly, whilst *D. sanguinalis* responded strongly to smoke-water application no dormancy breaking effect was observed upon KAR_1 application (Fig. 3.9f). This supports the work of previous authors that have suggested that smoke-water contains additional compounds, likely cyanohydrins or additional karrikinolides, which have germination stimulant activities (Flematti *et al.*, 2011).

The *A. myosuroides* batch initially tested (LH150-D) showed a small response to the highest concentration of KAR_1 applied (Fig. 3.9j). To determine if this effect was consequential, we monitored the responses of five additional *A. myosuroides* batches to KAR_1. The dormant batch LH170-D responded strongly to KAR_1 application and all concentrations tested increased germination over the controls however the highest concentration (100 µM) was less effective (Fig. 3.10a). Interestingly, after-ripening this dormant batch for 364 days (LH170-AR_{364}) reversed its response to KAR_1 when applied at concentrations above 10 µM (Fig. 3.10b). In the additional batches with intermediate dormancy levels (LH128, LH216 and LH312) KAR_1 had no stimulatory effect on germination, except at lower concentrations (<50 µM) in LH312, and had an inhibitory effect at high concentrations (>50 µM) (Fig. 3.10c-e). These results

demonstrate that the effect of KAR_1 is dependent on the dormancy level of the seed batches.

In the initial screen for species that responded to smoke-water and KAR_1, *A. fatua* was the only species that responded to both treatments (Fig. 3.9c,h). To further investigate the relationship between sensitivity to KAR_1 and response to smoke-water both treatments were applied across three additional *A. fatua* seed batches with differing levels of dormancy. Whilst the dormant batch (LH840-D) responded moderately to both smoke-water and KAR_1, after-ripening this batch for 110 days (LH840-AR_{110}) to produce a fully after-ripened batch caused smoke-water to inhibit germination at all concentrations tested (Fig. 3.11b). After-ripening however did not cause KAR_1 to become inhibitory to this seed batch (Fig. 3.11f). In the additional *A. fatua* seed batches, smoke-water slightly promoted germination at low concentrations and inhibited germination under higher concentrations (Fig. 3.11c,d). In both these seed batches KAR_1 was a less potent stimulator of germination than smoke-water, however did not inhibit germination at high concentrations (Fig. 3.11g,h). This again indicates the presence of additional inhibitory compounds in smoke-water, such as TMB, as described by other authors (Light *et al.*, 2010).

KAR_1 inhibits germination in fully after-ripened *A. myosuroides* (Fig. 3.10b) but not in fully after-ripened *A. fatua* (Fig. 3.11f). Interestingly, *A. myosuroides* seeds, in the dormant or after-ripened state, produced abnormal seedlings when seeds were imbibed on high concentrations (>50 µM) of KAR_1. During the germination process of these seeds, although coleorhiza emergence is promoted in the dormant state by high KAR_1 concentrations (Fig. 3.10a), radicle emergence was delayed and at 100 µM and coleoptile emergence was completely inhibited (Fig. 3.12c) leading to an 'arrested germination' phenotype (Fig. 3.12d) Seedlings exposed to 50 µM KAR_1 had both primary and secondary radicles emerged when only the primary radicle was present in

the control and these radicals were stunted (Fig. 3.12d). KAR_1 at 50 µM also caused abnormalities in coleoptile and plumule growth and 100 µM KAR_1 caused a complete inhibition of root growth and an unusual swollen and hooked coleoptile (Fig. 3.12d).

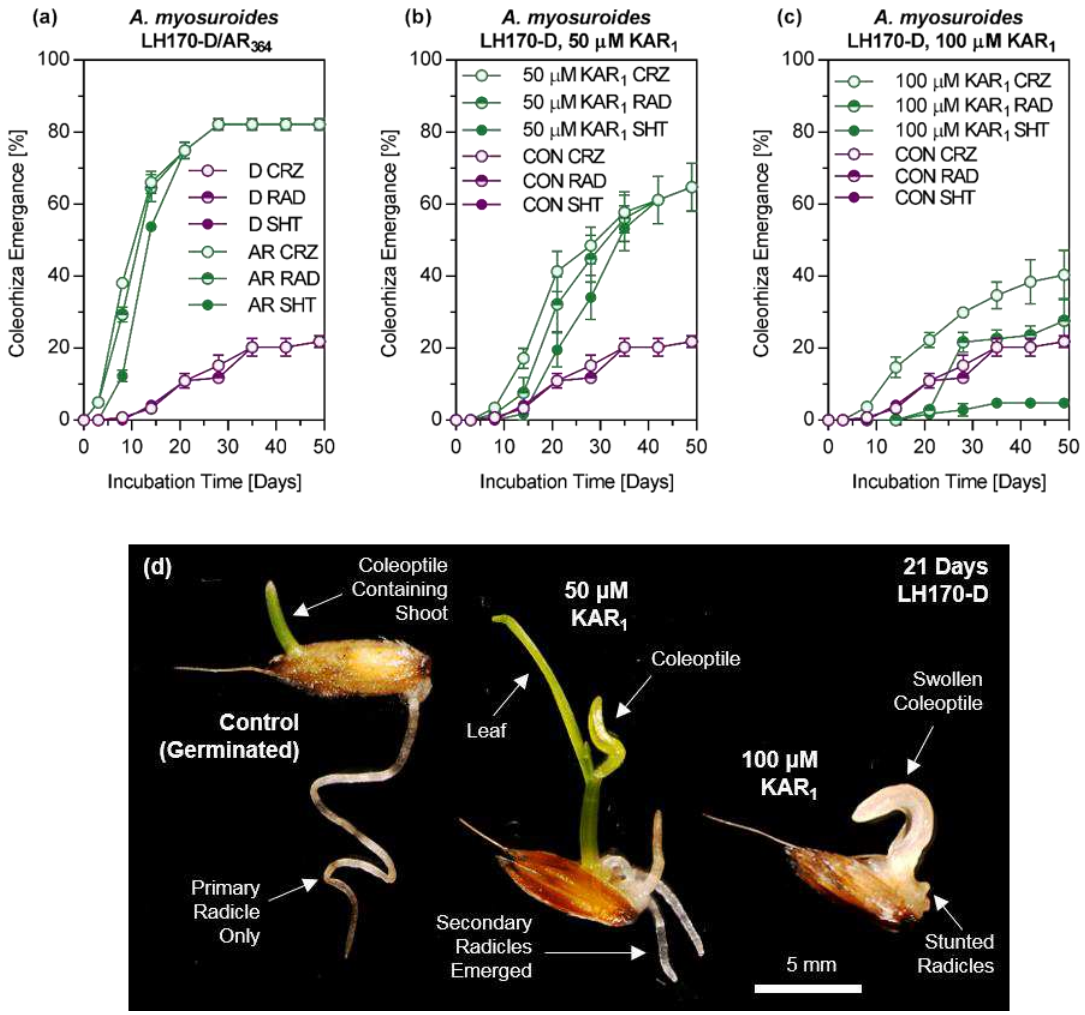


Fig. 3.12. KAR_1 causes seedling abnormalities in *A. myosuroides*. (a) Visible events during the germination of dormant 'D' (LH170-D) and fully after-ripened 'AR' (LH170-AR_{364}) batches. (b,c) The effect of (b) 50 µM and (c) 100 µM KAR_1 on visible events during the germination process compared to LH170-D. (d) Micrographs showing morphological abnormalities 21 days after imbibition when treated with 50 and 100 µM KAR_1. Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light. Scale bar represents 5 mm. CRZ, coleorhiza emergence; RAD, radicle emergence; SHT shoot emergence.

Strigolactones and KAR_1 share many common signalling components in plants (Morffy *et al.*, 2016). To determine if strigolactone signalling also provides a target for germination stimulants in *A. myosuroides*, a series of strigolactone analogs that had shown activity in *in vitro* activity assays (data not shown) were tested against a dormant *A. myosuroides* batch (LH170-D). Three of these analogs (RH109, RH192 and RH129) caused a small increase in maximum germination only at concentrations lower than 10 µM (Fig. 3.13).

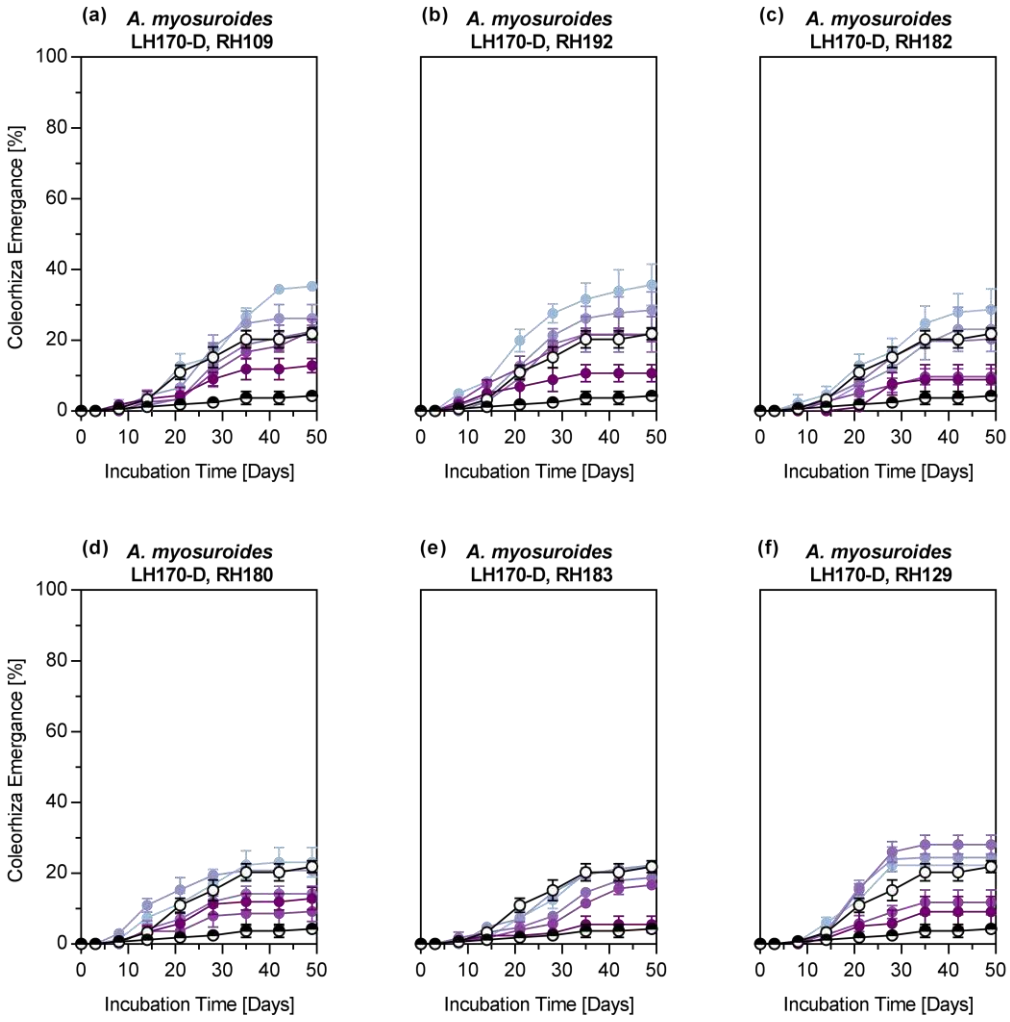


Fig. 3.13. Effect of strigolactone analogs on dormant *A. myosuroides*. Strigalactone analogs that showed activity in in vitro assays screened against dormant *A. myosuroides* (LH170-D). (○, H2O; half-shaded circle, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light.

These results demonstrate that strigolactones may have a role in the regulation of germination in *A. myosuroides* and may provide an additional feasible target for the development of dormancy breaking compounds.

3.4 Discussion

3.4.1 Identification of targets for the 'Flush and Kill' approach

By screening germination stimulants identified in crop and model species a number of compounds have been identified that have a dormancy breaking effect in grass weed species. However no one compound that was tested was able to break dormancy in all the species. This indicates that there are diversity of mechanisms enforcing dormancy amongst grass weeds and demonstrates a need to screen germination stimulants across multiple species to determine if they are applicable for WSB management.

The GID1 agonist AC-94377 was the most effective compound identified in terms of the number of species that responded and the size of the effect (Fig. 3.4). Indeed in *A. myosuroides* and *P. annua* AC-94377 was a more effective dormancy breaking treatment than gibberellin application (GA_3 and GA_4). This observation suggests that either AC-94377 has a greater affinity for the GID1 receptors of these species or alternatively that these species are in fact more sensitive to GA_1, another endogenous gibberellin that was not tested due to cost limitations. Nevertheless, the dormancy breaking effect of AC-94377 demonstrates that the gibberellin signalling machinery is present even in the dormant state, making GID1 agonism an attractive target for the development of WSB management compounds.

Fluridone, a PDS inhibitor, was also an effective dormancy breaking compound in *A. myosuroides* (Fig. 3.5-7). PDS acts early in carotenogenesis to convert phytoene into ζ-carotene, therefore inhibition of PDS has many biochemical implications besides ABA biosynthesis inhibition, such as phytoene overaccumulation (Sprecher *et al.*, 1998) or reactive oxygen species (ROS) generation due to a lack of ROS scavenging carotenoids. The accumulation of apoplastic superoxide is associated with cell elongation during cress germination (Muller *et al.*, 2009). Interestingly, fluridone treated *A. myosuroides* caryopses evolved 3-fold more apoplastic superoxide than GA_{4+7} treated caryopses that followed the same germination kinetics (Holloway, 2016). Therefore it is likely that fluridone's germination stimulant activity does not come solely from ABA biosynthesis inhibition and it can't be assumed that *de novo* ABA biosynthesis is involved in the regulation of *A. myosuroides* germination. Regardless of the MOA, fluridone was a successful dormancy breaking compound and PDS inhibition is another target suitable for WSB management.

Germination responses to smoke-derived treatments were again species-specific. *Avena fatua* responded partially to both smoke-water and KAR_1 (Fig. 3.9c,h) whereas *D. sanguinalis* responded strongly to smokewater but not KAR_1. *Alopecurus myosuroides* on the other hand responded to KAR_1 but not to smoke-water. Bioassay driven fraction of smoke-water identified KAR_1 as the most potent germination stimulant in smoke (Flematti *et al.*, 2004b) and since, numerous other compounds in smoke have been identified as having germination modifying activity (Flematti *et al.*, 2013). The different responses of grass weed species to either smoke-water or KAR_1 demonstrate that there are different sensitivities to these distinct smoke-derived compounds (Table 3.6) across species. In the example of *D. sanguinalis*, which responds only to smoke-water, it is likely that this response is due to sensitivity to cyanohydrins, a group of cyanogenic smoke-derived compounds that can break dormancy at low concentrations

through respiratory inhibition (Flematti *et al.*, 2013). Alternatively, *D. sanguinalis* could be sensitive to non-cyanogenic smoke-derived compounds, such as hydroquinone, that have a potent dormancy breaking effect in lettuce (Kamran *et al.*, 2017).

KAR_1 is a highly potent germination stimulant, breaking dormancy in Arabidopsis in the nanomolar range, partly through increasing the expression of gibberellin biosynthetic genes (Nelson *et al.*, 2009). In *A. myosuroides* and *A. fatua* a 1000-fold greater concentration of KAR_1 was required to break dormancy (Fig. 3.10 & 3.11). In Arabidopsis, KAR_1 application also has secondary effects on seedling morphology at this low concentration range, such as a decrease hypocotyl elongation and cotyledon expansion (Morffy *et al.*, 2016). Interestingly, high KAR_1 concentrations caused severe seedling abnormalities in *A. myosuroides* but not *A. fatua* (Fig. 3.12, data not shown). It is not clear what is causing these symptoms, however these results suggest that KAR_1 may have secondary MOAs perhaps through the interaction with other strigolactone signalling processes. Regardless of the MOA, the seedling abnormalities caused by KAR_1 generate an arrest in *A. myosuroides* seedling development that eventually leads to seedling mortality with a degree of specificity to other species (*A. fatua* does not have such symptoms) making KAR_1 a possible candidate herbicide for *A. myosuroides*.

3.4.2 Factors that influence grass weed seed responses to compounds

Temperature plays a key role in the regulating the depth of dormancy in physiologically dormant species and as dormancy is released the temperature range under which they germinate becomes wider (Baskin & Baskin, 2004). Previous work has shown that temperature plays a role in the response of non-dormant seed batches to germination stimulants. For example KAR_1 has little effect on tomato seeds imbibed at an optimal

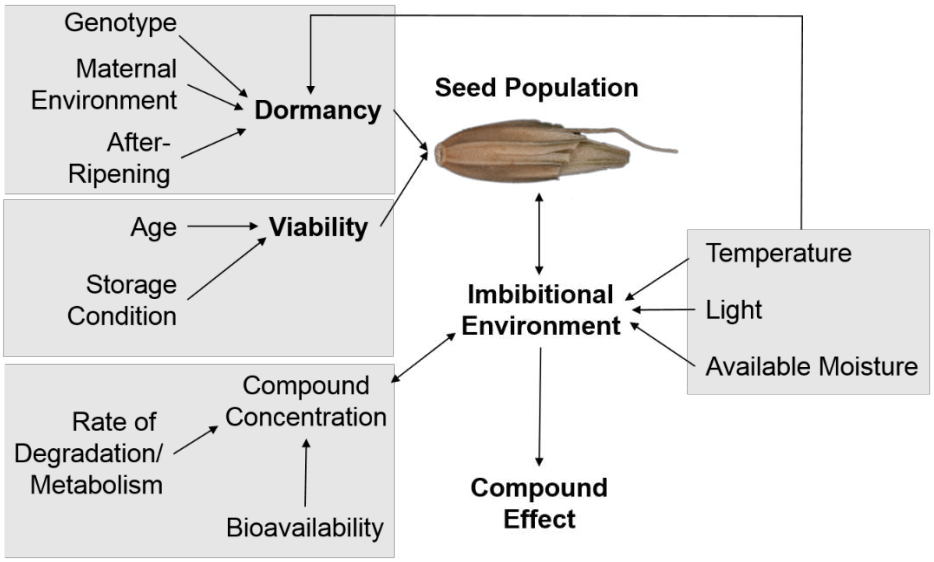


Fig. 3.14. The factors affecting the response of a seed population to a germination stimulant. The response of a seed population is dependent on the interaction between the imbibitional environment and the dormancy of the batch as well as the bioavailability of the compound.

temperature, however at sub- and superoptimal temperatures KAR_1 restores germination towards that of the optimal temperature (Jain & Van Staden, 2006). A similar trend was observed in *A. myosuroides that* responded to KAR_1 at suboptimal and not optimal temperatures (Holloway, 2016). In this work a temperature dependant relationship was identified with the PDS inhibitor fluridone. At 16 and 20°C only the higher concentrations of (≥ 50 µM at 16°C, ≥ 10 µM at 16°C) fluridone stimulated germination in dormant *A. myosuroides* (Figs. 3.6 & 3.7), however after-ripened seed at a supraoptimal temperature of 28°C germination was stimulated at concentrations above 0.1 µM. Conversely dormant *A. myosuroides* only responded minimally to the highest concentration of KAR_1 (100 µM) at 20°C (Fig. 3.9j), however at the lower temperature of 16°C lower concentrations (≥ 10 µM) had a germination stimulant effect on dormant seeds (Fig. 3.10a). It is clear that temperature plays an important role in determining the sensitivity of both dormant and after-ripened seed batches to compounds with different MOAs and therefore incubation temperature is an important

consideration when designing compound screening experiments to control the stringency of the screen.

The response of different species to smoke-water and KAR_1 also demonstrates the importance of dormancy level in determining the effect of germination stimulants. In dormant *A. myosuroides* KAR_1 behaves as a germination stimulant, and all concentrations tested promoted germination in dormant seeds (Fig. 3.10a) however in a fully after-ripened seed batch KAR_1 only had an inhibitory effect on germination (Fig. 3.10b). The same pattern was observed in the response dormant and fully after-ripened *A. fatua* to smoke-water (Fig. 3.11a,b). Additionally the dose-response relationships of these treatments in *A. myosuroides* and *A. fatua* were non-monotonic in the dormant state. Lower concentrations of either smoke-water or KAR_1 promoted germination until a certain point above which they were inhibitory (Figs. 3.10 & 3.11). This indicates the possibility of secondary MOAs for KAR_1 and other smoke-derived compounds in these species.

When comparing the effect of compounds across different seed batches it is difficult to separate the effects of genotype, dormancy level and ageing when seed batches have been produced under different conditions and have a differing storage histories. For example, in the effect of fluridone across multiple *A. myosuroides* batches, dormancy was broken in a freshly harvested batch (Fig. 3.8a) however no effect was seen on a fully after-ripened seed batch at an optimal temperature (Fig. 3.8b). Additional *A. myosuroides* batches with differing depths of dormancy failed to respond to fluridone (Fig. 3.8c-e). It is not clear if this is the result of differences in dormancy or genotype. This is reflective of a wider problem in the germination stimulants literature where for some compounds, such as KAR_1, both stimulatory and inhibitory effects are reported for the same species in different studies (Jefferson *et al.*, 2014). To combat this problem it would be necessary to propagate and store seeds from multiple weed

biotypes under equivalent conditions to understand the extent to which genotype contributes to the differences in the effects of compounds across different seed batches.

3.4.3 Identification of research needs for developing better germination stimulants

If a compound with a specific MOA breaks dormancy in a particular species it is apparent that the MOA of this compound is a useful target for the development of additional germination stimulants. However is not clear if a particular MOA can be ruled out if such a compound does not produce a physiological effect. This is because the effectiveness of any compound depends on a number of factors besides the importance of the target in regulating germination. The vast majority of germination stimulants have been identified through high-throughput screening of compound libraries or biological extracts on model species (Flematti *et al.*, 2004b; Sergeant *et al.*, 2009; Takeuchi *et al.*, 2014, 2015; Ye *et al.*, 2017b). Some germination stimulants, such as the ABA signalling antagonist AS_6, have been developed through rational design against the x-ray structures of receptor complexes in model species (Takeuchi *et al.*, 2014) yet do not have any dormancy breaking effect in weed species such as *A. myosuroides* (Fig. 3.5b). This is not an indication that ABA signalling is not involved in dormancy in weed species, since the carotenoid biosynthesis inhibitor fluridone is an effective dormancy breaking treatment in *A. myosuroides* (Fig. 3.7a). In order to focus the development of germination stimulants for WSB management, it will be important to understand the molecular processes regulating dormancy and germination in weed

species rather than relying on inferences from model species given the diversity of responses to germination stimulants seen across species.

The fate of compounds applied to seeds is also unclear and may be a source of differences in the effectiveness of germination stimulants between species. Differences in the uptake and translocation as well as metabolism and sequestration are key factors affecting the potency and selectivity of herbicides applied to plants (Zimdahl, 2018), yet this is a relatively unexplored area for germination stimulants. For example, a number of HAs have been identified as having high NCED inhibitory activity *in vitro* however their activity does not necessarily correlate to physiological effects *in vivo* (Awan *et al.*, 2017). Studies using fluorescent tracers have demonstrated differential uptake and localisation across the seed coat for different chemistries across species (Salanenka & Taylor, 2011). Grass seeds are particularly complex in this respect, with multiple distinct barriers in diaspores, such as the presence of maternally derived glumes, pericarp and testa that may provide multiple discriminatory barriers to the uptake of germination stimulants. These limitations illustrate the need for greater understanding of the mechanisms regulating germination in weed seeds in order to develop effective germination stimulants for WSB management.

3.5 Conclusions

By conducting targeted compound screens for dormancy breaking effects across grass weed species we have identified a number of targets and dormancy breaking treatments that have potential for WSB management (Table 3.8). The effects of some compounds are highly species-specific and dependent on the interaction between dormancy level and imbibitional temperature. This indicates that a 'silver bullet' compound, that can break the dormancy of all weed species, is probably unrealistic. However related species, that are weeds of similar cropping systems, respond similarly to specific germination stimulants. A key limitation to the development of weed seed-specific germination stimulants is the fundamental lack of knowledge surrounding the mechanisms regulating dormancy and germination in these species. Future work should aim to develop a deeper understanding of the diversity of these mechanisms, as well as the pharmacokinetics of germination stimulants, across a broad range of weed species.

Table 3.8 Summary of compounds with a dormancy breaking effect. Effect size relates in maximum germination (gMAX) relative to solvent control: Small, 10-30% increase; Intermediate, 30-50% increase; large, > 50% increase.

Compound Class	Compound	Species	Effect Size	Figure(s)
GID1 agonists	GA_3	*A. myosuroides*	Small	3.1a
	GA_{4+7}	*A. myosuroides*	Small	3.1b, 3.3
		A. fatua	Large	3.4f
		P. annua	Small	3.4c
	AC-94377	*A. myosuroides*	Large	3.2e, 3.3
		A. fatua	Large	3.2g
		P. annua	Large	3.4h
PDS inhibitors	Fluridone	*A. myosuroides*	Large	3.5d, 3.6h, 3.7a
	RH153	*A. myosuroides*	Intermediate	3.7b
Smoke-derived	Smokewater	*A. fatua*	Intermediate	3.9c
		D. sanguinalis	Large	3.9a
	KAR_1	*A. myosuroides*	Large	3.10a
		A. fatua	Intermediate	3.9h
Strigolactone analogs	RH192	*A. myosuroides*	Small	3.13b
	RH219	*A. myosuroides*	Small	3.13f

3.6 References

Adkins SW, Peters NCB. **2001**. Smoke derived from burnt vegetation stimulates germination of arable weeds. *Seed Science Research* **11**: 213–222.

Ali-Rachedi S, Bouinot D, Wagner M-H, Bonnet M, Sotta B, Grappin P, Jullien M. **2004**. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta* **219**: 479–488.

Awan SZ, Chandler JO, Harrison PJ, Sergeant MJ, Bugg TDH, Thompson AJ. **2017**. Promotion of germination using hydroxamic acid inhibitors of 9-*cis*-Epoxycarotenoid Dioxygenase. *Frontiers in Plant Science* **8**: 1–13.

Bartels P, Watson C. **1978**. Inhibition of carotenoid synthesis by Fluridone and Norflurazon. *Weed Science* **26**: 198–203.

Baskin JM, Baskin CC. **2004**. A classification system for seed dormancy. *Seed Science Research* **14**: 1–16.

Baskin C, Baskin J. **2014**. *Seeds*. Oxford: Academic Press.

Beaudoin N, Serizet C, Gosti F, Giraudat J. **2007**. Interactions between abscisic acid and ethylene signaling cascades. *The Plant Cell* **12**: 1103.

Bethke PC, Libourel IGL, Jones RL. **2007**. Nitric oxide in seed dormancy and germination. In: Bradford KJ, Nonogaki HN, eds. Seed Development, Dormancy and Germination. London: Blackwell Publishing Ltd., 153–175.

Bond BW, Burch PJ. **1990**. Stimulation of weed seed germination by 1-(3-chlorophthalirnido)cyclohexanecarboxamide (AC 94377). *Annals of Applied Biology* **116**: 119–130.

Brown JK, Smith JK (Eds.). **2000**. *Wildland Fire in Ecosystems: Effects of Fire on*

Flora. Fort Collins: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station.

Calvo AP, Nicolás C, Nicolás G, Rodríguez D. **2004**. Evidence of a cross-talk regulation of a GA 20-oxidase (*FsGA20ox1*) by gibberellins and ethylene during the breaking of dormancy in *Fagus sylvatica* seeds. *Physiologia Plantarum* **120**: 623–630.

Carmona R, Murdoch AJ. **1995**. Interactions of temperature and dormancy-relieving compounds on the germination of weed seeds. *Seed Science Research* **5**: 227–236.

Cembrowska-Lech D, Kępczyński J. **2017**. Plant-derived smoke induced activity of amylases, DNA replication and β-tubulin accumulation before radicle protrusion of dormant *Avena fatua* L. caryopses. *Acta Physiologiae Plantarum*. **39**: 1-12.

Chandler J. **2015**. Chemical Genetics of Seed Germination. Thesis: University of Warwick, School of Life Sciences. UK.

Chiwocha SDS, Dixon KW, Flematti GR, Ghisalberti EL, Merritt DJ, Nelson DC, Riseborough J-AM, Smith SM, Stevens JC. **2009**. Karrikins: A new family of plant growth regulators in smoke. *Plant Science* **177**: 252–256.

Cook C, Whichard L, Turner B, Wall M. **1966**. Germination of Witchweed (*Striga lutea* Lour.): Isolation and Properties of a Potent Stimulant. *Science* **154**: 4–6.

Corbineau F, Xia Q, Bailly C, El-Maarouf-Bouteau H. **2014**. Ethylene, a key factor in the regulation of seed dormancy. *Frontiers in Plant Science* **5**: 1–13.

Daws MI, Davies J, Pritchard HW, Brown NAC, Van Staden J. **2007**. Butenolide from plant-derived smoke enhances germination and seedling growth of arable weed species. *Plant Growth Regulation* **51**: 73–82.

Daws MI, Pritchard HW, Van Staden J. **2008**. Butenolide from plant-derived smoke functions as a strigolactone analogue: Evidence from parasitic weed seed germination. *South African Journal of Botany* **74**: 116–120.

Debeaujon I, Koornneef M. **2000**. Gibberellin requirement for arabidopsis seed germination is determined both by testa characteristics and embryonic abscisic acid. *Plant Physiology* **122**: 415–424.

Debeaujon I, Léon-Kloosterziel KM, Koornneef M. **2000**. Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. *Plant physiology* **122**: 403–414.

Diehl R, Walworth B. **1977**. Pthalimide Derivatives as Plant Growth Regualtors. United States Patent. American Cyanamid Company.

Donald WW, Tanaka FS. **1993**. The germination stimulant AC94377 reduces seed survival of wild mustard (*Sinapis arvensis*). *Weed Science* **41**: 185–193.

Dyer WE. **1995**. Exploiting weed seed dormancy and germination requirements through agronomic practices. *Weed Science* **43**: 498–503.

EPA. **1995**. *Pesticide Registration: Ethephon: PB89-109427*.

Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD. **2004a**. Molecular weight of a germination-enhancing compound in smoke. *Plant and Soil* **263**: 1–4.

Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD. **2004b**. A compound from smoke that promotes seed germination. *Science* **305**: 977.

Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD. **2005**. Synthesis of the seed germination stimulant 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one. *Tetrahedron Letters* **46**: 5719–5721.

Flematti GR, Goddard-Borger ED, Merritt DJ, Ghisalberti EL, Dixon KW, Trengove RD. **2007**. Preparation of 2*H*-furo[2,3-*c*]pyran-2-one derivatives and evaluation of their germination-promoting activity. *Journal of Agricultural and Food Chemistry* **55**: 2189–2194.

Flematti GR, Merritt DJ, Piggott MJ, Trengove RD, Smith SM, Dixon KW,

Ghisalberti EL. **2011**. Burning vegetation produces cyanohydrins that liberate cyanide and stimulate seed germination. *Nature Communications* **2**: 360–366.

Flematti GR, Scaffidi A, Goddard-Borger ED, Heath CH, Nelson DC, Commander LE, Stick R V., Dixon KW, Smith SM, Ghisalberti EL. **2010**. Structure-activity relationship of karrikin germination stimulants. *Journal of Agricultural and Food Chemistry* **58**: 8612–8617.

Flematti GR, Waters MT, Scaffidi A, Merritt DJ, Ghisalberti EL, Dixon KW, Smith SM. **2013**. Karrikin and cyanohydrin smoke signals provide clues to new endogenous plant signaling compounds. *Molecular Plant* **6**: 29–37.

Gamble PE, Mullett JE. **1986**. Inhibition of carotenoid accumulation and abscisic acid biosynthesis in fluridone-treated dark-grown barley. *European Journal of Biochemistry* **160**: 117–121.

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, Mccourt P. **2000**. Regulation of abscisic acid signaling by the ethylene response pathway in arabidopsis. *The Plant Cell* **12**: 1117–1126.

Goggin DE, Powles SB. **2014**. Fluridone: A combination germination stimulant and herbicide for problem fields? *Pest Management Science* **70**: 1418–1424.

Gott KA, Thomas TH. **1986**. Comparative effects of gibberellins and N-substituted pthalimide on seed germination and extension growth of celery (*Apium graveolens* L.). *Plant Growth Regulation* **279**: 273–279.

Graeber K, Linkies A, Müller K, Wunchova A, Rott A, Leubner-Metzger G. **2010**. Cross-species approaches to seed dormancy and germination: Conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae *DOG1* genes. *Plant Molecular Biology* **73**: 67–87.

Grappin P, Bouinot D, Sotta B, Miginiac E, Jullien M. **2000**. Control of seed

dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta* **210**: 279–285.

Hamiaux C, Drummond RSM, Janssen BJ, Ledger SE, Cooney JM, Newcomb RD, Snowden KC. **2012**. DAD2 is an α/β hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. *Current Biology* **22**: 2032–2036.

Han S-Y, Kitahata N, Sekimata K, Saito T, Kobayashi M, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K, Yoshida S, Asami T. **2004**. A novel Inhibitor of 9-cis-epoxycarotenoid dioxygenase in abscisic acid biosynthesis in higher plants. *Plant Physiology* **135**: 1574–1582.

Harrison PJ. **2014**. Biochemical investigations of the carotenoid cleavage dioxygenase enzyme family.Thesis: University of Warick, Department of Chemistry, United Kingdom.

Hartweck LM. **2008**. Gibberellin signaling. *Planta* **229**: 1–13.

Hashimoto T, Tamusa S. **1967**. Physiological activities of helminthosporol and helminthosporic acid: III Effects on seed germiantion. *Plant and Cell Physiology* **8**: 35–45.

Hayashi K, Kinoshita T. **2014**. Plant signaling: abscisic acid receptor hole-in-one. *Nature chemical biology* **10**: 414–5.

Holloway T. **2016**. Comprehensive physiological charachterisation of *Alopecurus myosuroides* Huds. germiantion as affected by karrikin 1 application. Master's Thesis: Royal Holloway University of London, School of Biological Sciences. London, United Kingdom.

Jain N, Van Staden J. **2006**. A smoke-derived butenolide improves early growth of tomato seedlings. *Plant Growth Regulation* **50**: 139–148.

Jefferson L, Pennacchio M, Havens-Young K. **2014**. *Ecology of Plant-Derived Smoke: Its Use in Seed Germination*. Chicago: Oxford University Press.

Jiang K, Otani M, Shimotakahara H, Yoon J, Park S, Miyaji T, Nakano T, Nakamura H, Nakajima M, Asami T. **2017a**. Substituted phthalimide AC94377 is a selective agonist of the gibberellin receptor GID1. *Plant Physiology* **173**: 825–835.

Jiang K, Shimotakahara H, Luo M, Otani M, Nakamura H. **2017b**. Chemical screening and development of novel gibberellin mimics. *Bioorganic & Medicinal Chemistry Letters* **27**: 3678–3682.

Kamran M, Khan AL, Ali L, Hussain J, Waqas M, Al-Harrasi A, Imran QM, Kim Y-H, Kang S-M, Yun B-W, *et al.* 2017. Hydroquinone; a novel bioactive compound from plant-derived smoke can cue seed germination of lettuce. *Frontiers in Chemistry* **5**: 1–8.

Kępczyński J, Białecka B, Light ME, Van Staden J. **2006**. Regulation of *Avena fatua* seed germination by smoke solutions, gibberellin A3 and ethylene. *Plant Growth Regulation* **49**: 9–16.

Kitahata N, Han SY, Noji N, Saito T, Kobayashi M, Nakano T, Kuchitsu K, Shinozaki K, Yoshida S, Matsumoto S, *et al.* 2006. A 9-*cis*-epoxycarotenoid dioxygenase inhibitor for use in the elucidation of abscisic acid action mechanisms. *Bioorganic and Medicinal Chemistry* **14**: 5555–5561.

Kitahata N, Ito S, Kato A, Ueno K, Nakano T, Yoneyama K, Yoneyama K, Asami T. **2011**. Abamine as a basis for new designs of regulators of strigolactone production. *Journal of Pesticide Science* **36**: 53–57.

Koornneef M, van der Veen JH. **1980**. Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) heynh. *Theoretical and Applied Genetics* **58**: 257–263.

Kucera B, Cohn MA, Leubner-Metzger G. **2005**. Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**: 281–307.

Kulkarni MG, Light ME, Van Staden J. **2011**. Plant-derived smoke: Old technology with possibilities for economic applications in agriculture and horticulture. *South African Journal of Botany* **77**: 972–979.

de Lange JH, Boucher C. **1990**. Autecological studies on *Audouinia capitata* (Bruniaceae). I. Plant-derived smoke as a seed germination cue. *South African Journal of Botany* **56**: 700–703.

Li C, Liu Z, Zhang Q, Wang R, Xiao L, Ma H, Chong K, Xu Y. **2012**. *SKP1* is involved in abscisic acid signalling to regulate seed germination, stomatal opening and root growth in *Arabidopsis thaliana*. *Plant, Cell and Environment* **35**: 952–965.

Light ME, Burger B V., Staerk D, Kohout L, Van Staden J. **2010**. Butenolides from plant-derived smoke: natural plant-growth regulators with antagonistic actions on seed germination. *Journal of Natural Products* **73**: 267–269.

Linkies A, Leubner-Metzger G. **2012**. Beyond gibberellins and abscisic acid: How ethylene and jasmonates control seed germination. *Plant Cell Reports* **31**: 253–270.

Linkies A, Mu K, Morris K, Turec V, Strnad M, Lynn JR, Finch-savage WE. **2009**. Ethylene interacts with abscisic acid to regulate endosperm rupture during germination : a comparative approach using *Lepidium sativum* and *Arabidopsis thaliana*. *The Plant Cell* **21**: 3803–3822.

Long RL, Stevens JC, Griffiths EM, Adamek M, Gorecki MJ, Powles SB, Merritt DJ. **2011**. Seeds of Brassicaceae weeds have an inherent or inducible response to the germination stimulant karrikinolide. *Annals of Botany* **108**: 933–944.

Matilla AJ, Carrillo-Barral N, Rodríguez-Gacio M del C. **2015**. An update on the role of *NCED* and *CYP707A* ABA metabolism genes in seed dormancy induction and the response to after-ripening and nitrate. *Journal of Plant Growth Regulation*. **34(2)**: 274-293.

Matilla AJ, Matilla-Vázquez MA. **2008**. Involvement of ethylene in seed physiology. *Plant Science* **175**: 87–97.

McCarty DR. **1995**. Genetic Control and Integration of Maturation and Germination Pathways in Seed development. *Annual reviews in Plant Physiology* **46**: 71–93.

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK. **1991**. The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**: 895–905.

Mitchell G, Bartlett DW, Fraser TEM, Hawkes TR, Holt DC, Townson JK, Wichert RA. **2001**. Mesotrione: A new selective herbicide for use in maize. *Pest Management Science* **57**: 120–128.

Miyazaki S, Jiang K, Kobayashi M, Asami T, Nakajima M. **2017**. Helminthosporic acid functions as an agonist for gibberellin receptor. *Bioscience, Biotechnology and Biochemistry* **81**: 2152–2159.

Mojzes A, Csontos P, Kalapos T. **2015**. Is the positive response of seed germination to plant-derived smoke associated with plant traits? *Acta Oecologica* **65**–**66**: 24–31.

Morffy N, Faure L, Nelson DC. **2016**. Smoke and hormone mirrors: action and evolution of karrikin and strigolactone signaling. *Trends in Genetics* **32**: 176–188.

Muller K, Linkies A, Vreeburg RAM, Fry SC, Krieger-Liszkay A, Leubner-Metzger G. **2009**. *In vivo* cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. *Plant Physiology* **150**: 1855–1865.

Müller K, Tintelnot S, Leubner-Metzger G. **2006**. Endosperm-limited Brassicaceae seed germination: Abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant and Cell Physiology* **47**: 864–877.

Nakayama I, Kamiya Y, Kobayashi M, Abe H, Sakurai A. **1990a**. Effects of a plant-

growth regulator, prohexadione, on the biosynthesis of gibberellins in cell-free systems derived from immature seeds. *Plant and Cell Physiology* **31**: 1183–1190.

Nakayama I, Miyazawa T, Kobayashi M. **1990b**. Effects of a new plant growth regulator prohexadione calcium (BX-112) on shoot elongation caused by exogenously applied gibberellins in rice (*Oryza sativa* L.) seedlings. *Plant Cell Physiology* **31**: 195–200.

Nambara E, Marion-Poll A. **2005**. Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56**: 165–185.

Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, Kamiya Y. **2010**. Abscisic acid and the control of seed dormancy and germination. *Seed Science Research* **20**: 55–67.

Nelson DC, Riseborough JA, Flematti GR, Stevens J, Ghisalberti EL, Dixon KW, Smith SM. **2009**. Karrikins discovered in smoke trigger arabidopsis seed germination by a mechanism requiring gibberellic acid synthesis and light. *Plant Physiology* **149**: 863–873.

Nishimura N, Hitomi K, Avari AS, Rambo RP, Hitomi C, Cutler SR, Schroeder JI, Getzoff ED. **2009**. Structural mechanism of abscisic acid binding and signalling by dimeric PYR1. *Science* **326**: 1373–1380.

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S. **2003**. Gibberellin biosynthesis and response during Arabidopsis seed germination. *The Plant Cell* **15**: 1591–1604.

Otani M, Yoon JM, Park SH, Asami T, Nakajima M. **2010**. Screening and characterization of an inhibitory chemical specific to Arabidopsis gibberellin 2-oxidases. *Bioorganic and Medicinal Chemistry Letters* **20**: 4259–4262.

Papenfus HB, Kulkarni MG, Posta M, Finnie JF, Van Staden J. **2015**. Smoke-

isolated trimethylbutenolide inhibits seed germination of different weed species by reducing amylase activity. *Weed Science* **63**: 312–320.

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TFF, *et al.* 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071.

Pepperman A, Bradow J. **2016**. Strigol analogs as germination regulators in weed and crop seeds. *Weed Science* **36**: 719–725.

Pons TL. **1989**. Breaking of seed dormancy by nitrate as a gap detection mechanism. *Annals of Botany* **63**: 139–143.

Pošta M, Light ME, Papenfus HB, Van Staden J, Kohout L. **2013**. Structure-activity relationships of analogs of 3,4,5-trimethylfuran-2(5*H*)-one with germination inhibitory activities. *Journal of Plant Physiology* **170**: 1235–1242.

Qin G, Gu H, Ma L, Peng Y, Deng XW, Chen Z, Qu LJ. **2007**. Disruption of *Phytoene Desaturase* gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. *Cell Research* **17**: 471–482.

Rademacher W. **2000**. growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. *Annual Review of Plant Physiology & Plant Molecular Biology* **51**: 501–531.

Rademacher W. **2016**. Chemical regulators of gibberellin status and their application in plant production. *Annual Plant Reviews* **49**: 359–403.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E. **2010**. ABA perception and signalling. *Trends in Plant Science* **15**: 395–401.

Roberts EH. **1973**. Oxidative processes and the control of seed germination. In: Heydecker W, ed. Seed Ecology. London: Butterworth & Co., 189–218.

Roberts HA, Chancellor RJ, Hill TA. **1982**. The Biology of Weeds. In: Roberts HA,

ed. Weed Control Handbook: Principles. Oxford: Blackwell, 1–36.

Roth-Bejerano N, Sedee NJA, van der Meulen RM, Wang M. **1999**. The role of abscisic acid in germination of light-sensitive and light-insensitive lettuce seeds. *Seed Science Research* **9**: 129–134.

Rzewuski G, Sauter M. **2008**. Ethylene biosynthesis and signaling in rice. *Plant Science* **175**: 32–42.

Salanenka YA, Taylor AG. **2011**. Seedcoat permeability: Uptake and post-germination transport of applied model tracer compounds. *HortScience* **46**: 622–626.

Santiago J, Dupeux F, Round A, Antoni R, Park SY, Jamin M, Cutler SR, Rodriguez PL, Márquez JA. **2009**. The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature* **462**: 665–668.

Sato S, Esashi Y. **1980**. a-Aminoisobutyric acid : A probable competitive inhibitor of conversion of 1-aminocyclopropane-l-carboxylic acid to ethylene. *Plant & Cell Physiology* **21**: 939–949.

Schaller GE, Binder BM. **2017**. Inhibitors of ethylene biosynthesis and signaling. In: Ethylene Signalling. *Humana Press*, New Jersey, USA. 223–235.

Sergeant MJ, Li JJ, Fox C, Brookbank N, Rea D, Bugg TDH, Thompson AJ. **2009**. Selective inhibition of carotenoid cleavage dioxygenases. Phenotypic effects on shoot branching. *Journal of Biological Chemistry* **284**: 5257–5264.

Sisler E, Blankenship S. **1998**. Method of counteracting an ethylene response in plants. Australian Patent AU-B-26457/95.

Sisler EC, Blankenship SM, Guest M. **1990**. Competition of cyclooctenes and cyclooctadienes for ethylene binding and activity in plants. *Plant Growth Regulation* **9**: 157–164.

Sisler EC, Reid MS, Yang SF. **1986**. Effect of antagonists of ethylene action on

binding of ethylene in cut carnations. *Plant Growth Regulation* **4**: 213–218.

Soós V, Sebestyén E, Posta M, Kohout L, Light ME, Van Staden J, Balázs E. **2012**. Molecular aspects of the antagonistic interaction of smoke-derived butenolides on the germination process of Grand Rapids lettuce (*Lactuca sativa*) achenes. *New Phytologist* **196**: 1060–1073.

Sprecher SL, Netherland MD, Stewart AB. **1998**. Phytoene and carotene response of aquatic plants to fluridone under laboratory conditions. *Journal of Aquatic Plant Management* **36**: 111–120.

Van Staden J, Drewes FE, Jager AK. **1995**. The search for germination stimulants in plant-derived smoke extracts. *South African Journal of Botany* **61**.

Van Staden J, Jäger AK, Light ME, Burger B V. 2004. Isolation of the major germination cue from plant-derived smoke. *South African Journal of Botany* **70**: 654–659.

