A handbook for standardised measurements of regenerative plant functional traits

Peter Poschlod[1], Tereza Mašková[1,2,3], Si-Chong Chen[4,5], Shyam S. Phartyal[6], Sergey Rosbakh[7,1], Fernando A. O. Silveira[8], Arne Saatkamp[9], James W. Dalling[10,11], Emma Dalziell[12,13], John B. Dickie[5], Eduardo Fernández-Pascual[14], Lydia Guja[15,16], Borja Jiménez-Alfaro[14], David J. Merritt[12,13], Mark K. J. Ooi[17], Filip Vandelook[18,19]

[1]Institute of Plant Sciences, Ecology and Conservation Biology, University of Regensburg, Regensburg, Germany

[2]Institute of Ecology and Evolution with Herbarium Haussknecht and Botanical Garden, Plant Biodiversity Group, Friedrich Schiller University Jena, D-07743 Jena, Germany

[3]Senckenberg Institute for Plant Form and Function Jena (SIP), D-07743 Jena, Germany

[4]State Key Laboratory of Plant Diversity and Specialty Crops, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, China

[5]Millennium Seed Bank, Royal Botanic Gardens Kew, Wakehurst, UK

[6]School of Ecology and Environment Studies (SEES), Nalanda University, Rajgir, Bihar, India

[7]Department of Plant and Environmental Sciences, University of Copenhagen, Denmark

[8]Department of Genetics, Ecology and Evolution, Federal University of Minas Gerais, Brazil

[9]Aix Marseille Université, Université Avignon, CNRS, IRD, UMR IMBE, Marseille, France

[10]Department of Plant Biology, University of Illinois, Urbana-Champaign, Urbana, Illinois, USA

[11]Smithsonian Tropical Research Institute, Ancon, Panama City, Republic of Panama

[12]Kings Park Science, Department of Biodiversity, Conservation and Attractions, Kings Park, WA, Australia

[13]School of Biological Sciences, The University of Western Australia, Crawley, WA, Australia

[14]Biodiversity Research Institute (IMIB), University of Oviedo - CSIC - Principality of Asturias, Mieres, Spain

[15]Centre for Australian National Biodiversity Research (a joint venture between CSIRO and Parks Australia), CSIRO, Canberra, ACT, Australia

[16]National Seed Bank, Australian National Botanic Gardens, Parks Australia, Canberra, ACT, Australia

[17]Centre for Ecosystem Science, School of Biological, Earth and Environmental Sciences, University of New South Wales, UNSW Sydney 2052, Australia

[18]Research Department, Meise Botanic Garden, 1860 Meise

[19]Biology department, Ecology, Evolution and Biodiversity Conservation, Katholieke Universiteit Leuven, 3000 Leuven, Belgium

*** Correspondence**:

Peter Poschlod, email: Peter.Poschlod@ur.de

ORCIDs:

Peter Poschlod: 0000-0003-4473-7656

Tereza Mašková: 0000-0001-8386-5146

Si-Chong Chen: 0000-0002-6855-2595

Shyam S. Phartyal: 0000-0003-3266-6619

Sergey Rosbakh: 0000-0002-4599-6943

Fernando A. O. Silveira: 0000-0001-9700-7521

Arne Saatkamp: 0000-0001-5638-0143

James W. Dalling: 0000-0002-6488-9895

Emma Dalziell: 0000-0003-4463-9984

John Dickie: 0000-0002-8792-8113

Eduardo Fernández-Pascual: 0000-0002-4743-9577

Lydia Guja: 0000-0001-5945-438X

Borja Jiménez-Alfaro: 0000-0001-6601-9597

David J. Merritt: 0000-0002-3250-6861

Mark K. J. Ooi: 0000-0002-3046-0417

Filip Vandelook: 0000-0003-4591-5557

Organ/Stage	Function	Trait
1. Gametophyte	1.1 Flowering and fertilization	1.1.1 Flowering phenology
		1.1.2 Flower longevity
		1.1.3 Pollen dispersal vector/pollination mode
		1.1.4 Pollen production per flower
		1.1.5 Ovule production per flower
		1.1.6 Pollen longevity
		1.1.7 Ovule longevity
		1.1.8 Stigma receptivity
		1.1.9 Self-incompatibility
		1.1.10 Pollen thermotolerance
2. Fruit	2.1 Attraction/ defence	2.1.1 Fruit size
		2.1.2 Fruit crop size
		2.1.3 Dry pulp-dry seed mass-ratio
		2.1.4 Fruit colour
		2.1.5 Fruit scent
		2.1.6 Fruit chemical compounds
3. Seed	3.1 Attraction/ defence	3.1.1 Seed colour
		3.1.2 Seed surface
		3.1.3 Seed coat thickness
		3.1.4 Seed coat chemical compounds
	3.2 Seed dispersal/ dispersal potential	3.2.1 Dispersule type/syndrome
		3.2.2 Dispersule structure
		3.2.3 Dispersule exposure
		3.2.4 Seed production
		3.2.5 Seed mass
		3.2.6 Seed size and shape
		3.2.7 Seasonality of seed release
		3.2.8 Dispersal vector
		3.2.9 Seed releasing height
		3.2.10 Terminal velocity
		3.2.11 Buoyancy
		3.2.12 Epizoochory
		3.2.13 Endozoochory
	3.3 Seed persistence	3.3.1 Serotiny
		3.3.2 Seed longevity (in the lab)
		3.3.3 Soil seed bank longevity
		3.3.4 Seed coat water permeability
		3.3.5 Desiccation tolerance