Stevens JC, Merritt DJ, Flematti GR, Ghisalberti EL, Dixon KW. **2007**. Seed germination of agricultural weeds is promoted by the butenolide 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one under laboratory and field conditions. *Plant and Soil* **298**: 113–124.

Takeuchi J, Mimura N, Okamoto M, Yajima S, Sue M, Akiyama T, Monda K, Iba K, Ohnishi T, Todoroki Y. **2018**. Structure-based chemical design of abscisic acid antagonists that block PYL-PP2C receptor interactions. *ACS Chemical Biology* **13**: 1313–1321.

Takeuchi J, Ohnishi T, Okamoto M, Todoroki Y. **2015**. Conformationally restricted 3′-modified ABA analogs for controlling ABA receptors. *Organic and Biomolecular Chemistry* **13**: 4278–4288.

Takeuchi J, Okamoto M, Akiyama T, Muto T, Yajima S, Sue M, Seo M, Kanno Y, Kamo T, Endo A, *et al.* 2014. Designed abscisic acid analogs as antagonists of PYL-

PP2C receptor interactions. *Nature chemical biology* **10**: 477–82.

Thomas T. **1984**. Gibberellin-like stimulation of celery (*Apium graveolens* L.) seed germination by N-substituted phthalimides. *Scientia Horticulturae* **23**: 113–117.

Thompson AJ, Jackson AC, Symonds RC, Mulholland BJ, Dadswell AR, Blake PS, Burbidge A, Taylor IB. **2000**. Ectopic expression of a tomato 9-*cis*-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. *Plant Journal* **23**: 363–374.

Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Hanada A, Aso Y, Ishiyama K, Tamura N, *et al.* 2008. High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in arabidopsis seeds. *Plant Physiology* **146**: 1368–1385.

Toh S, Kamiya Y, Kawakami N, Nambara E, Mccourt P, Tsuchiya Y, Biology S, Ms C. **2012**. Thermoinhibition uncovers a role for strigolactones in arabidopsis seed germination. *Plant and Cell Physiology* **53**: 107–117.

Ueguchi-Tanaka M, Ashikari M, Nakajima M, Itoh H, Katoh E, Kobayashi M, Chow TY, Hsing YIC, Kitano H, Yamaguchi I, *et al.* 2005. GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature* **437**: 693–698.

Villedieu-percheron E, Lachia M, Jung PMJ, Screpanti C, Fonné-pfister R, Wendeborn S, Zurwerra D, Mesmaeker A De. **2014**. Chemicals inducing seed germination and early seedling development. *Chimia* **68**: 654–663.

Waters MT, Nelson DC, Scaffidi A, Flematti GR, Sun YK, Dixon KW, Smith SM. **2012a**. Specialisation within the DWARF14 protein family confers distinct responses to karrikins and strigolactones in Arabidopsis. *Development* **139**: 1285–1295.

Waters MT, Scaffidi A, Flematti GR, Smith SM. **2012b**. Karrikins force a rethink of strigolactone mode of action. *Plant Signaling and Behavior* **7**: 969-972.

Yalpani N, Suttle JC, Hultstrand JF, Rodaway SJ. **1989**. Competition for *in vitro* [^{3}H] gibberellin A_4 binding in cucumber by substituted phthalimides comparison with *in vivo* gibberellin-like activity. *Plant Physiology* **91**: 823–828.

Yamaguchi S. **2008**. Gibberellin metabolism and its regulation. *Annual Review of Plant Biology* **59**: 225–251.

Yang SF, Hoffman NE. **1984**. Ethylene biosynthesis and its regulation in higher plants. *Annual Reviews in Plant Physiology* **35**: 165–175.

Ye Y, Zhou L, Liu X, Liu H, Li D, Cao M, Chen H, Xu L, Zhu J, Zhao Y. **2017**. A novel chemical inhibitor of aba signaling targets all ABA receptors. *Plant Physiology*.

Yoshida S, Asami T. **2006**. Abscisic Acid Biosynthesis Inhibitor. United States Patent US 7,098,365 B2.

Yoshioka T, Endo T, Satoh S. **1998**. Restoration of seed germination at supraoptimal temperatures by fluridone, an inhibitor of abscisic acid biosynthesis. *Plant and Cell Physiology* **39**: 307–312.

Yu Y-B, Yang SF. **1979**. Auxin-induced ethylene production and its inhibition by aminoethyoxyvinylglycine and cobalt ion. *Plant Physiology* **64**: 1074–1077.

Zimdahl R. **1988**. The concept and application of the critical weed-free period. In: Altieri MA, Liebman M, eds. Weed Management in Agroecosystems: Ecological Approaches. Florida: CRC Press, 145–155.

Zimdahl R. **2018**. *Fundamentals of Weed Science*. Colorado: Academic Press.

4. Molecular mechanisms of seed dormancy in blackgrass (*Alopecurus myosuroides*) as targets for management of the agricultural weed seed bank

Manuscript prepared for submission to 'New Phytologist' (Online ISSN: 1469-8137).

4.1 Author contributions

Thomas Holloway (TH), Kazumi Nakabayashi (KN), David Stock (DS), Anne Seville (AS) and Gerhard Leubner-Metzger (GL-M) planned and designed the research; TH and Lucio Garcia (LG) performed experiments; TH, KN, KS, Jonathan Cohn (JoC) and Jake Chandler (JaC) analysed and interpreted the data; DS and AS provided compounds. TH wrote manuscript and all authors revised and approved the final article. Physiological experiments and RNA extraction were performed by TH (Fig. 1 & 6). Transcriptome analysis by RNAseq from library preparation to raw data bioinformatics, *de novo* transcriptome assembly and initial differential gene expression (DEG) analysis was conducted by LG, KS and JoC (Syngenta Crop Protection LLP., Research Triangle Park, Durham, NC, USA). TH conducted the in-depth analysis of the transcriptome results and derived Fig. 2 from the *de novo* transcriptome assembly and DEG results. Functional annotation of the assembly (Fig. 2c), gene ontology enrichment analysis (Fig. 3) and targeted analysis of gene expression (Figs. 4 & 5) were performed by TH. Clustering analysis (Fig. 3) was performed by TH and JaC. The compound library for Fig. 6 was provided by DS and AS from Syngenta Ltd. (Jealott's Hill International Research Centre, UK).

The concept, experimental design and narrative of the manuscript were conceived by TH. Project supervision was provided by KN and GL-M (Royal Holloway University of London, UK) and by DS and AS (Syngenta Ltd., Jealott's Hill International Research Centre, UK). TH is the first (lead) author on the manuscript, prepared all the figures and

wrote the first draft. TH coordinated the internal revision process with contributions from JoC, KS, AS, JaC, KN and GL-M. The manuscript is in the advanced stages of preparation for submission to New Phytologist as a 'Full Paper Original Research Article'. Submission is anticipated before the end of December 2019.

Molecular Mechanisms of Seed Dormancy in Blackgrass (*Alopecurus myosuroides*) as Targets for Management of the Agricultural Weed Seed Bank

Thomas Holloway[1], Jake O. Chandler[1], Lucio Garcia[3], Jonathan Cohn[3], Kelly Schilling[3], David Stock[2], Anne Seville[2], Kazumi Nakabayashi[1a] & Gerhard Leubner-Metzger[1a]

[1]Department of Biological Sciences, Royal Holloway University of London, Egham, TW20 0EX, United Kingdom.

[2]Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, RG42 6EY, United Kingdom.

[3]Syngenta Crop Protection LLP, Research Triangle Park, Durham, NC 27703, United States of America.

[a]Shared corresponding authors' emails and phones:

Correspondence and requests for materials should be addresses to K.N. or G.L.M. at: School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom.

URL: www.seedbiology.eu

Corresponding Author Details:

Gerhard Leubner Metzger: gerhard.leubner@rhul.ac.uk, +44 1784 44 3895,

URL: https://pure.royalholloway.ac.uk/portal/en/persons/gerhard-leubner(b07cd3da-9c1d-4167-8d52-199a13d54351).html

Kazumi Nakabayashi: kazumi.nakabayashi@rhul.ac.uk, +44 1784 44 3769

URL: https://pure.royalholloway.ac.uk/portal/en/persons/kazumi-nakabayashi(e0fa28c2-6185-44d1-966c-192c95e86a70).html

ORCID *Thomas Holloway*: 0000-0002-8753-7841

ORCID *Jake O. Chandler:* 0000-0003-0955-9241

ORCID *Kazumi Nakabayashi*: 0000-0002-4186-541X

ORCID *Gerhard Leubner-Metzger*: 0000-0002-6045-8713

ORCID *Anne Seville*: 0000-0001-8024-7959

New Phytologist

Received :

Accepted :

Open access : CC BY Type

Article Type : Regular Article

Summary

• Seed dormancy is the key factor determining weed emergence in agricultural fields and there is growing interest in weed seeds as a target for weed management. A foremost limitation to this approach is the lack of molecular insight into the mechanisms regulating dormancy in weed seeds.

• *Alopecurus myosuroides* (blackgrass) seeds were collected from wheat field in the UK. At low temperatures, dormant (D) and after-ripened (AR) seeds germinate similarly and at warm temperatures dormancy is enforced. RNAseq analysis at both temperatures for D and AR seeds demonstrated distinct mechanisms, involving ethylene and gibberellin signaling, are involved in after-ripening and cold-induced dormancy release.

• An untargeted analysis revealed cluster-specific processes, such as seed defense responses and anaerobic respiration, which may have potential as targets for weed seedbank management. A targeted analysis of hormone biosynthesis and signaling pathways identified targets that could be exploited by dormancy breaking compounds. These targets were validated by screening a target catalogue compound library.

• Our findings suggest that dormancy breaking can occur via multiple independent but connected hormone-mediated mechanisms and provide a proof-of-concept for the use of next generation sequencing for identifying novel targets for germination stimulants.

Key words

Alopecurus myosuroides (blackgrass), after-ripening, ecophysiology, germination stimulants, RNAseq, seed dormancy, weed seedbank.

Introduction

Agricultural weeds present a serious threat to food security and sustainable intensification worldwide (Bridges, 1994). Weeds have become adapted to high-input agricultural systems by mimicking crops to avoid detection (Barrett, 1983) and by developing increasing levels of herbicide resistance to multiple herbicide modes of action (Heap, 2019). Regulatory restraints (Chauvel *et al.*, 2012) and a slowing of herbicide discovery (Duke, 2012) have caused the chemical 'crop protection toolbox' to shrink, necessitating the development of alternative weed control strategies that less reliant on herbicides.

Seed dormancy is a highly adaptive trait in agricultural weeds that allows them to synchronise their emergence with the cropping cycle to maximise competitiveness with the crop and to avoid weed control measures (Zimdahl, 2018). Adult weeds typically shed their seeds with a high degree of primary dormancy (Mohler *et al.*, 2007) that limits the range of conditions under which the seed will germinate (Baskin & Baskin, 2004). The depth and duration of this dormancy is influenced by environmental factors, particularly temperature, on both the mother plant and the seed (Bewley *et al.*, 2013). The transition from dormancy to germination, as studied in model systems, is regulated by the interplay of these environmental cues and plant hormones, centred around the antagonistic relationship between gibberellin (GA) and abscisic acid (ABA) biosynthesis and signalling (Finch-Savage & Leubner-Metzger, 2006). However, beyond model and crop systems, very little is known about the mechanisms regulating dormancy and germination in agricultural weeds.

Blackgrass (*Alopecurus myosuroides* L.) has been considered the most destructive cereal weed in Europe (Lutman *et al.*, 2013). Blackgrass plants, as with most weeds, are highly fecund and can produce up to 500 viable diaspores (dispersal units, hereafter seeds) that are typically dispersed at crop harvest from June-July (Clarke *et al.*, 2015).

After a period of dormancy, blackgrass seedlings typically emerge from October to December during the establishment of winter cereal crops (Colbach *et al.*, 2006) consistent with a winter annual lifecycle.

Seed dormancy is a relatively unexplored stage of the weed lifecycle as a target for weed management. 'Flush and kill' is an emerging concept that aims to manipulate the dormancy of the weed seedbank using germination stimulants in order induce weed emergence before the planting of the crop, allowing a broader range of control methods to be used. Previous attempts to achieve this, using compounds identified as germination stimulants in model species, show highly species-specific responses, indicating that there are diverse dormancy mechanisms across weed species (Metzger, 1983; Adkins & Peters, 2001; Goggin & Powles, 2014).

In this study, we use advances in next-generation sequencing (NGS) technology to develop a transcriptomic resource in order to identify the processes involved in the environmental regulation of seed dormancy in blackgrass. Using an ecophysiological approach we found that at low temperatures dormant (D) and after-ripened (AR) non-dormant blackgrass seeds germinate similarly, however under warm temperatures germination in the D seeds is inhibited. Both untargeted clustering-based approaches and targeted analyses of phytohormone biosynthesis and signalling genes were employed to address the following questions: Does loss of dormancy during after-ripening (controlled dormancy release by dry storage) result from changes in the dry seed transcriptome? Are the processes that regulate germination at low temperatures equivalent between D and AR states? Are the processes associated with dormancy and germination similar between blackgrass and model species? Finally we identified and tested potential targets for chemical manipulation of blackgrass dormancy and germination the weed seedbank.

Materials and Methods

Seed Material and Germination Kinetics

Alopecurus myosuroides diaspores (hereafter seeds) were collected at maturity in June 2017 at a farm in Bracknell (UK). This freshly harvested 'D' population was cleaned, dried to ~4% moisture content and stored at -20ºC in airtight jars above silica gel to preserve their physiological status. All germination assays were conducted in 60 mm petri dishes with two filter papers and >30 diaspores in triplicate. Cumulative germination was counted by the emergence of the coleorhiza through the margins of the glumes. Sub-samples of the FH population were after-ripened at 20ºC at 53% equilibrium relative humidity (~20% moisture content) and sequentially removed and assayed for germinability at 20ºC under constant light. For treatments comparing light and dark, plates were wrapped in two layers of aluminium foil. Distinct sets of replicates were used for each time-point to avoid light exposure during the assessment of germination. To assess the effect of a range of temperatures on germination, standard germination conditions were used for plates incubated in a two dimensional thermogradient plate (GRANT GRD1-LH, Grant Instruments Ltd., Cambridge, UK) under constant light.

Compound Screening

A target catalogue screening library was developed at Syngenta (Jealott's Hill International Research Centre, UK). The modes of action of these compounds were inferred both from structural similarity to compounds with known activity and *in vitro* enzyme activity assays. To protect the confidentiality of the compound structures, compound names were anonymised and their structures are not presented. Compounds were solubilised in dimethyl sulfoxide (DMSO) and exogenously applied at 5 concentrations (0.1, 1, 10, 50, 100 µM) in the germination medium following the method described above with the D seed batch at 16ºC. Plate assessment order and position in

the incubator were randomised at each assessment to control for temperature differences in the incubator. For each compound a dose-response curve was generated based on maximum cumulative germination for each of the three replicates. The area under the curve of each dose-response was used to compare the dormancy-breaking effect between compounds. A one-way ANOVA with a post-hoc Dunnett's Multiple Comparisons Test was used to calculate the significance of each compound response in comparison to the dose-response for DMSO at each compound concentration.

Light Microscopy

Caryopses at differing times after imbibition were fixed in 4% paraformaldehyde and then dehydrated in an ethanol series (Ruzin, 1999). Caryopses were then imbedded in 2-hydroxyethyl methacrylate polymerised with 1% benzoyl peroxide (Matsushima *et al.*, 2014). 5 µm sections were cut on a rotary microtome, stained with 1% toluidine blue (stains nuclei and polysaccharides blue) and counterstained with 1% safranin O (stains lignin red). Bright-field images were taken with an ECLIPSE Ni-E microscope (Nikon Corporation, Tokyo, Japan).

RNA Extraction, Library Preparation & Sequencing

FH and AR_{50} diaspores (~40 mg dry weight, ~30 diaspores) were imbibed following standard germination conditions at either 8 or 16^{o}C in constant light for 90 or 180 hours. These samples, along with dry seeds, were homogenised in liquid nitrogen and extracted in a hexadecyltrimethylammonium bromide (CTAB) buffer containing 1% β-mercaptoethanol following Graeber *et al.*, (2011) with the following modification: variable volumes of CTAB buffer were ground into a frozen powder with the sample. RNA quantity and quality was assessed using a NanoQuant^{TM} system (Tecan, Männedorf, Switzerland) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara,

California, USA). Only samples with an RNA integrity number (RIN) greater than 7.0 were progressed to sequencing (Annex Fig. 9.6). Messenger RNA was enriched by polyA isolation using an NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs (NEB), Massachusetts, USA). Libraries were prepared using NEBNext® Ultra™ II Directional RNA Library Prep Kit (NEB) with Sample Purification Beads and in-house indexes. A total of 50 libraries (5 replicates per treatment) were sequenced at 8 libraries per lane using an Illumina HiSeq X platform (Illumina Inc., California, USA) generating ~40 million 150 bp paired end reads (~80 million total) per sample (Annex Fig. 9.7).

De novo Assembly

De novo transcriptome assembly was performed using the rBPA pipeline (v.2.1.0) from the National Centre for Genome Resources (NCGR, Santa Fe, New Mexico). To reduce the amount of input sequence while still capturing the complexity of all conditions and replicates, only R1 of each pair was used for the assembly. Unitigs were assembled separately for each treatment in ABySS using the read pairing information (v.2.1.0) (Simpson *et al.*, 2009). Unitigs were then collapsed into a single sequence set using CD-HIT-EST (Fu *et al.* 2012) and Collapsed unitigs were assembled using MIRA (v.4.0) (Chevreux *et al.* 2004). Resultant contigs and untigs were scaffolded in ABySS. Transcriptome completeness was assessed using the Benchmarking Universal Single-Copy Orthologs (BUSCO) (Simao *et al.*, 2015) (Annex Fig. 9.8).

Functional Annotation

Functional annotation was performed using Blast2GO (v.5.2.5) (Götz *et al.* 2008) using a translated assembly. The top 20 BLAST hits for each scaffold (E-value < $1x10^{-3}$) were retrieved from UniProt/Swiss-Prot (v. 5) (The UniProt Consortium, 2019) using BLASTp

(version 2.1.7+, word size:6; HSP Length Cutoff:33). The 'Cloud InterProScan' (IPS) tool was run to identify structural domains and motifs. Gene ontology (GO) terms (Ashburner *et al.* 2011) were mapped from BLAST and IPS results using the Gene Ontology Database (GOA version 2018.02) (Gene Ontology Consortium, 2004) and annotated using default parameters. Scaffolds with a non-land plant top BLAST hit were removed.

Expression, Clustering and GO Enrichment Analysis

Reads were mapped to the assembly using the Burrows-Wheeler Aligner (BWA) (v.0.7.17) (Li & Durbin, 2010). Counts were generated for each transcript contig using HTseq (v.0.11.0) (Anders *et al.*, 2015) in default mode. Normalisation factors were calculated using EDAesq (v. 3.6) (Risso *et al.*, 2011) and differential expression analysis was conducted using the edgeR package (v. 3.22.0) (Robinson *et al.*, 2009). False discovery rate (FDR) was calculated following Benjamini & Hochberg (1995). Transcripts were considered differentially expressed if the absolute value of the log2 ratio of the mean of contrasted samples was >1 and FDR was < 0.001.

Clustering analysis was performed using MORPHEUS (Morpheus, 2019) for all transcript contigs where at least one replicate had an FPKM >5 and normalised by scaffold to generate z-scores. A K-means algorithm was used to generate 8 clusters using a correlation matrix of one minus Spearman's Rank for 1000 iterations (Annex Fig. 9.11). GO enrichment analysis using Fischer's Exact Test was conducted in Blast2GO to identify overrepresented 'Biological Process' GO terms between individual clusters with a subset of the assembly that had a minimum FPKM > 5 as the reference set.

Results and Discussion

Germination Kinetics in A. myosuroides

The dispersal unit (diaspore, hereafter 'seed') of *A. myosuroides* consists of a caryopsis (grain) comprising of the embryo and endosperm surrounded by a testa and pericarp, held within the maternally derived glumes of the spikelet (Hubbard, 1968). Approximately 25% of diaspores did not contain a caryopsis. When imbibed at 20°C, freshly harvested seeds are dormant (D), and this dormancy can be released by a period of dry storage (after-ripening) to produce seed batches with defined dormancy levels (Fig. **1e**). Germination of the seed occurs in a three step process beginning with the expansion of the coleorhiza that forms an outpouching of large vacuolated cells that ruptures the testa, pericarp and glumes (Fig. 1**a,c**). The radicle expands into this outpouching (Fig. 1**a,d**) and finally the shoot, composed of the plumule and coleoptile emerge from the distal margins of the gulmes (Fig. 1**a,d**). Since the first visible sign of germination is the emergence of the coleorhiza, we used coleorhiza emergence (Fig. 1**a**) to distinguish between germinated and ungerminated seeds.

D and AR batches respond differently to a gradient of imbibitional temperatures.

Dormant blackgrass seeds are limited in the range of temperatures under which they will germinate, having an optimum temperature between 6 and 9°C and a ceiling temperature of between 20 and 22°C (Fig **1f**). After-ripening increases both the optimum and ceiling temperatures in a dose-dependent manner so that in the fully after-ripened seed batch (AR_{100}) temperature has little effect on maximum germination proportion (Fig. **1f**). At 8°C there is only a small difference in the germination speed, but no difference in the maximum germination between D and AR_{50} batches however at 16°C, germination in the D state is inhibited while germination speed is increased in the AR_{50} batch. The difference in temperature response of D and AR batches indicates that dormancy in *A. myosuroides*

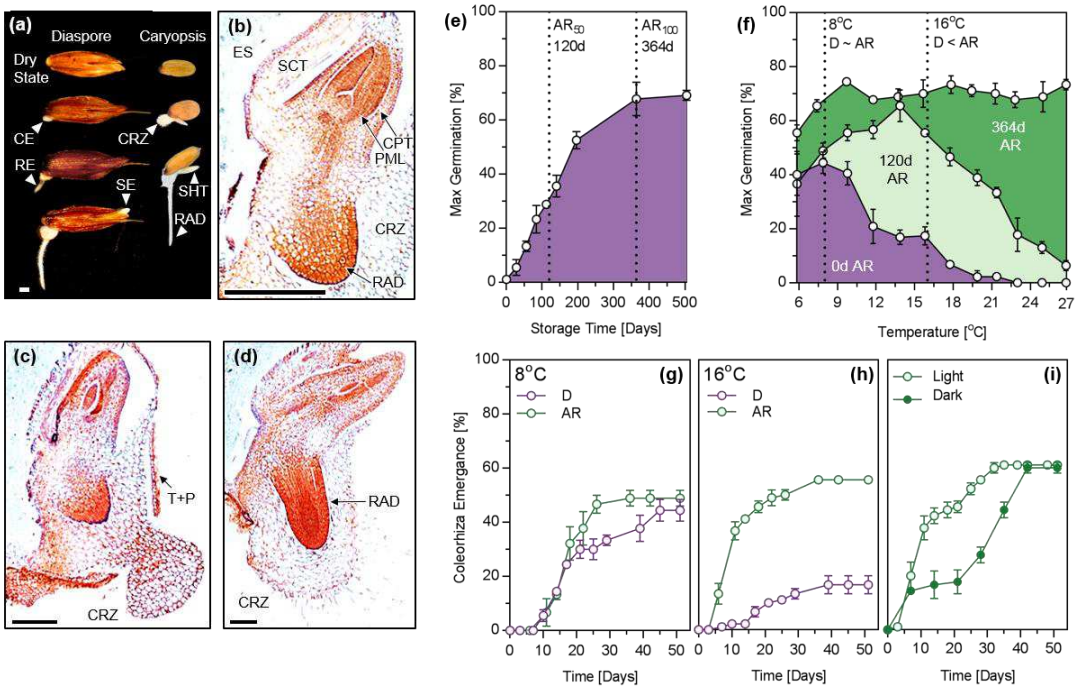


Fig. 1.Germination kinetics and ecophysiology of *A. myosuroides* germination. (a) Visible events during the germination of diaspores and caryopses showing coleorhiza, radicle and shoot emergence. (b-d) micrographs showing longitudinal sections of the embryo at (b) 24 hours after imbibition, (c) coleorhiza emergence and (d) immediately preceding radicle emergence. Scale bar in (a-d) represents 3 mm. (e) the effect of post-harvest storage (after-ripening) time at 50% relative humidity and 20°C on the mean germination final percentage. (f) The relationship between imbibitional temperature and mean final germination percentage on batches after-ripened for 0, 120 and 364 days. (g,h) Germination curves showing the differential effect of (g) 8°C and (h) 16°C on the cumulative coleorhiza emergence dormant (D) and 120 day after-ripened (AR) batches. (i) Germination curve showing the effect of incubation in the light and dark on coleorhiza emergence of the AR batch. Error bars represent standard error of the mean for three replicates of ~30 diaspores. CE, coleorhiza emergence; CPT, coleoptile; CRZ, coleorhiza; ES, endosperm; PML, plumule; RAD, radicle; RE, radicle emergence; SCT, scutellum, SE, shoot emergence; SHT, shoot; T+P, testa and pericarp.

functions to inhibit germination under the warm conditions when the seed is dispersed during the summer consistent with its winter annual habit (Wellington & Hitchins, 1966).

At both temperatures in the D and AR_{50} batches the first instances of coleorhiza emergence occur between 168 and 264 hours after imbibition (Fig. **1g,h**), therefore 180 and 90 hours after imbibition were chosen as comparable time points before the initiation of germination *sensu stricto*. This sampling scheme assumes that transcriptional changes determining the fate of the seeds occur during early imbibition as previously described by Weitbrecht *et al.* (2011).

Dormancy is not induced by changes in transcript abundance in the dry state

After-ripening is a period of dry storage at ambient temperatures and intermediate relative humidities that is commonly used as a controlled dormancy releasing treatment (Bewley *et al.*, 2013). However despite the pervasiveness of after-ripening in seed dormancy research, the molecular mechanisms of after-ripening still remain a partial mystery (Nelson *et al.*, 2017). In the dry state a pool of stored mRNA is present in Arabidopsis (Nakabayashi *et al.*, 2005). Studies using RNA polymerase inhibitors have demonstrated that this pool of stored mRNA is sufficient to complete germination but not subsequent growth in Arabidopsis, whereas chemical inhibition of translation inhibits germination completely (Rajjou *et al.*, 2004). Many hypotheses for the molecular mechanism of after-ripening focus on the modification of this pool of stored mRNA. Whilst some authors have proposed that *de novo* transcription occurs in the dry state (Bove *et al.*, 2005) perhaps due to the presence of pockets of high humidity within specific seed structures (Leubner-Metzger, 2005), much of the work on after-ripening focuses on modification of stored mRNA affecting translation efficiency (Bazin *et al.*, 2011), differential stability of dormancy- and germination- related transcripts (Nelson *et al.*, 2017) and stored mRNA degradation by reactive oxygen species (Bailly *et al.*, 2008).

In the total RNA that was extracted from dry and imbibed D and AR_{50} *A. myosuroides* seeds, we saw no difference in RNA integrity as measured by capillary electrophoresis. Illumina sequencing generated libraries of >40 million 150 bp paired-end reads. BUSCO analysis indicated that the assembly had a high degree of completeness (C:94.2% [S:65.3%,D:28.9%], F:3.1%,M:2.7%, n:1440).

Differential expression analysis for all pairwise comparisons between dry D and AR seeds and imbibed treatments demonstrated that up to 40% of the transcriptome was significantly perturbed after 180 hours of imbibition at either temperature (absolute LogFC >1, FDR <0.001). However only one significant differentially expressed gene (DEG) was found upregulated in a pairwise comparison between AR and D dry seeds, with homology to a *Brachypodium distachyon uncharacterised G protein* (UniProt KB I1HNR9). It seems unlikely that this gene has any role in dormancy breaking since a certain number of false positives are expected and this transcript has no homology to previously described dry seed after-ripening related DEGs (Meimoun *et al.*, 2014). In agreement with this is the tight clustering of D and AR dry samples demonstrated by principal component analysis (PCA) (Fig. **2a**) This finding supports the hypothesis that *de novo* transcription in the dry state is not the major mechanism releasing dormancy during after-ripening.

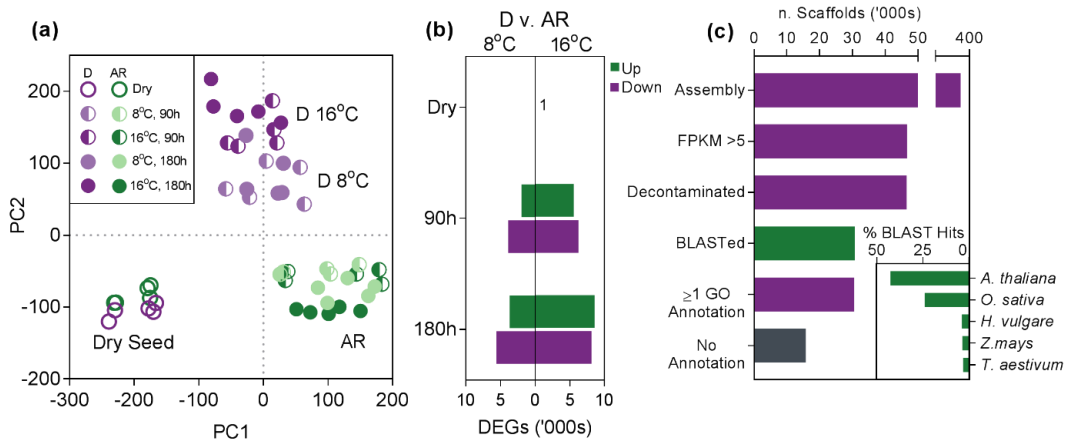


Figure 2. Summary of differential expression analysis and functional annotation. (a) Principal component analysis (PCA) plot showing the first and second principal components (PC1 & PC2) for all conditions and replicates analysed. (b) The number of differentially expressed genes (DEGs) identified by pairwise comparisons between dormant (D) and after-ripened (AR) at both temperatures and across the time points using edgeR (logFC >2, FDR<0.001). (c) Summary of the filtering and functional annotation steps to generate the annotated assembly performed using Blast2GO. The subfigure shows the distribution of species for BLAST top-hits used for functional annotation

Identification of processes associated with dormancy enforcement

In order to gain insight into the broad spectrum of processes associated with the interaction between dormancy and temperature a clustering approach was employed to generate gene lists with similar expression patterns (Fig. **3a**) and overrepresented GO terms were inferred using Fischer's Exact Test. A representative selection of GO terms that are significantly enriched ($P<0.05$, $n<10$) in at least one cluster are represented as a heatmap (Fig. **3b**).

In general, in clusters where AR treatments are more highly expressed, biological processes involving development, metabolism and growth are overrepresented (Fig. **3b**). In 'Cluster 3', where AR 16^{o}C, AR 8^{o}C and D 8^{o}C treatments are more highly expressed, cell cycle related processes were enriched. Regulatory elements of the cell cycle machinery such as *Cyclin-Dependant Kinases* (*CDKs*) and the *Anaphase Promoting Complex* (*APC*) are specifically expressed in the imbibed AR state and not in the dry seed, whereas inhibitors of cell cycle progression such as *Cyclin-Dependant Kinase Inhibitors* are expressed in the imbibed D state (Fig. **3c**). This indicates that cell cycle progression is associated with germination and arrest of the cell cycle may occur during dormancy enforcement. This observation complements a number of reverse genetics studies that demonstrate modified germination behaviours in lines overexpressing cell cycle regulatory genes in both bud and seed dormancy (Velappan *et al.*, 2017).

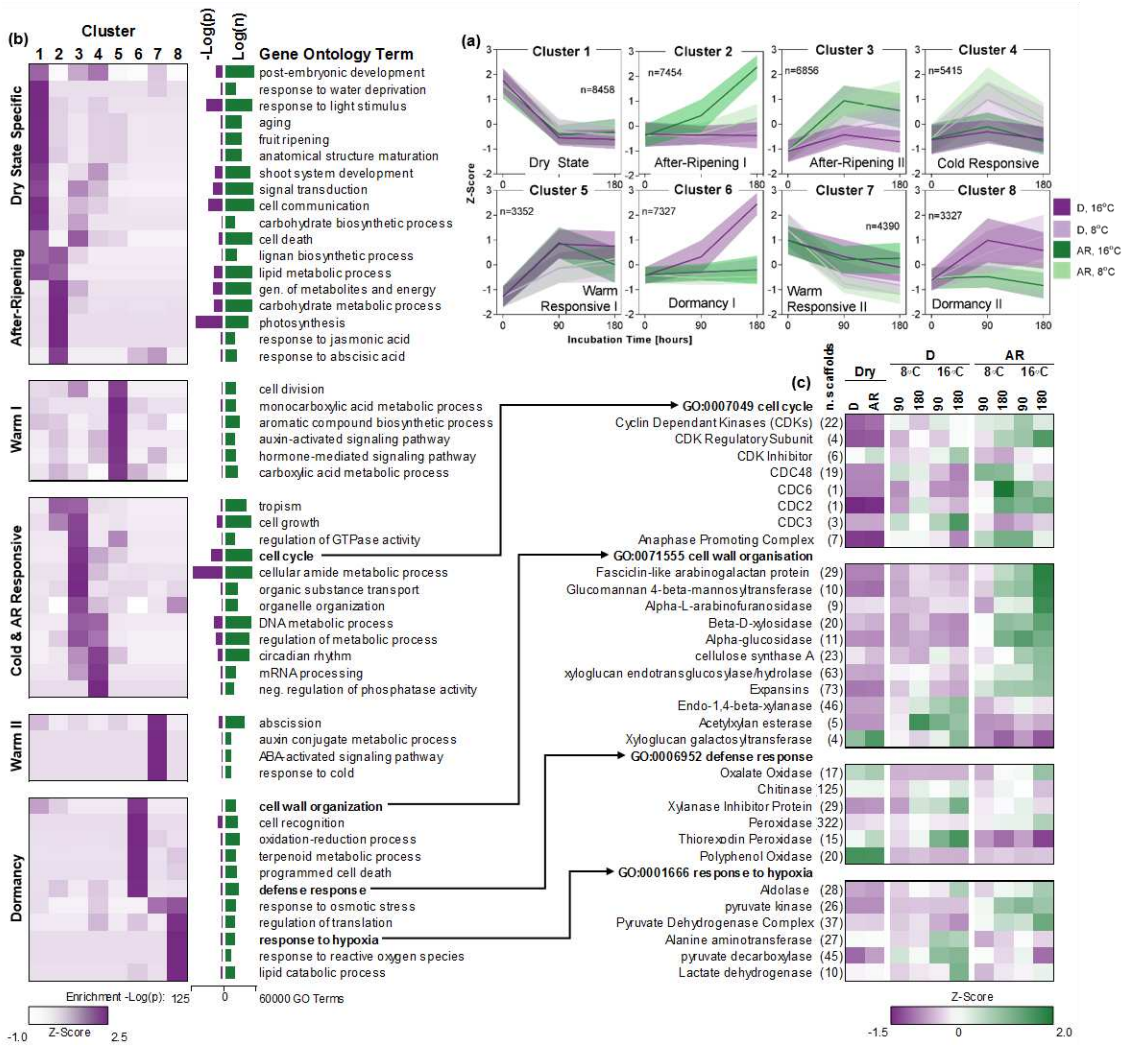


Fig. 3. Clustering and gene ontology (GO) enrichment analysis. (a) Expression profile plots of mean Z-score (line) and 90% confidence intervals (shaded area) for clusters generated by K-means (K=8) clustering implemented in MORPHEUS. (b) Heatmap of the enrichment of a representative selection of GO terms across the clusters visualised as a z-score of enrichment values (-Log(p)) calculated from Fischer's Exact Test (n terms >10, $p < 0.01$) implemented in Blast2GO. Bars next to each GO term show the maximum enrichment score (-Log(p), purple) and the log of number of associated terms analysed (Log(n), green). (c) Heatmaps showing the mean z-score of the expression of different groups of genes associated with enriched GO terms in different clusters where green represents upregulation and purple shows downregulation. Colour-blind safe colour palette was generated using ColorBrewer 2.0 (Brewer, 2019).

Dormancy upregulates plant defence responses

Seed decay through the activity of pathogens and predators is one of the key mechanisms regulating seed persistence in the soil (Long *et al.*, 2014). The interactions between weed seeds and fungi have the potential to be exploited for the purposes of weed seedbank management (Pollard, 2018) yet this area of research has to date been

limited by the molecular tools available to study seed-microbe interactions (Müller-Stöver *et al.*, 2016).

Amongst the most common fungal species in *A. myosuroides* seeds were (by BLAST top-hit, number of contaminating sequences with FPKM >5) were *Metarhizium sp., Podospora sp., Talaramyces sp.* & *Amylomyces sp*. In the cluster where genes are highly expressed in the D state at 16ºC ('Cluster 6'), the 'defence response' GO term was enriched. Whereas some pathogenic response genes such as *Polyphenol Oxidase* are specifically expressed in the dry seed, *Xylanase Inhibitor Proteins* and *Thioredoxin Peroxidases*, that are associated with fungal defence responses in seeds (Pollard, 2018), are expressed in the D seeds at 16ºC (Fig. **3c**). Antimicrobial defence peptides, such as *Vicillin* and *Defensin-like 1*, as well as *Superoxide Dismutase* (*SOD*) and were also highly expressed in the D 16ºC state. This suggests that alongside the inhibition of germination, dormancy also regulates processes that prepare the seed for long term survival against pathogen attack in the soil seed bank.

Anaerobic respiration genes are expressed in the D state

The 'response to hypoxia' GO term was enriched in this high temperature enforced dormancy-specific cluster. Investigation of the enzymes contributing to this enrichment revealed that genes encoding enzymes associated with aerobic respiration, such as *Pyruvate Kinase* and elements of the *Pyruvate Dehydrogenase Complex* are expressed in the AR state whereas those involved in anaerobic respiration, such as *Pyruvate Decarboxylase* and *Lactate Dehydrogenase* were expressed specifically in the D state at 16°C. This indicates that distinct respiratory mechanisms are involved in dormancy compared to during germination, and that D seeds may be relying on fermentation as a means of regenerating ATP.

The ability of some semiaquatic species, such as rice or the paddy weed *Echinochloa crus-galli*, to germinate under hypoxic conditions is a well-known phenomenon attributed to alcoholic and lactic fermentation (Bewley *et al.*, 2013) and anoxia can induce secondary dormancy in a number of grass species (Simpson, 1990). It is not clear what the advantage of performing anaerobic respiration in the D state could be, given the energetic inefficiency of fermentation (2 ATP glucose^{-1} anaerobic vs. 36+ ATP glucose^{-1} aerobic) and the accumulation of the toxic by-products acetaldehyde, ethanol and lactate. A possible explanation is that the energy requirements of the D seed may be low, and bypassing the mitochondria may avoid oxidative damage (Ratajczak *et al.*, 2019) that could cause a reduction in viability during a long period of dormancy.

Distinct hormonal processes are involved in dormancy release by cold temperatures and after-ripening

At the lower temperature of 8ºC, D and AR batches have similar germination kinetics, reaching the same maximum germination after ~40 days (Fig. **1g**). However PCA of all D vs. AR DEGs shows a cluster of replicates in the D 8ºC condition more closely associated with the D 16ºC condition than with the after-ripened treatments (Fig. **2a**). Analysis of transcription factors enriched in the cold responsive 'cluster 4' revealed that ethylene responsive transcription factors such as *ETHYLENE RESPONSE ELEMENT BINDING PROTEIN 1 & 13* (*EREBP1, EREBP13*) and *AP2-LIKE ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR* (*AIL1*) show a cold specific expression pattern in both D and AR batches (Fig. **4d**). It is likely that the large number of these isoforms identified reflect minor sequence variants or natural variation in the seed populations, rather than true paralogues. The expression of elements of the ethylene signalling pathway such as *ETHYLENE RECEPTOR 2* (*ETR2*) and *REVERSION-TO-ETHYLENE-INSENSITIVITY* (*RTE*), a positive regulator of ethylene receptor activity

(Resnickt *et al.*, 2014), are also positively regulated by the cold (Fig. **4c**). However transcription of ethylene biosynthetic genes was not considerably altered (Fig. **4b**).

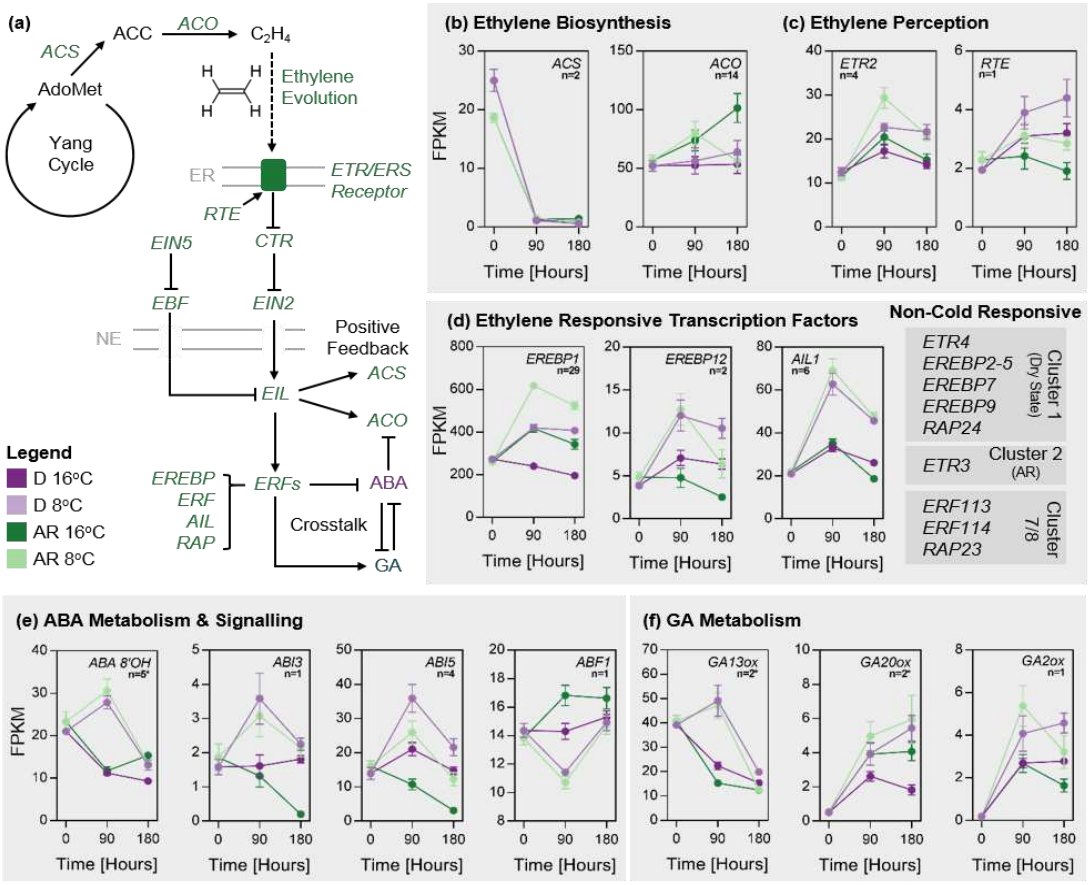


Fig. 4. Cold-induced changes in gene expression. (a) Ethylene biosynthesis and signalling pathway in rice drawn based on Rzewuski & Sauter (2008). (b-f) expression plots showing a change in normalised read counts (fragments per kilobase of transcript per million mapped reads, FPKM) for (b) ethylene biosynthesis genes, (c) genes involved in ethylene perception, (d) ethylene-responsive transcription factors, (e) genes involved in abscisic acid (ABA) metabolism and signalling and (f) genes involved in gibberellin (GA) metabolism and signalling. Genes are named by their nearest homolog in rice or Arabidopsis. Numbers under gene names represent the number of scaffolds summed. An asterisk next to this number indicates that dry-seed specific transcripts were not included. Error bars show standard error of the mean for 5 replicates. ABA, Abscisic acid; ABA 8'OH, *Abscisic acid 8'-hydroxylase*; ACC, 1-Amino-cyclopropane-1-carboxylic acid; ACO, *1-Aminocyclopropane-1-Carboxylic Acid Oxidase*; ABF, *ABA Response Element Binding Factor*; ABI3/5; *Abscisic Acid Insensitive 3/5*; AIL1, *AP2-Like Ethylene-Responsive Transcription Factor*; AdoMet, S-adenosyl-methionine; CTR1, *Constitutive Triple Response 1*; EBF, *EIN Binding F-Box*; EIL, *EIN-3-Like*; EIN2/5, *Ethylene Insensitive 2/5*; EREBP, *Ethylene-Responsive Element Binding Protein*; ERF, *Ethylene Response Factor*; ETR, *Ethylene Receptor*; GA, bioactive gibberellins; GA13ox, *Gibberellin 13-Oxidase*; GA20ox, *Gibberellin 20-Oxidase*; RAP23/24, *Related To AP2 3/4*; RTE, *Reversion-To-Ethylene-Insensitivity 1*.

Ethylene signalling plays an important role in the regulation of dormancy and germination through its interaction with GA and ABA signalling (Linkies & Leubner-Metzger, 2012).

In Arabidopsis, ethylene receptor mutants are hypersensitive to ABA (Beaudoin *et al.*, 2007) and GA application increases the expression of *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO*). Downstream of ethylene signalling, the expression of *ABA 8'OH,* encoding the main ABA degrading enzyme ABA 8'hydroxylase, was induced by the cold (Fig. **4e**) and other genes of the ABA signalling cascade, such as *ABA INSENSITIVE 3* and *5* (*ABI3&5*) along with *ABA BINDING FACTOR 1* (*ABF1*) showed cold-responsive expression patterns (Fig. **4e**). This contradicts pervious microarray data that show an after-ripening dependant increase in *ABA 8'OH* expression (Barrero *et al.* 2009). Interestingly, *gibberellin 13-oxidase* (*GA13ox*), encoding an enzyme that reduces gibberellin activity in rice (Magome *et al.*, 2013), also showed a cold-responsive expression pattern (Fig. **5f**).

Whilst expression of ABA biosynthetic genes, including the rate limiting *9-cis-epoxycarotenoid dioxygenases* (*NCEDs*), followed a dry state-specific expression pattern (Fig **5a**), genes involved in the biosynthesis of gibberellins, such as *kaurene synthase* (*KS*), *kaurene oxidase* (*KO*), *kaureneoic acid oxidase* (*KAO*) and the rate limiting gene *gibberellin 3-oxidase* (*GA3ox*), were expressed in an after-ripening dependant manner and not expressed in the D 8ºC condition (Fig. **5b**). The gibberellin receptor gene *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*) was constitutively expressed across all the treatments. Genes encoding some *GIBBERELLIC ACID-INSENSITIVE* (*GAI*) DELLA proteins, which represses the expression of GA-responsive genes in the absence of bioactive GAs, expression was higher in the D 16ºC condition. Known downstream germination-specific GA-responsive genes (Chen & Bradford, 2000; Jan & Komatsu, 2006), such as *xyloglucan endotransglycosylase/hydrolase 8* (*XTH8*) and *β-expansins* show AR-specific expression patterns (Fig. **5b**).

Taken together, distinct hormonal processes appear to be involved in dormancy release by cold temperatures and after-ripening. In the cold, upregulation of ethylene-responsive transcription factors and *ABA 8' hydroxylase* in the absence of *GA3ox* expression

indicate a potential GA-independent mechanism for dormancy release. Conversely, the AR-specific expression pattern of key GA biosynthetic genes and known GA-responsive genes suggests an important role for gibberellin in regulating after-ripening dependant dormancy release.

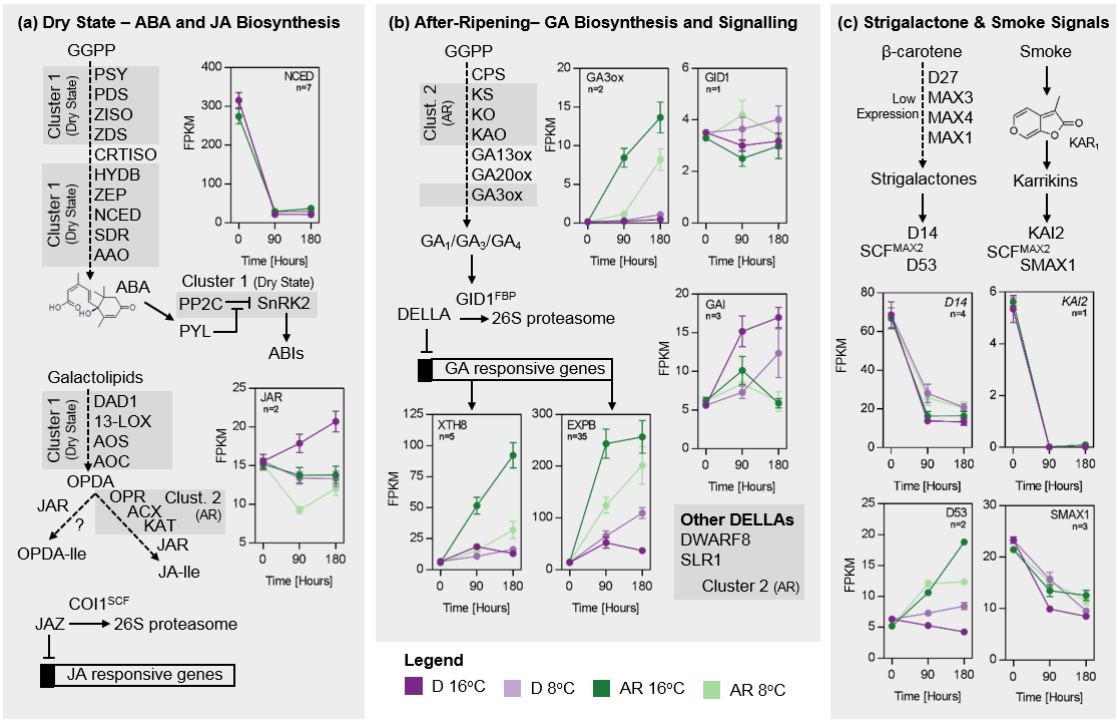


Fig. 5. Dormancy- and after-ripening-specific changes in gene expression. Expression patterns of hormone biosynthesis and signalling genes with pathway components grouped into the clusters in which they are mostly highly represented. (a) Abscisic acid and jasmonate biosynthesis genes are highly expressed in the dry seed. (b) GA biosynthesis genes and GA responsive genes are expressed in an after-ripening dependant manner. (c) Genes in the strigalactone/smoke signal transduction pathway show dormancy and after-ripening specific expression patterns. Genes are named by their nearest homolog in rice or Arabidopsis. Numbers under gene names represent the number of scaffolds summed. Error bars show standard error of the mean for 5 replicates. 13-LOX, *13-Lipoxygenase*; AAO, *Abscisic Aldehyde Oxidase*; ABA, abscisic acid; ACX, *Acyl CoA Oxidase*; COI1, *Coronatine Insensitive1*; CPS, *ent-Copalyl Diphosphate Synthase*; CRTISO, *Carotene Isomerase*; DAD1, *Defective In Anther Dehiscense 1*; EXPB, *β-Expansin*; GA13ox, *Gibberellin 13-Oxidase* ; GA20ox, *Gibberellin 20-Oxidase*; GA3ox, *Gibberellin 3-Oxidase*; GAI, *GA Insensitive*; GID1, *Gibberellin Insensitive Dwarf1*; GGPP, Geranylgeranyl Diphosphate; HYDB, *β-Ring* Carotene *Hydroxylase*; JA, jasmonic acid; JA-Ile, jasmonoyl isoleucine; JAR, *Jasmonoyl Isoleucine Conjugate Synthase*; JAZ, *JASMONATE ZIM DOMAIN Proteins*; KAI2, *Karrikin Insensitive2*; KAO, *ent-Kaurenoic Acid Oxidase*; KAR1, karrikin 1; KAT, *L-3-Ketoacyl-Coa-Thiolase*; KO, *Ent-Kaurene Oxidase*; KS, *ent-Kaurene Synthase*; NCED, *9-cis-Epoxycarotenoid Dioxygenases*; OPDA, *cis*-(+)-12-oxophytodienoic acid; OPDA-Ile, isolucene conjugate of OPDA; OPR, *OPDA Reductase*; PDS, *Phytoene Desaturase*; PP2C, *Type 2C Protien Phosphatases*; PSY, *Phytoene Synthase*; PYL, *PYRABACTIN* RESISTANCE-Like; SCF, *SKP Cullin F-box*; SDR, *Short-Chain Dehydrogenase/ Reductase*; SLR1, *Slender Rice 1*; SnRK2, *SNF1-Related Protein Kinase 2*; XTH8, xyloglucan endotransglycosylase/hydrolase 8; ZEP, *Zeaxanthin Epoxidase*; ZDS, *Z-Carotene Desaturase*; ZISO, *Z-Carotene Isomerase*.

Changes in hormonal pathways associated with dormancy

In a targeted analysis of the transcript abundance of hormone biosynthesis and signalling pathways, it is interesting that very few significantly up-regulated transcripts were identified in the D 16ºC state, the condition under which dormancy is enforced. For example, there is no overall pattern of expression to suggest that D seeds may be more sensitive to ABA or less sensitive to GA. However there were upregulation of some jasmonate- and strigolactone-related genes in the D 16ºC state.

Early jasmonate biosynthetic genes such as *13-lipoxygenases* (*13-LOX*), *allene oxide synthase* (*AOS*) and *allene oxide cyclase* (*AOC*) were specifically expressed in the dry seed indicating a potential accumulation of 12-oxo-phytodienoic acid (OPDA) in the dry state (Fig. **5a**). *OPDA reductase* (*OPR*) and genes involved in the peroxisomal β-oxidation of the resultant 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) are more highly expressed in the AR state, yet *JA-amino acid synthetase* (*JAR1*), that catalyses the activating conjugation of Jasmonic acid (JA) to JA-Ile, is more highly expressed in the D 16ºC. Whilst OPDA and JA-Ile both have some regulatory roles in dormancy and germination in model species (Dave *et al.*, 2011; Linkies & Leubner-Metzger, 2012) it is also possible that OPDA or JA-Ile have some role in the enrichment in defence response GO terms seen in the D 16ºC state (Fig. 3**b,c**).

Expression levels of strigolactone biosynthetic genes such as *D27*, *MAX3*, *MAX4* and *MAX1* were very low across all the treatments, however genes of the strigalactone receptor complex such as *D14* (in the dry state) and *D53* (in the AR state) had relatively high expression levels (Fig. 5c). The receptor *KARRIKIN INSENSITIVE 2*, that perceives karrikin produced by smoke, had a dry seed-specific expression pattern.

Identification and assessment of potential targets for weed seedbank management

In the context of an agricultural weed with a winter annual habit, targets for the management of the weed seedbank should exploit processes that are active in the D state under warm conditions. For the example of compounds targeting endogenous hormone signalling, the presence of a receptor and downstream signalling components is required. This is the case for the gibberellin receptor *GID1* and the karrikin receptor *KAI2*. Using a target catalogue compound library, we assessed a number of targets for dormancy breaking and germination stimulation. Of the 6 compounds that had a significant dormancy breaking effect, three were GID1 agonists, two were Phytoene Desaturase (PDS) inhibitors and one was a KAI2 agonist (Fig. **6a-g**). Interestingly, the

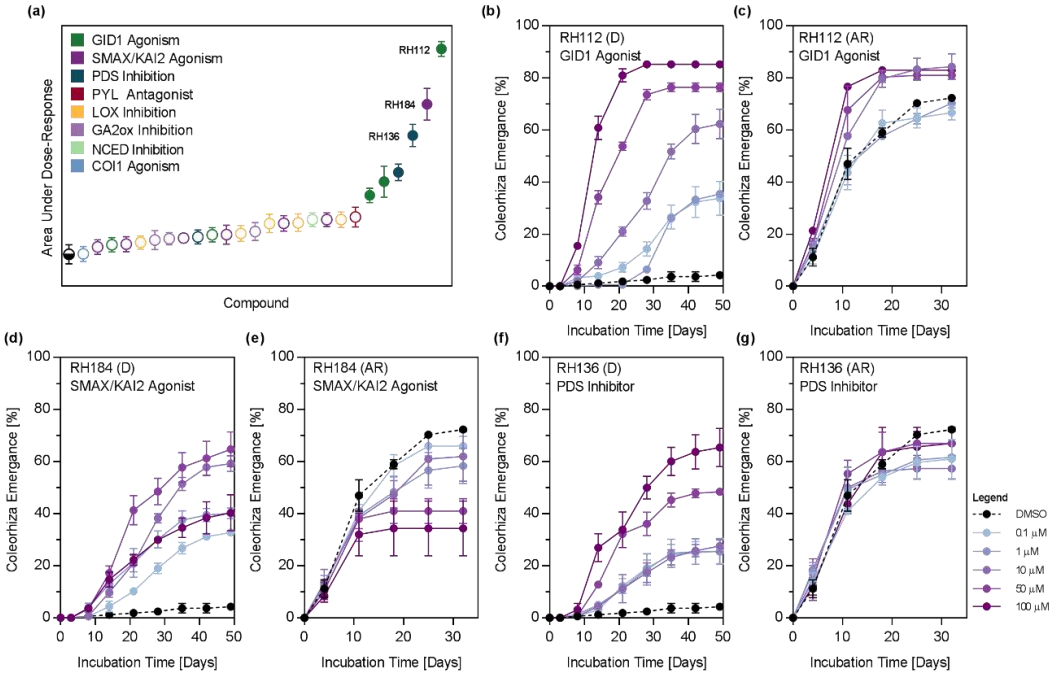


Fig. 6. Chemical screen to identify targets for dormancy breaking compounds. (a) Summary of the compound screen, showing the area under the curve of the dose-response generated from maximum germination percentage for triplicates of ~30 seeds. Individual points are coloured by mode of action, inferred from structural similarity or *in vitro* activity. Filled circles show significant responses resulting from one-way ANOVA with a post-hoc Dunnett's Multiple Comparisons Test (P<0.001). (b-g) germination curves showing the dose-response effect of the most potent dormancy-breaking compounds on D (b,d,f) and AR (c,e,g) batches. Error bars represent standard error of the mean for three replicates of ~30 diaspores

effect of the KAI2 agonist (RH184) was dependant on the dormancy status of the seed batch where in the D state dormancy was broken but in the AR state after-ripening germination was inhibited. The expression pattern of *KAI2* was dry seed specific (Fig. 5c) suggesting that this KAI2 accumulates in the dry state and remains during imbibition.

Conclusions

Seed dormancy in *A. myosuroides* is a highly complex trait regulated by the interaction of multiple hormone signalling pathways. Dormancy, rather than a state of quiescence, is characterised by the expression of many dormancy-specific genes involved in defence against pathogens, alternative respiration pathways and inhibition of the cell cycle. Cold temperature-release from primary dormancy of *A. myosuroides* is associated with changes in ethylene signalling components whilst after-ripening mediated dormancy release involves the expression of gibberellin biosynthesis genes. Our targeted analysis of the hormonal regulation of dormancy and germination in *A. myosuroides* has identified a number of targets that were validated using a compound screening approach. This work will inform the design and selection of novel targets for agricultural weed seedbank management.

Acknowledgements

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) Research Grants (BB/M02203X/1, BB/R505730/1) to G.L.-M. We thank Mark Levy, Diane Grant and Sarah Rabjohn for assistance with seed processing and Dr. Marta Pérez for technical assistance with RNA extraction. We are grateful to Richard Dale and Racella McNair (Syngenta Ltd.) for logistical support with RNA samples and to An Hu and Mariana Franco (Syngenta Ltd.) for assistance with the library preparation. We are also grateful to Katrin Hermann and Jutta Boehmer (Syngenta Ltd.) for provision of the compound library.

Author contributions

T.H., K.N., D.S., A.S., and G.L.-M. planned and designed the research; T.H. performed experiments; L.G. was responsible for sequencing library preparation and K.S. produced the *de novo* transcriptome assembly; T.H., J. Ch. and J. Co. analysed the gene expression data; A.S. provided access to materials; T.H, K.N. and G.L.-M. wrote the manuscript with contributions from all authors. T.H., K.N. and G.L.-M. contributed equally to this work.

References

Adkins SW, Peters NCB. **2001**. Smoke derived from burnt vegetation stimulates germination of arable weeds. *Seed Science Research* **11**: 213–222.

Anders S, Pyl PT, Huber W. **2015**. HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**: 166–169.

Bailly C, El-Maarouf-Bouteau H, Corbineau F. **2008**. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *Comptes Rendus - Biologies* **331**: 806–814.

Barrero, J.M. et al., 2009. Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant Physiology*, **150**(2), pp.1006–1021.

Barrett SCH. **1983**. Crop mimicry in weeds. *Economic Botany* **37**: 255-282.

Baskin JM, Baskin CC. **2004**. A classification system for seed dormancy. *Seed Science Research* **14**: 1–16.

Bazin J, Langlade N, Vincourt P, Arribat S, Balzergue S, El-Maarouf-Bouteau H, Bailly C. **2011**. Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *The Plant Cell* **23**: 2196–2208.

Beaudoin N, Serizet C, Gosti F, Giraudat J. **2007**. interactions between abscisic acid and ethylene signaling cascades. *The Plant Cell* **12**: 1103.

Benjamini Y, Hochberg Y. **1995**. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**: 289–300.

Bewley J, Bradford K, Hilhorst H, Nonogaki H. **2013**. *Seeds - Physiology of Development, Germination and Dormancy*. London: Springer.

Bove J, Lucas P, Godin B, Ogé L, Jullien M, Grappin P. **2005**. Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. *Plant Molecular Biology* **57**: 593–612.

Brewer C. ColorBrewer 2.0. www.colorbrewer.org [accessed 14 August 2019]

Bridges D. **1994**. Impact of weeds on human endeavours. *Weed Technology* **8**: 392–395.

Chauvel B, Guillemin J, Gasquez J, Gauvrit C. **2012**. History of chemical weeding from 1944 to 2011 in France: Changes and evolution of herbicide molecules. *Crop Protection* **42**: 320–326.

Chen F, Bradford KJ. **2000**. Expression of an expansin is associated with endosperm weakening during tomato seed germination. *Plant Physiology* **124**: 1265–1274.

Clarke J, Ginsburg D, Clare K, Tonguc L. **2015**. *The Encyclopaedia of Arable Weeds*. AHDB.

Colbach N, Durr C, Roger-Estrade J, Chauvel B, Caneill J. **2006**. AlomySys: Modelling black-grass (*Alopecurus myosuroides* Huds.) germination and emergence, in interaction with seed characteristics, tillage and soil climate: I. Constructon. *European Journal of Agronomy* **24**: 113–128.

Dave A, Hernandez ML, He Z, Andriotis VME, Vaistij FE, Larson TR, Graham IA. **2011**. 12-Oxo-Phytodienoic acid accumulation during seed development represses seed germination in Arabidopsis. *The Plant Cell* **23**: 583–599.

Duke SO. **2012**. Why have no new herbicide modes of action appeared in recent years? *Pest Management Science* **68**: 505–512.

Finch-Savage WE, Leubner-Metzger G. **2006**. Seed dormancy and the control of germination. *New Phytologist* **171**: 501–523.

Gene Ontology Consortium. **2004**. The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Research* **32**: 258–261.

Goggin DE, Powles SB. **2014**. Fluridone: A combination germination stimulant and herbicide for problem fields? *Pest Management Science* **70**: 1418–1424.

Graeber K, Linkies A, Wood AT, Leubner-Metzger G. **2011**. A guideline to family-wide comparative state-of-the-art quantitative RT-PCR analysis exemplified with a Brassicaceae cross-species seed germination case study. *The Plant cell* **23**: 2045–2063.

Heap I. **2019**. International Survey of Herbicide Resistance. www.weedscience.org [accessed 14 August 2019].

Hubbard C. **1968**. *Grasses*. Bungay, Sussex: The Chaucer Press.

Jan A, Komatsu S. **2006**. Functional characterization of gibberellin-regulated genes in rice using microarray system ga-regulated gene expression. *Genomics, Proteomics & Bioinformatics* **4**: 137–144.

Leubner-Metzger G. **2005**. b-1,3-Glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *The Plant Journal* **41**: 133–145.

Li H, Durbin R. **2010**. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**: 589–595.

Linkies A, Leubner-Metzger G. **2012**. Beyond gibberellins and abscisic acid: How ethylene and jasmonates control seed germination. *Plant Cell Reports* **31**: 253–270.

Long RL, Gorecki MJ, Renton M, Scott JK, Colville L, Goggin DE, Commander LE, Westcott DA, Cherry H, Finch-Savage WE. **2014**. The ecophysiology of seed persistence: A mechanistic view of the journey to germination or demise. *Biological Reviews*.

Lutman PJW, Moss SR, Cook S, Welham SJ. **2013**. A review of the effects of crop agronomy on the management of *Alopecurus myosuroides*. *Weed Research* **53**: 299–313.

Magome H, Nomura T, Hanada A, Takeda-kamiya N, Ohnishi T, Shinma Y, **Katsumata T, Kawaide H, Kamiya Y, Yamaguchi S. 2013**. CYP714B1 and CYP714B2 encode gibberellin 13-oxidases that reduce gibberellin activity in rice. **110**.

Matsushima R, Masahiko M, Miyako K, Kondo H, Kaoko F, Kawagoe Y, Sakamoto W. **2014**. Amyloplast-Localized SUBSTANDARD STARCH GRAIN4 protein influences the size of starch grains in rice endosperm. *Plant physiology* **164**: 623–636.

Meimoun P, Mordret E, Langlade NB, Balzergue S, Arribat S, Bailly C, El-Maarouf-Bouteau H. **2014**. Is gene transcription involved in seed dry after- ripening? *PLoS ONE* **9**: 1–6.

Metzger J. **1983**. Promotion of germination of dormant weed seeds by substituted phthalimides and gibberellic acid. *Weed Science* **31**: 285–289.

Mohler C, Liebman M, Staver C. **2007**. Weed life history: identifying vulneribilities. In: Ecological Management of Agricultural Weeds. Cambridge University Press.

Morpheus. **2019**. MORPHEUS Versatile matrix visualization and analysis software. URL https://software.broadinstitute.org/morpheus/ [accessed 21st June 2019].

Müller-Stöver D, Nybroe O, Baraibar B, Loddo D, Eizenberg H, French K, Sønderskov M, Neve P, Peltzer DA, Maczey N, *et al.* 2016. Contribution of the seed microbiome to weed management. *Weed Research* **56**: 335–339.

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E. **2005**. Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: Epigenetic and genetic regulation of transcription in seed. *Plant Journal* **41**: 697–709.

Nelson SK, Ariizumi T, Steber CM. **2017**. Biology in the dry seed: transcriptome changes associated with dry seed dormancy and dormancy loss in the Arabidopsis GA-insensitive *sleepy1-2* mutant. *Frontiers in Plant Science* **8**: 1–21.

Pollard AT. **2018**. Seeds vs fungi: an enzymatic battle in the soil seedbank. *Seed Science Research* **28**: 197–214.

Rajjou L, Gallardo K, Debeaujon I, Job C, Job D. **2004**. The effect of α-Amanitin on the Arabidopsis seed proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination. *Plant Physiology* **134**: 1598–1613.

Ratajczak E, Małecka A, Ciereszko I, Staszak AM. **2019**. Mitochondria are important determinants of the aging of seeds. *International Journal of Molecular Sciences* **20**: 1-12.

Resnickt JS, Went C, Shockey JA, Changs C. **2014**. REVERSION-TO-ETHYLENE SENSITIVITY1, a conserved gene that regulates ethylene receptor function in Arabidopsis. *PNAS* **103**: 7917–7922.

Risso D, Schwartz K, Sherlock G, Dudoit S. **2011**. GC-Content normalization for RNA-Seq data. *BMC Bioinformatics* **12**: 1-17.

Robinson MD, McCarthy DJ, Smyth GK. **2009**. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.

Ruzin SE. **1999**. *Plant Microtechnique and Microscopy*. Oxford University Press.

Rzewuski G, Sauter M. **2008**. Ethylene biosynthesis and signaling in rice. *Plant Science* **175**: 32–42.

Simao FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. **2015**. Genome analysis BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* **31**: 3210–3212.

Simpson G. **1990**. *Seed Dormancy in Grasses*. Cambridge: Cambridge University Press.

Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJM, Birol I. **2009**. ABySS: A parallel assembler for short read sequence data. *Genome Research* **19**: 1117–1123.

The UniProt Consortium. **2019**. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Research* **47**: D506–D515.

Velappan Y, Signorelli S, Considine MJ. **2017**. Cell cycle arrest in plants: What distinguishes quiescence, dormancy and differentiated G1? *Annals of Botany* **120**: 495–509.

Weitbrecht K, Müller K, Leubner-Metzger G. **2011**. First off the mark: Early seed germination. *Journal of Experimental Botany* **62**: 3289–3309.

Wellington PS, Hitchins S. **1966**. Seed doramncy and the winter annual habit in blackgrass (*Alopecurus myosuroides* Huds.). *Journal of the National Institute of Agricultural Botany* **10**: 628–643.

Zimdahl R. **2018**. *Fundamentals of Weed Science*. Colorado: Academic Press.

5. Coleorhiza-enforced seed dormancy: a novel mechanism regulating germination in grasses

Manuscript prepared for submission to 'Nature Plants' (Online ISSN: 2055-0278).

5.1 Author contributions

Thomas Holloway (TH), Kazumi Nakabayashi (KN), David Stock (DS), Anne Seville (AS) and Gerhard Leubner-Metzger (GL-M) planned and designed the research; TH and Tina Steinbrecher (TS) performed experiments; TH and TS analysed and interpreted the data; TH wrote the manuscript; all authors revised and approved the final article. Specifically: Germination kinetics (Figs. 1 & 6), microscopy (Fig. 2), microarray data analysis (Fig. 3), sample preparation, enzyme activity assays (Fig. 5) and isoelectric focusing (Fig. 4) were performed by TH. The method development and measurement of coleorhiza puncture force was performed by TH and TS. The hypothesis that the coleorhiza becomes reinforced during dormancy enforcement was formulated by TH based on analysis of publically available microarray data.

The concept, experimental design and narrative of the manuscript were conceived by TH. Project supervision was provided by KN and GL-M (Royal Holloway University of London, UK) and by DS and AS (Syngenta Ltd., Jealott's Hill International Research Centre, UK). TH is the first (lead) author on the manuscript, prepared all the figures and wrote the first draft. TH coordinated the internal revision process with contributions from AS, TS, KN and GL-M. The manuscript is in the advanced stages of preparation for submission to Nature Plants as an 'Article'. Submission is anticipated before the end of November 2019.

Coleorhiza-enforced seed dormancy: a novel mechanism regulating germination in grasses.

Thomas Holloway[1], Tina Steinbrecher[1], Anne Seville[2], David Stock[2], Kazumi Nakabayashi[1] & Gerhard Leubner-Metzger[1]

[1]School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom

[2]Syngenta, Jealott's Hill International Research Centre, Warfield, Bracknell RG42 6EY, United Kingdom

Correspondence and requests for materials should be addresses to K.N. or G.L.M. at: School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom.

URL: www.seedbiology.eu

Corresponding Author Details:

Gerhard Leubner Metzger: gerhard.leubner@rhul.ac.uk, +44 1784 44 3895,

URL: https://pure.royalholloway.ac.uk/portal/en/persons/gerhard-leubner(b07cd3da-9c1d-4167-8d52-199a13d54351).html

Kazumi Nakabayashi: kazumi.nakabayashi@rhul.ac.uk, +44 1784 44 3769

URL: https://pure.royalholloway.ac.uk/portal/en/persons/kazumi-nakabayashi(e0fa28c2-6185-44d1-966c-192c95e86a70).html

ORCID *Thomas Holloway*: 0000-0002-8753-7841

ORCID *Tina Steinbrecher*: 0000-0003-3282-6029

ORCID *Kazumi Nakabayashi*: 0000-0002-4186-541X

ORCID *Gerhard Leubner-Metzger*: 0000-0002-6045-8713

ORCID *Anne Seville*: 0000-0001-8024-7959

Nature Plants

Received:

Accepted:

Published Online:

Abstract

The tissues that surround the embryo in dicotyledonous seeds have a well-studied role in enforcing seed dormancy and regulating germination through the modulation of their cell wall mechanical properties. Here we report that the coleorhiza, a tissue that surrounds the radicle in grass embryos, performs the same role during dormancy and germination in *A. fatua* (common wild oat). Using novel biomechanical techniques, we show that the coleorhiza becomes strengthened during the enforcement of dormancy and weakened during germination. Ablation experiments demonstrate that the coleorhiza is required for the maintenance of dormancy. Analysis of publically available microarray data indicate that xyloglucan endotransglycosylase/hydrolases (XTH) may have a role in coleorhiza reinforcement. Separation of XTH isozymes identify XTHs that are specific to the dormant coleorhiza. Our study supports the idea that physical restraint of the radicle is an important mechanism regulating germination and that monocots and dicots have independently evolved similar mechanism to achieve this.

Introduction

In recent years there has been a growing interest in how mechanical forces shape plant interactions with the environment[1]. This is particularly true in plant dormancy research, where organs physically blocking the growth and expansion of tissues have been found to play an important role in both bud[2] and seed dormancy[3]. Seeds are structurally complex and different organs of the seed provide different contributions to seed dormancy[4]. From a biomechanical perspective a seed consists of two functional compartments: the embryo that expands in response to environmentally-mediated hormonal signals, and the covering layers that restrain the growth of the embryo up to a threshold point, above which germination occurs[3]. In eudicot seeds, the key tissue in restraining the growth of the radicle is the micropylar endosperm (MPE) that surrounds

the tip of the radicle (embryonic root)[3]. The force required to puncture this tissue, and hence complete germination, is changed in response to temperature and hormone application[5].

Weakening of the MPE is a prerequisite for the completion germination of many eudicot species[6]. This weakening is directed by environmentally-mediated hormonal mechanisms involving reactive oxygen species, gibberellins (GAs) and ethylene[5,7–9] through the increased activity of cell wall remodeling proteins (CWRPs) such as expansins, glucanases, mannanases and transglycosylates that loosen the polysaccharide bonds within the MPE cell walls[7,10–12]. Biomechanical techniques have been used to confirm that hormone-mediated upregulation of these CWRPs results in a decrease in the force required to puncture the MPE[5,13–16]. Monocotyledonous seeds, such as cereals, lack the MPE that plays this important role in dicot seed dormancy and germination. Instead, the radicle is surrounded by a non-vascularised embryonic tissue called the coleorhiza that expands upon imbibition and is thought to have some role in protecting the growing embryo during the germination process[17]. It has recently been suggested that the coleorhiza of monocots and the MPE of dicots may be functionally related tissues, and that the coleorhiza may have an important role to play in the regulation of dormancy in monocot seeds[18,19].

In this study we use the agricultural weed *Avena fatua* (wild oat) to demonstrate that the coleorhiza and MPE are indeed functionally related, in so far as the coleorhiza modulates its mechanical properties to restrain or permit the growth of the radicle during dormancy and germination. We also provide an explanation for how this process may be mediated by the xyloglucan endotransglycosylase/hydrolase family of CWRPs.

Results

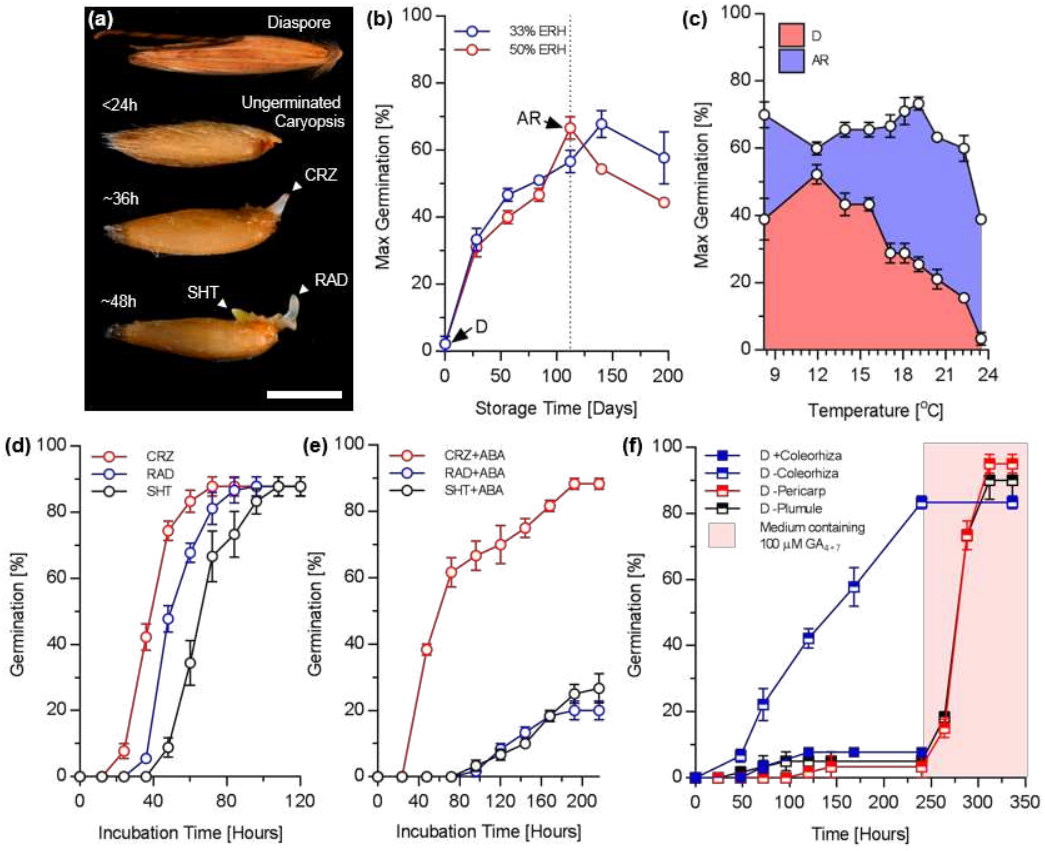


Fig. 1. Morphophysiology of *A. fatua* germination. a, Photographs of *A. fatua* diaspore and caryopses at different stages of the germination process, showing the emergence of the coleorhiza, the rupture of the coleorhiza by the radicle and the emergence of the shoot. **b**, After-ripening assau showing the effect of storage time on the maximum germination of D diaspores at 33 and 50% equilibrium relative humidity. **c**, The effect of different temperatures on the maximum germination of D and AR diaspores. **d**, Germination curve showing the timing of these events at an optimal temperature in the after-ripened (AR) state. **e**, the effect of 50 µM *cis*-S(+)-Abscisic acid (ABA) on the timing of germination events. **f**, the effect of ablation of the coleorhiza from dormant caryopses in comparison to other wounding controls such as the removal of the embryonic shoot and pericarp. After 240 hours, these wounding controls were transferred to a germination promoting medium containing 100 µM GA_{4+7} to confirm that ablation did not affect germinability. Scale bar 3 mm. Error bars show SEM for 3 independent replicates of 30 individuals. D, dormant; AR, after-ripened; CRZ, coleorhiza emergence; RAD, radicle emergence; SHT, shoot emergence.

Germination kinetics in *A. fatua*. Dormancy in *A. fatua* acts to inhibit germination under warmer temperatures, a mechanism common in winter annuals (Fig. 1c). After a period of dry storage ('after-ripening'), dormancy is released and after-ripened (AR) caryopses will germinate under warm conditions (Fig. 1b). Germination in *A. fatua*, as with other cereals, occurs as a three step process, beginning with the emergence of

the coleorhiza (Fig. 1a) caused by expansion of the cells of the coleorhiza that ruptures the covering layers of the testa and pericarp (Fig. 2b). This mechanism of expansion occurs independently of the cell cycle as coleorhizal cells do not proceed through the cell cycle (Appendix. Fig. 9.13). The expansion of coleorhiza cells occurs before the growth of the radicle that later ruptures the coleorhiza (Appendix. Fig. 9.12). Application of 50 µM *cis*-S(+)-ABA (hereafter ABA), a phytohormone associated with the acquisition and maintenance of dormancy, has a disproportional effect on radicle and shoot emergence in after-ripened (AR) caryopses (Fig. 1e), indicating that different embryonic organs have autonomous regulation of their rate of expansion. This retardation of radicle emergence under ABA treatment also occurs in dicot species, such as the endosperm weakening model *Lepidium sativum*, as the result of an inhibition of MPE weakening[5]. This was the first indication that the coleorhiza may have a role in restraining the radicle under conditions unfavourable for germination.

Ablation of the coleorhiza breaks dormancy. If the coleorhiza restrains the growth of the radicle to maintain dormancy, we hypothesised that ablation of the coleorhiza from dormant caryopses would induce radicle growth. This is indeed the case, however the initiation of radicle growth occurs at a slower rate than during germination in the AR state, suggesting that physical restraint is not the only factor regulating the growth of the radicle. We are confident that the dormancy breaking effect of coleorhiza ablation is not a wounding response, since severe wounding treatments, such as the removal of the entire embryonic shoot, did not induce germination (Fig. 1f). Removal of the testa and pericarp, concurrent with coleorhiza ablation, do not contribute to this effect either, since ablation of both had no dormancy breaking effect. Transferal of these ablation treatments onto a medium containing 100 µM Gibberellin A_{4+7}, a strong dormancy breaking treatment for *A. fatua*, demonstrated that the ablation treatments did not have any effect on the germinability of the caryopses (Fig. 1f).

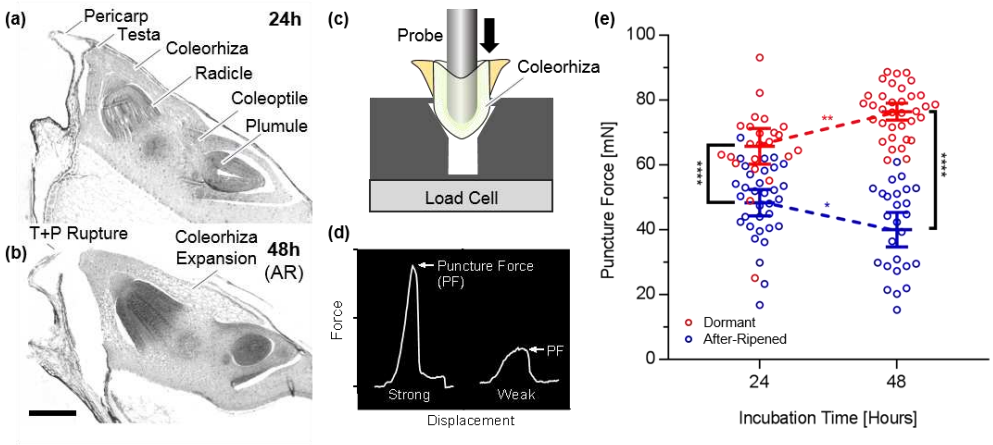


Fig. 2. Mechanical properties of the coleorhiza. a,b, Brightfield micrographs of the *A. fatua* embryo at (a) 24 and (b) 48 hours after imbibition showing the expansion of the cells of the coleorhiza and the rupture of the covering layers of the testa and pericarp. **c,d** Graphical representation of the biomechanics approach used to measure coleorhiza puncture force where (d) shows example force-displacement curves for strong and weak coleorhizae. **e**, Puncture force measurements for D and AR coleorhizae at 24 and 48 hours after imbibition were each point represents a single measurement. AR 24h, *n*=33; D 24h, *n*=23; AR 48h, *n*=26; D 48h, *n*=36. Significance was inferred using a 2-way ANOVA and between treatment comparisons were made using Tukey's Multiple Comparisons Test. **P* < 0.05, ***P* < 0.01, ****P* < 0.005 and *****P* < 0.001. Scale bar, 200 µm. PF, puncture force; T+P, testa and pericarp.

Dormancy level affects the biomechanical properties of the coleorhiza. We hypothesised that, like the MPE of many eudicots[3], the restraint imposed by the coleorhiza on the expansion of the radicle was mechanical in nature. In order to test this hypothesis we adapted a method previously used to measure the tissue resistance of the MPE[3] to quantify the force required by the radicle to rupture the coleorhiza (Puncture Force, PF) using a custom-made biomechanics platform. This involved pushing a probe with the same dimensions as the radicle through the coleorhiza and measuring the resultant forces using a load cell (Fig. 2c,d). In the AR state, PF was reduced between 24 and 48 hours after imbibition (Fig. 2e). After 24 hours of imbibition, there was a significant difference between D and AR coleorhizae, and after 48 hours of imbibition PF in the D state was further increased. That is to say the AR coleorhiza becomes weakened over time and the D coleorhiza is reinforced. The difference between PF in the AR state (~40 mN) and the D state (~80 mN) are likely to be physiologically relevant, since they are similar to the values measured in models for

MPE weakening using equivalent methods; for example 120 mN (D State) to 50 mN (AR state) in *L. sativum*[20].

Different XTHs have tissue- and dormancy-specific patterns of activity. Xyloglucan endotransglycosylase/hydrolases (XTHs) are cell wall remodelling enzymes that modulate cell wall mechanical properties through the remodelling of the hemicellulose xyloglucan[21]. These enzymes may have both a xyloglucan endotransglycosylase activity (XET, EC 2.4.1.207) and hydrolase activity (XEH, EC 3.2.1.151)[21]. In a microarray expression experiment using the Barley1 GeneChip, Barrero and colleagues identified a probe targeting XTHs that was highly expressed in the D coleorhiza of *H. vulgare*[18]. We have expanded this analysis by identifying more XTHs in their microarray dataset by searching for the conserved motif in the active site of XTHs ('DEIDFEFLG'). This search yielded 15 probes that correspond to *H. vulgare* XTHs. Multiple alignment of these probe exemplar sequences identified two clades of XTHs (Fig. 3a). Mapping relative expression data onto this phylogenetic tree shows that these clades have either an expression pattern more associated with dormancy or germination. For example, one probe (Barley1_02670) is highly expressed specifically in the dormant coleorhiza, whilst others (e.g. Barley1_02672) are specifically expressed in the AR coleorhiza. This approach has demonstrated that there may be a division of function within the XTH gene family.

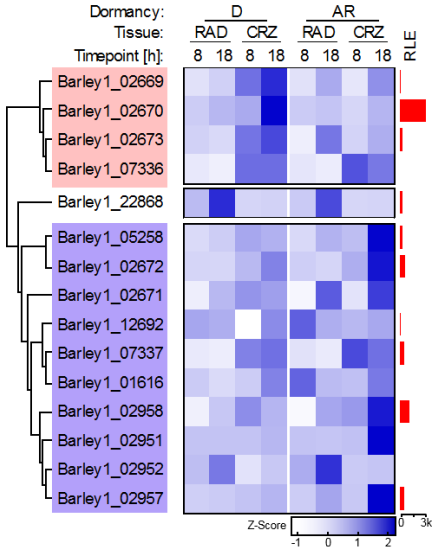


Fig. 3. Tissue- and dormancy-specific expression patterns of XTHs. Heatmap of XTHs identified using a search for the conserved motif 'DEIDFEFLG' across Barley1 GeneChip probe sequences. Sequences are arranged according to a tree built from a probe sequence alignment. Relative mean expression values (RLE) for D and AR *H. vulgare* coleorhizae and radicles were extracted from a publically available dataset[18] and are normalised by row as z-scores. The relative expression levels for each probe are shown by red bars.

In order to test the hypothesis that there are dormancy- and germination- specific XTHs present in different embryonic tissues in *A. fatua* we used an isoelectric focusing separation coupled with a zymographic visualisation (Fig. 4). In the radicle we see that, in the D and AR states, and an ABA treatment, XET isozymes are similar – one major isozyme at pH 7.4 and a minor isozyme in the AR and ABA treatments at pH 8.2. The same pattern is seen in the AR coleorhiza, however the D coleorhiza is has a different pattern of isozymes with lower pIs (isozymes at pH 6.2, 6.9). ABA treated AR coleorhizae had the major isozyme at pH 7.4 as the other AR treatments with an additional isozyme at pH 7.6. These results demonstrate there are XTHs specific to the dormant coleorhiza, and that changes in XTH isozyme profile are occurring in the coleorhiza and not the radicle.

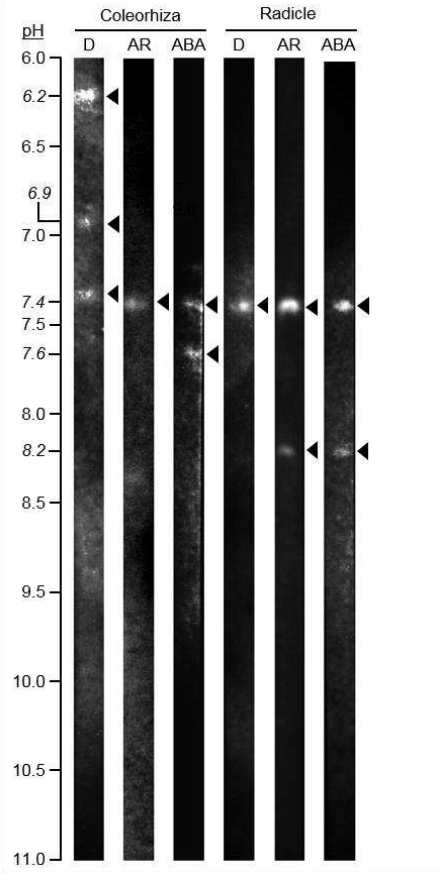


Fig. 4. Tissue and dormancy specific activity patterns of XET isozymes. Isoelectric focusing zymograms showing the different XET isozymes present in D, AR and ABA treated radicles and coleorhizae after 48 hours of imbibition. White spots show UV fluorescence of a transglycosylated labelled xyloglucan oligosaccharide visualising XET activity at the isoelectric point of specific XET isozymes (indicated by arrows). In each lane the total protein load was normalised by relative XET activity. For D tissues, *n*=~300 individuals; for AR tissues *n*=~150 individuals.

XET activity differs between D and AR embryos. To further test the hypothesis that the XET isozyme profile in D and AR states are functionally different, we quantified the XET activity of tissue lysate from whole D and AR embryos across a spectrum of pHs. We hypothesised that XET activity in the germinating AR state would be optimal at lower pHs since apoplastic pH is typically lower in expanding tissues[22]. The XETs in the AR state were indeed optimally active at lower pHs than those present in the D state (Fig. 5a).

Total XET activity against the substrate used (XLLG-SR) was lower in the D state. ABA application reduced XET activity only in the coleorhiza but not the radicle (Fig. 5b). XET activity was not directly related to the expansion of tissues as measured by water uptake, since after 72 hours in the coleorhiza and 48 hours in the radicle, XET activity levels were reduced even though expansion of the tissues continued (Fig. 5c,d). The peak of XET activity in the coleorhiza was closer in timing to the emergence of the radicle than the expansion of the coleorhiza, indicating that XET activity may play more of a role in the weakening of the coleorhiza observed from the puncture force measurements rather than in the expansion of the coleorhiza itself.

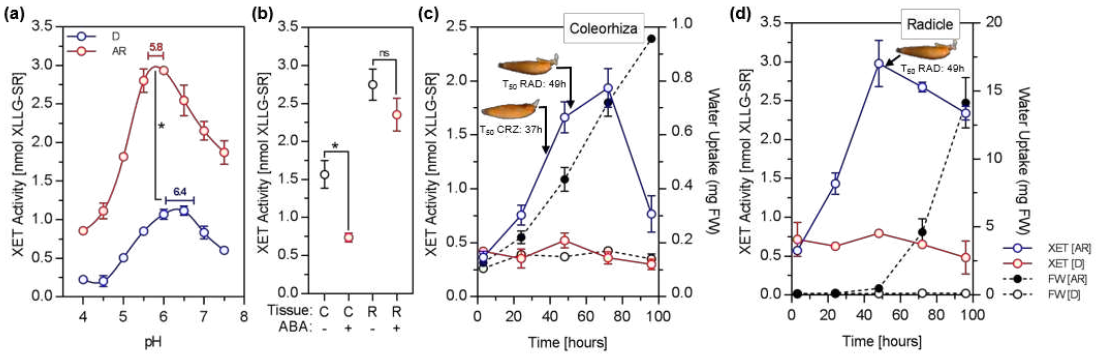


Fig. 5. Dynamics of XET activity from tissue lysates. a, the effect of pH on XET activity in lysates of isolated D and AR embryos imbibed for 48 hours. Horizontal error bars show the 95% confidence interval for the optimum pH generated through a segmental linear regression model. **b**, the effect of ABA treatment on XET activity in AR coleorhizae and radicles. Significance was calculated using a one-way ANOVA. **cd**, Dynamics of XET activity over time in D and AR coleorhizae (c) and radicles (d) plotted against the change in fresh weight (water uptake). The time taken for the AR populations to reach 50% cumulative coleorhiza and radicle emergence (T_{50} CRZ/T_{50} RAD) are shown by arrows. Error bars show standard error of the mean for triplicates of ~50 individuals.

Xyloglucan oligosaccharides modify germination behaviour. In the absence of *XTH* mutants in monocots or specific XET inhibitors[23] we aimed to test if XTHs were implicated in coleorhiza reinforcement by applying an excess of a xyloglucan oligosaccharide (XXXG) and observing a change in germination behaviour. Previous authors have suggested a role for XGOs in regulating cell wall loosening as a negative feedback mechanism preserving cell wall integrity[24]. Application of XXXG to AR

caryopses had a small inhibitory effect on germination rate, however when applied in combination with ABA, radicle emergence was completely inhibited (Fig. 6a) and germination was arrested at the coleorhiza emergence stage (Fig. 6b). This germination arrest indicates some role for XTHs in the inhibition of radicle emergence during germination.

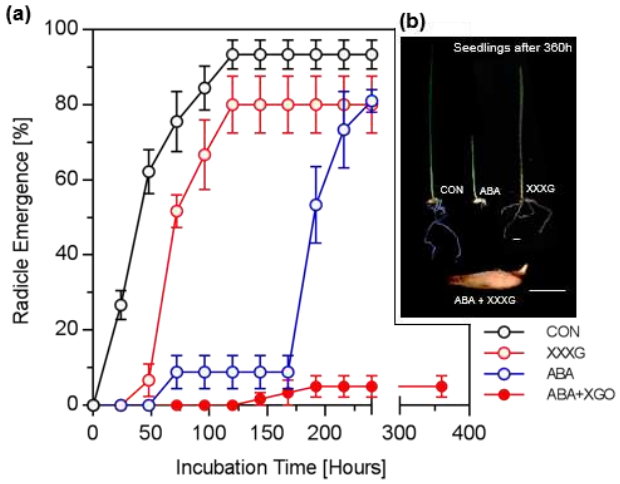


Fig. 6. The effect of the xyloglucan oligosaccharide XXXG on germination kinetics. a, Germination curve showing the effect of 50 µM ABA, 0.1 % (w/v) XXXG and a mixture of both on radicle emergence of AR caryopses. **b**, photographs of the seedling growth symptoms produced by the treatments in the germination assay. A combination of XXXG and ABA causes germination arrest at coleorhiza emergence. Scale bar = 5 mm. Images of individual seedlings are representative of the symptoms seen in the different treatments. Error bars show standard error of the mean for triplicates of 30 caryopses.

Discussion

Convergent evolution between monocots and dicots in the regulation of germination. Our measurement of changes in the tissue resistance of the coleorhiza in D and AR states demonstrates that the coleorhiza, like the MPE of dicots, modulates its mechanical properties to regulate the growth of the radicle. Though they share similar anatomical compartments, as organs surrounding the radicle in both monocot and dicot diaspores, the MPE and coleorhiza have distinct developmental origins. The MPE is a triploid endospermic tissue formed from the double fertilisation of polar nuclei[25] whereas the coleorhiza is homologous to the hypocotyl and basal Poales do

not necessarily possess a coleorhiza[26]. This convergent evolution of mechanical restraint lends support to the concept that mechanical interactions are important determinants of seed dormancy and germinability.

XTHs as candidates for coleorhiza reinforcement genes. Whilst only weakening has been reported in the MPE, here we report that the coleorhiza becomes reinforced during the maintenance of dormancy. We suggest that this reinforcement is the result of active cell wall remodelling processes such as xyloglucan remodelling. Many species have large families of XTHs, (e.g. 33 in *A. thaliana*[21]) and GUS reporter lines have demonstrated that individual XTHs have highly tissue-specific expression patterns[26] that are likely to play a role in pH, temperature and substrate specificity[28–33]. Across the conditions tested, we identified a unique XET isozyme (pI ~6.2) that was only present in the dormant coleorhiza. Similarly in *A. thaliana,* a seed-specific XTH (*XTH25, at5g57550*) is specifically expressed only in the MPE of D seeds of the Cape Verde Islands (CVI) ecotype[34]. The physiological result of XET activity is substrate-dependant, with loosening and reinforcement effects observed depending on exogenous application of different xyloglucan oligosaccharides[35]. We found that exogenous application of XXXG (a xyloglucan nonasacharide) has little effect on coleorhiza emergence or rupture in AR diaspores, however when applied together with ABA, germination is arrested at coleorhiza emergence. Whilst our results do not directly implicate XTHs in coleorhiza cell wall reinforcement or weakening, the presence of ABA inducible and dormancy-specific XET isozymes in the coleorhiza and the observation that xyloglucan oligosaccharide application can arrest germination at the coleorhiza emergence stage lends support to the idea that XTHs are involved in coleorhiza reinforcement.

Conclusion

In this study we used a novel biomechanical approach to demonstrate mechanical reinforcement in the coleorhiza of dormant *A. fatua* caryopses. Ablation experiments also showed that the coleorhiza is required for the maintenance of dormancy. XET activity assays demonstrated that specific XET isozymes are present in the dormant coleorhiza. We propose that these isozymes may have a role in coleorhiza reinforcement. Our work cannot directly implicate XTHs in this process, so further investigation is required to determine the precise cell wall remodelling mechanisms involved in the process of coleorhiza enforced dormancy. An understanding of the convergent function of the MPE and coleorhiza will contribute to an improved understanding of key agronomic issues such as pre-harvest sprouting[36], germplasm quality and seedling vigour.

Methods

Seed material and germination assays. *A. fatua* diaspores were collected in August 2017 from a wheat field in Hampshire, UK. Half of this population was air-dried and stored at -20°C ('D' population) and the other half was stored at 50% relative humidity (above a saturated solution of $Ca(NO_3)_2$) at 20°C. Periodically subpopulations were removed from the humid condition and a germination assay conducted under standard conditions: in a 90 mm petri-dish containing 2 filter papers and 5 mL of autoclaved ultrapure water with 30 diaspores in triplicate. All germination experiments were carried out at 20°C under constant light (fluorescent light at 100 µmol m^{-2} s^{-1}) in a Panasonic MLR-352 Environmental Test Chamber. This was repeated monthly until no further increase in maximum germination was recorded (112 days) to generate a fully after-ripened ('AR') population. In order to determine the optimal temperature condition for

germination assays, D and AR diaspores were imbibed on a purpose-built thermogradient plate with a temperature range between 8 and 23°C under constant light. 20°C was because this condition gave the greatest difference in germination between D and AR diaspores with minimal evaporation of the germination medium.

Exogenous application of phytohormones and oligosaccharides. For experiments involving ABA application, 50 µM cis-S(+)-Abscisic Acid (Duchefa Biochemie, Haarlem, The Netherlands) was prepared in 1N KOH and adjusted to a pH 7 using HCl. For experiments involving GA application, 100 µM of a mixture of gibberellin A_4 and A_7 (Duchefa Biochemie, Haarlem, The Netherlands), containing 0.1% (v/v) DMSO was prepared to a neutral pH using KOH. The xyloglucan nonosacharide XXXG (Megazyme, Leinster, Ireland) was prepared in ultrapure water to a concentration of 0.1% (w/v) and filter-sterilised.

Flow cytometry. 20 AR caryopses were imbibed at 20°C under constant light for 3, 24 and 48 hours and dissected into coleorhiza and plumule tissues on ice. Nuclei were extracted in 100 µL of Cystain UV Precise P extraction buffer (Sysmec Partec GmbH, Görlitz, Germany) by maceration with a razor blade. Samples were stained with 1 mL of Cystain UV Precise P DAPI fluorescent buffer (Sysmec Partec GmbH, Görlitz, Germany) and filtered through a 30-µm filter. >9,000 nuclei were analysed using a Partec PAS Flow Cytometer (Sysmex Partec GmbH, Germany). Data analysis and noise reduction was performed using Flowing Software 2.5.1 (Terho 2017).

Ablation experiments. For experiments involving he ablation of the coleorhiza, a fine grade silica-based sandpaper was used to remove the coleorhiza from dry caryopses. For the ablation of the plumule a fine razor blade was used to bisect the embryo to the depth of the scutellum and the plumule removed with fine forceps. In pericarp ablation experiments, the pericarp was peeled away from the embryo using fine forceps without

disturbing the underlying tissues. After 240 hours, pericarp and plumule ablated treatments were transferred to a plate containing 100 µM GA_{4+7} to test for germinability.

Microscopy. Partially dissected embryos that had been imbibed for 24 or 48 hours under standard conditions were fixed in 4% (w/v) paraformaldehyde and subjected to an ethanol dehydration gradient. The samples were embedded in 2-hydroxyethyl methyl acrylate polymerised with 1% (v/v) benzoyl peroxide (Technovit 7100 cold curing resin system, Kulzer Technique, Wehrheim, Germany)[37] following the manufacturers recommendations with mofidicatons[38]. Sections of 5 µm were cut on a rotary microtome (Microtom HM 355S, Thermo Scientific, Massachusetts, USA) and progressively stained with 1% (w/v) safranin O (to stain cell wall lignin counterstained with 1% (w/v) toluidine blue (to stain nuclei and polysaccharides)[38]. Bright-field images were taken using a Nikon ECLIPSE Ni-E stereomicroscope (Nikon, Tokyo, Japan) and processed using GIMP[40] (version 2.8.16) to desaturate the images and improve the contrast. Macroscopic images of germination stages were taken using a Stereomicroscope (Leica MZ125, Leica Biosystems, Wetzlar, Germany) and Images were processes using the inbuilt Leica Application Suite Software (version 4.1).

Puncture force measurements. Puncture force was measured directly for isolated coleorhizae using a purpose built device[20]. Custom-made metal holders held the coleorhizae in place while a metal probe (0.2 mm diameter) was driven into it at a rate of 0.7 mm min^{-1} while the resultant forces were measured by a load cell (F_{max} = 1 mN). An in-house software platform recorded the resultant force-displacement data and logged puncture force for each sample based on the maximum force sustained by the sample until its rupture. Statistical differences between treatments was inferred using a 2-way ANOVA and between-treatment comparisons were made using Tukey's Multiple Comparisons Test implemented in GraphPad Prism (version 7.05, Graphpad Software, California, USA).

XET activity assay. Preparations of 50 coleorhizae or radicles were dissected from D or AR caryopses, weighed and frozen in liquid nitrogen. Total protein was extracted from isolated tissues by micropestle homogenisation in 5x (w/v) of an extraction buffer containing 200 mM succinate (Na+) buffer (pH 5.5) containing 2.5 mg/mL bovine serum albumin (BSA)[41] and protein was quantified using the Bradford method[42]. Total protein concentrations were adjusted to 20 ug using the extraction buffer. 5 uL of sample was applied to 6 mm diameter discs of a matrix (EDIPOS, Edenborough, UK) composed of Whatman No. 1 filter paper impregnated with tamarind seed xyloglucan (2.5 g m^{-2}) and a sulforhodamine conjugate of the xyloglucan oligosaccharide XLLG (1 µmol m^{-2}), held within a 96 well microtitre plate. This plate was incubated at 20°C in the dark for 3 hours in container at saturated relative humidity to prevent evaporation. After incubation, the discs were washed repeatedly with ethanol:formic acid:water (1:1:1) followed by water to remove unbound labelled substrate[41] and dried in an oven at 60°C. Fluorescence was measured in a multimode plate reader (SPARK, Tecan Trading AG, Switzerland) by using an excitation filter at 570 nm and emission filter at 615 nm as an mean of 24 independent readings per well in triplicates. Readings were taken before and after sample application, the fraction of substrate bound per well was calculated and blank measurements were subtracted. Based on the known amount of substrate on the membrane, XET activity was calculated as nmol of XLLG transglycosylated. For the XET activity assay involving a gradient of pHs, the same protocol was followed except for the sample preparation, where individual replicates of 30 isolated embryos were prepared in extraction buffer with pHs adjusted with 10M NaOH. After extraction, pH was checked using universal indicators strips.

Separation of XET isozymes by isoelectric focusing. D or AR caryopses were imbibed for 48 hours under standard conditions. For D caryopses 300 caryopses were dissected into coleorhiza and radicles and for AR caryopses (or those treated with ABA) 150 caryopses were dissected on ice. These samples were homogenised using a

micropestle in 350 mM succinate (Na+) at pH 5.5 on ice and centrifuged at 13 krpm to remove cell debris. The supernatant was filtered through a 0.22 µM cellulose acetate spin column (Agilent Technologies, California, USA) and desalted against distilled water using a BioRad Bio-Spin 6 column (BioRad Laboratories, California, USA). Total protein concentration was measured using the Bradford method[41]. Samples were loaded onto dehydrated isoelectric focusing strips (Immobiline DryStrip pH 6-11, 13 cm, GE Healthcare, Illinois, USA) according to their relative XET activity as measured at 48 hours from the XET activity assay (AR(coleorhiza 225 ug, radicle 120 ug), ABA(coleorhiza 300 ug, radicle 120 ug), D(coleorhiza 720 ug, radicle 520 ug) in a solution containing 5% glycerol and 2% IPG buffer (pH 6-11, GE Healthcare, Illinois, USA). Samples in rehydrated gel strips were subjected to an isoelectric focusing program following the manufacturer's recommendations (8000V, 16 kVh, current limit 50 µA and power 200 mW) in a flatbed isoelectric focusing apparatus (3100 OFFGEL fractionator, Agilent Technologies, California, USA). Gel strips were then equilibrated for 10 minutes in a buffer containing 50 mM succinate (Na+, pH 5.5), 10 mM $CaCl_2$ and 1 mM Dithiothreitol[43]. Equilibrated gels were transferred to strips of the substrate matrix previously described and sandwiched between parafilm and two sheets of glass. This sandwich was incubated in the dark for 24 hours. After incubation, unbound fluorescent substrate was removed by washing as previously described[43] and visualised using a UV transilluminator with an orange filter. Images were processed using GIMP[40] (version 2.8.16) to improve the contrast.

References

1. Moulia, B. Plant biomechanics and mechanobiology are convergent paths to flourishing interdisciplinary research. *J. Exp. Bot.* **64,** 4617–4633 (2013).

2. Lee, Y. *et al.* Photoperiodic Regulation of Growth-Dormancy Cycling through Induction of Multiple Bud – Shoot Barriers Preventing Water Transport into the Winter Buds of Norway Spruce. *Front. Plant Sci.* **8,** 1–16 (2017).

3. Steinbrecher, T. & Leubner-metzger, G. The biomechanics of seed germination. *J. Exp. Bot.* **68,** 765–783 (2017).

4. Linkies, A., Graeber, K., Knight, C. & Leubner-Metzger, G. The evolution of seeds. *New Phytol.* **186,** 817–831 (2010).

5. Müller, K., Tintelnot, S. & Leubner-Metzger, G. Endosperm-limited Brassicaceae seed germination: Abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiol.* **47,** 864–877 (2006).

6. Bewley, J., Bradford, K., Hilhorst, H. & Nonogaki, H. *Seeds - Physiology of Development, Germination and Dormancy*. (Springer, 2013).

7. Leubner-Metzger, G. Functions and regulation of β-1,3-glucanases during seed germination, dormancy release and after-ripening. *Seed Sci. Res.* **13,** 17–34 (2003).

8. Zhang, Y. *et al.* Involvement of reactive oxygen species in endosperm cap weakening and embryo elongation growth during lettuce seed germination. *J. Exp. Bot.* **65,** 3189–3200 (2014).

9. Muller, K. *et al. In Vivo* Cell Wall Loosening by Hydroxyl Radicals during Cress Seed Germination and Elongation Growth. *Plant Physiol.* **150,** 1855–1865 (2009).

10. Chen, F. & Bradford, K. J. Expression of an Expansin Is Associated with Endosperm Weakening during Tomato Seed Germination. *Plant Physiol.* **124,** 1265–1274 (2000).

11. Chen, F., Nonogaki, H. & Bradford, K. J. A gibberellin-regulated xyloglucan endotransglycosylase gene is expressed in the endosperm cap during tomato seed germination. *J. Exp. Bot.* **53,** 215–223 (2002).

12. Martínez-Andújar, C. *et al.* Mechanisms of hormonal regulation of endosperm cap-specific gene expression in tomato seeds. *Plant J.* **71,** 575–586 (2012).

13. Lee, K. J. D. *et al.* Distinct Cell Wall Architectures in Seed Endosperms in Representatives of the Brassicaceae and Solanaceae. *Plant Physiol.* **160,** 1551–1566 (2012).

14. Toorop, P. E., van Aelst, A. C. & Hilhorst, H. W. M. The second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA. *J. Exp. Bot.* **51,** 1371–1379 (2000).

15. Oracz, K. *et al.* Myrigalone a inhibits lepidium sativum seed germination by interference with gibberellin metabolism and apoplastic superoxide production required for embryo extension growth and endosperm rupture. *Plant Cell*

Physiol. **53,** 81–95 (2012).

16. Leubner-Metzger, G., Kucera, B. & Müller, K. Emerging and established model systems for endosperm weakening. *Seeds Biol. Dev. Ecol.* 195–204 (2007).

17. Sargent, J. A. & Osborne, D. J. A comparative study of the fine structure of coleorhiza and root cels during the early hours of germination of rye embryos. *Protoplasma* **103,** 91–103 (1980).

18. Barrero, J. M., Talbot, M. J., White, R. G., Jacobsen, J. V & Gubler, F. Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant Physiol.* **150,** 1006–1021 (2009).

19. Millar, A. A. *et al.* Seed dormancy and ABA metabolism in Arabidopsis and barley: The role of ABA 8'-hydroxylase. *Plant J.* **45,** 942–954 (2006).

20. Graeber, K. *et al.* DELAY OF GERMINATION 1 mediates a conserved coat-dormancy mechanism for the temperature- and gibberellin-dependent control of seed germination. *PNAS* **111,** E3571–E3580 (2014).

21. Rose, J. K. C., Braam, J., Fry, S. C. & Nishitani, K. The XTH Family of Enzymes Involved in Xyloglucan Endotransglucosylation and Endohydrolysis: Current Perspectives and a New Unifying Nomenclature. *Plant Cell Physiol.* **43,** 1421–1435 (2002).

22. Rayle, D. L. & Cleland, R. E. The Acid Growth Theory of Auxin-induced Cell Elongation Is Alive and Well. *Plant Physiol.* **99,** 1271–1274 (1992).

23. Chormova, D., Franková, L., Defries, A., Cutler, S. R. & Fry, S. C. Phytochemistry Discovery of small molecule inhibitors of xyloglucan endotransglucosylase (XET) activity by high-throughput screening. *Phytochemistry* **117,** 220–236 (2015).

24. McDougall G., & Fry, S. Anti-auxin activity of xyloglucan oligosacharides: the role of groups other than the terminal a-L-fucose residue. *Journal of Experimental Botany*. **40**, 233-238 (1989).

25. Brown, R. C., Lemmon, B. E., Nguyen, H. & Olsen, O. A. Development of endosperm in Arabidopsis thaliana. *Sex. Plant Reprod.* **12,** 32–42 (1999).

26. Tillich, H. J. Seedling diversity and the homologies of seedling organs in the order Poales (monocotyledons). *Ann. Bot.* **100,** 1413–1429 (2007).

27. Becnel, J., Natarajan, M., Kipp, A. & Braam, J. Developmental expression patterns of Arabidopsis XTH genes reported by transgenes and Genevestigator. *Plant Mol. Biol.* **61,** 451–467 (2006).

28. Vaaje-kolstad, G., Farkaš, V., Fincher, G. B. & Hrmova, M. Barley xyloglucan xyloglucosyl transferases bind xyloglucan-derived oligosaccharides in their acceptor-binding regions in multiple conformational states. *Arch. Biochem. Biophys.* **496,** 61–68 (2010).

29. Hrmova, M., Farkas, V., Lahnstein, J. & Fincher, G. B. A Barley Xyloglucan Xyloglucosyl Transferase Covalently Links Xyloglucan, Cellulosic Substrates, and (1,3;1,4)-b -D-Glucans. *J. Biol. Chem.* **282,** 12951–12962 (2007).

30. Hrmova, M. *et al.* Substrate specificity and catalytic mechanism of a xyloglucan xyloglucosyl transferase HvXET6 from barley (*Hordeum vulgare* L .). *FEBS J.*

276, 437–456 (2009).

31. Maris, A., Suslov, D., Fry, S. C., Verbelen, J. & Vissenberg, K. Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of Arabidopsis and their effect on root growth and cell wall extension. *J. Exp. Bot.* **60,** 3959–3972 (2009).

32. Maris, A. *et al.* Differences in enzymic properties of five recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of Arabidopsis thaliana. *J. Exp. Bot.* **62,** 261–271 (2011).

33. Schunmann, P., Smith, R., Lang, V., Matthews, P. & Chandler, P. Expression of XET-related genes and its relation to elongation in leaves of barley (*Hordeum vulgare* L .). *Plant, Cell Environ.* **20,** 1439–1450 (1997).

34. Dekkers, B. J. W. & Pearce, S. P. Dormant and after-Ripened Arabidopsis thaliana Seeds are Distinguished by Early Transcriptional Differences in the Imbibed State. *Front. Plant Sci.* **7,** 1–15 (2016).

35. Takeda, T. *et al.* Suppression and acceleration of cell elongation by integration of xyloglucans in pea stem segments. *Proceeding Natl. Inst. Sci.* **99,** 9055–9060 (2002).

36. Vetch, J. M., Stougaard, R. N., Martin, J. M. & Giroux, M. J. Review: Revealing the genetic mechanisms of pre-harvest sprouting in hexaploid wheat (*Triticum aestivum* L.). *Plant Sci.* **281,** 180–185 (2019).

37. Yeung, E. C. T., Stasolla, C., Sumner, M. J. & Huang, B. Q. in *Plant Microtechniques and Protocols* 1–572 (2015). doi:10.1007/978-3-319-19944-3

38. Matsushima, R. *et al.* Amyloplast-Localized SUBSTANDARD STARCH GRAIN4 Protein In fl uences the Size of Starch Grains in Rice Endosperm. *Plant Physiol.* **164,** 623–636 (2014).

39. Ruzin, S. E. *Plant Microtechnique and Microscopy*. (Oxford University Press, 1999).

40. Team, T. G. GIMP 2.8.10. Available at: www.GIMP.org.

41. Fry, S. C. Novel 'dot-blot'' assays for glycosyltransferases and glycosylhydrolases: optimization for xyloglucan endotransglycosylase (XET) activity. *Plant J.* **11,** 1141–1150 (1997).

42. Bradford, M. M. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **254,** 248–254 (1976).

43. Iannetta, P. P. M. & Fry, S. C. Visualization of the activity of xyloglucan endotransglycosylase (XET) isoenzymes after gel electrophoresis. *Phytochem. Anal.* **10,** 238–240 (1999).

Acknowledgements

We thank Mark Levy, Diane Grant and Sarah Dean for assistance with seed processing and Prof. Paul Fraser and Chris Gerrish for materials and technical assistance with isoelectric focusing. This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) Research Grants (BB/M02203X/1, BB/R505730/1) to G.L.-M. The development of methods for analysing coleorhiza biomechanics was supported by the a BBSRC Research Grant (BB/M000583/1) to T.S. and G.L.-M.

Author Contribution Statement

T.H, T.S., K.N., D.S., A.S. and G.L.-M. planned and designed the research; T.H and T.S. performed experiments; A.S. provided access to materials; T.H., T.S. K.N. and G.L.-M. analysed and interpreted that data; T.H., K.N. and G.L.-M. wrote the manuscript with contributions from all authors.

6. Vernalisation enforces seed dormancy in the agricultural weed *Alopecurus myosuroides* (Huds.)

Manuscript prepared for submission to 'Seed Science Research' (Online ISSN: 1475-2735).

6.1 Author contributions

Experiments were conducted by Thomas Holloway (TH) and Marta Pérez (MP). Propagation of the seed batches and physiological experiments were conducted by TH and DNA extraction and the quantification of global methylation was conducted by MP.

The concept, experimental design and narrative of the manuscript were conceived by TH. Project supervision was provided by K. Nakabayashi (KN) and G. Leubner-Metzger (GL-M) (Royal Holloway University of London, UK) and by David Stock (DS) and Anne Seville (AS) (Syngenta Ltd., Jealott's Hill International Research Centre, UK). TH was the lead author on the manuscript, analysed the data, prepared all the figures and wrote the text. The manuscript is in the advanced stages of preparation for submission to Seed Science Research as an 'Article'. Submission is anticipated before the end of December 2019.

Vernalisation enforces seed dormancy in the agricultural weed *Alopecurus myosuroides* (Huds.)

Thomas Holloway[1], *Marta Pérez*[1], *Anne Seville*[2], *David Stock*[2], *Kazumi Nakabayashi*[1] & *Gerhard Leubner-Metzger*[1]

[1]School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom

[2]Syngenta, Jealott's Hill International Research Centre, Warfield, Bracknell RG42 6EY, United Kingdom

Correspondence and requests for materials should be addresses to K.N. or G.L.M. at: School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom.

URL: www.seedbiology.eu

Corresponding Author Details:

Gerhard Leubner Metzger: gerhard.leubner@rhul.ac.uk, +44 1784 44 3895,

URL: https://pure.royalholloway.ac.uk/portal/en/persons/gerhard-leubner(b07cd3da-9c1d-4167-8d52-199a13d54351).html

Kazumi Nakabayashi: kazumi.nakabayashi@rhul.ac.uk, +44 1784 44 3769

URL: https://pure.royalholloway.ac.uk/portal/en/persons/kazumi-nakabayashi(e0fa28c2-6185-44d1-966c-192c95e86a70).html

ORCID *Thomas Holloway*: 0000-0002-8753-7841

ORCID *Marta Pérez*: 0000-0002-6802-205X

ORCID *Kazumi Nakabayashi*: 0000-0002-4186-541X

ORCID *Gerhard Leubner-Metzger*: 0000-0002-6045-871

ORCID *Anne Seville*: 0000-0001-8024-7959

Seed Science Research

Received:

Accepted:

Published Online:

Abstract

Seed dormancy is the key factor determining weed emergence patterns in the field. *Alopecurus myosuroides* (blackgrass) is a serious cereals weed in Europe that experiences two emergence peaks affecting winter and spring cereals respectively. Seedlings that emerge in autumn experience a period of cold winter temperatures, whereas those that emerge in spring do not. In this work we investigated the effects of this overwintering during vegetative growth on the primary seed dormancy of the offspring. *Alopecurus myosuroides* plants were propagated under controlled conditions where a proportion of the population was subjected to a simulated winter period (vernalisation) as seedlings. The offspring produced by vernalised plants was significantly more dormant, requiring longer after-ripening and cold stratification treatments to germinate at warm temperatures. However there was no difference in the range of temperatures under which dormant seeds germinate. We hypothesised that this difference in dormancy was the result of an epigenetic memory of vernalisation. In the absence of techniques to identify changes in epigenetic status at the gene level, global changes in methylation were quantified using an ELISA-based approach. Imbibition in dormant seeds produced by vernalised plants was associated with a global demethylation event that was not observed in the offspring of plants that had not been vernalised. Taken together these results demonstrate the importance of temperature at different stages of the plant lifecycle in determining dormancy levels and consequently weed emergence patterns in the field.

Introduction

Alopecurus myosuroides Huds. (blackgrass) is often cited as the most destructive agricultural weed in Europe (Lutman *et al.*, 2013) due to its high fecundity (Moss, 1985), complex herbicide resistance traits (Heap, 2019) and competitive ability against cereal crops (Maréchal & Henriet, 2012). *Alopecurus myosuroides* has a typical winter annual life history (Fig. 1). Diaspores (hereafter seeds) are dispersed in the summer with a high degree of primary dormancy that prevents their germination until colder winter temperatures and consequently a major peak of emergence occurs in winter cereals. A minor emergence also occurs in spring, which coincides with the planting of spring cereals (Clarke *et al.*, 2015). In this way, *A. myosuroides* synchronises its emergence with the cropping cycle to avoid herbicide applications and maximize its competitiveness with the crop (Maréchal & Henriet, 2012).

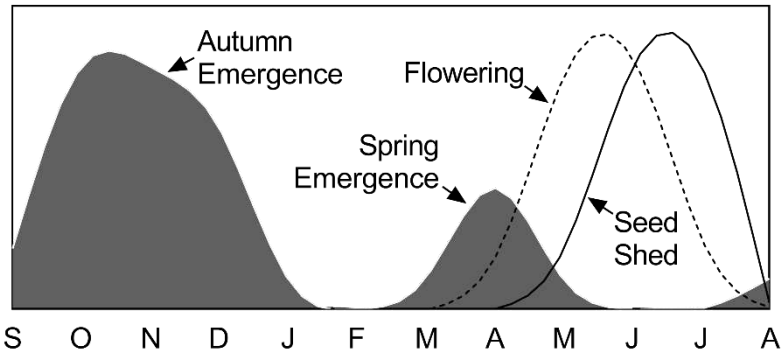


Figure 1. The lifecycle of *A. myosuroides*. Grey shaded area represents emergence; dotted and solid lines shows the period during which flowering and seed dispersal occur respectively. Modified from Clarke *et al.* (2015).

A long term trend for earlier sowing of winter wheat has led to increased *A. myosuroides* infestations (Moss, 2017) hence recent *A. myosuroides* management practices have shifted from winter cereal production to spring cereals and oil seed rape (OSR) in an attempt to avoid the peak winter *A. myosuroides* emergence (AHDB, 2016,

2017, 2018). This system has been successful in managing *A. myosuroides* infestations (Lutman *et al.*, 2013), however recent evidence has suggested that *A. myosuroides* is adapting to this strategy by delaying emergence until the spring, leading to similar levels of emergence in the winter and spring (Taylor-Davies, 2017). Models of *A. myosuroides* emergence recognise the importance of seed dormancy in determining the timing and scale of emergence in field (Colbach *et al.*, 2006a,b) however these models focus on the autumn emergence of *A. myosuroides* and do not take into account a potential shift to spring emergence.

There is a large body of evidence in crop and model species to suggest that the environment experienced by the mother plant modulates seed dormancy in the offspring (Penfield & MacGregor, 2017). These effects are divided into those that affect the developing zygote whilst it is maturing on the mother plant ('zygotic environment effects'), and those effects that are strictly maternal before fertilisation occurs, that are mediated by the maternal genome or epigenome ('true maternal effects') (Penfield & MacGregor, 2017). Whilst zygotic environmental effects are well studied even in *A. myosuroides* (Swain *et al.*, 2006), studies on true maternal effects are much less common. Interestingly in *A. myosuroides*, although there are two peaks of emergence, flowering and seed dispersal occur only once across the year, suggesting that these two emerged populations of *A. myosuroides* have similar zygotic environment however the true maternal environmental conditions differ. The autumn emerged population experiences winter and the spring emerging population does not.

In this study we demonstrate that vernalisation of young *A. myosuroides* seedlings, or mimicking of winter under controlled conditions, has a consierable impact on the seed dormancy of their offspring. This difference in dormancy affects the after-ripening requirement and temperature preferences for germination in the after-ripened state. We propose that this mechanism generates heterogeneity in the soil seed bank that contributes to the adaptability and weediness of *A. myosuroides*.

Materials and Methods

Seed Material

A field collected parental population of *A. myosuroides* seeds from Germany that was harvested in May 2015, was germinated at 20ºC under constant light on moist filter papers for 10 days. The resultant seedlings were then transferred to 6 cm diameter pots containing a 10:1 mixture of John Innes Number 1 compost and perlite and grown for 14 days in an 18/6h day night cycle at 18ºC. Half of this crop was transferred to a Conviron™ Environmental Test Chamber (Conviron Adaptis CMP6010) set at the same conditions, where the temperature and photoperiod was gradually reduced over the course of 6 days from 18ºC to a 8/16h day/night cycle at 6ºC and 4ºC respectively whilst the other half of the population was propagated under the original warm conditions. This condition was maintained for 35 days, and then the temperature and day/night cycles were increased up to the initial condition. Vernalised (V) and non-vernalised (NV) plants were then transferred to a glasshouse bay maintained at 18ºC with a 16/8 hour photoperiod with supplemental lighting from sodium lamps. The onset anthesis was counted over time as the emergence of the tip of the panicle from the culm. Both batches were harvested on the same day (21.09.2017) by agitating the mature panicles to ensure only mature seeds were collected. These seeds were weighed for each plant and the resultant seed batches were dry-stored using silica gel (<10% moisture content by weight) and stored at -20ºC. To determine the 100 seed weight for each batch, triplicates of 100 randomly selected freshly harvested seeds were weighed using a fine balance.

Germination Kinetics

Germination assays were conducted using the following standard conditions unless otherwise specified: seeds were incubated in 3 mL distilled water in petri dishes with

two filter papers (Machery-Nagel™ MN-713), and incubated at 20°C under constant light in environmental test chambers (Panasonic™ MLR-352-PE). The emergence of the coleorhiza (coleorhiza emergence, CE) through the margin of the glumes was counted over time until there was no further increase in the number of emerged coleorhizae. This value was reported as the maximum number of germinated seeds within the population (gMAX). For the production of seed batches with different levels of dormancy, dormant freshly harvested seeds stored in porous paper bags were incubated in a hermetically sealed chamber containing saturated calcium nitrate, that generated an equilibrium relative humidity of 53%. Incubation under these conditions resulted in a seed moisture content of 19.3% (w/w). Seeds were periodically removed and germination was tested using standard conditions. For experiments involving the endogenous application of gibberellin A_{4+7} or fluridone (Duchefa Biochemie, Haarlem, The Netherlands) compounds were first solubilised in absolute dimethyl sulfoxide (DMSO) and consequently a solvent control of 0.1% (v/v) DMSO was included in the control for these experiments. To investigate the effect of imbibitional temperature on germination seeds of freshly harvested or 196 day after-ripened V and NV batches were incubated across a gradient of temperatures from 5°C to 28°C under constant light conditions using a two dimensional thermogradient plate (GRANT GRD1-LH, Grant Instruments Ltd., Cambridge, UK) and germination was scored using the standard conditions procedure.

Seed Viability Testing

Vernalised and non-vernalised batches that had been after-ripened for 196 days were imbibed for 24 hours at 20°C under constant light as previously described. One hundred diaspores in triplicate were dissected and the number of diaspores that contained a fully formed caryopsis were recorded. Caryopses were then transferred to

a petri-dish containing filter papers as described before, with a medium containing 1% (w/v) 2,3,5- triphenyltetrazolium chloride (TTC). As negative (dead) control, another set of caryopses were incubated at 90ºC in ultrapure water for an hour, were also transferred to TTC plates. These plates were then incubated for a further 24 hours at 20ºC in the dark. Staining was assessed using a stereomicroscope. Embryos were considered stained when the entirety of the visible external portion of the embryo (underneath the testa and pericarp) was fully stained dark red. Caryopses with weak of patchy staining were considered unstained.

Cold Stratification

Triplicates of 30 freshly harvested V and NV diaspores were imbibed in petri dishes as previously described with 1 mL of autoclaved distilled water. These plates were incubated in the dark at 4ºC using a laboratory refrigerator for 3, 7, 14 and 21 days. After this time, an additional 2 mL of autoclaved distilled water was added and the plates were transferred to an incubator at 20ºC under constant light and germination was scored as previously described.

ELISA based quantification of DNA methylation

Forty milligrams (dry weight) of *A. myosuroides* seeds that were either dry, or had been imbibed for 90 hours under standard germination conditions, were homogenised by pestle and mortar in liquid nitrogen. DNA was extracted using a DNeasy™ Plant Mini Kit (Qiagen, Venlo, Netherlands) following the manufacturer's recommendations with modifications (5% (v/v) β-mercaptoethanol in lysis buffer). DNA yield and quality were quantified using a NanoQuant Plate™ (Tecan, Männedorf, Switzerland). Quantification of 5-methylcytosine (5-mC) was performed using a fluorometric enzyme-linked immunosorbent assay (ELISA) kit (MethylFlash™ Methylated DNA Quantification Kit,

Epigentek, New York, USA) following the manufacturer's instructions for 10 ng of genomic DNA. Fluorescence proportional to 5-methylcytosine (5-mC) concentration was measured for 3 biological and two technical replicates using a florescence microplate reader (Tecan SPARK^{TM}, Männedorf, Switzerland) at $530_{EX}/590_{EM}$ nm and %5-mC was calculated from a standard curve of synthetic 5-mC.

Results

Vernalisation of plants had a strong impact on *A. myosuroides* plant morphology, flowering and seed production. Vernalised plants were shorter than NV plants with more dense culms (data not shown). The initiation of flowering in both V and NV plants

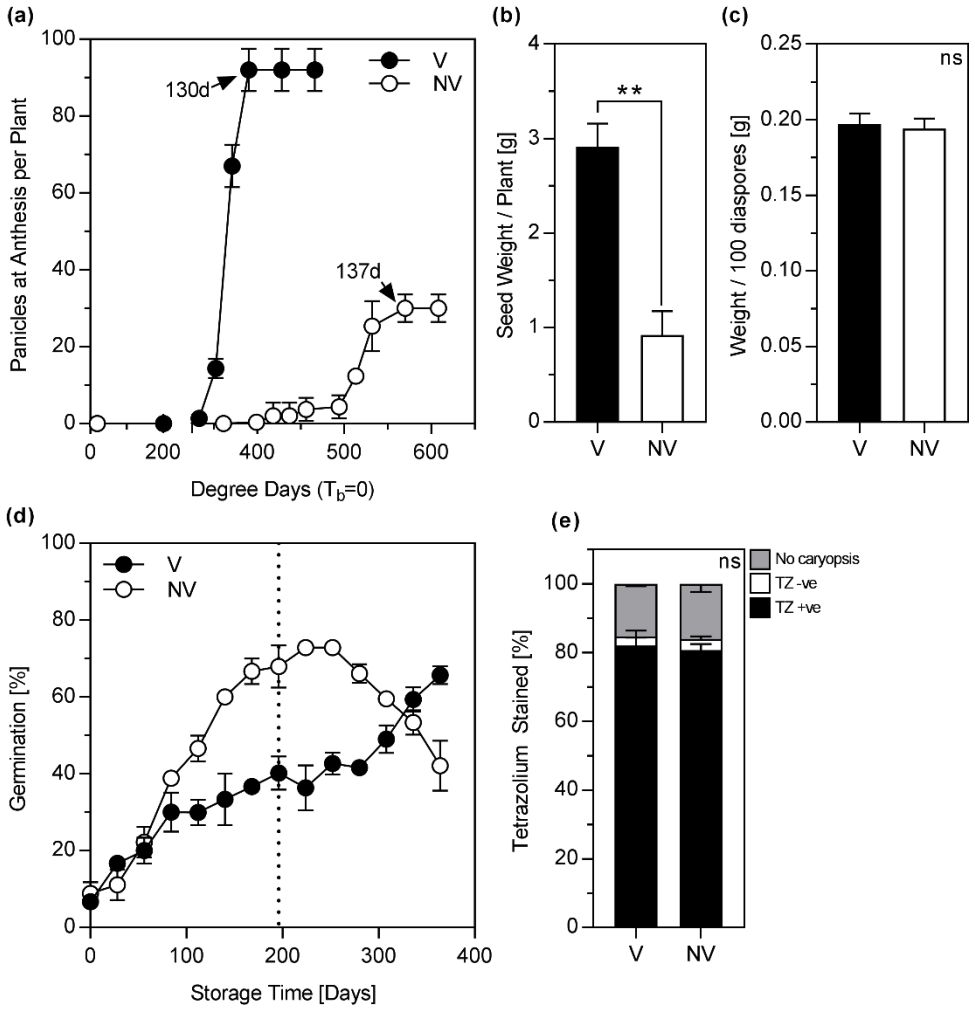


Figure 2. Propagation and description of seed batches. (a) Assessment of flowering time for V and NV *A. myosuroides* plants. Accumulated day degrees were calculated with a base temperature of 0°C. Flowering was counted as by the emergence of panicles from the culms. **(b)** The difference in the seed mass produced by each plant for V and NV treatments. Statistical significance from a Mann-Whitney test is represented by asterisks (**, p=0.0079). **(c)** Seed mass for triplicates of 100 seeds in V and NV batches. **(d)** After-ripening curves showing the change in maximum germination over storage time at 53% equilibrium relative humidity for V and NV batches. **(e)** Viability testing of freshly harvested V and NV batches showing the proportion of diaspores containing no caryopsis (grey) and that had embryos stained (black) or non-stained (white) by incubation in 2,3,5- triphenyltetrazolium chloride (TTC) solution. Error bars show standard error of the mean.

occurred simultaneously (130-140 days after germination), however when expressed as degree days there was a delay in the flowering of NV plants (Fig. 2a) yet V plants produced approximately three fold more panicles (Fig. 2b) which equated to a three-fold increase in seed mass per plant (Mann Whitney U(40,15), p=0.0079) (Fig. 2b). There was no significant difference in the 100 seed weight of the plants produced by V and NV plants (Mann Whitney U(12,9), p=0.7) and there was no difference in the viability of seeds produced by either treatment as measured by presence of a caryopsis or by tetrazolium staining (two-way ANOVA, F (2,12) = 0.225, p=0.8018). The absence of a caryopsis contributed more to the non-viable population than a lack of staining by incubation in TTC solution (Fig. 2e).

The seeds produced by NV plants after-ripened more rapidly than seeds produced by V plants, reaching a fully after-ripened state after approximately 200 days. In the fully after-ripened state, seeds from NV plants reached ~75% maximum germination (Fig. 2d), reflecting the number of tetrazolium stained embryos (~80%) (Fig. 2e). The vernalised seed batch did not reach a fully after-ripened state even after a year of after-ripening treatment (Fig. 2d). Interestingly, after 250 days of after-ripening the maximum germination of seeds produced by NV plants was steadily reduced, indicating that seeds were losing viability during after-ripening (Fig. 2d).

In the dormant state, seeds produced by V and NV plants germinated over a similar range of temperatures between 5 and 15^{o}C (Fig. 3a). A low proportion of seeds (~20%) from the V batch germinated under this temperature range whereas a large proportion of seeds (~50%) from NV plants germinated under these conditions (Fig. 3a). This effect was most pronounced at 7^{o}C (Fig. 3c). After 196 days of after-ripening the temperature range permissive to germination was changed in seeds produced by both V and NV plants. (Fig. 3b). Seeds from NV plants reached high germination percentages (60-75%) over a broad range of temperatures between 5 and 22^{o}C, whereas this level of germination was only achieved in after-ripened seeds produced

by V plants at a single temperature of 12°C (Fig. 3e). After-ripened seeds from NV plants also germinated to a higher maximum proportion at 5°C, the coldest temperature tested (Fig. 3d).

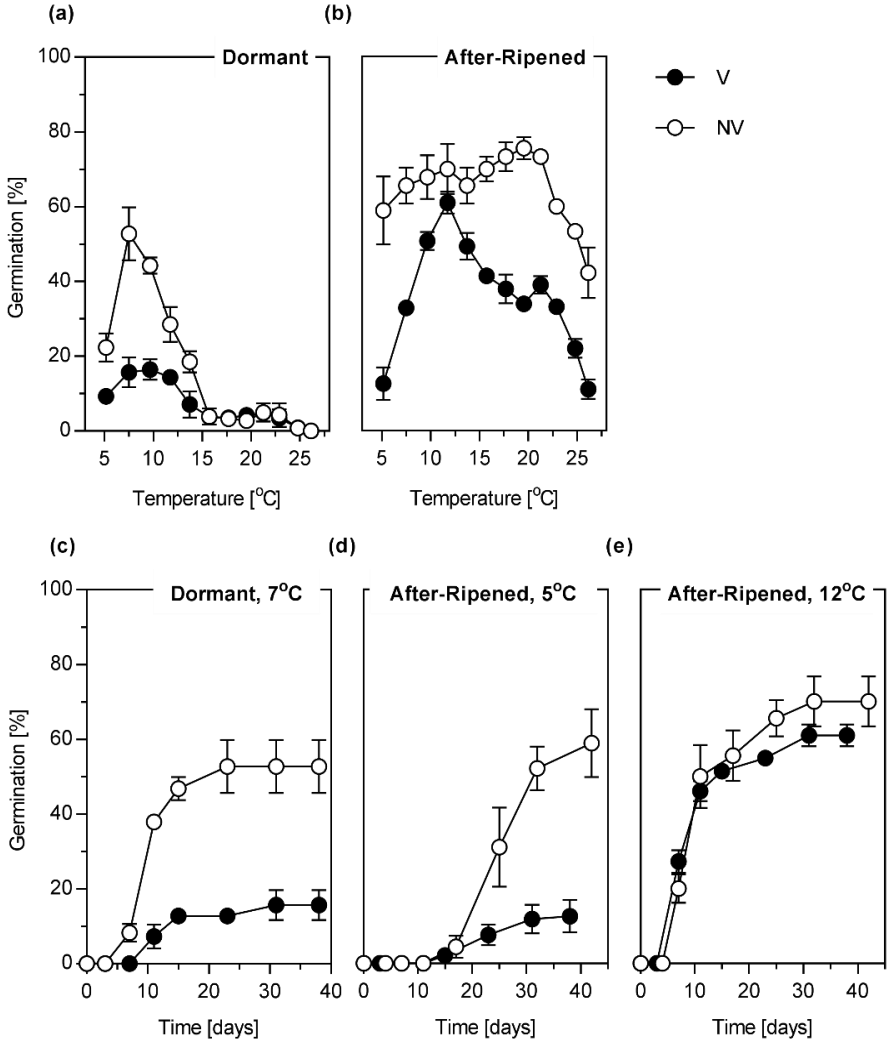


Figure 3. The effect of dormancy and vernalisation on the response of *A. myosuroides* seeds to temperature. **(a,b)** the effect of imbibition of dormant **(a)** and 196 day after-ripened **(b)** V and NV seeds across a gradient of temperatures. **(c-e)** germination curves for V and NV batches at selected temperatures **(c)** dormant seeds incubated at 7°C, **(d)** after-ripened seeds incubated at 5°C and **(e)** after-ripened seeds incubated at 12°C. Germination was scored as coleorhiza emergence for triplicates of > 30 seeds. Error bars show standard error of the mean.

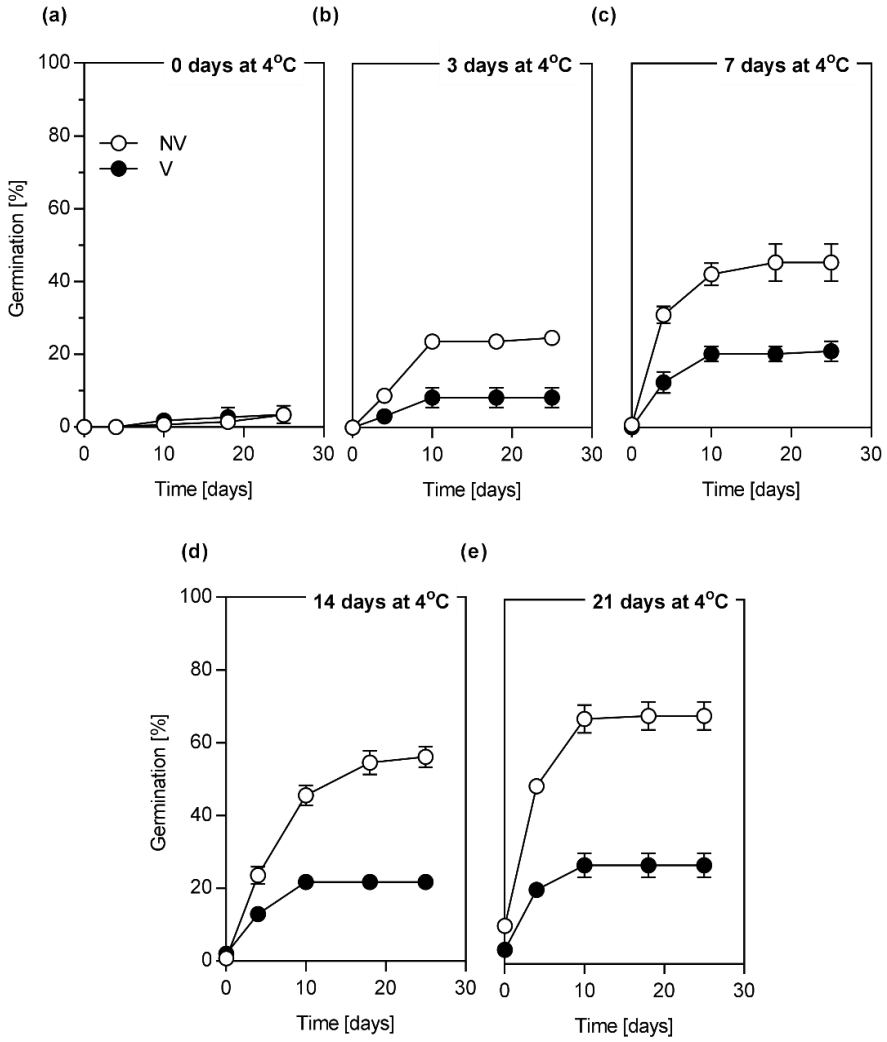


Figure 4. Cold stratification of dormant *A. myosuroides* seeds produced by V or NV plants. The effect of imbibition of seeds produced by V and NV plants at 4°C in constant darkness prior to transferal to 20°C in constant light where seeds were incubated in the cold for **(a)** 0 days, **(b)** 3 days, **(c)** 7 days, **(d)** 14 days and **(e)** 21 days. Germination was scored as cumulative coleorhiza emergence. Error bars show standard error of the mean.

Seeds produced by V and NV plants also differed in their response to cold stratification. Whilst in the dormant state there was low germination of both batches when imbibed at 20°C under constant light (<5%), cold stratification at 4°C in the dark increased the germination of both batches when transferred to 20°C under constant light in a dose-dependent manner (Fig. 4). However the effect of cold stratification was consistently ~2-fold greater in seeds from NV plants (Fig. 4). After 21 days of cold stratification, the NV treatment reached similar final germination proportions (mean=68%) as their

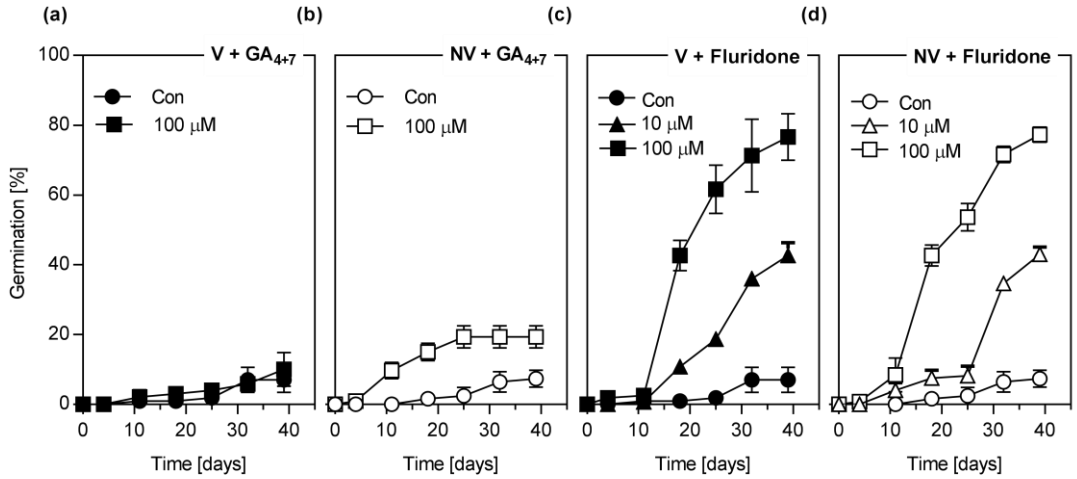


Figure 5. The effect of gibberellin and fluridone application on dormant seeds from V and NV plants. **(a,b)** The effect of 100 μM gibberellin A_{4+7} on dormant seeds from **(a)** V and **(b)** NV plants. **(c,d)** the effect of application of 10 or 100 μM fluridone on dormant seeds from **(c)** V and **(d)** NV plants. Germination was counted as counted as cumulative coleorhiza emergence for triplicates of >30 seeds incubated at 20°C under constant light. Error bars show standard error of the mean.

respective fully after-ripened seed batch (mean=73%). Cold stratification of seeds produced by V plants for 21 days reached lower germination proportions (mean=27%) particularly when compared to their respective 196 day after-ripened batch (mean=40%) (Fig. 4e).

Incubation of seeds produced from V or NV plants in gibberellin A_{4+7} (GA_{4+7}) and fluridone had no substantial differential effect on germination (Fig. 5). Gibberellin application slightly increased the maximum germination of seeds produced by NV plants, however this effect seems unsubstantial given that the dose applied was high. Conversely both batches did respond to fluridone application (Fig. 5c,d) and this response was very similar for both batches.

Quantification of global DNA methylation levels in both dormant and after-ripened batches of seeds produced from V or NV plants identified a pattern of differential methylation only in the V condition. Whilst there was no change in methylation between 0 and 90 hours in the after-ripened batch, a significant demethylation was observed in the dormant batch (two-way ANOVA, $F(1,15)=5.12$, $p=0.0377$). No Significant changes

in methylation were observed in seeds from the NV batch associated with after-ripening or imbibition.

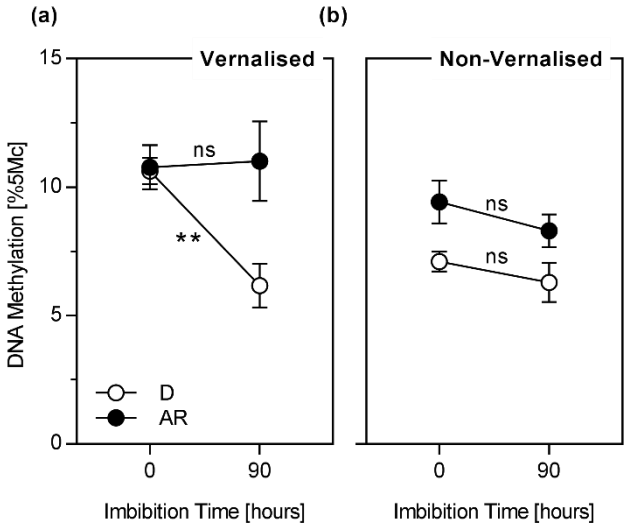


Figure 6. Changes in DNA methylation associated with the enforcement of dormancy. Results from ELISA-based quantification of global DNA methylation from dormant (D) and after-ripened (AR) batches of seeds produced by **(a)** vernalised and **(b)** non-vernalised plants either dry or after 90 hours of imbibition at 20°C under constant light. Error bars show standard error of the mean for >3 biological replicates of 30 seeds. Asterisks shows significance at *p<0.01* from a two-way ANOVA where 'ns' shows no significant effect.

Discussion

Vernalisation has a clear effect on *A. myosuroides* morphology and seed production as previously discussed by other authors (Chauvel *et al.*, 2002). The original result from this study is that vernalisation modulates the dormancy of the offspring of *A. myosuroides* plants, as demonstrated by an increase in after-ripening and cold stratification requirement. The implication of this finding is that winter emerging *A. myosuroides* may produce offspring that have higher dormancy levels than spring emerging populations. Both of these populations flower and set seed at the same time in the field (Clarke *et al.*, 2015), yet the seed returned to the seedbank is likely to be heterogeneous. This could serve as a bet-hedging strategy in the constantly changing environment of an agricultural field where herbicide application, cultivation and crop rotation could drastically change year on year. Bet-hedging strategies, that spread the risk of reproductive failure, have been reported for heteromorphic seeds, where the fitness benefit of heteromorphy correlates with the unpredictability of the environment (Arshad *et al.*, 2019).

Interestingly although seeds produced from V and NV plants differed in their dormancy levels, the range of temperatures under which these batches germinate in the dormant state did not differ in contrast to established physiologically dormant species, where differences in dormancy level are typically associated with changes in the temperature window permissive to germination (Baskin & Baskin, 2004). Additionally these two dormant batches did not differ in their responses to the classical dormancy breaking treatments of exogenous gibberellin and fluridone application. Fluridone is an inhibitor of phytoene desaturase, an enzyme involved in the biosynthesis of the carotenoid precursors of abscisic acid (ABA), the phytohormone associated with the enforcement of dormancy (Bartels & Watson, 1978; Nambara *et al.*, 2010). These results indicate some involvement of *de novo* ABA biosynthesis in the enforcement of dormancy in *A.*

myosuroides however this process cannot explain the difference we see in the dormant seeds from V and NV plants.

Examples of true maternal effects on seed dormancy are sparse in the literature, however they typically involve changes in temperature during the vegetative phase in plant growth (Thomas & Raper, 1975; Sawhney *et al.*, 1985; Chen *et al.*, 2014; Auge *et al.*, 2017). These effects have been linked to the expression patterns of genes that are also responsible for controlling the timing of flowering such as *FLOWERING LOCUS C* (*FLC*), circadian clock genes, *FLOWERING LOCUS T* (*FT*) and *MOTHER OF FLOWERING TIME* (*MFT*) that also control the response to vernalisation and day length in *Arabidopsis thaliana* (Chen *et al.*, 2014; Penfield & MacGregor, 2017; Chen & Penfield, 2018). Disruption of these genes cause*s* altered seed dormancy phenotypes (Penfield *et al.*, 2005; Chono *et al.*, 2006; Nakamura *et al.*, 2011), modified seed hormone metabolism (Chiang *et al.*, 2009; Chen *et al.*, 2014) and changes in seed coat pigmentation (Macgregor *et al.*, 2015).

Signals during vegetative development are 'remembered' through epigenetic gene silencing, and the vernalisation response of *Arabidopsis* is becoming an increasingly well understood example of this process (Song *et al.*, 2012). In *Arabidopsis* FLC is quantitatively downregulated in the cold by a polycomb based switching mechanism whereby histone modifications regulate the balance between *FLC* expression and transcription of the *FLC* antisense transcripts *COOLAIR* and *COLDAIR* (Song *et al.*, 2012). DNA methylation also has an important role in vernalisation induced flowering (Burn *et al.*, 1993), embryo maturation and cold-stratification induced bud dormancy breaking in forestry (Santamaría *et al.*, 2009; Viejo *et al.*, 2010; Pérez *et al.*, 2015), whereby patterns of global DNA demethylation precede developmental transitions.

The analysis of patterns of histone modifications and DNA methylation at the gene-level that are performed in model species are unfeasible for agricultural weed species

such as *A. myosuroides* that have very limited publically available genetic resources. What we sought to achieve with the quantification of 5-mC was to investigate the general trends in genomic methylation that are associated with the interaction between vernalisation, dormancy level and imbibition. Our results for the V batch show demethylation in response to imbibition only in the D state and no changes in the NV batch. This indicates that, rather than seed dormancy being a 'quiescent state' in *A. myosuroides*, a developmental program may be induced to prevent germination and that this program may be associated with environmental cues throughout the plant lifecycle.

The response of *A. myosuroides* to vernalisation is a strong example of phenotypic plasticity in agricultural weeds and a mechanism by which *A. myosuroides* can increase the heterogeneity of it soil seedbank as a potential bet-hedging strategy against the constantly changing environment of the agricultural field. Models for predicting weed emergence should take into account the plasticity of weed seed dormancy, especially in the context of changing weed management practice and climate change.

Conclusions

Vernalisation of *A. myosuroides* plants increased the after-ripening and cold stratification requirement for germination but had no effect on the window of temperatures permissive to germination in the dormant state. After-ripening widened the temperature window more slowly in seeds produced from vernalised plants. The enforcement of dormancy in seeds from vernalised plants was associated with a global DNA demethylation event that was not observed in seeds produced by non-vernalised plants. In the context of autumn and spring emergence peaks in the lifecycle of *A. myosuroides*, these results indicate that vernalisation acts to increase the

heterogeneity of dormancy in the weed seedbank. Consequently, models should take into account the effect of temperature throughout the lifecycle of weeds to more effectively predict weed emergence in the field.

Acknowledgements

We thank Mark Levy, Diane Grant and Sarah Rabjohn for assistance with seed processing This work was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) Research Grant (BB/R505730/1) to G.L.-M.

Author Contribution Statement

T.H, M.P., K.N., D.S., A.S. and G.L.-M. planned and designed the research; T.H and M.P. performed experiments; A.S. provided access to materials; T.H., K.N. and G.L.-M. analysed and interpreted that data; T.H., K.N. and G.L.-M. wrote the manuscript with contributions from all authors.

References

AHDB. **2016**. Cereals and Oilseeds Report: Fall in winter cropping across England and Wales.

AHDB. **2017**. Cereals and Oilseeds Report: Planting Survey Results: Regional split for 2017 OSR area.

AHDB. **2018**. Cereals and Oilseeds Report: Oilseed rape area bounces back in England and Scotland.

Arshad W, Sperber K, Steinbrecher T, Nichols B, Jansen VAA, Leubner-Metzger G, Mummenhoff K. **2019**. Dispersal biophysics and adaptive significance of dimorphic diaspores in the annual *Aethionema arabicum* (Brassicaceae). *New Phytologist* **221**: 1434–1446.

Auge GA, Blair LK, Neville H, Donohue K. **2017**. Maternal vernalization and vernalization-pathway genes influence progeny seed germination. *New Phytologist* **216**: 1-13.

Bartels P, Watson C. **1978**. Inhibition of carotenoid synthesis by fluridone and norflurazon. *Weed Science* **26**: 198–203.

Baskin JM, Baskin CC. **2004**. A classification system for seed dormancy. *Seed Science Research* **14**: 1–16.

Burn J, Bagnall D, Metzger J, Dennis E, Peacock W. **1993**. DNA methylation, vernalization, and the initiation of flowering. *PNAS* **90**: 287–291.

Chauvel B, Munier-Jolain NM, Grandgirard D, Gueritaine G. **2002**. Effect of vernalization on the development and growth of *Alopecurus myosuroides*. *Weed Research* **42**: 166–175.

Chen M, Moore K, Florance H, MacGregor DR, Smirnoff N, Paszkiewicz K, Penfield S, Graham IA, Dave A. **2014**. Maternal temperature history activates Flowering Locus T in fruits to control progeny dormancy according to time of year. *PNAS* **111**: 18787–18792.

Chen M, Penfield S. **2018**. Feedback regulation of *COOLAIR* expression controls seed dormancy and flowering time. *Science* **360**: 1014–1017.

Chiang G, Barua D, Karmer E, Amasino R, Donohue K, Koornneef M. **2009**. Major flowering time gene, *FLOWERING LOCUS C*, regulates seed germination in *Arabidopsis thaliana*. *PNAS* **106**: 11661–11666.

Chono M, Honda I, Shinoda S, Kushiro T, Kamiya Y, Nambara E, Kawakami N, Kaneko S, Watanabe Y. **2006**. Field studies on the regulation of abscisic acid content and germinability during grain development of barley: Molecular and chemical analysis of pre-harvest sprouting. *Journal of Experimental Botany* **57**: 2421–2434.

Clarke J, Ginsburg D, Clare K, Tonguc L. **2015**. The Encyclopaedia of Arable Weeds. AHDB.

Colbach N, Busset H, Yamada O, Dürr C, Caneill J. **2006a**. AlomySys: Modelling black-grass (*Alopecurus myosuroides* Huds.) germination and emergence, in interaction with seed characteristics, tillage and soil climate: II. Evaluation. *European Journal of Agronomy* **24**: 113–128.

Colbach N, Durr C, Roger-Estrade J, Chauvel B, Caneill J. **2006b**. AlomySys: Modelling black-grass (*Alopecurus myosuroides* Huds.) germination and emergence, in interaction with seed characteristics, tillage and soil climate: I. Constructon. *European Journal of Agronomy* **24**: 113–128.

Heap I. **2019**. International Survey of Herbicide Resistance. URL: www.weedscience.org (Last Accessed: 14.10.2019)

Lutman PJW, Moss SR, Cook S, Welham SJ. **2013**. A review of the effects of crop agronomy on the management of *Alopecurus myosuroides*. *Weed Research* **53**: 299–313.

Macgregor DR, Kendall SL, Florance H, Fedi F, Moore K, Paszkiewicz K, Smirnoff N, Penfield S. **2015**. Seed production temperature regulation of primary dormancy occurs through control of seed coat phenylpropanoid metabolism. *New Phytologist* **205**: 642–652.

Maréchal P, Henriet F. **2012**. Ecological review of black-grass (*Alopecurus myosuroides* Huds.) propagation abilities in relationship with herbicide resistance. *Biotechnology, Agronomy, Society and Environment* **16**: 103–113.

Moss SR. **1985**. The survival of *Alopecurus myosuroides* Huds. seeds in soil. *Weed Research* **25**: 201–211.

Moss S. **2017**. Black-grass (*Alopecurus myosuroides*): Why has this weed become such a problem in western europe and what are the solutions? Outlooks on Pest Management.

Nakamura S, Abe F, Kawahigashi H, Nakazono K, Tagiri A, Matsumoto T, Utsugi S, Ogawa T, Handa H, Ishida H, et al. 2011. A Wheat Homolog of *MOTHER OF FT* and *TFL1* acts in the regulation of germination. *The Plant Cell* **23**: 3215–3229.

Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, Kamiya Y. **2010**. Abscisic acid and the control of seed dormancy and germination. *Seed Science Research* **20**: 55–67.

Penfield S, Josse EM, Kannangara R, Gilday AD, Halliday KJ, Graham IA. **2005**. Cold and light control seed germination through the bHLH transcription factor *SPATULA*. *Current Biology* **15**: 1998–2006.

Penfield S, MacGregor DR. **2017**. Effects of environmental variation during seed production on seed dormancy and germination. *Journal of Experimental Botany* **68**: 1–7.

Pérez M, Viejo M, LaCuesta M, Toorop P, Cañal MJ. **2015**. Epigenetic and hormonal profile during maturation of *Quercus Suber* L. somatic embryos. *Journal of Plant Physiology* **173**: 51–61.

Santamaría ME, Hasbún R, Valera MJ, Meijón M, Valledor L, Rodríguez JL, Toorop PE, Cañal MJ, Rodríguez R. **2009**. Acetylated H4 histone and genomic DNA methylation patterns during bud set and bud burst in *Castanea sativa*. *Journal of Plant Physiology* **166**: 1360–1369.

Sawhney R, Quick WA, Hsiaof AI. **1985**. The Effect of Temperature during Parental Vegetative Growth on Seed Germination of Wild Oats (*Avena fatua* L.). Annals of Botany **55**: 25-28.

Song J, Angel A, Howard M, Dean C. **2012**. Vernalization - a cold-induced epigenetic switch. *Journal of Cell Science* **125**: 3723–3731.

Swain AJ, Hughes ZS, Cook SK, Moss SR. **2006**. Quantifying the dormancy of *Alopecurus myosuroides* seeds produced by plants exposed to different soil moisture and temperature regimes. *Weed Research* **46**: 470–479.

Taylor-Davies B. **2017**. Nuffield Report: System BEN offers alternative solutions to resistance management. URL: https://www.nuffieldscholar.org/news/ben-taylor-davies-report-published (Last Accessed: 14.10.2019).

Thomas T, Raper C. **1975**. Seed germinability as affected by the environmental temperature of the mother plant. *Tobacco Science* **19**: 98–100.

Viejo M, Rodríguez R, Valledor L, Pérez M, Cañal MJ, Hasbún R. **2010**. DNA methylation during sexual embryogenesis and implications on the induction of somatic embryogenesis in *Castanea sativa* Miller*. Sexual Plant Reproduction* **23**: 315–323.

7. General Discussion and Conclusions

7.1 Summary of results

The overall objective of this work was to develop an understanding of the mechanisms of grass weed seed dormancy and how these mechanisms could be influenced for the purposes of weed seed bank management. The germination responses of five economically important grass weeds to a gradient of temperatures were characterised at differing levels of primary dormancy. Release of the physiological dormancy of these grass weeds was achieved by after-ripening storage, and was associated with a widening of the temperature window for germination differently in summer and winter annual weeds. In winter annuals, dormancy release was associated with an increase in the maximum temperature (T_c) permissive for germination, whereas in summer annuals dormancy release was associated with a reduction in the minimum temperature (T_b) for germination. In *A. myosuroides*, cold exposure during vegetative growth was found to increase the after-ripening requirement of the progeny but did not affect the window of temperatures permissive to germination in the dormant state.

The winter annual weed *A. myosuroides* was selected for further analysis of the molecular mechanisms of dormancy and germination due its predictable change in temperature response at defined dormancy levels, its diploid genome (facilitating reference-free assembly) and its economic importance as a major weed of European cereals agriculture. Transcriptome analysis revealed a conservation of the core ABA:GA regulatory module between *A. myosuroides* and model species, however distinct mechanisms were involved in dormancy release by after-ripening and cold temperature. After-ripening was associated with the expression of GA biosynthesis genes whereas cold-induced dormancy release was associated with the expression of ABA degradation and ethylene-responsive transcription factor genes. The *A. myosuroides* ortholog of the gibberellin receptor GID1 was identified as a potential

target for germination stimulants, and this observation was validated when a synthetic GID1 agonist was identified as the top-hit in a dormancy-breaking compound screen. Screening of this compound across the five weed species showed a similar effect amongst winter annual, but not summer annual grass weeds.

This transcriptome study, as well as analysis of other publicly available transcriptome datasets, identified tissue-specific cell wall remodelling transcripts, such as *Xyloglucan Endotransglycosylase/Hydrolases* (*XTHs*), which were upregulated in the dormant coleorhiza. This observation led to the hypothesis that the coleorhiza may have become mechanically reinforced during dormancy enforcement. Application of biomechanical techniques, developed for measuring puncture force in the micropylar endoderm, were used to confirm this hypothesis. Separation of XTH isozymes by isoelectric focusing identified an XTH isoform that was present only in the dormant coleorhiza.

7.2 Do the lifecycle habits of grass weeds predict germination response to temperature in the dormant state?

7.2.1 How does dormancy affect the response of weeds seeds to the thermal environment?

All five species in this work exhibited primary dormancy when freshly harvested. After-ripening storage was used as a method to release the primary dormancy of grass weed species in a controlled manner to generate fully after-ripened ($AR_{100\%}$) and half after-ripened ($AR_{50\%}$) seed batches. Primary dormancy restricted the range of temperatures under which seeds germinated. In winter annuals, dormancy restricted germination to cooler temperatures and dormancy release increased the maximum temperature for

germination (T_c). Conversely in summer annuals dormancy restricted germination to warmer temperatures and dormancy release reduced the minimum temperature for germination (Chapter 2). Whilst it is inappropriate to make generalisations about life history and dormancy from just five weed species, particularly when the two species in this work come from the same tribe (Paniceae), other authors have made this link between life-history and the function of dormancy in restricting the window of conditions permissive to germination (reviewed by Baskin & Baskin, 2014).

If dormancy is the key factor regulating emergence patterns in nature, then winter and summer annuals are achieving the opposite outcome in terms of emergence phenology, from their physiological dormancy (autumn or spring germination). We might therefore hypothesise that the mechanisms through which dormancy is regulated may be distinct between summer and winter annuals. Screening of germination stimulants demonstrated differences in the response of our winter annuals (*A. myosuroides, A. fatua* and *P. annua*) and summer annuals (*D. sanguinalis* and *S. faberi*) to different germination stimulants. For example whilst winter annual species all responded to both GAs and a synthetic GID1 agonist, neither summer annual species responded to either compound (Chapter 3). Summer annual grasses also responded differently to after-ripening than winter annuals. In *D. sanguinalis* a 50 day period of after-ripening was required before any germination was observed at 20ºC, in contrast to winter annual species where at this temperature even in the dormant state some germination was observed. *Setaria faberi* did not respond predictably to after-ripening as other species did, a response previously described in its weedy close relative (Tribe Paniceae) *Echinochloa crus-galli* (Honek *et al.*, 1999).

In studies comparing dormancy cycling in winter and summer Arabidopsis ecotypes, similar mechanisms involving gibberellin biosynthesis were observed at temperatures permissive to germination and expression of the gibberellin receptor GID1 was positively correlated with dormancy level in the summer annual ecotype (Huang *et al.*,

2014; Footitt *et al.*, 2017). Attempts have been made by some authors to link life-history traits to the response to germination stimulants (Chen, 2014; Mojzes *et al.*, 2015), however the life-cycle phenology (winter vs. summer annual) of plants has yet to investigated. From this work, conclusions cannot be drawn about the role of life history phenology in determining the response of weed seeds to germination stimulants due to the limited number of species used. Future work in developing an understanding of the association between life-history traits and the responses to different germination stimulants would be valuable in selecting germination stimulants and predicting the behaviour of these compounds on seedbanks with diverse populations of weed seeds.

7.2.2 Is after-ripening storage an appropriate method for generating seed batches with defined dormancy levels?

This work has aimed to understand the role of seed dormancy in grass weeds by comparison to non-dormant seeds of the same genotype. After-ripening storage was effective in releasing dormancy in a controlled manner in most species (with the exception of *S. faberi*) (Chapter 2). Whilst after-ripening time is unsuitable for comparing the dormancy levels of between species due to differences in sorption isotherm properties (Chapter 2), after-ripening was used to demonstrate differences in dormancy in seeds produced by vernalised and non-vernalised *A. myosuroides* plants, a result that was confirmed by a difference in their cold stratification requirement (Chapter 6). However the use of after-ripening in this work has had two main limitations. Firstly, for some species (e.g. *A. myosuroides*), the generation of fully after-ripened seed batches took up to a year of after-ripening storage, imposing time restrictions on the planning of follow-up experiments. Additionally in *S. faberi* after-ripening did not produce a controlled and predictable release in dormancy. After-ripening storage may also not be representative of the natural dormancy releasing

processes that occur in nature. Winter annuals shed their seed in the summer, when soil conditions are dry and natural after-ripening is proposed to occur (Bewley *et al.*, 2013). Conversely, summer annuals shed seed in the cooler winter months when soil moisture is likely to be less limiting and summer annual species are typically more sensitive to cold stratification than after-ripening (Baskin & Baskin, 2014). Further studies into germination stimulants in weeds should therefore take into consideration the environmental conditions causing dormancy loss in weeds in their agronomic contexts in order to have findings that are more applicable in the field.

7.2.3 Do the conditions experienced by seeds in petri dishes reflect field conditions?

Seeds in the WSB exist in a 'dormancy continuum' where the dormancy levels of seeds fluctuate depending on seasonal cues (Baskin & Baskin, 1985). However this work has focused solely on a comparison between differing levels of primary dormancy. The majority of studies on seed dormancy mechanisms have focused on primary dormancy, since primary dormant seed batches can be generated in a reproducible manner and primary dormancy can be preserved by cold-storage of dry seeds after-harvest, facilitating both mutant screening and molecular analysis (Finch-Savage & Footitt, 2017). However in the field, many of the seeds that are shed with primary dormancy will not germinate in the first season and develop secondary dormancy. The relationship between primary and secondary dormancy has been studied in the volunteer weed *Brassica napus* (oil seed rape), where the depth of primary dormancy correlates with the likelihood of entrance into secondary dormancy (Soltani *et al.*, 2019) and the same relationship has also been observed in Arabidopsis (Auge *et al.*, 2015). Advances in '-omics' technologies have allowed untargeted comparisons of the transcriptome profiles of seeds in both primary and secondary dormancy states. In

Arabidopsis the enforcement and release from of both dormancy types are associated with similar changes in gene expression, notably for genes responsible for the dynamics of the ABA:GA balance (Cadman *et al.*, 2006). However some secondary dormancy-specific processes have also been identified in dormancy cycling experiments, such as differences in the expression of clock and flowering time genes (Footitt *et al.*, 2017).

Numerous experiments have demonstrated that levels of seed dormancy fluctuate in response to changes in environmental conditions and these fluctuations are associated with changes in the transcription of genes with a role in the regulation of germination (Cadman *et al.*, 2006; Footitt *et al.*, 2013, 2015, 2017; Lee *et al.*, 2017). In this work seed dormancy has been investigated by simulation experiments using constant environmental conditions (continuous water, light and temperature) in order to simplify and standardise results across species. In nature seed dormancy is often negatively regulated in many species by daily fluctuations in temperature or photoperiod and this phenomenon has been proposed as the mechanism by which seeds perceive their burial depth (reviewed by Baskin & Baskin, 2014). Seeds in the WSB are also exposed to cycles of imbibition and drying that can have an effect on dormancy and the subsequent germination of seeds. Under controlled conditions, cycles of drying and rehydration can either enforce or release dormancy differently across grass species (reviewed by Simpson, 1990). However whilst these factors are important determinants of dormancy in the field, the responses of different species to these factors is highly variable. The use of solely primary dormant seed under constant environmental conditions in this work is justified by a need to reduce the complexity of factors influencing dormancy and the observation that the core mechanisms of dormancy release are conserved across dormancy types in model species.

7.3 Are the mechanisms regulating dormancy and germination conserved between model species and grass weeds?

7.3.1 Does the maternal environment of weeds regulate the dormancy of their offspring?

Seed dormancy allows seeds to incorporate information from environmental conditions experienced by previous generations in order to adjust emergence times in the progeny (Auge *et al.*, 2017b). In doing so, seeds increase the heterogeneity of the seed bank that serves as a strategy to spread the risk of reproductive failure in changeable environments (Cohen, 1966). Studies on 'true maternal effects' on dormancy are limited in the literature (Penfield & MacGregor, 2017) with only few studies demonstrating the effect of temperature and photoperiod on the dormancy of the progeny. *A. myosuroides* has two peaks of emergence; a major peak in the autumn and a minor peak in the spring (Maréchal & Henriet, 2012), meaning that plants that emerge in the autumn experience winter (vernalisation) whereas those that emerge in spring do not. By replicating these two maternal environments under controlled conditions, seed batches produced by vernalised and non-vernalised plants were generated. Vernalised plants produced more seeds, confirming previous reports in *A. myosuroides* (Chauvel *et al.*, 2002). Seeds produced by parents that had been vernalised took longer to after-ripen and had a longer cold-stratification requirement to break dormancy (Chapter 6). The after-ripening time required to generate an $AR_{100\%}$ batch in seeds from vernalised plants (~1 year) was similar to the time to reach $AR_{100\%}$ in a field collected *A. myosuroides* batch (Chapter 2). Interestingly this observation contradicts findings in different Arabidopsis ecotypes, where vernalisation reduced dormancy in ecotypes where there was a significant effect (Auge *et al.*, 2017a).

The observation that the maternal environment affects seed dormancy in weeds has important implications for weed management at the seed bank level. Firstly, the responses of seeds to different germination stimulants are dependent on the dormancy levels of the seeds. For example, where the smoke derived germination stimulant KAR_1 promotes germination in dormant *A. myosuroides*, in the after-ripened state KAR_1 acts as a germination inhibitor (Chapter 3). Therefore the response of seeds to germination stimulants may also be affected by the influence of environmental conditions of their parents. This hypothesis was tested using fluridone, an ABA biosynthesis inhibitor, however no significant difference was found (Chapter 6) and KAR_1 was not tested. An understanding of how the maternal environment influences dormancy would be valuable to make better forecasts for weed emergence that would improve weed management practises in the following season.

7.3.2 Are mechanisms of dormancy and germination conserved across species?

In model species the interaction between ABA and GA is at the core of the regulation of dormancy and germination (Finch-Savage & Leubner-Metzger, 2006) and more recently additional phytohormones such as jasmonates and ethylene have been found to play a role in influencing the balance of ABA and GA signalling (Linkies & Leubner-Metzger, 2012; Corbineau *et al.*, 2014). In Arabidopsis, the dry seed transcriptome has an overrepresentation of ABA-responsive genes containing an ABRE *cis* element in the promoter sequence (Nakabayashi *et al.*, 2005). In *A. myosuroides*, transcripts encoding NCEDs, the rate limiting step in ABA biosynthesis, were specifically expressed in the dry state (Chapter 4) supporting a previously described role for ABA in seed maturation (Bewley *et al.*, 2013). In both Arabidopsis and barley, after-ripening is associated with an increased expression of the genes encoding enzymes responsible for ABA

degradation upon imbibition (*AtCYP707A2* and *HvABA8'OH-1*) (Millar *et al.*, 2006). However this was not the case in *A. myosuroides*, where there was no difference in the expression of *ABA8'OH* between dormant and after-ripened seeds at a warmer temperature (Chapter 4). Instead *ABA8'OH* expression was greater in both dormant and after-ripened seeds imbibed at a cold temperature.

Germination is associated with an increase in the expression of genes encoding gibberellin synthesis enzymes in imbibed seeds and a consequent increase in the accumulation of bioactive gibberellins in Arabidopsis and barley (Jacobsen *et al.*, 2002; Ogawa *et al.*, 2003). In *A. myosuroides* gibberellin biosynthesis genes, such as *GA3oxidase*, were expressed in after-ripened seeds regardless of temperature but not in dormant seeds at the cold temperature that also germinate (Chapter 4). The gibberellin biosynthesis inhibitors flurprimidol and paclobutrazol also inhibited germination of after-ripened *A. myosuroides* diaspores at a warm temperature (Chapter 3) further supporting a role for gibberellin biosynthesis in after-ripening mediated dormancy release. Gibberellin degradation by GA2oxidase has also been implicated in the maintenance of dormancy (Footitt *et al.*, 2011) however in *A. myosuroides GA2oxidase* relative gene expression was low and there were no differences between dormant and after-ripened seeds (Chapter 4). In addition specific GA2oxidase inhibitors had no germination stimulant effect (Appendix 9.5).

Gibberellin perception, through the GID1 receptor, is also regulated by dormancy level and temperature in Arabidopsis (Footitt *et al.*, 2011; Hauvermale *et al.*, 2014). In *A. myosuroides* we found a GID1 homolog (Appendix 9.9b) that unlike in Arabidopsis, did not change expression level due to differences in dormancy (Chapter 4). Across the grass weed species investigated, responses to both natural and synthetic GID1 agonists were highly variable. In *A. myosuroides, A. fatua* and *P. annua* GA_3 and GA_{4+7} had a dormancy breaking effect, however in *A. myosuroides* and *P. annua* a synthetic GID1 agonist was a more potent dormancy breaking treatment. Conversely *S. faberi*

and *D. sanguinalis* did not respond to either GID1 agonist (Chapter 3) and in *A. myosuroides* GA_{4+7} had a greater dormancy breaking effect than GA_3. Since only one GID1 ortholog was robustly expressed in dormant *A. myosuroides* (Chapter 4) these results suggest that different synthetic agonists have different affinities (or availability) for the GID1 receptor, possible as a result of allosteric binding, as described by Gazara *et al.*, (2018). Indeed agonist specificity has been identified in Arabidopsis GID1 receptors (GID1a-c), where GID1b has the greatest affinity for GA_4 only under a limited range of pHs (Nakajima *et al*. 2006). It would be interesting to check if this pattern of GA responsiveness across species is also seen upon exogenous GA_1 application, since this is the bioactive gibberellin which accumulates during barley germination (Jacobsen *et al.*, 2002) however such a GA_1 application is currently cost prohibitive. This observation also suggests that GID1 affinity limits the activity of endogenous gibberellins and therefore structure optimisation of GID1 agonists could lead to more potent germination stimulants.

Taken together our results show that the core hormonal signalling mechanisms involved in the hormonal regulation of dormancy and germination are conserved between model species, such as Arabidopsis and barley, and *A. myosuroides* however hormone signalling appears to differ substantially. Distinct species-specific mechanisms are also likely to be involved in the regulation of germination across the grass weeds, since their responses to endogenous application of hormones differ considerably. This diversity in the regulation of germination indicates that it is unlikely that a single germination stimulant will be discovered that will break dormancy in all weed species.

7.3.3 Have grasses and eudicots convergently evolved a physical restraint to regulate germination?

Dormancy is not only regulated by hormonal interactions, but also by the physical barriers that surround the embryo to prevent germination. The structure of the dispersal unit of grasses is quite different from that of eudicots. Whilst the eudicot embryo is typically surrounded by an endosperm (not present in all species, e.g. most members of the family Fabaceae) and testa (seed coat) and possibly a pericarp (fruit coat, e.g. many members of family Asteraceae), grass seeds (diaspores containing a caryopsis) are typically dispersed with additional covering layers of the glumes that have a well described role in the regulation of germination in many species (reviewed by Simpson, 1990). Additionally, instead of being surrounded by endosperm, the radicle in grasses is enclosed by a structure called the coleorhiza that is common to all grasses (Rudall *et al.*, 2005). In many eudicot species the endosperm surrounding the radicle (MPE) regulates germination through modification of its mechanical properties (reviewed by Steinbrecher & Leubner-Metzger, 2017). In initial compound screens applying ABA to after-ripened *A. myosuroides* diaspores a differential effect on different visible germination stages was observed (Chapter 3), where further germination was delayed at coleorhiza emergence. This germination arrest was reminiscent the effect of ABA application to Arabidopsis and *L. sativum* seeds where high concentrations of ABA arrest germination after testa rupture due to a lack of weakening of the MPE (Müller *et al.*, 2006).

Previous work had suggested that the coleorhiza may have some role in the regulation of germination analogous to the MPE through ABA metabolism and potentially through weakening by cell wall remodelling processes (Millar *et al.*, 2006; Barrero *et al.*, 2009; González-Calle *et al.*, 2015). In order to investigate these processes, *A. fatua* was chosen due to its large size that facilitates dissection and tissue preparation. Ablation

of the coleorhiza from dormant caryopses released dormancy (Chapter 5) indicating a role for the coleorhiza in the maintenance and release from dormancy. To test if this role in the release from dormancy was mechanical, a technique was optimised to measure the puncture force of dormant and after-ripened coleorhizae. In after-ripened coleorhizae progress towards germination was associated with a decrease in coleorhiza puncture force as seen in the MPE during germination in many species (reviewed by Steinbrecher & Leubner-Metzger, 2017). Interestingly unlike in the MPE in dormant eudicots, the coleorhiza became reinforced during imbibition in the dormant state (Chapter 5).

To investigate the mechanism of coleorhiza reinforcement, both publicly available microarray datasets and the *A. myosuroides* transcriptome in this work were probed for cell wall remodelling genes upregulated in the dormant state. The upregulation of a *Xyloglucan Endotransglycosylase/Hydrolase* (*XTH*) transcripts specifically in the coleorhiza was reported in the barley microarray of Barrero *et al.* (2009). By searching for the conserved sequence of the active site of this family of genes in microarray probe sequences, 14 additional putative *XTHs* were identified and alignment of these sequences revealed a phylogenetic relationship between *XTHs* expressed in the dormant and after-ripened states. Additionally those *XTHs* that were highly expressed in the dormant state were localised in the coleorhiza (Chapter 5). Analysis of the *A. myosuroides* transcriptome identified a number of additional genes encoding CWRPs that were differentially expressed in the dormant state such as *xyloglucan galactosyltransferases, endo-1,4-beta-xylanases and acetylxylan esterases* (Chapter 4). Performing a search for the XTH motif in the *A. myosuroides* assembly identified a large number of putative *XTH* genes, that when aligned showed dormancy- and germination-specific expression patterns that was reflected in their phylogeny (Appendix 9.10). Interestingly an *XTH* (*AtXTH25*) is also specifically expressed in the MPE of dormant Arabidopsis Cvi seeds during imbibition (Dekkers & Pearce, 2016).

Dormancy-specific XTH isozymes were also demonstrated to be present in the *A. fatua* coleorhiza using an isoelectric focusing approach (Chapter 5).

Taken together these results indicate that the MPE and coleorhiza share a common function in restraining the growth of the radicle to regulate germination. Since the coleorhiza and MPE have a different developmental origin, this mechanism is an example of convergent evolution in the regulation of germination between monocots and dicots. These findings also lend support to the concept that mechanical interactions are important determinants of seed dormancy and germinability. Additionally, a previously unreported division of labour within the *XTH* gene family was identified that will inform the study of xyloglucan remodelling in a broader sense.

7.4 Was the transcriptomic approach successful in identifying targets for WSB management?

7.4.1 What are the challenges and limitations of RNAseq in non-model species?

Despite the power of NGS technologies to reveal global transcriptional responses to different treatments without a requirement for genome sequence information, these technologies have some important assumptions and limitations that need to be understood in order to put NGS experiments in perspective. Firstly, library preparation relies on reverse transcription and amplification via the polymerase chain reaction (PCR) that can introduce bias into cDNA libraries (Hansen et al., 2010). After-sequencing, reference-free assembly can cause the missassembly of transcripts due to gene duplication, transposable elements and contamination from non-target species (Hölzer & Marz, 2019). Read counting and normalisation is another source of error, where many normalisation methods are available that vary considerably in their output (Dillies et al., 2013). Functional annotation of assembled transcript contigs relies on the

assumption that sequence similarity to annotated genes in model species is a good predictor of molecular function. Additionally, there is not a one-to-one relationship between normalised transcript and protein abundance. Studies directly comparing transcriptomes and proteomes have observed lower than expected correlations between transcript abundances attributed to post-transcriptional regulation prior to translation (Stare et al., 2017; Bathke et al., 2019). All of these challenges need to be taken into account when interpreting gene expression data from NGS experiments in non-model species.

With these limitations in mind, the aim of our transcriptomic analysis of environmentally controlled dormancy in *A. myosuroides* was not to understand specific molecular mechanisms of dormancy but to probe the broader processes involved in the regulation of germination in *A. myosuroides* to inform the selection of dormancy breaking compounds. The transcriptome presented in this work was designed with additional biological replicates (5 per treatment) to be run across multiple sequencing lanes to minimise bias with a high sequencing depth (40 million 150 bp paried-end (or 80 million total) reads/sample). Reference-free (*de novo*) assembly of a subset of these reads generated a larger than expected number of scaffolds (347,138) with a mean length of 916 bp (Appendix 9.7), however a number of chimeric scaffolds were also generated (max scaffold length 56,256 bp). A high proportion of reads mapped back to the assembly (91-98%) however properly paired alignment rates were lower (54-76%) reflecting the large number of putatively assembled isoforms. It is likely that these isoforms reflect diversity in the seed population and minor variants of genes, rather than true paralogs. Scaffolds with low normalised expression (FPKM <5) were removed due to a high degree of within-replicate variation. However, benchmarking of assembly completeness using the BUSCO method (Simao *et al.*, 2015) demonstrated a high degree of completeness (94.2% complete BUSCOs). Functional annotation of the filtered assembly was performed using BLAST and InterProScan (Gotz *et al.*, 2008),

and for key hormone biosynthesis and signalling genes BLAST annotations were validated using an alignment and tree building approach against manually curated reference sequences (Appendix 9.9). Taken together, these results show that the transcriptome presented in this work is not without limitations, however the assembly has a high degree of completeness, sufficient coverage to conduct expression analysis and in the majority of cases validation of BLAST annotations confirmed a high degree of annotation accuracy.

7.4.2 Was the RNAseq approach successful in identifying novel targets for WSB management?

Targeted analysis of hormone synthesis and signalling gene expression revealed a partly conserved transcriptional response for the core ABA:GA signalling module in *A. myosuroides* when compared to model species (Chapter 4). Identification of novel molecular mechanisms regulating dormancy in *A. myosuroides* is limited by the current state of knowledge in model species, where much remains to be discovered about the mechanisms regulating seed dormancy. However comparisons between compound screening results (Chapter 3) and the transcriptomes of dormant and germinating *A. myosuroides* seeds (Chapter 4) provides some useful insights about targets for germination stimulants. For example a number of putative *GID1* isoforms were identified in *A. myosuroides*, however only one was strongly expressed in the dormant state and phylogenetically confirmed (TC_749302) (Appendix 9.9) indicating that this *GID1* is the target for the effective GID1 agonists that were identified through compound screening in *A. myosuroides* (Chapter 3). Phylogenetic analysis of this putative *GID1* identified close homology with a barley *GID1* whose mutant (*gse1*) that has a strongly diminished response to the GID1 agonist (AC94377), with respect to other gibberellin insensitive mutants (Chandler *et al.*, 2008). This GID1 agonist was

identified in *A. myosuroides* as having a strong dormancy effect, supporting that hypothesis that the GID1 that was expressed in the transcriptome was the target of this compound. Information about the relative proportions of targets could therefore be useful in understanding the responses of different species to specific germination stimulants.

Another potential target found in the transcriptome was a putative homolog of the *KAI2* receptor for the smoke-derived germination stimulants karrikinolides. This transcript was expressed at low levels but showed dry seed-specific expression pattern, indicating that imbibed seeds may be sensitive to KAR_1 application due to accumulation of the receptor (Chapter 4). KAR_1 was indeed an effective dormancy breaking compound in *A. myosuroides* however in after-ripened seeds KAR_1 acted as a germination inhibitor (Chapter 3). Additionally at high concentrations, KAR_1 treated seeds produced seedlings with severe morphological abnormalities (Chapter 3). Inhibition of hypocotyl extension at high KAR_1 concentrations have been demonstrated in Arabidopsis (Nelson *et al.,* 2010), however it appears these seedling effects are stronger in *A. myosuroides* at similar concentrations.

Targeted analysis of known genes regulating dormancy and germination can only reveal information about the regulation of germination in grass weeds in the context of what is known from model species through forward and reverse genetics studies. However the use of untargeted approaches identified a number of interesting dormancy-related processes that had not previously been described in the literature (Chapter 4). Differential expression analysis revealed a vast number of DEGs across the treatments; 40% of the assembled transcript contigs were differentially expressed between all the comparisons even with a relativity stringent DEG-calling threshold (Chapter 4) therefore looking at individual DEG lists was not a practical approach to analysing the transcriptional responses of *A. myosuroides* seeds. Instead a clustering approach (Appendix 9.11) was employed to create gene lists with similar expression

profiles and the overrepresentation of functional annotations was used to infer differences in processes occurring in different clusters. Two clusters showing dormancy-associated expression patterns (Cluster 6 & 8) were generated and these clusters were found to be enriched in genes involved in, for example, cell wall organisation, defence responses and the response to hypoxia.

Dormant *A. myosuroides* seeds imbibed at high temperatures had higher expression levels of pathogenic response genes such as *Xylanase Inhibitor Proteins* and *Thioredoxin Peroxidases* that have a previously described role in the seed defences to pathogens (reviewed by Pollard, 2018). In the plant defence literature, such pathogenic defence genes are typically regulated by jasmonate (JA) signalling (Jung *et al.*, 2007). In *A. myosuroides* the rate limiting step of bioactive JA-Ile biosynthesis (*JAR1*) was upregulated in the dormant state (Chapter 4). I hypothesised that dormancy, in addition to the inhibition of germination, also acts to enhance processes which contribute to the longevity of *A. myosuroides* seeds in the soil seed bank, such as defence against pathogenic fungi. If these processes are regulated by JA signalling in seeds, then this could provide an interesting novel target for the management of the WSB where inhibitors of JA biosynthesis, such as Jarin-1 (Meesters *et al.*, 2014), could be used to repress seed defence responses and make weed seeds more vulnerable to fungal pathogenesis to deplete the WSB.

Untargeted analysis of the *A. myosuroides* transcriptome also identified an upregulation of genes related to anaerobic "respiration" (fermentation), such as *Pyruvate Decarboxylase* and *Lactate Dehydrogenase* in the dormant state, whereas genes involved in aerobic respiration were specifically expressed in treatments that promoted germination (Chapter 4). This phenomenon has not been observed in model species, however anaerobic conditions can induce secondary dormancy in a number of grass species (reviewed by Simpson, 1990) and some weeds of paddy rice are able to germinate under-water due through alcoholic and lactic fermentation (Rumpho &

Kennedy, 1981). The adaptive significance of anaerobic respiration during dormancy is not clear, however this process may provide an additional non-hormonal target for WSB management worthy of further investigation in *A. myosuroides*. For example inhibition of ATP generation through anaerobic fermentation could cause an energy deficit and exhaustion in seeds or alternatively inhibition of lactate or alcohol dehydrogenase activity could cause an accumulation of phytotoxic intermediates.

Results of our untargeted analysis of dormancy and germination in *A. myosuroides* demonstrate that dormancy is a state associated with more processes than just the inhibition of germination. Whilst most of the recent research into germination stimulants has focused on targeting the hormonal processes involved in the regulation of germination (Villedieu-percheron *et al.*, 2014), untargeted analysis of transcriptomics data provide the opportunity to identify additional dormancy-related process that might be manipulated for the purposes of WSB management.

7.5 Future challenges in the study of germination stimulants in grass weeds.

7.5.1 Will there be a 'silver bullet' germination stimulant that works for all weed seeds?

This work has investigated the germination responses of a range of germination stimulants across five grass species. The germination responses of these species to different compounds was highly species-specific and often compounds with activity in Arabidopsis did not have an activity on grass weeds (Chapter 3). This may be the result in differences in the regulation of dormancy, target-site affinity but also compound uptake and metabolism. It therefore seems unlikely that a 'silver bullet' compound that is able to break the dormancy of all the weed seeds in the WSB will be

identified in the immediate future. Instead it may be useful to develop an understanding of the patterns in the responses to compounds across different species. In the screen of GID1 agonists in this work, weed species in tribe Poeae with a winter annual lifecycle responded to GID1 agonists and two weed species from tribe Paniceae with a summer annual lifecycle did not (Chapter 3). It is not clear if this observation reflects a true pattern related to phylogeny or life cycle due to the limited number of species used however both are weeds from different agronomic contexts (winter cereals and maize respectively), and show similar germination responses to imbibitional temperature as dormancy is released (Chapter 2). It may therefore be more constructive to look for compounds that can break dormancy in weed species that are a problem in specific cropping contexts. This approach would benefit from a greater understanding of the patterns of compound responses across different species, and how these responses can be related to life history traits.

7.5.2 Will the germination stimulants identified in petri-dish assays be effective in the field?

In many ways, constant laboratory conditions do not reflect field conditions as previously discussed. In the petri-dish style germination assay, compounds are applied to dry seeds that rapidly take up the solution during imbibition potentially offering an easy route of entry for germination stimulants. Conversely in a WSB situation, seeds may be at varying levels of hydration and compound uptake may not be so easily facilitated by differences in water potential between soil and the seed. The differential uptake of compounds across seed coats has already been probed in the literature to a limited extent (Salanenka & Taylor, 2011) however little is known about the behaviour of germination stimulants in soil. Studies of the stability of germination stimulants for parasitic weed seeds have identified soil stability as a key factor limiting the success of germination stimulants for ‘suicidal germination’ (Zwanenburg *et al.*, 2016). An

understanding of how compounds are taken-up by seeds in different growth media will therefore be essential in developing germination stimulants for WSB management.

7.5.3 Could weeds become resistant to germination stimulants?

Successful weed management strategies involve strong selective pressures typically involving mass-mortality events where a few individuals survive either by chance or by adaptive fitness. Reliance on a limited range of herbicide MOAs has driven the evolution of herbicide resistance in weed populations globally (Chapter 1). There is no reason to assume that germination stimulants used to manage the WSB would be exempt from the effects of this selective pressure. The analysis of the *A. myosuroides* transcriptome presented in this work demonstrated that distinct mechanisms regulate the release from dormancy during after-ripening including the interaction of distinct hormonal signalling pathways (Chapter 4). Dormancy breaking can also occur through multiple independent mechanisms, such as GID1 agonism, the inhibition of ABA biosynthesis and from smoke cues (Chapter 3). In contrast herbicides typically have monogenic non-redundant targets that are vital for survival in susceptible weeds. It might therefore be hypothesised that resistance would develop more rapidly to germination stimulants than it has for many herbicide MOAs. It may therefore be important to test the effects of germination stimulants with different MOAs as mixtures to avoid applying strong selective pressure on a single one of the many regulatory pathways involved in the regulation of germination.

7.5.4 Is there a future for 'Flush and Kill' in weed management?

In this chapter many of the limitations and knowledge gaps to chemical manipulation of the WSB have been discussed. However, despite these limitations, the 'Flush and Kill'

concept has great potential in bring the complex dynamics of the WSB under the control of farmers. This is particularly relevant in the context of a shift away from tillage practises that have historically been used to control weed emergence. Future research should focus both on the biology of seed dormancy in weeds to better understand their response to the environment. An understanding of the diversity of dormancy regulation and responses to germination stimulants across species will also be important in selecting the best targets for germination stimulants in different agronomic contexts.

7.6 Project conclusions

7.6.1 Overall Conclusion

Chemical genetics and transcriptomics approaches have demonstrated a diversity of mechanisms involved in the regulation of dormancy and germination across species. This dormancy acts to restrict the range of temperatures in which grass weed seeds germinate in a manner that reflects their life history and field emergence patterns. The mechanical restraint of the radicle as a means of regulating germination is a mechanisms that is conserved across mono- and dicotyledonous plant classes. The ongoing potential of the 'Flush and Kill' concept will depend on a broadening understanding of the diversity of mechanisms controlling weed seed responses to germination stimulants and to the environment.

7.6.2 Addressing the specific aims of the project

Aim 1: *Develop an understanding of the role of temperature on the germination of grass weed seeds with defined levels of dormancy and investigate how these patterns of germination can be explained by patterns of gene expression.*

After-ripening was able to release the dormancy in dose-dependent manner in all but one species. After-ripening rate was dependent on both the dormancy level of seeds and their differing sorption isotherm properties. After-ripening caused an increase in the maximum temperature permissive to germination in winter annuals and a decrease in minimum temperature in summer annuals. Vernalisation of *A. myosuroides* plants increased their dormancy as well as affecting seed production. Transcriptomic profiling of the effect of after-ripening and imbibitional temperature in *A. myosuroides* diaspores identified differential regulation of dormancy release by after-ripening and the cold. Dry after-ripening did not cause significant differences in expression in the dry state. When

imbibed, dormancy release by after-ripening was associated with the expression of gibberellin biosynthesis genes while under cold temperatures dormancy release was associated by the expression of abscisic acid catabolism genes and ethylene responsive transcription factors.

Aim 2: *Using information and experimental conditions obtained from Aim 1, select and test putative germination stimulants on seed batches with defined dormancy levels.*

Published and novel compounds from a target catalogue compound library were screened against dormant and after-ripened *A. myosuroides* seed batches. GID1 agonists, PDS inhibitors and KAR_1 were effective dormancy breakers in *A. myosuroides*. A selection of hit compounds were further tested across four other species, and multiple seed batches with differing genotypes or storage histories. Hit compounds had diverse effects across the species and in some species responded differently depending on the dormancy level of batches.

Aim 3: *Investigate the hypothesis that the coleorhiza and MPE share a common function in the physical restraint of the radicle during dormancy and germination.*

Ablation of the coleorhiza from dormant *A. fatua* caryopses promoted germination when compared to wounding controls. Biomechanical measurements of the coleorhiza demonstrated weakening over time when imbibed in the after-ripened state and a reinforcement in the dormant state. The coleorhiza and MPE therefore share a conserved function in the restraint of radicle growth in dormant seeds. Since these two tissues have a distinct developmental origin, this is an example of convergent evolution in the regulation of germination between dicotyledonous and monocotyledonous plants. Analysis of multiple transcriptome datasets identified candidate CWRPs associated with coleorhiza strengthening or weakening, such as XTHs. The XET activity of XTHs

was profiled across embryonic tissues and dormancy states. A peak of XET activity in the after-ripened state was associated with radicle emergence and separation of XET isozymes using isoelectric focusing identified dormancy-specific XETs as hypothesised by the phylogenetic analysis of transcriptome data. These results confirm the hypothesis that the coleorhiza plays a key role in the regulation of germination.

7.7 References

Auge GA, Blair LK, Burghardt LT, Coughlan J, Edwards B, Leverett LD, Donohue K. **2015**. Secondary dormancy dynamics depends on primary dormancy status in *Arabidopsis thaliana*. *Seed Science Research* **25**: 230–246.

Auge GA, Blair LK, Neville H, Donohue K. **2017a**. Maternal vernalization and vernalization-pathway genes influence progeny seed germination. *New Phytologist* **216**: 388–400.

Auge GA, Leverett LD, Edwards BR, Donohue K. **2017b**. Adjusting phenotypes via within- and across-generational plasticity. *New Phytologist* **216**: 343–349.

Barrero JM, Talbot MJ, White RG, Jacobsen J V, Gubler F. **2009**. Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant Physiology* **150**: 1006–1021.

Baskin JM, Baskin CC. **1985**. The annual dormancy cycle in buried weed seeds: A continuum. *BioScience* **35**: 492–498.

Baskin C, Baskin J. **2014**. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. Oxford: Academic Press.

Bathke J, Konzer A, Remes B, McIntosh M, Klug G. **2019**. Comparative analyses of the variation of the transcriptome and proteome of *Rhodobacter sphaeroides* throughout growth. *BMC Genomics* **20**: 1–13.

Bewley J, Bradford K, Hilhorst H, Nonogaki H. **2013**. *Seeds - Physiology of Development, Germination and Dormancy*. London: Springer.

Cadman CSC, Toorop PE, Hilhorst HWM, Finch-Savage WE. **2006**. Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant Journal* **46**: 805–822.

Chandler PM, Harding CA, Ashton AR, Mulcair MD, Dixon NE, Mander LN. **2008**. Characterization of gibberellin receptor mutants of barley (*Hordeum vulgare* L.). *Molecular Plant* **1**: 285–294.

Chauvel B, Munier-Jolain NM, Grandgirard D, Gueritaine G. **2002**. Effect of vernalization on the development and growth of *Alopecurus myosuroides*. *Weed Research* **42**: 166–175.

Chen Y. **2014**. Thesis: The Effect of Smoke on Seed Germination: Global Patterns and Regional Prospects for the Southern High Plains. Texas Tech University, USA.

Cohen D. **1966**. Optimizing reproduction in a randomly varying environment. *Journal of Theoretical Biology* **12**: 119–129.

Corbineau F, Xia Q, Bailly C, El-Maarouf-Bouteau H. **2014**. Ethylene, a key factor in the regulation of seed dormancy. *Frontiers in Plant Science* **5**: 1–13.

Dekkers BJW, Pearce SP. **2016**. Dormant and after-ripened *Arabidopsis thaliana* seeds are distinguished by early transcriptional differences in the imbibed state. *Frontiers in Plant Science* **7**: 1–15.

Dillies MA *et al.* 2013. A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. *Briefings in Bioinformatics* **14**: 671–683.

Finch-Savage WE, Footitt S. **2017**. Seed dormancy cycling and the regulation of dormancy mechanisms to time germination in variable field environments. *Journal of Experimental Botany* **68**: 843–856.

Finch-Savage WE, Leubner-Metzger G. **2006**. Seed dormancy and the control of germination. *New Phytologist* **171**: 501–523.

Footitt S, Douterelo-Soler I, Clay H, Finch-Savage WE. **2011**. Dormancy cycling in Arabidopsis seeds is controlled by seasonally distinct hormone-signaling pathways.

PNAS **108**: 20236–20241.

Footitt S, Huang Z, Clay HA, Mead A, Finch-Savage WE. **2013**. Temperature, light and nitrate sensing coordinate Arabidopsis seed dormancy cycling, resulting in winter and summer annual phenotypes. *Plant Journal* **74**: 1003–1015.

Footitt S, Müller K, Kermode AR, Finch-Savage WE. **2015**. Seed dormancy cycling in Arabidopsis: Chromatin remodelling and regulation of DOG1 in response to seasonal environmental signals. *Plant Journal* **81**: 413–425.

Footitt S, Ölçer-Footitt H, Hambidge AJ, Finch-Savage WE. **2017**. A laboratory simulation of Arabidopsis seed dormancy cycling provides new insight into its regulation by clock genes and the dormancy-related genes *DOG1, MFT, CIPK23* and *PHYA*. *Plant Cell and Environment* **40**: 1474–1486.

Gazara RK, Moharana KC, Bellieny-Rabelo D, Venancio TM. **2018**. Expansion and diversification of the gibberellin receptor *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*) family in land plants. *Plant Molecular Biology* **97**: 435–449.

González-Calle V, Barrero-Sicilia C, Carbonero P, Iglesias-Fernández R. **2015**. Mannans and endo-β-mannanases (*MAN*) in *Brachypodium distachyon*: Expression profiling and possible role of the *BdMAN* genes during coleorhiza-limited seed germination. *Journal of Experimental Botany* **66**: 3753–3764.

Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Garcı JM, Nagaraj SH, Valencia D, Geno C De. **2008**. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research* **36**: 3420–3435.

Hansen KD, Brenner SE, Dudoit S. **2010**. Biases in Illumina transcriptome sequencing caused by random hexamer priming. *Nucleic Acids Research* **38**: 1–7.

Hauvermale AL, Tuttle KM, Takebayashi Y, Seo M, Steber CM. **2014**. Loss of *Arabidopsis thaliana* seed dormancy is associated with increased accumulation of the

GID1 GA hormone receptors. *Plant and Cell Physiology* **56**: 1773–1785.

Hölzer M, Marz M. **2019**. *De novo* transcriptome assembly: A comprehensive cross-species comparison of short-read RNA-Seq assemblers. *GigaScience* **8**: 1–16.

Honek A, Martinkova Z, Jarosik V. **1999**. Annual cycles of germinability and differences between primary and secondary dormancy in buried seeds of *Echinochloa crus-galli*. *Weed Research* **39**: 69–79.

Huang Z, Footitt S, Finch-Savage WE. **2014**. The effect of temperature on reproduction in the summer and winter annual *Arabidopsis thaliana* ecotypes Bur and Cvi. *Annals of Botany* **113**: 921–929.

Jacobsen J V, Pearce DW, Poole AT, Pharis RP, Mander LN. **2002**. Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. *Physiologia Plantarium* **115**: 428–441.

Jefferson L, Pennacchio M, Havens-Young K. **2014**. *Ecology of Plant-Derived Smoke: Its Use in Seed Germination*. Chicago: Oxford University Press.

Jung C, Lyou SH, Yeu S, Kim MA, Rhee S, Kim M, Lee JS, Choi Y Do, Cheong JJ. **2007**. Microarray-based screening of jasmonate-responsive genes in *Arabidopsis thaliana*. *Plant Cell Reports* **26**: 1053–1063.

Lee Y, Karunakaran C, Lahlali R, Liu X, Tanino KK, Olsen JE. **2017**. Photoperiodic regulation of growth-dormancy cycling through induction of multiple bud – shoot barriers preventing water transport into the winter buds of norway spruce. *Frontiers in Plant Science* **8**: 1–16.

Linkies A, Leubner-Metzger G. **2012**. Beyond gibberellins and abscisic acid: How ethylene and jasmonates control seed germination. *Plant Cell Reports* **31**: 253–270.

Maréchal P, Henriet F. **2012**. Ecological review of black-grass (*Alopecurus myosuroides* Huds.) propagation abilities in relationship with herbicide resistance.

Biotechnology, Agronomy, Society and Environment **16**: 103–113.

Meesters C, Mönig T, Oeljeklaus J, Krahn D, Westfall CS, Hause B, Jez JM, Kaiser M, Kombrink E. **2014**. A chemical inhibitor of jasmonate signaling targets JAR1 in *Arabidopsis thaliana*. *Nature Chemical Biology* **10**: 830–836.

Millar AA, Jacobsen J V., Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F. **2006**. Seed dormancy and ABA metabolism in Arabidopsis and barley: The role of ABA 8'-hydroxylase. *Plant Journal* **45**: 942–954.

Mojzes A, Csontos P, Kalapos T. **2015**. Is the positive response of seed germination to plant-derived smoke associated with plant traits? *Acta Oecologica* **65**–**66**: 24–31.

Müller K, Tintelnot S, Leubner-Metzger G. **2006**. Endosperm-limited Brassicaceae seed germination: Abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant and Cell Physiology* **47**: 864–877.

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E. **2005**. Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: Epigenetic and genetic regulation of transcription in seed. *Plant Journal* **41**: 697–709.

Nakajima *et al.* 2006. Identification and characterization of Arabidopsis gibberellin receptors. *Plant Journal* **46**: 880-889.

Nelson D, Flematti G, Riseborough JA, Ghisalberti EL, Dixon KW, Smith SM. 2010. Karrikins enhance light responses during germination and seedling development in *Arabidopsis thaliana*. *PNAS* **107**: 7095-7100.

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S. **2003**. Gibberellin biosynthesis and response during Arabidopsis seed germination. *The Plant Cell* **15**: 1591–1604.

Penfield S, MacGregor DR. **2017**. Effects of environmental variation during seed

production on seed dormancy and germination. *Journal of Experimental Botany* **68**: 1–7.

Pollard AT. **2018**. Seeds vs fungi: An enzymatic battle in the soil seedbank. *Seed Science Research* **28**: 197–214.

Rudall PJ, Stuppy W, Cunniff J, Kellogg EA, Briggs BG. **2005**. Evolution of reproductive structures in grasses (Poaceae) inferred by sister-group comparison with their putative closest living relatives, Ecdeiocoleaceae. *American Journal of Botany* **92**: 1432–1443.

Rumpho ME, Kennedy RA. **1981**. Anaerobic metabolism in germinating seeds of *Echinochloa crus-galli* (barnyard grass). *Plant Physiology* **68**: 165–168.

Salanenka YA, Taylor AG. **2011**. Seedcoat permeability: Uptake and post-germination transport of applied model tracer compounds. *HortScience* **46**: 622–626.

Simao FA, Waterhouse RM, Ioannidis P, Kriventseva E V, Zdobnov EM. **2015**. Genome analysis BUSCO : assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* **31**: 3210–3212.

Simpson G. **1990**. *Seed Dormancy in Grasses*. Cambridge: Cambridge University Press.

Soltani E, Baskin JM, Baskin CC. **2019**. A review of the relationship between primary and secondary dormancy, with reference to the volunteer crop weed oilseed rape (*Brassica napus*). *Weed Research* **59**: 5–14.

Stare T, Stare K, Weckwerth W, Wienkoop S, Gruden K. **2017**. Comparison between proteome and transcriptome response in potato (*Solanum tuberosum* L.) leaves following potato virus Y (PVY) infection. *Proteomes* **5**.

Steinbrecher T, Leubner-Metzger G. **2017**. The biomechanics of seed germination. *Journal of Experimental Botany* **68**: 765–783.

Villedieu-percheron E, Lachia M, Jung PMJ, Screpanti C, Fonné-pfister R, Wendeborn S, Zurwerra D, Mesmaeker A De. **2014**. Chemicals Inducing Seed Germination and Early Seedling Development. *Chimia* **68**: 654–663.

Zwanenburg B, Mwakaboko AS, Kannan C. **2016**. Suicidal germination for parasitic weed control. *Pest Management Science* **72**: 2016–2025.

8. Materials and Methods

8.1 Seed collection, production and storage

The seed batches used in this thesis come either from field collections in the UK, propagation in the glasshouse at Royal Holloway University or as contributions from the seed bank at Jealott's Hill International Research Center (Bracknell, UK). Information about the provenance of individual seed batches can be found in Chapter 2 (Table 2.2). All the seed batches were subjected to the same storage conditions upom arrival to the university.

8.1.1 Collection of seed material in the field

Seed collection in the field was conducted following the recommendations in Baskin & Baskin, (2014). *Alopecurus myosuroides* LH170 was collected from a wheat field in Jealott's Hill (Bracknell, Berkshire, UK, 51°27'19.2"N 0°45'44.8"W) in June 2017 and *A. fatua* LH840 was collected from a barley field in Kingsclere (Hampshire, UK, 51°15'29.8"N 1°19'36.1"W) in August 2016. To ensure seeds were collected only at maturity, seed bearing culms were cut and agitated in a plastic bag. Culms bearing seeds that did not detach were discarded.

8.1.2 Propagation of plants for seed in controlled environments

All seed batches that were produced from plants grown under controlled environment conditions were subjected to the same temperature and supplementary lighting regime during seed maturation. After-ripened or non-dormant seeds were germinated in 90 mm Petri dishes containing 5 mL of water at 20ºC under constant light for 2 weeks (see 8.2.1). Seedlings were then transferred to 40-cell seed trays (LBS Horticultural Ltd.,

Lancashire, UK) containing a 10:1 (v/v) mixture of J. Arthur Bower's John Innes No. 1 compost (Westland Horticulture Ltd., Dungannon, County Tyrone, Northern Ireland) and Standard. These seed trays were incubated for two weeks in a phytochamber with a 18/6 hour day/night cycle under fluorescent white light at 20/18°C for two weeks and regularly watered. After two weeks seedlings were potted into round 15 cm diameter pots (*A. myosuroides* and *P. annua*) or 30 cm diameter pots (*D. sanguinalis* and *S. faberi*) containing a 10:1 (v/v) mixture of J. Arthur Bower's John Innes No. 3 compost (Westland Horticulture Ltd.) and Standard Perlite (Sinclair Pro, Westland Horticulture Ltd.) and 300 g m^{-3} of Exemptor® (10% w/w Thiacloprid insecticide, Bayer AG. Leverkusen, Germany) for the control of soil-borne insect pests. Plants were transferred to a glasshouse bay with supplemental sodium lighting on a 16/8 hour day/night cycle at 20/18°C and watered regularly. Plants were fed approximately every two weeks with a water soluble fertiliser (Peters® Professional Grow-Mix, ICL Ltd. Ipswitch, UK) at 1 g L^{-1} until flowering. To control mildew on *A. myosuroides* plants a commercial fungicide (Trifloxystrobin + Tebuconazole, Fungus Fighter Plus®, Bayer AG) was applied using a trigger spray bottle following the manufacturer's recommendations. After flowering and seed maturation, the watering regime was reduced and plants were allowed to desiccate before seed harvest. To ensure seeds were collected only at maturity, seed bearing culms were cut and agitated in a plastic bag. Culms bearing seeds that did not detach were discarded.

8.1.3 Vernalisation

To assess the effects of vernalisation on progeny dormancy, the propagation method above (8.1.2) was followed, except that half of the seedlings were kept in seed trays and incubated in an environmental test chamber (Conviron™ Adaptis® CMP6010) set to 16/8 hour day/night cycle at 20/18°C with the same photoperiodicity as the

phytochamber. The temperature in this chamber was gradually reduced over the course of 6 days to an 8/16 hour day/night cycle at 6/4°C (Table 8.1). This condition was maintained for 35 days and then temperatures and lighting were increased to the initial condition. In total 12 plants that were vernalised and 6 non-vernalised plants were grown to maturity. These plants were harvested following the method described in 8.1.2. The *P. annua* plants used to produce LH011 were also vernalised following this method. Attempts to vernalise *D. sanguinalis* and *S. faberi* following this method resulted in the death of the plants.

Table 8.1. Vernalisation program.

Day length (hours)	Day temp. (°C)	Night temp. (°C)	Day light level	Number of cycles	Cumulative days
16	18	16	3	2	2
14	14	10	2	2	4
12	8	6	1	2	6
8	6	4	1	35	41
12	8	6	1	2	43
14	14	10	2	2	45
16	18	16	3	2	47

8.1.4 Seed batch processing and cleaning

Immediately after harvest, for both field collected and propagated batches, seeds were spread evenly on trays and transferred to a seed drying room held between 10-20% relative humidity at 20°C in the dark for one week. Different seed cleaning processes were used for different species. For *A. fatua* glumes (but not lemma and palea) were removed using a laboratory seed thresher (STM-350, Seed Processing Holland Inc., Enkhuizen, The Netherlands) resulting in the removal of awns from most diaspores. For *A. myosuroides* a coarse metal sifter was used to remove large debris and a fine sifter was used to separate diaspores from anthers. For smaller seeded species (*D. sanguinalis, P. annua* and *S. faberi*) a 'Seed Blower' was used to separate anthers from diaspores (Seed blower Ø 100 mm, type 4110.10.00, Seed Processing Holland Inc.).

8.1.5 Measuring flowering and seed production by A. myosuroides plants

During the propagation of *A. myosuroides* plants for Chapter 6, the onset of anthesis was counted regularly over time by recording the emergence of the tip of the panicles from the culms for each plant individually. To measure seed production, diaspores were weighed for each plant separately after drying. To determine the 100 seed weight for both batches, triplicates of 100 seeds were randomly selected and weighed using a fine balance.

8.1.6 Seed provided by collaboration partner

In compound screens using multiple seed batches from the same species, seeds with unknown dormancy were provided by Syngenta Ltd. (Jealott's Hill International Research Centre, UK). These seeds had been stored for varying lengths of time in a dry seed storage facility in cotton seed bags. For these seed batches it is not clear if batches with low germination are dormant or aged.

8.1.7 Long term seed storage

All seeds in this work were subjected to the same seed storage protocol after arrival at the University. After harvest or collection, seeds were dried to < 20% equilibrium relative humidity in a purpose built seed drying room and processed following the methods described in 8.1.4. Seeds were transferred to 13 x 18 cm 40 g m^{-2} glassine bags (HERA Papierverarbeitung GmbH & Co KG, Nidda, Germany) and stored for one week above silica gel (Chameleon® C 2,5 - 6 mm drying agent, VWR International Ltd., Pennsylvania, USA) at room temperature. The silica gel contained a colour changing agent to indicate a loss of hygroscopic potential. When this color change was

observed, silica gel was refreshed and regenerated by heating in a drying oven (DRY-Line® 53, VWR International Ltd.) at 110°C for >7 hours or overnight.

After a week of storage above silica gel the water activity of the seed was tested using a dew point hygrometer (see 8.1.9). When seeds reached < 10% equilibrium relative humidity (%ERH) they were transferred in their bags to hermetically sealed 2 L 'Kilner Jars' (Item code: 0025.513, Rayware Ltd. Liverpool, UK) along with ~250 mL of silica gel and stored in a -20°C cold room. Monitoring germination of dormant seed batches (*A. fatua* LH840-D and *A. myosuroides* LH170-D) showed that there was minimal change in dormancy over a three year storage period when seeds were stored under this condition (Chapter 2).

8.1.8 After-ripening seed storage

After-ripening was used to release seed dormancy and generate seed batches with defined dormancy levels. For after-ripening assays sub-populations of ~200 seeds from dormant seed batches were portioned into aliquots in 5.3 x 7.8 cm 40 g m^{-2} glassine bags (HERA Papierverarbeitung GmbH & Co KG, Nidda, Germany). These bags were suspended above saturated aqueous solutions of $MgCl_2$ or $Ca(NO_3)_2$ in hermetically sealed containers generating ERHs of 32% and 53% respectively. Measurements of seed %ERH when stored under these conditions showed that seeds came to equilibrium with this humidity within one week (Chapter 2). Sub-populations were periodically removed every 4 weeks and a standard germination assay was conducted at 20°C under constant light following the method described in 8.2.1.

To generate seed batches with standardised after-ripening treatments, after-ripening curves (Chapter 2) were generated showing the change in maximum germination occurring over after-ripening storage time. A four parameter logistic curve was fitted using GraphPad Prism software (v7.0.5, GSL Biotech LLC, California, USA) to after-

ripening curves and used to interpolate the half-maximal ($AR_{50\%}$) and maximal ($AR_{100\%}$) after-ripening times under either humidity. Values for *S. faberi* were not interpolated because the relationship between after-ripening and maximum germination was ambiguous. Larger batches (~100 g) were incubated in 13 x 18 cm 40 g m^{-2} glassine bags (HERA Papierverarbeitung GmbH & Co KG, Nidda, Germany) under the described storage conditions and removed after reaching $AR_{50\%}$ or $AR_{100\%}$. These batches were then dried and stored at -20ºC as previously described.

8.1.9 Measurement of equilibrium relative humidity

A dew point hygrometer (LabMaster-aW, Novasina AG, Lachen, Switzerland) was used to measure the %ERH generated by seeds and aqueous solutions of different salts. This device was regularly calibrated using hygroscopic standards as described by the manufacturer. For measurements of seed %ERH, ~1 g of seeds were equilibrated to 20oC and %ERH was measured with a 5 minute stable observation time. Care was taken to minimise the exposure of samples to the ambient environment. For the measurement of solutions, 3 mLs of solution was pipetted into the sample cups and measurements with a 5 minute stable observation time and varied temperatures were used. Percentage saturation of the salt solutions was calculated relative to published solubility data on the PubChem Database (Kim et al., 2019).

8.1.10 Generation of seed moisture sorption isotherms

Approximately 1 g of seeds was equilibrated above SAL-T humidity standards (4, 6, 11, 33, 53, 75, 90 & 98 %ERH, Novasina AG, Lachen, Switzerland) on a filter paper cup inside a hermetically sealed container. These seeds were allowed to equilibrate at 20ºC for one week in a controlled environment chamber (MLR-352-PE, Panasonic

Corporation, Osaka, Japan). After equilibration seeds were weighed using a fine balance taking care to minimise exposure to the ambient environment. Samples were then heated to 110°C for 3 hours in a drying oven (DRY-Line® 53, VWR International Ltd.) and reweighed. Seed moisture content was calculated as the difference between equilibrated mass and dry mass as a percentage of the mass equilibrated at 100% ERH (above a solution of ultrapure water). These values were fitted to a five parameter asymmetric sigmoidal curve (Gottschalk & Dunn, 2005) using GraphPad Prism (v7.0.5, GSL Biotech LLC, California, USA) and the moisture contents generated from different storage humidities were interpolated.

8.1.11 Seed viability testing using the tetrazolium assay

Seeds were imbibed for 24 hours at 20°C in a controlled environment chamber (MLR-352-PE, Panasonic Corporation, Osaka, Japan) under constant light. One hundred diaspores in triplicate were dissected and the number of diaspores that contained a fully formed caryopsis was recorded. Caryopses were then transferred to a petri-dish containing two filter papers and an aqueous solution of 1% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC). As a negative (dead) control, another set of caryopses were incubated at 90°C in ultrapure water for an hour and then transferred to TTC plates. These plates were incubated for a further 24 hours at 20°C in the dark. Staining was assessed under a stereomicroscope. Embryos were considered stained when the entirety of the visible external portion of the embryo (underneath the testa and pericarp) was stained dark red (Appendix 9.16). Caryopses with weak or patchy staining were considered unstained and non-viable.

8.2 Germination kinetics

8.2.1 Standard germination assay conditions

For all germination assays and tissue preparations a standard germination medium and filter paper substrate was used with different Petri-dish sizes and incubation medium volumes depending on the species. For all species the incubation medium was reverse osmosis purified water (Purite® Select Fusion, Suez Recycling and Recovery UK Ltd., Berkshire, UK) with a resistivity of > 18.0 MΩ·cm that was sterilised by autoclaving at 121°C for 20 minutes. Filter papers, manufactured from cotton linters and refined pulp with a high level of α-cellulose, were acquired from Machery-Nagel™ (MN-713, Machery-Nagel GmbH & Co. KG, Oensingen, Switzerland).

A. fatua seeds were incubated in clear 9 cm Petri dishes with two 8 cm filter papers with 5 mL of water. *A. myosuroides* and *S. faberi* seeds were incubated in 6 cm Petri dishes with 5 cm filter papers and 2.5 mL of water. The small seeded species *D. sanguinalis* and *P. annua* were incubated in 6 cm Petri dishes with 5 cm filter papers and 2 mL of water. Petri dishes were sealed with Parafilm™ (Bemis™, Thermo Fisher Scientific Inc., Massachusetts, USA). When Petri dishes were stacked in incubators, a blank plate containing filter papers and water but no seeds was placed on top of the stack. In larger germination assays (>100 plates), the position of plates within the stack and the incubator was pseudorandomised at each assessment time point. Unless otherwise stated, germination assays consisted of biological triplicates containing >30 diaspores per Petri-dish. Unless otherwise stated, germination was counted as the emergence of the coleorhiza through the proximal margins of the glumes.

8.2.2 The use of two dimensional thermal gradient plates

Thermal gradient plates consist of a sheet of aluminum where a gradient of temperatures is generated by heating one end of the plate and cooling the other. Plates are divided into grids where each grid square represents the position of one Petri-dish. Each plate therefore experiences a small gradient of temperatures that we expect to be in the range of ± 1°C. The temperature in the center of each position was measured using a thermocouple temperature sensor (K type, ±0.1°C) embedded in a petri dish using a temperature data logger (VOLTCRAFT® PL-125-T4USB, Conrad Electronic AG, Wollerau, Switzerland). In this work two different thermal gradient plates were used. For *A. fatua*, a custom built plate (2D Thermogradiententisch, Albert-Ludwigs-Universität, Freiburg, Germany) with supplementary fluorescent lighting was used because it was more suitable for larger Petri dishes. For species that were incubated in 6 cm Petri dishes a GRANT™ GRD1-LH (Grant Instruments Ltd., Cambridge, UK) with supplementary fluorescent lighting was used because of its greater temperature-linearity across the plate. When incubating plates on a two dimensional thermal gradient plate the standard germination conditions described in 8.2.1 were used. Additionally, at temperatures above 24°C, supplemental water was added to replace water that was lost through evaporation.

8.2.3 Cold stratification

Triplicates of 30 freshly harvested *A. myosuroides* seeds produced by vernalised and non-vernalised plants (LH192V-D & LH192NV-D) were imbibed in Petri-dishes as described in 8.2.1 but with just 1 mL of water. These plates were incubated in the dark at 4°C using a laboratory refrigerator for 3, 7, 14, and 21 days. After this time, an additional 2 mL of water was added and the plates were transferred to an incubator at 20°C in constant light and germination was scored as described in 8.2.1.

8.2.4 Coleorhiza ablation experiments

For experiments involving ablation, a fine grade (P240) silica-based abrasive paper was used to remove the coleorhiza from dry caryopses. Coleorhiza ablation using this method produced similar results to surgical ablation of the coleorhiza from imbibed caryopses (data not shown). A number of wounding controls were also generated using imbibed caryopses. For the ablation of the plumule a fine razor blade was used to bisect the embryo to the depth of the scutellum and the plumule was removed using fine forceps. For pericarp ablation, the pericarp and testa were peeled away from the embryo using fine forceps without disturbing the underlying tissues. After 240 hours, pericarp and plumule ablated treatments were transferred to a plate containing 100 µM GA_{4+7} + 0.1 % (v/v) DMSO (Table 8.2) under standard germination conditions (8.2.1) to determine if tissue ablation had affected the germinability of the seeds.

8.2.5 Statistical analysis of thermal gradient table data

Estimation of cardinal temperatures from the germination data produced from thermogradient table experiments was conducted by calculating the area under the curve (AUC) for a point-to-point spline of the germination data for individual plates. For calculating base (T_b) and ceiling (T_c) temperatures, linear regression analysis (GraphPad Prism v7.0.5) was performed on the relationship between AUC and temperature. For T_b estimation, data points below an optimal temperature were included in the regression analysis for T_c estimation only data points above the optimal temperature were included. The intercept of the regression line and 95% confidence intervals with the x-axis was used to extrapolate the cardinal temperatures. For the estimation of optimal temperatures (T_o) a segmental linear regression analysis (GraphPad Prism v7.0.5) was performed with all AUC data points where the parameter ‘X0’ was considered the optimal temperature with a predicted 95% confidence interval.

8.3 Microscopy

8.3.1 Stereomicroscopy and imaging of whole seeds and isolated tissues

Images of whole and dissected diaspores were taken using a stereomicroscope fitted with a camera (Leica MZ125, Leica Biosystems, Wetzlar, Germany) and images were processed using the inbuilt Leica Application Suite Software (version 4.1). Images were further processed using GIMP v2.8.16 (The GIMP Team, 2019) to improve the contrast and remove noise.

8.3.2 Sample preparation for light microscopy

The light microscopy in this work was all performed using the same method. Diaspores or caryopses were imbibed for varying lengths of time under standard germination conditions (8.2.1) at 20ºC under constant light. Samples were then fixed in 4% (w/v) paraformaldehyde during three cycles of vacuum infiltration in a vacuum desiccator and overnight incubation at 4ºC. After fixation, samples were washed in an excess of phosphate buffered saline (PBS) and then subjected to the following ethanol (% v/v) dehydration gradient: 30% (2x 30 mins), 50% (2x 30 mins), 70% (2x 30 mins), 85% (2x 30 mins), 95% (2x 30 mins) and 100% (4x 30 mins). A resin based system was chosen for embedding after the failure of paraffin-based methods to infiltrate the endosperm. Samples were embedded in 2-hydroxyethyl methyl acrylate (HEMA) (Technovit® 7100 cold curing resin system, Kulzer Technique, Wehrheim, Germany) following Matsushima *et al.*, (2014) with modifications (Yeung *et al.*, 2015). The following dilution series of HEMA in 96% ethanol (% v/v) was used to embed samples: 25% (24 hours, 3 exchanges), 50% (24 hours, 3 exchanges), 75% (24 hours, 3 exchanges), 100% (96 hours, 4, exchanges). Samples were then polymerised in a heatsink blocking mold (Histoform™ Q, Kulzer Technique, Wehrheim, Germany) by the addition of 1% (v/v) benzoyl peroxide. After polymerisation, blocks were removed and trimmed with a

jewelers saw and re-embedded in the desired orientation. Samples were then mounted onto supports (Histoblock™ Q, Kulzer Technique, Wehrheim, Germany) using the Technovit™ 3040 polymer following the manufacturer's instructions.

Sectioning was performed using a rotary microtome (Microtom HM355S, Thermo Scientific, Massachusetts, USA) to cut 5 µm sections. Sections were mounted onto slides (VWR 631-0117, VWR International Ltd., Pennsylvania, USA) by flotation on water and dried at 45°C on a hotplate for 2 hours. Sections were progressively stained on the slide using in 1% (w/v) safranin O (to stain cell wall lignin) counterstained within 1% (w/v) toluidine blue (to stain nuclei and polysaccharides) (Ruzin, 1999). Coverslips were mounted using Canada Balsam (Merck KGaA, Darmstadt, Germany) and sealed using clear nail varnish. Bright-field images were taken using an ECLIPSE™ Ni-E stereomicroscope (Nikon, Tokyo, Japan) and images were processed using GIMP (v2.8.16) to improve the contrast.

8.3.3 Measuring organ expansion during germination

To measure the expansion of different embryonic tissues during the germination in *A. fatua*, caryopses that had been imbibed under standard germination conditions (8.2.1) were fixed in paraformaldehyde (8.3.2) and stored at 4°C. Caryopses were washed in PBS then laterally cut using a razor blade to produce ~0.5 mm thick sections. Before imaging, sections were briefly dipped in 1% (w/v) toluidine blue and gently washed in PBS. Sections were then imaged using a stereomicroscope (8.3.1).

8.4 Compound screening

8.4.1 Preparation of compound stocks

With the exception of ABA and compounds/mixtures that were supplied as liquids (Smoke-water and TMB) compounds were solubilised in DMSO (HPLC Grade (99.9+% Purity, Alfa Aesar GmbH & Co KG, Massachusetts, USA). For waxy or immiscible compounds, solubilisation was facilitated by heating to 45°C for one hour with vigorous shaking in sealed vials (Thermomixer HC, STARLAB Ltd., Milton Keynes, UK). Details of compound suppliers and solubilisation can be found in Table 8.2.

The ABA stock was prepared by dissolving *cis*-S(+)-ABA in 1N KOH at 100 mM, diluted in ultrapure water to 10 mM and adjustment of pH to 7.0 using 10N HCl. Gibberellin stocks (GA_3 and GA_{4+7}) were prepared first by solubilisation in DMSO at a 100 mM concentration, diluted in ultrapure water to 10 mM and pH adjustment to pH 7.0 using KOH. Both 10 mM stocks had a DMSO concentration of 10% (v/v). Gibberellin and ABA stocks were filter sterilised through nylon syringe filters (0.2 µm pore size). Stock solutions were stored at -20°C and working solutions for physiological assays were prepared on the same day as seeds were imbibed to minimise the degradation of compounds with unknown stability.

Table 8.2 Details of compound stock solutions. Supplier details: ChemSpace US Inc., New Jersey, USA; Duchefa Biochemie BV, Haarlem, The Netherlands; Fluka Analytical, Honeywell International Inc., North Carolina, USA; Grayson Australia, Tecnica Pty. Ltd., Victoria, Australia; Syngenta Ltd., Jealott's Hill International Research Centre, Berkshire, UK. For anonymised compounds a molecular weight is not shown to protect the confidentiality of the compound structures. *GA_{4+7} is a mixture of GA_4 and GA_7 in a ~60:40 ratio, the molecular weight (MW) for GA_7 is shown. '●' represents stock solutions that were solubilised by heating to 45°C for 1 hour.

Compound	MW (g mol^{-1})	Supplier	Product No.	Solvent	Solubility (● = heat)
AC94377	306.74	Syngenta Ltd.	-	DMSO	> 100 mM
AS_6	380.50	Syngenta Ltd.	-	DMSO	> 100 mM
cis-S(+)-ABA	264.30	Duchefa Biochemie	A0941	1N KOH	> 100 mM
Fluridone	329.30	Duchefa Biochemie	F0919	DMSO	~ 100 mM ●
Flurprimidol	312.29	Fluka Analytical	32523	DMSO	> 100 mM
GA_3	346.40	Duchefa Biochemie	G0907	DMSO	> 100 mM
*GA_{4+7}	330.38	Duchefa Biochemie	G0938	DMSO	> 100 mM
KAR_1	150.13	Syngenta Ltd.	-	DMSO	> 100 mM
Mesotrione	339.32	Syngenta Ltd.	-	DMSO	> 100 mM
Paclobutrazol	293.80	Duchefa Biochemie	P0922	DMSO	> 100 mM
RH102	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH104	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH109	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH112	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH119	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH129	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH129	-	Syngenta Ltd.	-	DMSO	> 100 mM ●
RH130	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH134	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH139	-	Syngenta Ltd.	-	DMSO	> 100 mM ●
RH147	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH150	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH153	-	Syngenta Ltd.	-	DMSO	> 100 mM ●
RH156	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH159	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH160	-	Syngenta Ltd.	-	DMSO	> 100 mM ●
RH179	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH180	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH182	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH182	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH183	-	Syngenta Ltd.	-	DMSO	> 100 mM ●
RH186	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH192	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH194	-	Syngenta Ltd.	-	DMSO	> 50 mM ●
RH196	-	Syngenta Ltd.	-	DMSO	> 100 mM
RH198	-	Syngenta Ltd.	-	DMSO	> 100 mM
Smoke-water	N/A	Grayson Australia	Regen2000	N/A	N/A
TMB	126.15	ChemSpace US Inc.	39735679	N/A	N/A

8.4.2 Incubation conditions

Compound screens were conducted using the standard germination conditions described in 8.2.1. To control for temperature and light variability within incubators,

plate position within stacks was pseudorandomised at each germination assessment. All compound testing was conducted under constant lighting conditions at different temperatures (Chapter 3). Compounds were exogenously applied at 5 concentrations (0.1, 1, 10, 50 and 100 µM) prepared in ultrapure water as described in 8.2.1. As a control for the effect of DMSO, the solvent used to solubilise many compounds (Table 8.2), treatments containing 0.1% (v/v) DMSO or a dilution series of DMSO representative of the concentrations present in the treatments (0.0001, 0.001, 0.01, 0.05 & 0.1% (v/v) DMSO) in ultrapure water were included as controls.

8.4.3 Statistical analysis of screening data

In order to distinguish compounds with a statistically significant germination stimulant effect on dormant *A. myosuroides* LH170-D seeds, the area under the dose-response (AUC) of compounds was used. The maximum germination achieved after 49 days incubation was plotted against the log concentration of the compound for each replicate separately and the area under a spline was calculated. This AUC of the dose-response was considered an appropriate measure of compound performance since many compounds had complex dose-response profiles (for example promotion at low and inhibition at high concentrations in the case of KAR_1, Chapter 3) making selection of a single concentration less representative of the compound across doses. A one-way analysis of variance (ANOVA) with a post-hoc Dunnett's Multiple Comparisons Test was used to infer the significance of differences between the DMSO dose-response (8.4.2) and the compound dose-responses for the three replicates. Compounds were considered to have a significant germination stimulating effect when $P<0.001$.

8.5 Preparation and sequencing of RNAseq samples

8.5.1 Preparation of A. myosuroides seed samples for RNA extraction

Forty milligrams (dry weight, ~ 30 diaspores) of dormant and after-ripened *A. myosuroides* LH170-D and LH170-AR_{120} (Table 2.2) were imbibed for 90 or 180 hours at either 8 or 16°C in environmental test chambers as described in 8.2.1. Monitoring incubator temperatures using a temperature data-logger (Testo™ 174H, Testo Ltd., Hampshire, UK) showed the incubator maintained stable temperatures (± 1°C) over the course of sample preparation (data not shown). After incubation, diaspores were surface dried using filter papers, flash frozen in liquid nitrogen and stored at -80°C until RNA extraction.

8.5.2 RNA extraction from A. myosuroides seeds

RNA extraction from *A. myosuroides* diaspores was challenging, possibly due to high starch and phenolic content. Attempts to extract RNA using commercial kits such as RNeasy® Plant Mini Kit (Qiagen NV, Hilden, Germany) or the Ambion® RNAaqueous™ Total RNA Isolation Kit (Thermo Fischer Scientific, Massachusetts, USA) following the manufacturers' instructions resulted in a low RNA yield. Instead an RNA extraction method was optimised following Graeber *et al.*, (2011) with many modifications. Samples were homogenised in liquid nitrogen with a frozen extraction buffer (CTAB buffer) containing 2% (w/v) hexadecyltrimethyl-ammonium bromide (CTAB), 2% (w/v) polyvinylpyrrolidone (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, and 2% (v/v) ß-mercaptoethanol. For dry seeds 1 mL of CTAB buffer was used, for imbibed seeds 3 mL was used except for dormant seeds imbibed at 16°C where 5 mL of CTAB buffer was required. The frozen homogenate was then thawed with constant mixing and 1 mL aliquots of homogenate were either transferred

to a 10 minute incubation in a water bath at 65°C or stored at -80°C. The homogenate the centrifuged to remove cell debris (13,000 rpm, 10 minutes, 4°C) and subjected to phase extractions with 900 µL of a mixture of chloroform:isoamylalcohol (24:1) where the aqueous (upper) phase was retained and the lower phase (chloroform) was discarded. This was repeated three times, with vigorous agitation and centrifugation (13,000 rpm, 10 minutes, 20°C) before each phase extraction. The aqueous phase was then subjected to an overnight (4°C) lithium chloride precipitation following Walker & Lorsch (2013). The resultant pellet was suspended in a SDS–Tris-HCl–EDTA (SSTE) buffer containing 1 M NaCl, 0.5% (w/v) sodium dodecyl sulfate (SDS), 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA with vigorous agitation. The samples were again subjected to three chloroform:isoamylalcohol phase extractions as previously described and subjected to an ethanol precipitation for 2 hours at -80°C following Walker & Lorsch (2013).

The resultant pellet was resuspended in 100 µL of Invitrogen™ Nuclease Free Water (Thermo Fischer Scientific, Massachusetts, USA) and subjected to an in-solution DNAase treatment following the manufacturer's instructions (RNase-Free DNase Set, Qiagen NV, Hilden, Germany). The samples were further purified using an RNeasy™ Mini Kit (Qiagen NV, Hilden, Germany) following the manufacturer's instructions with the exception that 1% ß-mercaptoethanol was added to the 'RLT buffer'. For samples where RNA yield was low (dormant diaspores imbibed at 16°C where the 5 mL extraction buffer volume was used), 200 µL of sample (resulting from two phase extractions) was loaded in the column, for all other samples 100 µL was loaded. The eluate was then subjected to a further lithium chloride precipitation following Walker & Lorsch (2013) and resuspended in 30 µL Nuclease Free Water.

8.5.3 Assessment of RNA quantity and quality

RNA quantity was measured using a Nanoquant™ plate read by a Spark™ Multimode Plate Reader (Tecan Group Ltd., Männedorf, Switzerland). The purity of RNA samples was assessed by the ratio of absorbance at 260:280 nm and 260:230 nm. For the 260:280 absorbance ratio, an indicator of protein contamination, ratios > 2.0 were considered pure enough for sequencing. For the 260:230 absorbance ratio, an indicator of polysaccharide and EDTA contamination, ratios > 2.4 were considered pure enough for sequencing.

RNA integrity was assessed by chip-based capillary electrophoresis using an Agilent Bioanalyzer™ 2100 with RNA 6000 Nano Chips following the manufacturer's instructions (Agilent Technologies Inc., California, USA). Electrophoretic traces were analysed using the accompanying 2100 Expert software (vB.02.08.SI648, Agilent Technologies Inc., California, USA) to derive RNA Integrity Numbers (RIN). Samples with a RIN > 7.0 were considered for sequencing, however most samples had a RIN of between 8.0 and 9.0.

8.5.4 RNA shipping

RNA extraction was conducted in the UK (Royal Holloway University) however cDNA library preparation and sequencing was conducted in the USA (Syngenta Crop Protection LLP, North Carolina, USA). Samples were shipped using RNAstable™ tubes (Biomatrica, Inc., California, USA) following the manufacturer's instructions. Three µg of total RNA in 30 µL of nuclease free water was loaded into RNAstable™ tubes that contain a matrix that stabilises RNA. Samples were then desiccated using a vacuum concentrator at 7.2 mbar at 20°C for 1.5 hours (CHRIST AVC 2-25 CD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Samples were

sealed in moisture-proof bags with silica gel and shipped at ambient temperatures to the USA.

8.5.5 Preparation of cDNA libraries

Sequencing libraries were prepared by collaborators at Syngenta Crop Protection LLP. (Research Triangle Park, North Carolina, USA). Messenger RNA was enriched by polyA isolation using an NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs (NEB), Massachusetts, USA). Libraries were prepared using NEBNext® Ultra™ II Directional RNA Library Prep Kit (NEB) with sample purification beads and in-house indexes.

8.5.6 High-throughput sequencing using Illumina™ HiSeq® technology

Illumina™ sequencing was performed by the service provider GENEWIZ (New Jersey, USA). A total of 50 libraries (5 replicates per treatment) were sequenced at 8 libraries per lane using an Illumina HiSeq X platform (Illumina Inc., California, USA) generating ~40 million 150 bp paired end reads (~80 million total) per sample.

8.6 Bioinformatics

8.6.1 Reference-free transcriptome assembly

De novo transcriptome assembly was performed by collaborators at Syngenta Crop Protection LLP. (Research Triangle Park, North Carolina, USA) using the rBPA pipeline (v.2.1.0) from the National Centre for Genome Resources (NCGR, Santa Fe, New Mexico). To reduce the amount of input sequence while still capturing the complexity of all conditions and replicates, only R1 of each read pair was used for the assembly.

Unitigs were assembled separately for each treatment in ABySS using the read pairing information (v.2.1.0) (Simpson *et al.*, 2009). Unitigs were then collapsed into a single sequence set using CD-HIT-EST (Fu *et al.*, 2012). Collapsed unitigs were assembled using MIRA (v.4.0) (Chevreux *et al.*, 2004). Resultant contigs and untigs were scaffolded in ABySS. Transcriptome completeness was assessed using the Benchmarking Universal Single-Copy Orthologs (BUSCO) (Simao *et al.*, 2015).

8.6.2 Functional annotation of the assembly

Functional annotation was performed using Blast2GO (v.5.2.5) (Gotz *et al.*, 2008) using the translated assembly. The top 20 BLAST hits for each scaffold (E-value < $1x10^{-3}$) were retrieved from UniProt/Swiss-Prot (v. 5) (The UniProt Consortium, 2019) using BLASTp (version 2.1.7+, word size:6; HSP Length Cutoff:33). The 'Cloud InterProScan' (IPS) tool was run to identify structural domains and motifs. Gene ontology (GO) terms (Ashburner *et al.*, 2011) were mapped from BLAST and IPS results using the Gene Ontology Database (GOA version 2018.02) (Gene Ontology Consortium, 2004) and annotated using default parameters. Scaffolds with a non-land plant top BLAST hit were removed. For a selection of genes that were discussed in the manuscript, the BLAST top-hit annotation was confirmed using an alignment and tree building approach with manually curated sequences from the Swiss-Prot database. Multiple sequence alignments were implemented in Geneious® (v8.0.5, Biomatters Ltd., Auckland, New Zealand) after manual trimming using the inbuilt MUSCLE alignment executable (Edgar, 2004) with default parameters. Trees were built with a Jukes-Cantor distance model using the Neighbor-Joining method in Geneious® with 100 bootstrap replicates.

8.6.3 Differential expression analysis

Differential expression analysis was conducted by collaborators at Syngenta Crop Protection LLP. (Research Triangle Park, North Carolina, USA). Reads were mapped to the assembly using the Burrows-Wheeler Aligner (BWA) (v.0.7.17) (Li & Durbin, 2010). Counts were generated for each transcript contig using HTseq (v.0.11.0) (Anders *et al.*, 2015) in default mode. Normalisation factors were calculated using EDAesq (v. 3.6) (Risso *et al.*, 2011) and differential expression analysis was conducted using the edgeR package (v. 3.22.0) (Robinson *et al.*, 2009) and false discovery rate (FDR) was calculated following Benjamini & Hochberg (1995). Transcripts were considered differentially expressed if the absolute value of the log2 ratio of the mean of contrasted samples was >1 and FDR was < 0.001.

8.6.4 Clustering and gene ontology enrichment analysis

Clustering analysis was performed using MORPHEUS (Morpheus, 2019) for scaffolds where at least one replicate had an FPKM >5 and normalised by scaffold to generate z-scores. A K-means algorithm was used to generate 8 clusters using a correlation matrix of one minus Spearman's Rank for 1000 iterations. GO enrichment analysis using Fischer's Exact Test was conducted in Blast2GO (Gotz *et al.*, 2008) to identify overrepresented 'Biological Process' GO terms between individual clusters with a subset of the assembly that had a minimum FPKM of 5 as the reference set. Enzyme commission (EC) annotations were used to generate lists of genes associated with a specific enzymatic activity (IUBMB, 1992). The mean expression across replicates for each treatment for selected enzyme classes was plotted in another heatmap as z-scores. For accessible visualisation of heatmaps a colourblind-friendly colour palette was generated using ColorBrewer 2.0 (Brewer, 2019).

8.6.5 Analysis of publically available microarray data

Probe sequences for the Barley1 22k GeneChip probe array (Close *et al.*, 2004) were acquired from the PLEXdb database (Wise *et al.*, 2007). A custom BLAST database of probe sequences was created using Geneious® (v8.0.5, Biomatters Ltd., Auckland, New Zealand). A BLASTx executable implemented in Geneious® was used to identify probes containing the conserved XTH active site motif 'DEIDFEFLG' with a maximum of two mismatches. This search yielded 15 probes that corresponded to barley XTHs. The identity of these probe sequences was confirmed by BLAST against the Swissprot database (Bairoch & Apweiler, 2000). Probe sequences were manually trimmed and aligned in Geneious® using the inbuilt MUSCLE alignment executable (Edgar, 2004) using the default parameters. Trees were built with a Jukes-Cantor distance model using the Neighbor-Joining method in Geneious® with 100 bootstrap replicates. Expression data from Barrero *et al.* (2009) for the identified probes was used to create a heatmap from the z-score of the means of each treatment.

8.7 Quantifying *A. fatua* coleorhiza biomechanical properties

8.7.1 Tissue preparation for puncture force experiments

Avena fatua LH840-D and LH840-AR_{112} diaspores were dehulled (lemma and palea removed) and the caryopses were imbibed embryo side up following 8.2.1 and incubated at 20°C under constant light for 24 or 48 hours. Coleorhizae were isolated in batches of 20 as this was the number of puncture force measurements that could be made during a period of an hour. Coleorhizae were isolated by removing the testa and pericarp surrounding the embryo, cutting the embryo transversely at the apex of the epiblast to the depth of the scutellum and removal of the radicle using a hooked

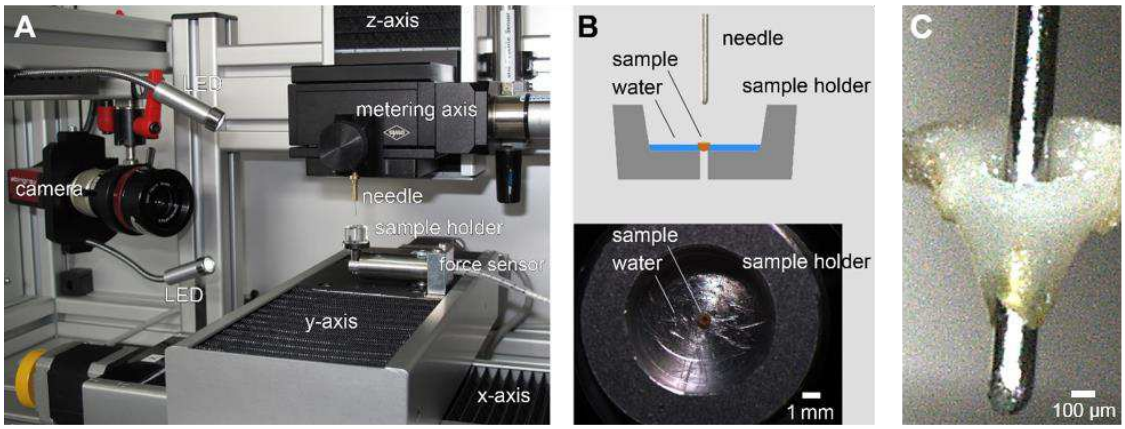


Figure 8.1. Puncture force device used to measure coleorhiza biomechanical properties. (a) The custom-built puncture force device used to measure coleorhiza biomechanical properties consisting of a force and displacement (metering axis) sensor, a camera, LED lights and an *x-y* positioning stage. A measuring tip (needle) with chosen tip diameters/geometry is driven into the sample while force and displacement are recorded. (b) Example of a sample holder used to measure tobacco micropylar endosperm weakening. (c) Image produced by the in-built camera of an *A. fatua* coleorhiza immediately after a puncture force measurement, showing the geometry of the needle used. Parts (a) and (b) are reproduced from Steinbrecher *et al.* (2017) with permission.

needle. Any potentially damaged coleorhizae were discarded. Isolated coleorhizae were transferred to a germination Petri-dish (8.2.1) with grid squares allowing individual coleorhizae to be identified by a unique number. Images of coleorhizae were taken following 8.3.1 and measurements of the length of the coleorhizae were performed using ImageJ1 v1.50i (Schneider *et al.*, 2012).

8.7.2 Puncture force apparatus

Puncture force measurements were conducted using a custom-made testing machine described in Hourston *et al.*, (2017). Samples were held in place using 3D-printed sample holders (Ultimaker 2+, Ultimaker BV, Utrecht, Netherlands) holding 0.5 mm thick aluminum discs with either a 0.3 or 0.4 mm diameter perforation. For dormant coleorhizae, the 0.3 mm holder was used and for after-ripened samples the 0.4 mm holder was used since after-ripened coleorhizae increased in diameter during imbibition. Sample holders were placed on a force sensor (load cell, F_{max} = 1 mN) and

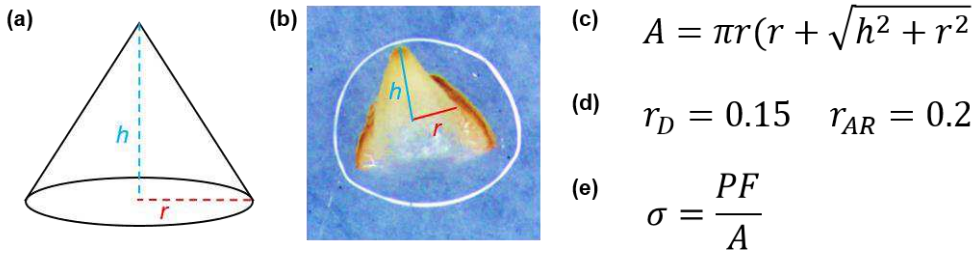


Figure 8.2. Calculation of stress from puncture force data. (a) A cone where *h* represents height from the base and *r* represents the radius of the base. (b) Coleorhiza were approximated to cones by taking the point at which the coleorhiza was the same width as the sample holder used and the length from the center of that line to the tip of the coleorhiza (*h*). (c) Formula for the surface area of a cone. (d) With the two sample holders, the radius for dormant coleorhizae (r_D) was 0.15 mm and for after-ripened coleorhizae (r_{AR}) was 0.2 mm. (e) formula for the calculation of stress (σ) from puncture force (*PF*) and estimated surface area (*A*).

positioned using an *x*-*y* stage (LTM80, OWISw GmbH, Germany). A needle with a 0.2 mm diameter and a hemispheric tip was driven through the coleorhizae at a rate of 0.7 mm min^{-1} in the *z*-axis powered by a servo-motor (Fig. 8.1a, 'metering axis') (HVM60, OWISw GmbH, Germany) whilst the resultant forces are measured by the load cell. In-house software recorded the force displacement data and logged the maximum force sustained by each sample until its rupture as the puncture force.

8.7.3 Analysis of puncture force data

A different number of puncture force measurements were made for each treatment since a number of measurements were discarded because the coleorhizae were pushed through the sample holders. The following numbers of coleorhizae were included in the analysis: Dormant 24 hours imbibed, n=23; dormant 48 hours imbibed, n=35; after-ripened 24 hours imbibed, n=33; after-ripened 48 hours imbibed, n=26. A Kolmogorov-Smirnov test demonstrated that the distribution of puncture force values was not significantly different from a normal distribution in any treatment ($p > 0.05$). An ordinary two-way ANOVA was employed to infer significant interactions between the treatments and Tukey's test was used to infer significant differences between pairs of treatments.

In order to check that the differences observed between treatments were not the result of the difference sized sample holders (8.7.2) used for dormant and after-ripened samples, the stress applied across the tissues was estimated. Coleorhizae surface area were approximated to a cone geometry based on the measurements taken in ImageJ (8.7.1), where the radius of the cone was half the diameter of the sample holder perforation (Fig. 8.2). A one-way ANOVA identified significant differences across the treatments ($F(3, 112) = 383.8$, $P<0.0001$) indicating that differences in puncture force were not the result of differences in sample holders.

8.8 Flow cytometry of *A. fatua* embryonic organs

8.8.1 Sample preparation and isolation of nuclei

Twenty LH840-AR_{112} caryopses were imbibed at 20°C under constant light for 3, 24 and 48 hours following 8.2.1 at 20°C under constant light and dissected into coleorhiza and plumule tissues on ice. Nuclei were extracted in 100 µL of Cystain UV Precise P extraction buffer (Sysmec Partec GmbH, Görlitz, Germany) by maceration with a razor blade. Samples were stained with 1 mL of Cystain UV Precise P DAPI fluorescent buffer (Sysmec Partec GmbH, Görlitz, Germany) and filtered through a 30 µm filter.

8.8.2 Analysis of nuclear DNA content

Greater than 9,000 nuclei were analysed for each treatment using a Partec PAS Flow Cytometer (Sysmex Partec GmbH, Germany). Data analysis and noise reduction was performed using Flowing Software v2.5.1 (Terho, 2017).

8.9 Measurement of xyloglucan endotransglycosylase (XET) activity

8.9.1 Preparation of native total protein samples

Populations of 50 *A. fatua* LH840-D and LH840-AR_{112} caryopses were imbibed for 3, 24, 48, 72 and 96 hours following 8.2.1 in triplicate at 20ºC under constant light. Caryopses were dissected into coleorhiza, radicle and shoot (plumule + coleoptile) tissues on ice, weighed using a fine balance, flash frozen in liquid nitrogen and stored at -80ºC. Native total protein samples were prepared following Fry (1997) with modifications. An extraction buffer was prepared containing 200 mM succinate (Na^+) adjusted to pH 5.5 with 10 M NaOH and 2.5 mg mL^{-1} bovine serum albumin (BSA, Albumin Fraction V, 411176809, Carl Roth GmbH & Co KG, Karlsruhe, Germany) then sterilised using 0.22 µm pore size nylon syringe filter. BSA was added to inhibit the property of xyloglucan endotransglycosalase/hydrolases (XTHs) to adhere to plastic and glassware (Sharples *et al.*, 2017). Extraction buffer was added to frozen samples in a ratio of 5:1 (buffer:sample, v/w) and homogenised on ice using a micropestle. Cell debris was removed by centrifugation (13,000 rpm, 4ºC, 10 minutes) and the supernatant stored at -80ºC.

8.9.2 Quantification of total protein using the Bradford method

Total protein was quantified following Bradford (1976) with modifications. All Bradford reactions were performed with 100 µL of Bradford Reagent (Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Inc., California, USA) and 1 µL of sample or a variable amount of BSA for the generation of standard curves, *Q.S.* to a 500 µL final reaction volume. After a 5 minute incubation at room temperature, absorbance at 595 nm was measured in 96-well microtiter plates (Costar® 3599, Corning Inc., New York, USA) using a Spark™ Multimode Plate Reader (Tecan Group Ltd., Männedorf,

Switzerland). A linear regression of the standard curve for BSA was used to interpolate protein concentration in the samples. If BSA was added to the sample during extraction, this known amount was subtracted from the calculated protein concentration.

8.9.3 Quantification and visualization of XET activity

An XET activity assay was developed based on a method published by Fry (1997). Samples are loaded onto a matrix composed a filter paper sheet (Whatman® No. 1, GE Healthcare Life Sciences Inc., Illinois, USA) impregnated with tamarind seed xyloglucan (2.5 g m^{-2}) and a sulphordamine labelled xyloglucan oligosaccharide (XLLG-SR, 1 µmol m^{-2}). This matrix, hereafter XET substrate matrix, was purchased from Prof. Steven Fry of the Edinburgh Cell Wall Group (EDIPOS Ltd., Institute of Molecular Plant Sciences, The University of Edinburgh, UK). The XET substrate matrix was cut into 6 mm diameter discs using a paper hole punch and placed in the wells of a ultraviolet (UV) transparent 96-well microtiter plate (Costar® 3635, Corning Inc., New York, USA).

Samples (5 µL) were loaded directly onto the XET substrate matrix in the wells with the plate on ice. Plates were the closed with a damp filter paper covering all the wells between the lid and microtiter plate and sealed in tightly fitting polyethylene grip-seal sample bags to prevent evaporation of the samples during incubation. For the assay shown in Chapter 5, 20 ug of total protein was loaded into the assay. This assembly was incubated in the dark for 3 hours for the assay in Chapter 5, or different periods of time for data shown in the appendix. As negative controls XET substrate matrix with only extraction buffer loaded or Whatman® No. 1 with no XLLG-SR loaded were included in triplicate. After incubation, the XET substrate matrix was washed three times in the microtiter plates with ethanol:formic acid:water (1:1:1) and then three times

with ultrapure water to remove un-bound fluorescent substrate. The assay was then dried in a drying oven (DRY-Line® 53, VWR International Ltd.) at 70°C for 20 minutes.

XET activity was quantified with a Spark™ Multimode Plate Reader (Tecan Group Ltd., Männedorf, Switzerland) using a custom method programmed in the SparkControl™ Software package. Epiexcitation of the wells at 570 nm with an emission filter at 615 nm was measured for 24 evenly-spaced positions across the sample well and averaged to account for localised differences in fluorescence across the XET substrate matrix. Dynamic range and sensitivity were improved through gain and sensor *Z*-position optimisation. Assay plates were measured before and after incubation and the proportion of XLLG-SR that had been transglycosylated to the xyloglucan substrate was calculated after blank measurements were subtracted. For visualisation of XET activity, assay plates were imaged in a UV transilluminator with an orange filter (U:Genius® 3, Syngene International Ltd., Bangalore, India) using the inbuilt 'saturation detect' function to avoid oversaturation of the images. Example visualisation and quantification plots are shown in the appendix (Fig. 9.14).

8.9.4 Quantifying XET activity over a range of pHs

A pH series of buffers was generated by adjusting the pH of a 400 mM solution of succinic acid with 10 M NaOH and periodically taking aliquots at defined pHs (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5). Buffers were adjusted to 200 mM with ultrapure water and BSA (2.5 mg mL^{-1}) was added. Populations of 20 *A. fatua* LH840-D and LH840-AR_{112} caryopses were imbibed for 48 hours following 8.2.1 in triplicate at 20°C under constant light. Native protein extraction of whole embryos was performed using the pH series buffers following 8.9.1. After extraction, the pH of the lysate was checked using Universal Indicator Paper. Total protein was quantified using the Bradford method following 8.9.2 and XET activity was quantified following 8.9.3.

8.10 Separation of XET isozymes using isoelectric focusing

8.10.1 Preparation of native total protein samples for isoelectric focusing

Populations of 300 *A. fatua* LH840-D and LH840-AR_{112} caryopses were imbibed for 48 hours following 8.2.1 in triplicate at 20°C under constant light. Caryopses were dissected into coleorhizae and radicles following 8.9.1 with the exception that the extraction buffer contained 350 mM succinate (Na^+) without BSA. The supernatant extracted after pelleting cell debris was filtered through a 0.22 µm pore size cellulose acetate spin filter (Part No. 5185-5990, Agilent Technologies Inc., California, USA) by centrifugation (13,000 rpm, 4°C, 5 minutes). The filtrate was then desalted using a buffer exchange column (Bio-Spin 6 column, Bio-Rad Laboratories, Inc., California, USA) against ultrapure water following the manufacturer's recommendations. Protein concentration of the purified total protein samples was quantified using the Bradford method following 8.9.2.

8.10.2 Separation of native total protein by isoelectric focusing

A rehydration solution was prepared containing 5% (v/v) glycerol and 0.5% of an ampholyte-containing buffer (Immobiline® IPG buffer pH 6-11, GE Healthcare Life Sciences Inc., Illinois, USA). Native protein samples were loaded relative to their XET activities estimated from the XET activity assay described in 8.9.3 (AR(coleorhiza 225 ug, radicle 120 ug), ABA(coleorhiza 300 ug, radicle 120 ug), D(coleorhiza 720 ug, radicle 520 ug) and *Q.S.* to 250 µL using ultrapure water. The rehydration solution was applied to dehydrated isoelectric focusing (IEF) gel strips (Immobiline® DryStrip® pH 6-11, 13 cm, GE Healthcare Life Sciences Inc., Illinois, USA), covered with mineral oil to prevent evaporation and left to rehydrate overnight at 4°C. Rehydrated gel strips were washed in ultrapure water and subjected to an IEF program following the

manufacturer's recommendations (8000 V, 16 kVh, 50 µA current limit and 200 mW power at 20°C) in a flatbed IEF device (3100 OFFGEL® Fractionator, Agilent Technologies Inc., California, USA).

8.10.3 Visualisation of xyloglucan endotransglycosylase isozymes

Visualisation of XET isozymes was performed following Iannetta & Fry (1999) with modifications. After separation, gel strips were washed in ultrapure water to remove mineral oil residues. Gel strips were then equilibrated in a buffer containing 50 mM succinate (Na+, pH 5.5), 10 mM $CaCl_2$ and 1 mM dithiothreitol (DTT) for 10 minutes at room temperature with gentle agitation. Equilibrated gel strips were transferred to a glass plate gel side up and a strip of XET substrate matrix (8.9.3) with the same dimensions as the gel strip was placed on top followed by a sheet of Parafilm™ (Bemis™ M, Thermo Fisher Scientific Inc., Massachusetts, USA) followed by another glass plate. This 'sandwich' assembly was sealed in a polyethylene bag containing a moist filter paper to prevent evaporation and incubated for 24 hours at 20°C in the dark with a 2 kg weight compressing the assembly. After incubation, the XET substrate matrix was removed and washed in an excess ethanol:formic acid: water (1:1:1) for two hours with gentle agitation to remove unbound XLLG-SR. Florescent bands, showing XET activity at specific isoelectric points, were visualised using a UV transilluminator with an orange filter (U:Genius® 3, Syngene International Ltd., Bangalore, India). Images were processed using GIMP v2.8.16 (GIMP, 2019) to improve the contrast. A pre-processed zymogram is shown in the appendix (Fig. 9.15).

8.11 Quantification of global DNA methylation

8.11.1 Purification of DNA from A. myosuroides diaspores

Forty milligrams (~30 diaspores) of the *A. myosuroides* batches LH192V-D, LH192V-AR_{196}, LH192NV-D and LH192NV-AR_{196} were imbibed for 90 hours following 8.2.1 at 20°C under constant light. DNA extraction was performed by Dr. M. Pérez (Royal Holloway University) using a DNeasy™ Plant Mini Kit (Qiagen NV, Hilden, Germany) following the manufacturer's recommendations with modifications (5% (v/v) β-mercaptoethanol in lysis buffer). DNA yield and quality were quantified using a NanoQuant Plate™ (Tecan, Männedorf, Switzerland).

8.11.2 Enzyme-linked immunosorbent assay for the quantification of DNA methylation

Quantification of 5-methylcytosine (5-mC) was performed by Dr. M. Pérez (Royal Holloway University) using a fluorometric enzyme-linked immunosorbent assay (ELISA) kit MethylFlash™ Methylated DNA Quantification Kit, Epigentek, New York, USA) following the manufacturer's recommendations for 10 ng of genomic DNA. Fluorescence proportional to 5-methylcytosine (5-mC) concentration was measured for 3 biological and two technical replicates using a florescence microplate reader (Tecan, Männedorf, Switzerland) with excitation filter at 530 nm and an emission filter at 590 nm. %5-mC was calculated from a standard curve of synthetic 5-mC.

8.12 References

Anders S, Pyl PT, Huber W. **2015**. HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**: 166–169.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, *et al.* 2011. Gene Ontology: A tool for the unification of biology. *Nature Genetics* **25**: 25–29.

Bairoch A, Apweiler R. **2000**. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucleic Acids Research* **28**: 45–48.

Barrero JM, Talbot MJ, White RG, Jacobsen J V, Gubler F. **2009**. Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant physiology* **150**: 1006–1021.

Baskin C, Baskin J. **2014**. *Seeds: ecology, biogeography, and Evolution of Dormancy and Germination*. Academic Press: San Diego.

Benjamini Y, Hochberg Y. **1995**. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**: 289–300.

Bradford MM. **1976**. A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **254**: 248–254.

Brewer C. **2019**. ColorBrewer 2.0. URL: http://colorbrewer2.org (last acessed 09.10.2019).

Chevreux B, Pfisterer T, Drescher B, Driesel AJ, Müller WEG, Wetter T, Suhai S. **2004**. Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. *Genome Research* **14**: 1147–1159.

Close TJ, Wanamaker SI, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing

RA, Muehlbauer GJ, Kleinhofs A, Wise RP. **2004**. A new resource for cereal genomics : 22K Barley GeneChip comes of age. *Bioinformatics* **134**: 960–968.

Edgar RC. **2004**. MUSCLE : multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792–1797.

Fry SC. **1997**. Novel 'dot-blot" assays for glycosyltransferases and glycosylhydrolases: optimization for xyloglucan endotransglycosylase (XET) activity. *The Plant Journal* **11**: 1141–1150.

Fu L, Niu B, Zhu Z, Wu S, Li W. **2012**. Sequence analysis CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* **28**: 3150–3152.

Gene Ontology Consortium. **2004**. The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Research* **32**: 258D–261.

Gottschalk PG, Dunn JR. **2005**. The five-parameter logistic: A characterization and comparison with the four-parameter logistic. *Analytical Biochemistry* **343**: 54–65.

Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Garcı JM, Nagaraj SH, Valencia D, Geno C De. **2008**. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research* **36**: 3420–3435.

Graeber K, Linkies A, Wood AT a, Leubner-Metzger G. **2011**. A guideline to family-wide comparative state-of-the-art quantitative RT-PCR analysis exemplified with a brassicaceae cross-species seed germination case study. *The Plant cell* **23**: 2045–2063.

Hourston JE, Ignatz M, Reith M, Leubner-Metzger G, Steinbrecher T. **2017**. Biomechanical properties of wheat grains: The implications on milling. *Journal of the Royal Society Interface* **14**.

Iannetta PPM, Fry SC. **1999**. Visualization of the activity of xyloglucan endotransglycosylase (XET) isoenzymes after gel electrophoresis. *Phytochemical*

Analysis **10**: 238–240.

IUBMB. **1992**. Classification and Nomenclature of Enzymes. In: Enzyme Nomenclature. San Diego: Academic Press, 5–22.

Kim S, Chen J, Cheng T, Gindulyte A, He J, He S, Li Q, Shoemaker BA, Thiessen PA, Yu B, *et al.* 2019. PubChem 2019 update: Improved access to chemical data. *Nucleic Acids Research* **47**: D1102–D1109.

Li H, Durbin R. **2010**. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**: 589–595.

Matsushima R, Masahiko M, Miyako K, Kondo H, Kaoko F, Kawagoe Y, Sakamoto W. **2014**. Amyloplast-localized SUBSTANDARD STARCH GRAIN4 protein influences the size of starch grains in rice endosperm. *Plant physiology* **164**: 623–636.

Morpheus. **2019**. MORPHEUS versatile matrix visualization and analysis software. URL: https://software.broadinstitute.org/morpheus/ (Last accessed 18.10.2019).

Risso D, Schwartz K, Sherlock G, Dudoit S. **2011**. GC-content normalization for RNA-Seq data. *BMC Bioinformatics* **12**: 1–17.

Robinson MD, McCarthy DJ, Smyth GK. **2009**. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.

Ruzin SE. **1999**. *Plant Microtechnique and Microscopy*. Oxford University Press: Oxford.

Schneider CA, Rasband WS, Eliceiri KW. **2012**. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**: 671–675.

Sharples SC, Nguyen-Phan TC, Fry SC. **2017**. Xyloglucan endotransglucosylase/hydrolases (XTHs) are inactivated by binding to glass and cellulosic surfaces, and released in active form by a heat-stable polymer from

cauliflower florets. *Journal of Plant Physiology* **218**: 135–143.

Simao FA, Waterhouse RM, Ioannidis P, Kriventseva E V, Zdobnov EM. **2015**. Genome analysis BUSCO : assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* **31**: 3210–3212.

The Gimp Team. 2019 GIMP v2.8.16. URL: www.gimp.org, (last accessed 09.10.2019)

Terho P. **2017**. Flowing Software v2.5.1, URL: www.flowingsoftware.btk.fi. (last accessed 09.10.2019)

The UniProt Consortium. **2019**. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Research* **47**: D506–D515.

Walker SE, Lorsch J. **2013**. RNA purification - Precipitation methods. In: Methods in Enzymology. Elsevier: Amsterdam.

Wise RP, Caldo RA, Hong L, Shen L, Cannon E, Dickerson JA. **2007**. BarleyBase/PLEXdb BT - Plant Bioinformatics: Methods and Protocols. In: Edwards D, ed. Totowa, NJ: Humana Press: Totowa, 347–363.

Yeung ECT, Stasolla C, Sumner MJ, Huang BQ. **2015**. The Glycol Methacrylate Embedding Resins—Technovit 7100 and 8100. In: Plant Microtechniques and Protocols. Springer: Cham.

9. Appendix

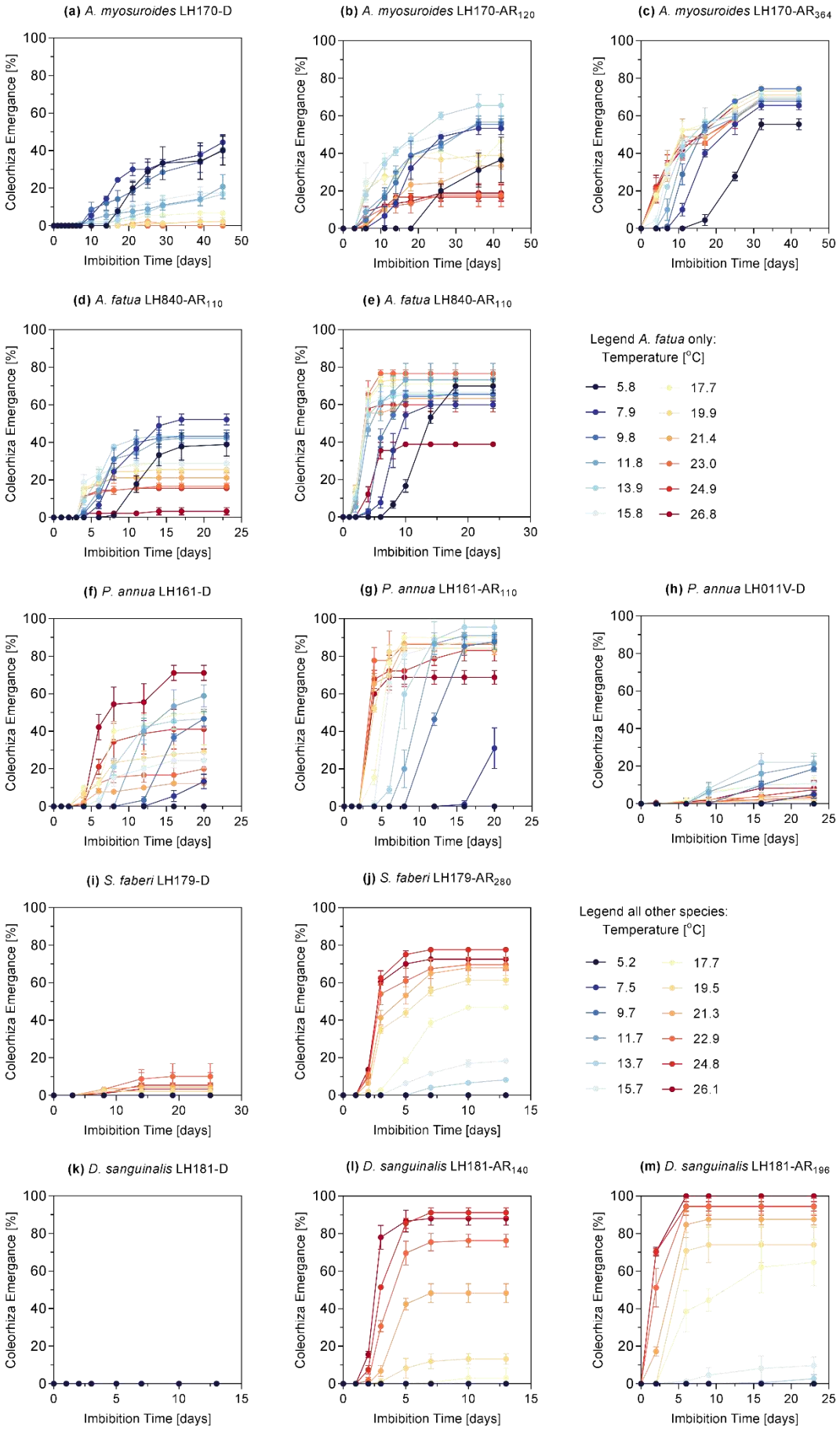


Fig. 9.1. Germination curves for the response of dormant and after-ripened seeds to a gradient of temperatures. Data show percentage cumulative coleorhiza emergence. Error bars show standard error of the mean for triplicates of >30 diaspores.

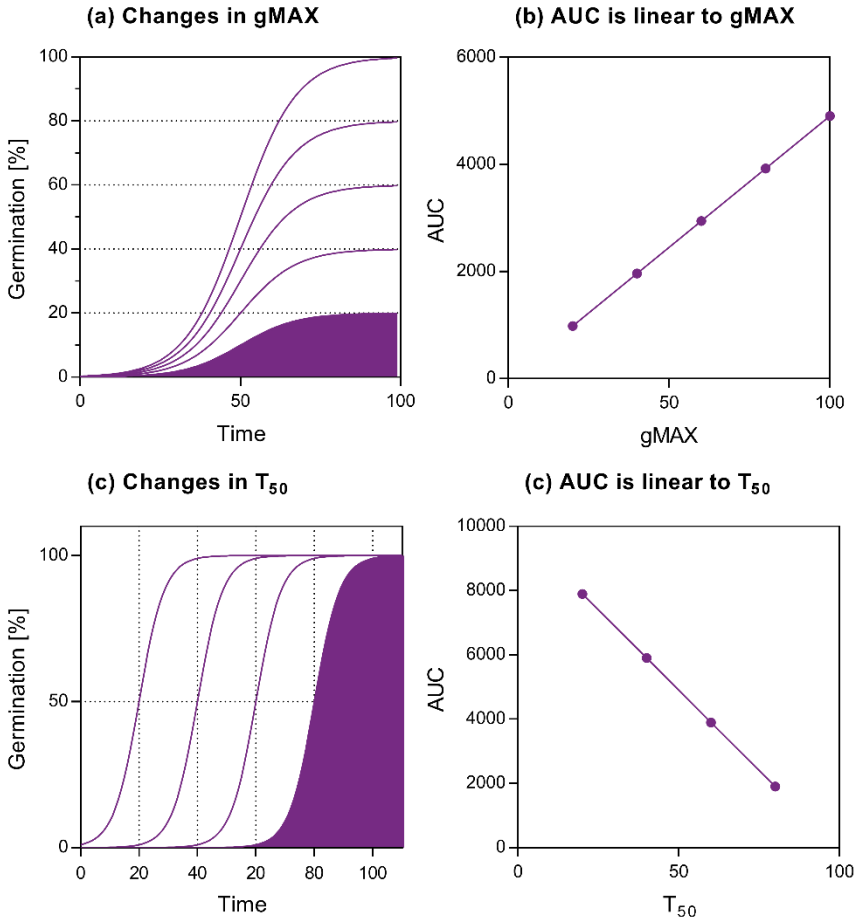


Fig. 9.2. The relationship between area under the curve (AUC) and other measures of germination rate. (a) Example germination curves plotted as hill functions with equal hill slopes and T_{50} and differing maxima (b) values calculated for the area under the hill functions in (a). (c) Example germination curves plotted as hill functions with equal hill slopes maxima but differing T_{50} (d) values calculated for the area under the hill functions in (c).

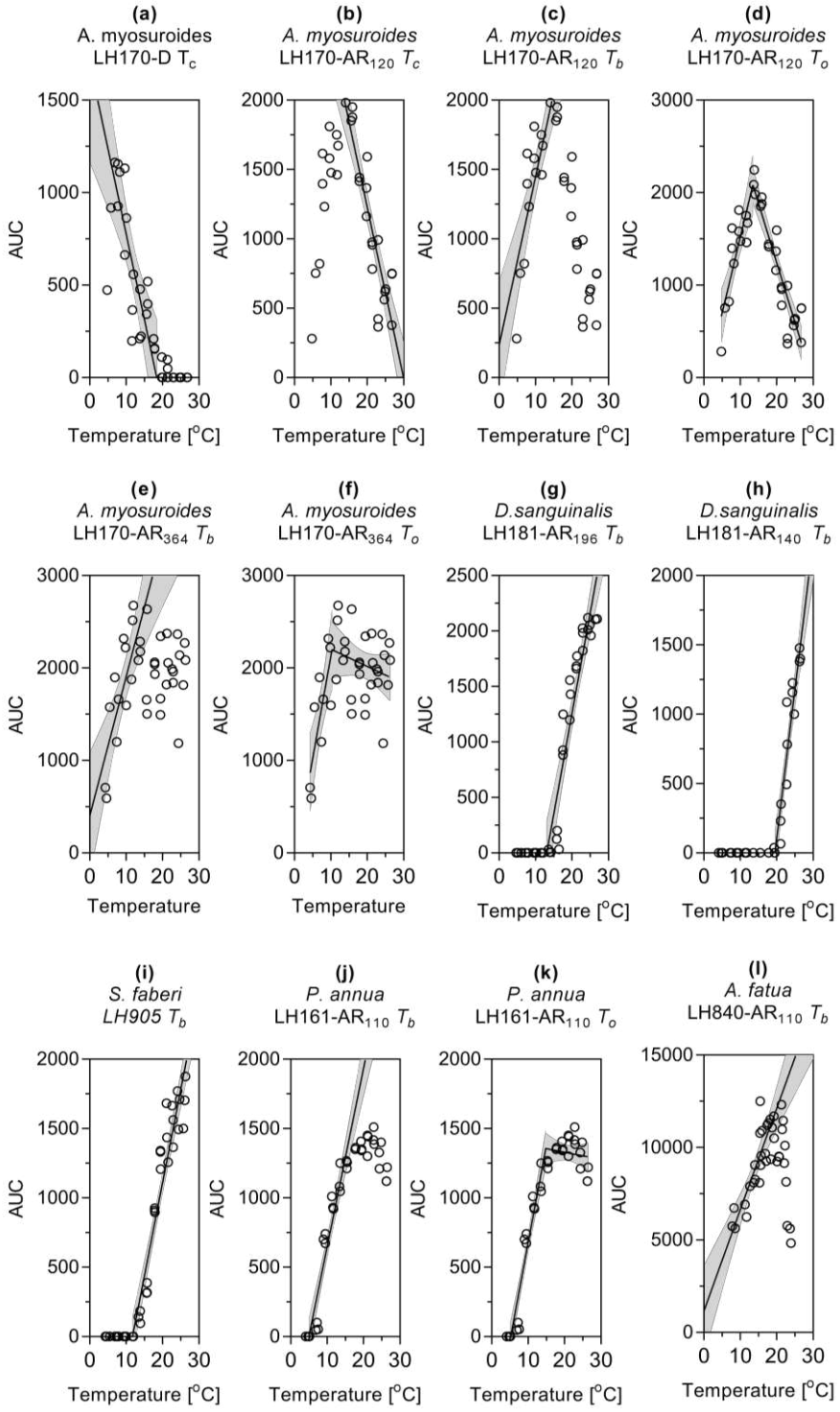


Fig. 9.3. Extrapolation of cardinal temperatures for germination. Each point on the plot represent the area under the germination curve for cumulative coleorhiza emergence (AUC) for a single replicate. Lines show the regression function used to interpolate the cardinal temperatures: T_b, base temperature; T_o, optimum temperature; T_c, ceiling temperature. Shaded area represents the 95% confidence interval for the regression lines.

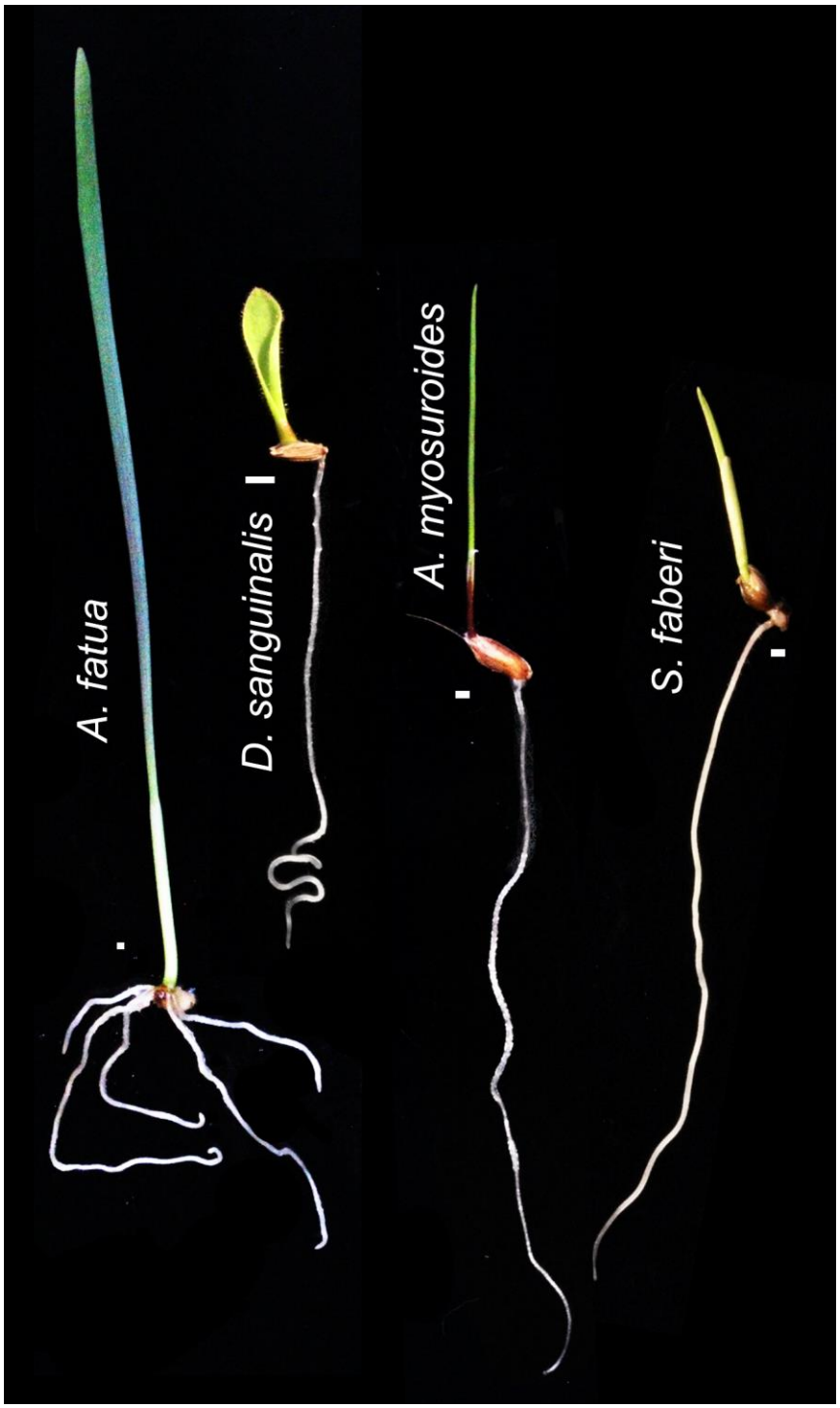


Fig. 9.4. Images of weed seedlings. Images of weed seedlings after two weeks of post-germination growth at 20°C under constant light. Scale bar represents 2 mm. Images were taken with an EOS Kiss X5 (Canon Inc, Tokyo, Japan): f/25, 1/60 second exposure, ISO-6400, 100mm focal length.

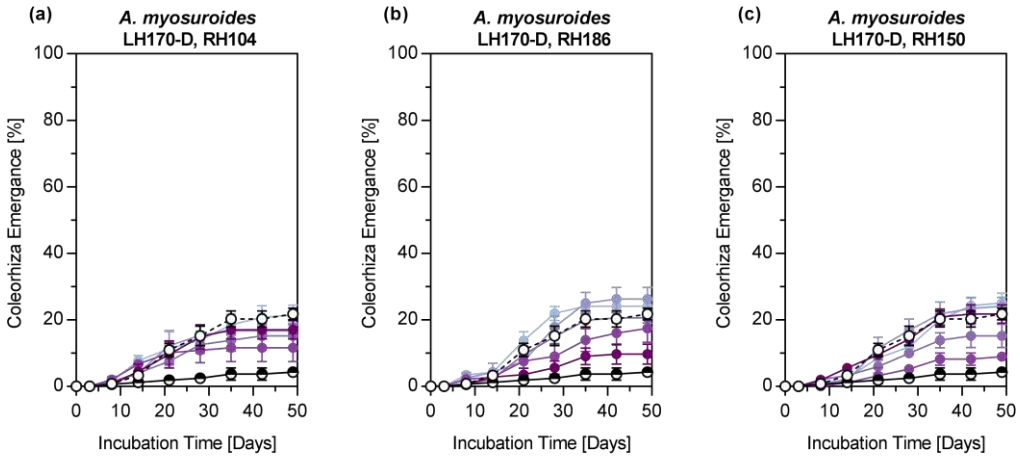


Fig. 9.5. Inhibitors of GA2-oxidase have no effect on dormant *A. myosuroides*. The effect of GA2oxidase inhibitors (a) RH104, (b) RH186 and (c) RH150 on the germination of dormant *A. myosuroides* LH170. (○, H_2O; ●, 0.1% (v/v) DMSO; ●, 0.1 µM; ●,1 µM; ●, 10 µM; ●, 50 µM; ●, 100 µM) Error bars show standard error of the mean for triplicates of ~30 seeds, incubated at 16°C under constant light.

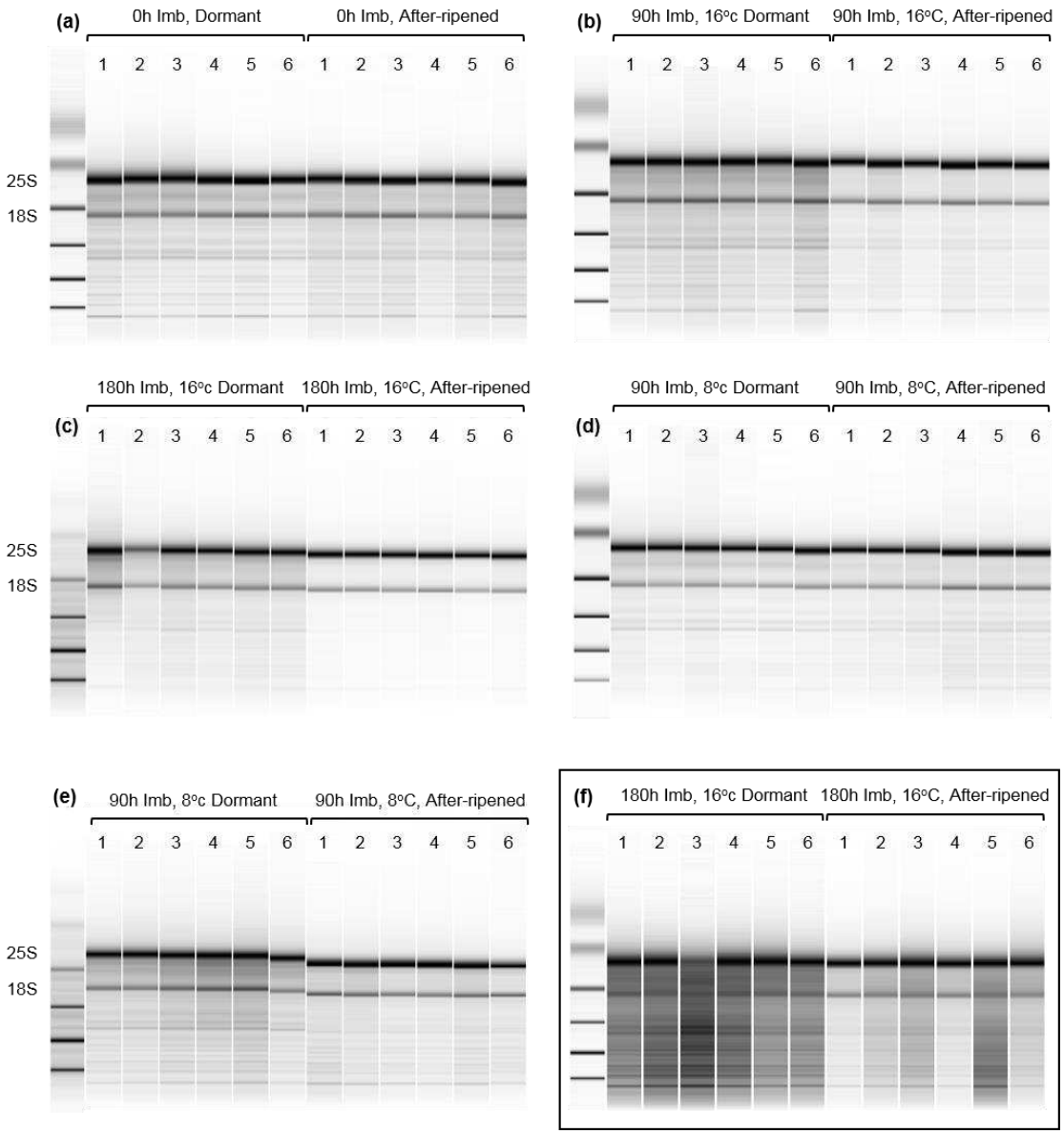


Fig. 9.6. Gel-like images from capillary electrophoresis of *A. myosuroides* RNA samples. Images produced from capillary electrophoresis traces using a Bioanalyzer 2100 RNA 6000 Nano chip with the 2100 expert software (Agilent Technologies Inc., California, USA). (a-e) Gel-like images for samples used for high-throughput sequencing. (f) Shows a gel-like image where the same extraction protocol with a lower extraction buffer volume (2 mL) has been used for both dormant and after-ripened samples imbibed at 16ºC for 180 hours, showing a decrease in RNA integrity in the dormant samples.

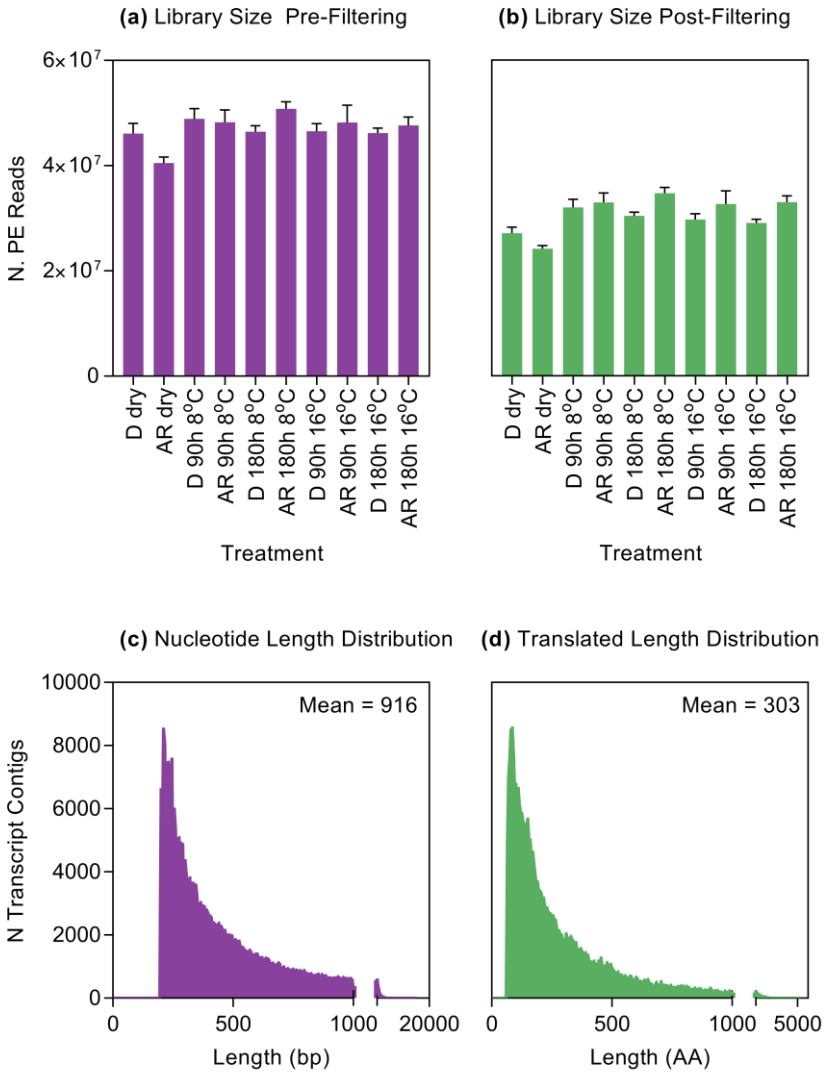


Fig. 9.7. Reference free assembly statistics. (a,b) library sizes (in number of paired-end (PE) reads for five replicates of each sample before (a) and after (b) quality control filtering. (c,d) Histograms showing the lengths of assembled transcript contigs for the nucleotide (c) and translated (d) assemblies. Error bars show standard error of the mean.

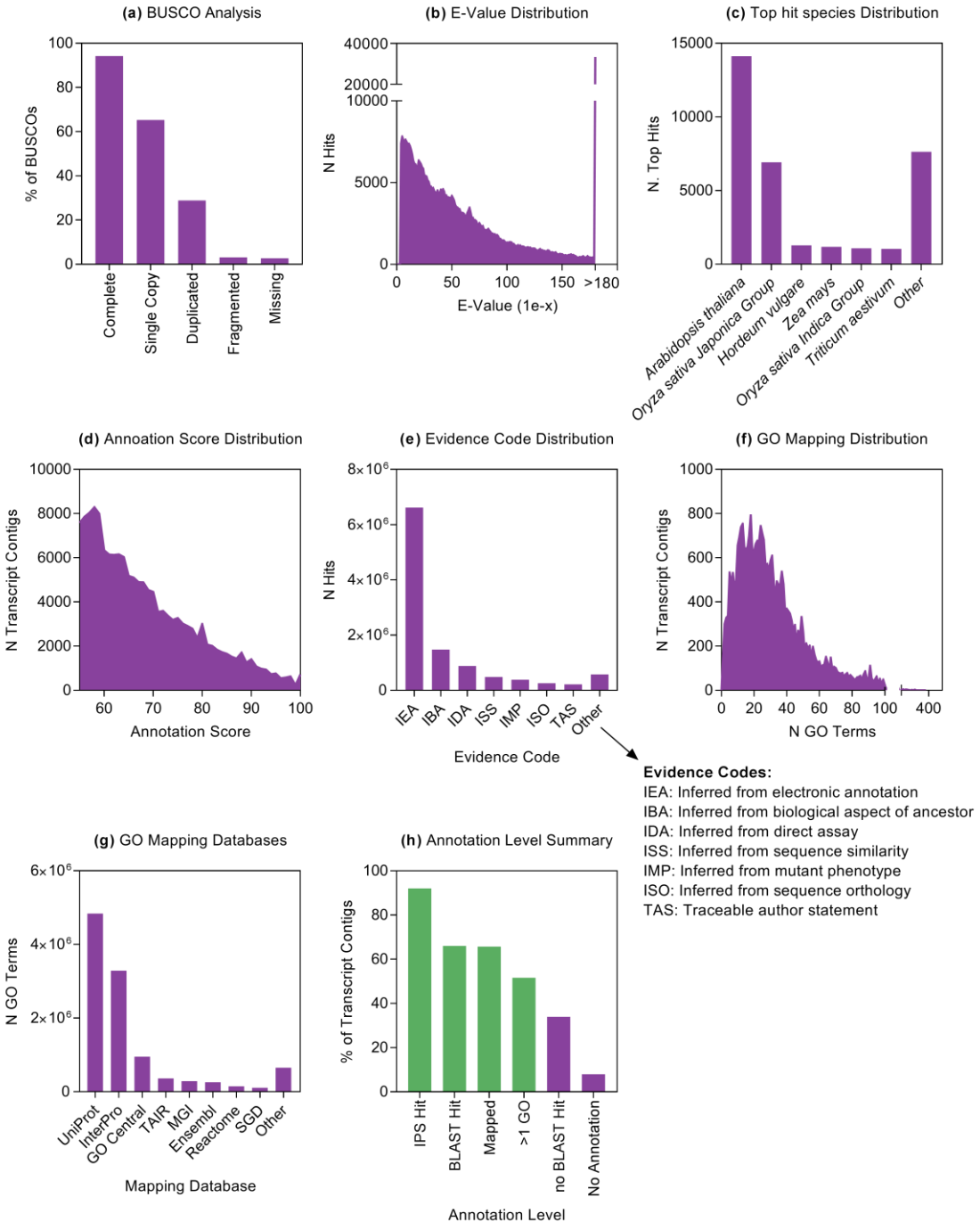


Fig. 9.8. Summary of assembly annotation. (a) BUSCO analysis showing high assembly completeness (94%) (b) E-value distribution for all BLAST hits for transcript contigs with an FPKM >5. (c) The species annotation distribution from the top BLAST hits for transcript contigs with an FPKM >5. (d) Distribution of annotation scores (a heuristic measure of annotation quality) for annotated transcript contigs with an FPKM >5. (e) Evidence code distribution for annotated transcript contigs with an FPKM >5. (f) Distribution of the Gene Ontology (GO) terms for annotated transcript contigs with an FPKM >5. (g) The databases which were used to map these GO terms. (h) A summary of the annotation level of the assembly for transcript contigs with an FPKM >5.

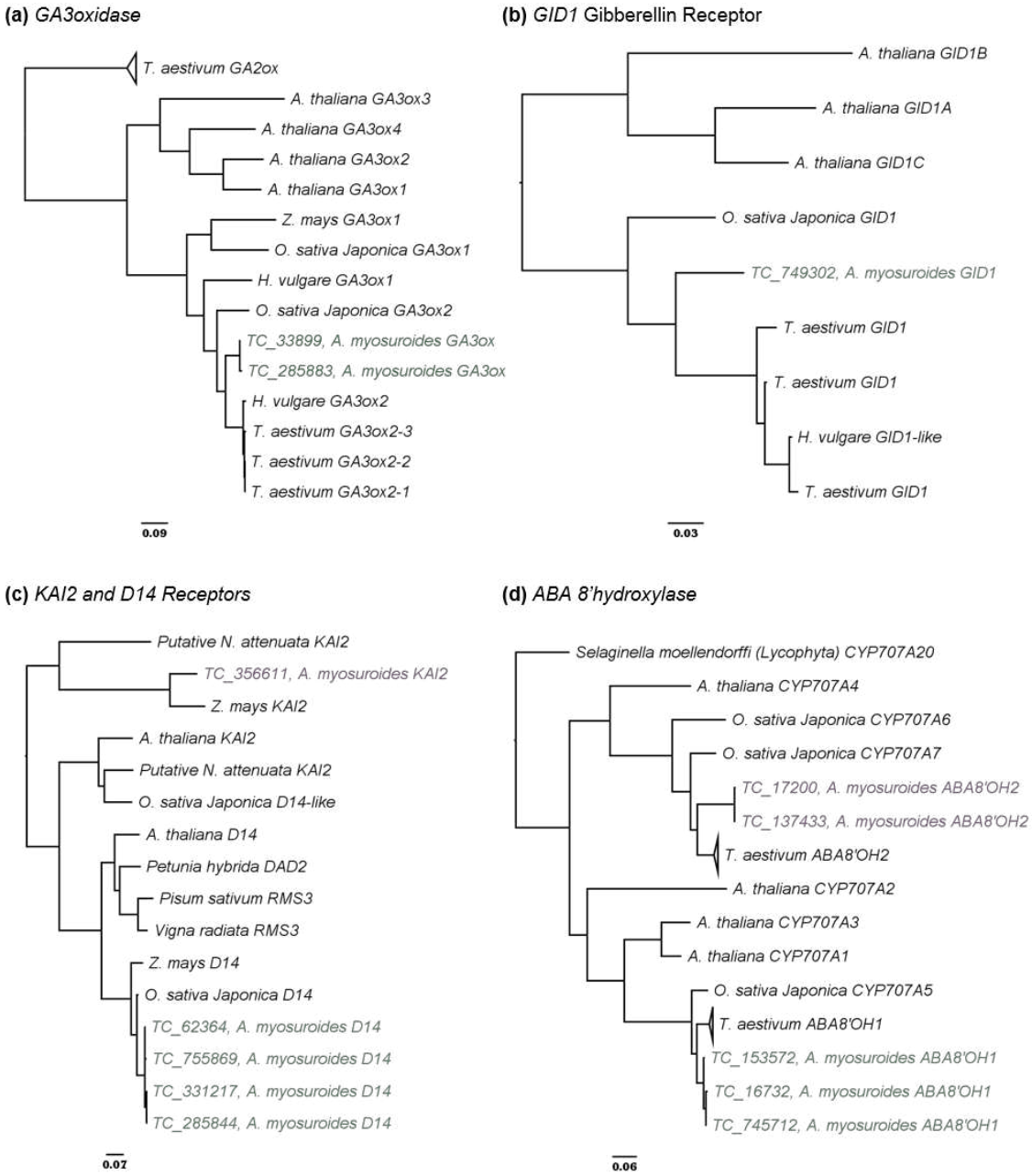


Fig. 9.9. Phylogenetic trees validating functional annotations. A selection of phylogenetic trees demonstrating how functional annotations were confirmed for key genes discussed in the manuscript for (a) *GA3-oxidase*, (b) *GID1*, (c) *KAI2* & *D14* and (d) *ABA 8'hydroxylases*. Trees were built in Geneious® (v8.0.5) from a manually trimmed MUSCLE alignment of RefSeq protein sequences and translated transcript contigs. Trees were built using the Neighbor-Joining method. Scale bar represents substitutions per position.

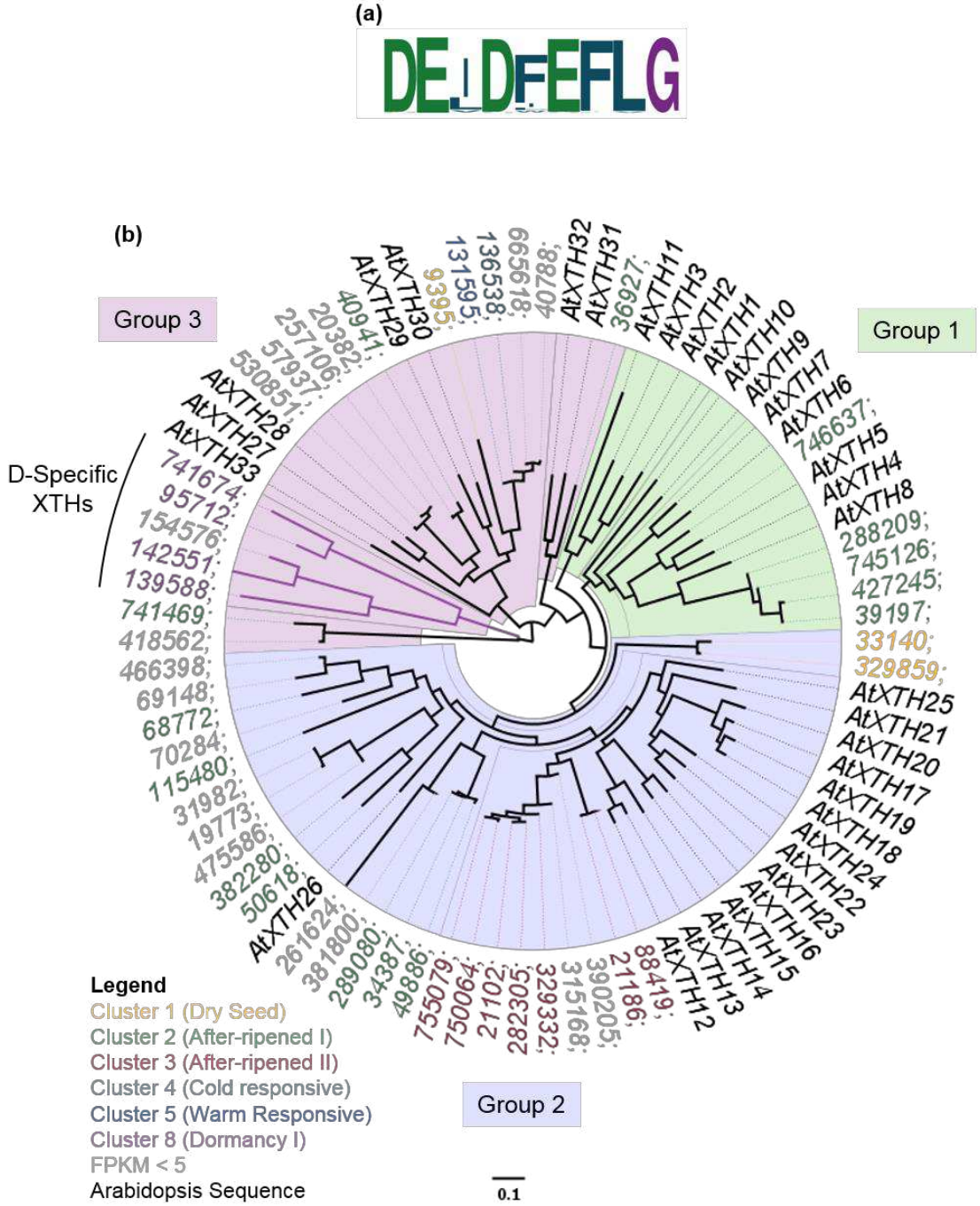


Fig. 9.10. Phylogeny and expression patterns of *A. myosuroides* and *A. thaliana* XTHs. (a) Conserved motif of XTH catalytic site across phylogeny (generated using www.weblogo.berkeley.edu). (b) Phylogeny of A. myosuroides translated transcript contigs with the motif in (a) and *A. thaliana* XTH sequences (SwissProt Database) showing groupings based on the established *A. thaliana* nomenclature (Rose *et al.,* 2002). The expression pattern of *A. myosuroides* transcript contigs are shown by the colour of the text based on their cluster identity. Scale bar represents 0.1 substitutions per position.

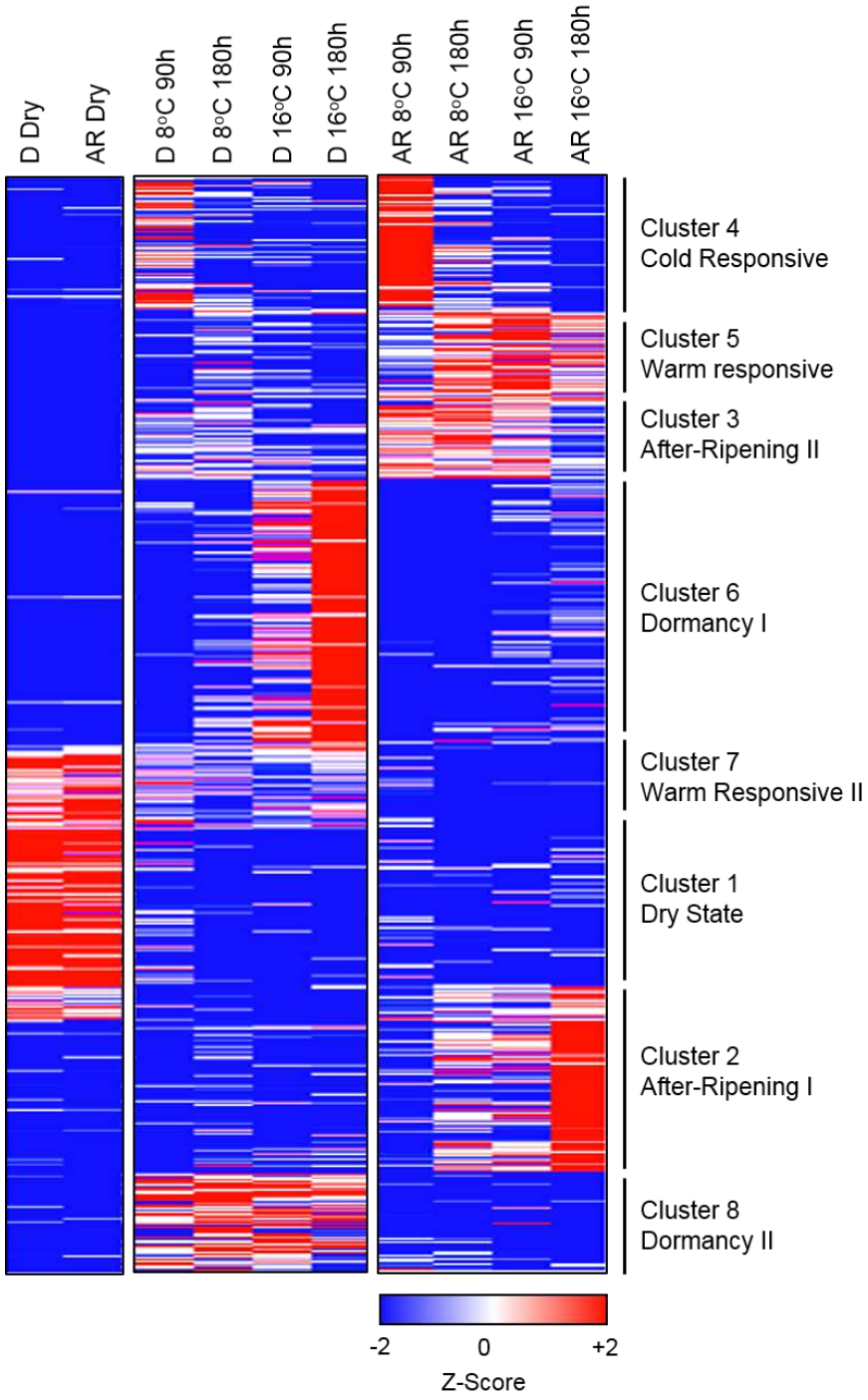


Fig. 9.11. Heatmap of K-means cluster analysis. Heatmap showing clustering output from MORPHEUS (https://software.broadinstitute.org/morpheus/) for k=8. Heatmap coloring based on Z-score for treatment means for each transcript contigs.

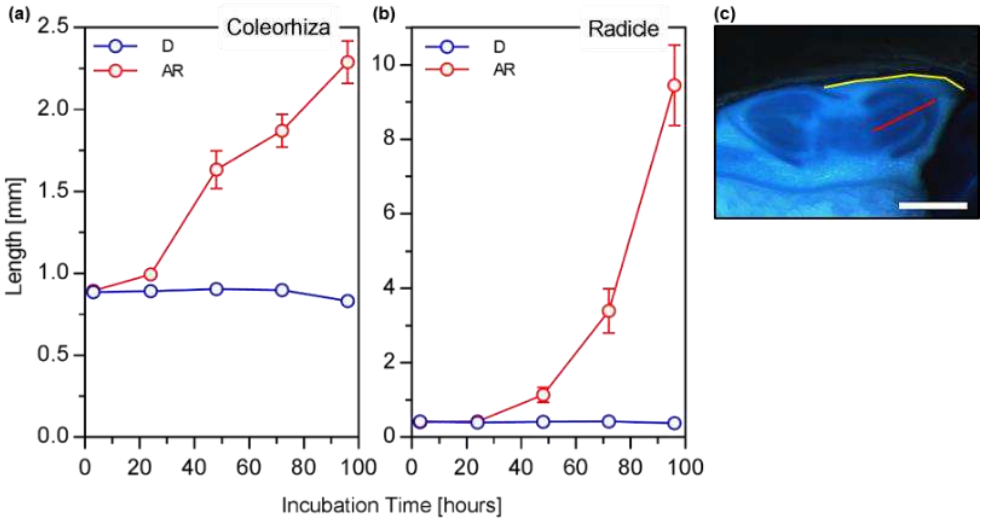


Fig. 9.12. Organ expansion in dormant and after-ripened *A. fatua* coleorhizae and radicles. (a,b) measurement of organ length over time for D and AR coleorhizae (a) and radicles (b). c, An example image showing an embryo stained with methylene blue. For radicle length measurements, the length from the center point of the base of the radicle to the apex of the root cap was measured (red line). For measurements of coleorhiza length, the periphery of coleorhiza was measured form the apex of the epiblast to the apex of the coleorhiza was measured (yellow line). Scale bar = 500 µm. Error bars show standard error of the mean for ~30 individuals per treatment. Seed batch LH840; D, dormant; AR, after-ripened.

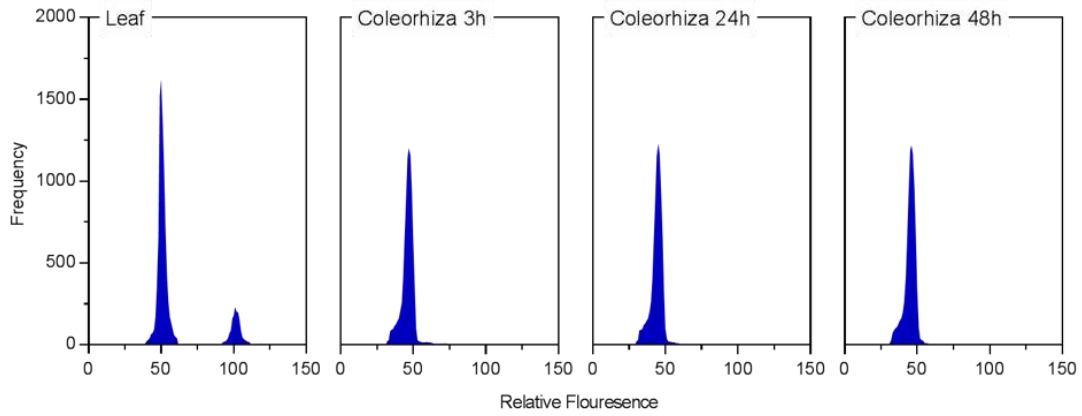


Fig. 9.13. Flow cytometric analysis of DNA contents in *A. fatua* leaf and coleorhiza tissue. Gain-adjusted frequency histograms showing embryonic leaf tissue and coleorhiza tissue isolated from LH840-AR_{112} caryopses imbibed for 3, 24 and 4 hours. The presence of two peaks in the leaf tissue shows nuclei with 2n DNA contents, indicating DNA replication is occurring and this tissue is proceeding through the cell cycle. The absence of this peak in the coleorhiza samples shows that this tissue is not proceeding through the cell cycle. Measurements were made for ~20 individuals on >9,000 nuclei per sample.

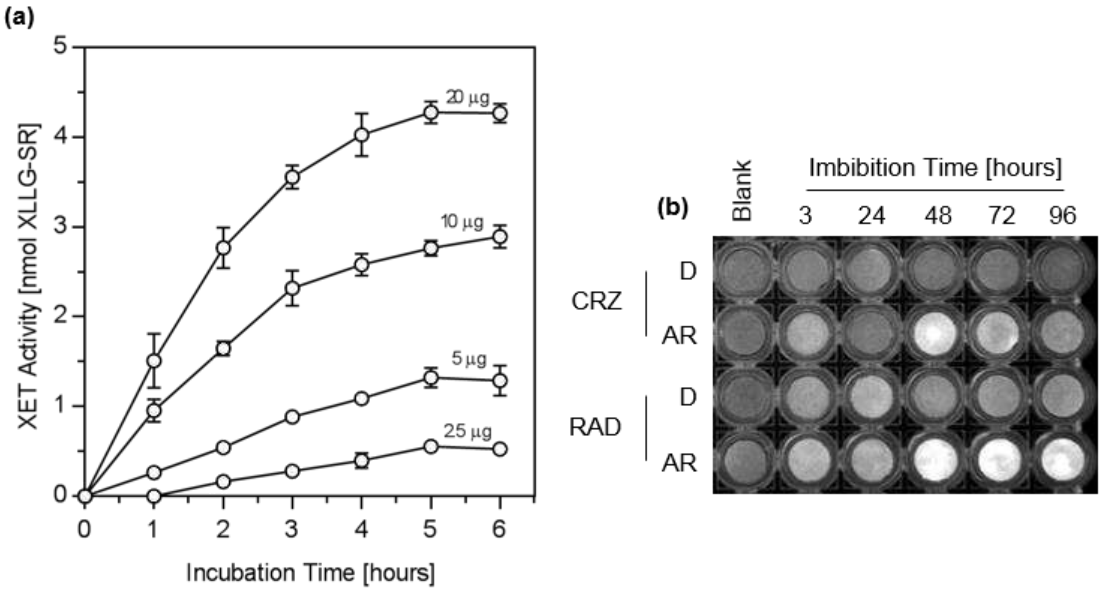


Fig. 9.14. Optimisation of the XET activity assay. (a) The effect of different total protein loadings and incubation times on measured XET activity from isolated *A. fatua* LH840-D and LH840-AR_{112} embryos from 48 hour imbibed AR caryopses. (b) Example UV transilluminator image of the XET activity assay showing 20 ug total protein loaded with a 3 hour assay incubation time from D and AR coleorhiza and radicles isolated from caryopses imbibed for different lengths of time. Error bars show standard error of the mean for triplicates of ~30 individuals.

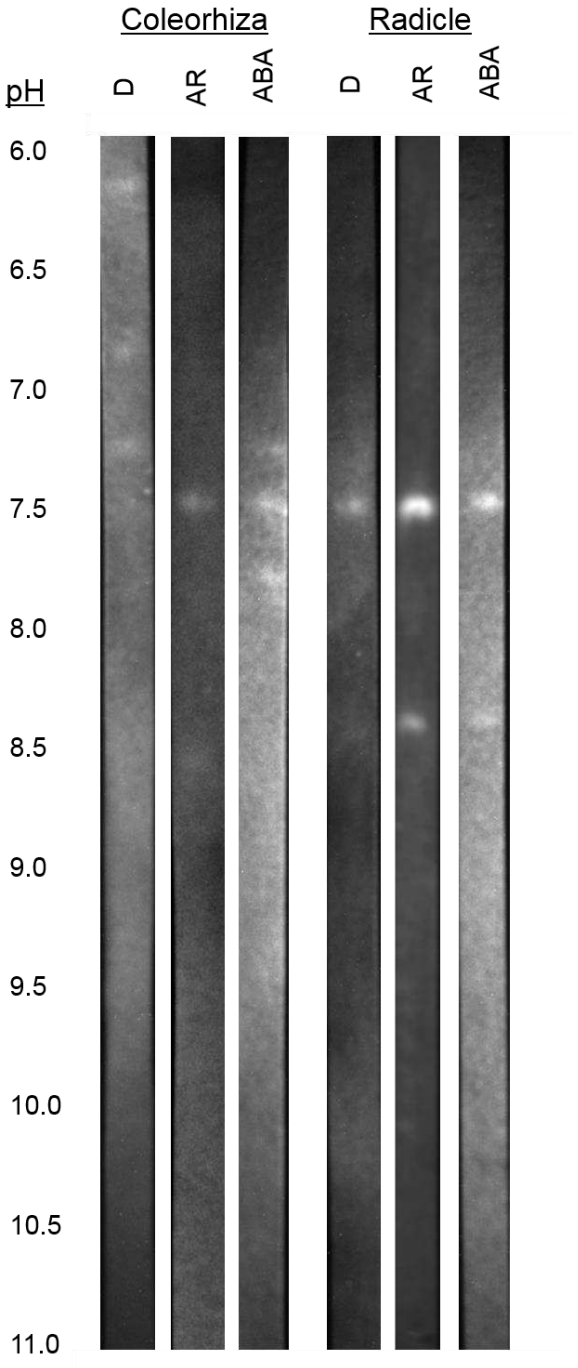


Fig. 9.15. Original zymogram images from isoelectric focusing of XET isozymes. Images of XET substrate matrix strips incubated with gel strips after isoelectric focusing. Fluorescent bands show areas where transglycosylation of a fluorescent substrate to the membrane has occurred indicating the presence of an XET isoform at that pH.

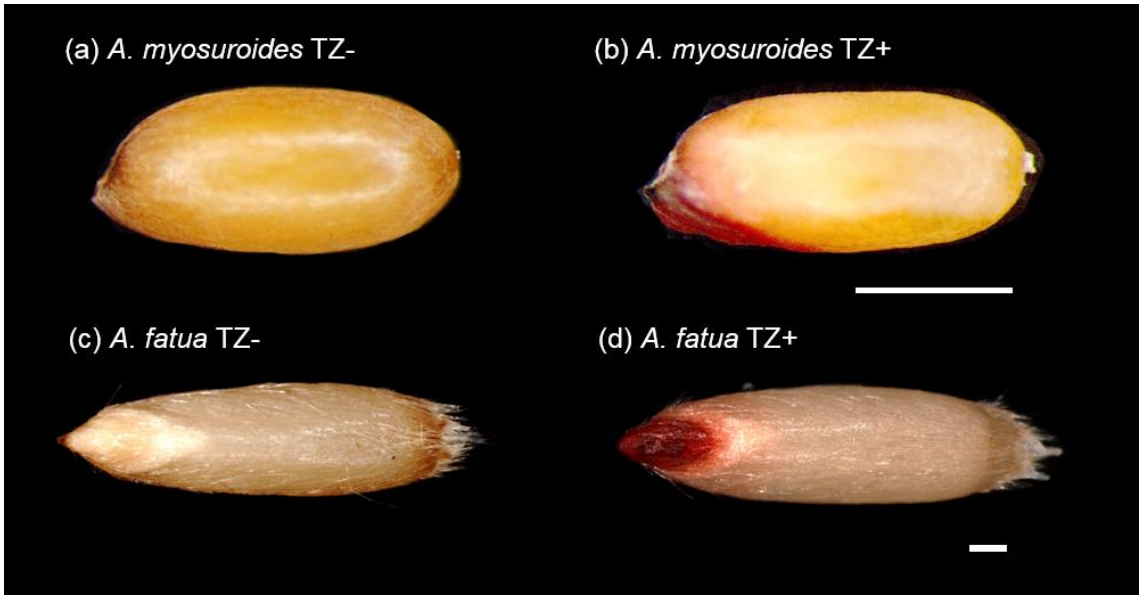


Fig. 9.16. Tetrazolium staining of *A. myosuroides* and *A. fatua* caryopses. Images of (a) *A. myosuroides* TZ negative (heat killed), (b) *A. myosuroides* TZ positive, (c) *A. fatua* TZ negative (heat killed), (d) *A. fatua* TZ positive. Scale bar = 1 mm.