		3.3.6 Seed metabolic rate 3.3.7 Seed water content
	3.4 Seed dormancy and germination	3.4.1 Embryo-to-seed size ratio 3.4.2 Post-dispersal embryo development 3.4.3 Seed dormancy and dormancy-breaking cues 3.4.4 Seed viability 3.4.5 Seed light requirements for germination 3.4.6 Seed temperature requirements for germination 3.4.7 Seed moisture requirements for germination 3.4.8 Seed oxygen requirements for germination 3.4.9 Response to heat 3.4.10 Response to chemical cues 3.4.11 Safe site/gap detection 3.4.12 Germination speed
4. Seedling	4.1 Seedling establishment	4.1.1 Seed nutrient content 4.1.2 Seedling morphology type 4.1.3 Seedling emergence depth 4.1.4 Seedling growth rate 4.1.5 Root elongation rate 4.1.6 Seedling resistance to abiotic stress (e.g., frost, drought, salinity)

Summary

A lack of standardised sampling protocols prevents functional traits from expressing their full potential to revolutionise plant ecology, biogeography, and evolutionary biology. Handbooks providing protocols for standardised measurements of plant functional traits allow researchers to tackle large-scale ecological questions but have traditionally focused on vegetative traits such as leaves, stems and roots. This handbook provides standardised protocols for 58 regeneration-related traits of flowers and gametophytes (10 traits), fruits (6 traits), seeds (36 traits) and seedlings (6 traits). It is the first effort to standardise sampling for relevant regeneration traits to understand processes, such as pollination, frugivory, seed dispersal, seed longevity, germination, and seedling establishment.

The protocols were designed to embrace the diversity of ecological contexts experienced by flowers, gametophytes, fruits, seeds, and seedlings and incorporate methods for temperate to tropical, dry to moist and fire-prone to fire-sensitive ecosystems.

We offer general guidelines for sampling, storing, and processing regenerative traits. Before laying out the protocol, we briefly describe each trait functionality, trade-offs, and sources of variability to give a broad context. Standardised protocols to estimate regenerative plant traits will unlock the full potential of plants to mitigate land use and climate change impacts, and restore destroyed ecosystems.

Key words: fruit; functional trait; handbook; pollen; protocol; regeneration; seed; seedling

Introduction

The uses and applications of plant functional traits in modern ecology

Trait-based approaches have revolutionised ecology, evolution, biogeography and conservation science in the last two decades. Using plant traits ('morphological, physiological, phenological or behavioural characteristics impacting individuals' fitness via their effects on growth, reproduction and survival' (Violle *et al*., 2007)) has significantly enhanced our mechanistic understanding and predictive capabilities of the processes that drive plant diversity patterns at various levels (Violle *et al*., 2014, Díaz *et al*., 2016, He *et al*., 2019, Carmona *et al*., 2021).

The widespread use of trait data across ecological scales relies fundamentally on sequential steps, including developing trait sampling protocols, trait ontologies, and analytical tools needed for data standardisation, harmonisation and statistical implementation. Such protocols and tools have become available more recently (e.g. Pérez-Harguindeguy *et al*., 2013; Garnier *et al*., 2017; Wigley *et al*., 2020; de Bello *et al*., 2021), and, in combination with the development of big data repositories, have now culminated in global plant trait databases such as the TRY database, which contained nearly 3 million trait entries in the first version increasing to nearly 12 million trait entries in the fifth version (Kattge *et al*., 2020). Yet, the key reproductive traits reflecting the variability of plant regeneration strategies along biotic and abiotic gradients are largely underrepresented in such global trait databases (Kattge *et al*., 2020, Visscher *et al*., 2022).

The (overlooked) role of regeneration traits in plant ecology

A closer examination of the TRY database shows that almost no seed ecological traits are represented. Key exceptions are for seed morphology, seed size, dispersal mode, seed longevity, and seed germination stimulation. By far the most entries are for seed size and dispersal mode provided primarily by BioPop (Poschlod *et al.*, 2003; Jackel *et al.*, 2006) and representing only the Central European flora. The fact that seed traits are widely neglected is also reflected by the first and second editions of the handbook for standardised measurement of plant functional traits worldwide (Cornelissen *et al.*,

2003; Pérez-Harguindeguy *et al.*, 2013). In the first edition, only four seed ecological traits were taken into account – dispersal mode, dispersule shape and size, and seed mass. Only two others – aspects of dispersal potential and functional seedling morphology - were added in the second edition.

The knowledge gap for regenerative traits is surprising, considering the increasing understanding of the importance of seed ecological traits. This has been highlighted in books and book chapters (e.g. Leck *et al.*, 1989; Thompson *et al.*, 1997; Gallagher, 2014), or in articles which show the role seed ecological traits may play in explaining the global and local distribution of species (Tweddle *et al.*, 2003; Carta *et al.*, 2024), local species assembly (Ozinga *et al.*, 2009; Poschlod *et al.*, 2013; Jiménez-Alfaro *et al.*, 2016, Larson & Funk 2016), vegetation and population dynamics (Larson *et al.*, 2015; Rosbakh & Poschlod, 2015), effects of climate change (Walck *et al.*, 2011) and changing land use (Kahmen *et al.*, 2002; Ozinga *et al.*, 2009), and other aspects such as functional connectivity between habitats (Fischer *et al.*, 1996; Römermann *et al.*, 2008; Schleicher *et al.*, 2011).

Consideration of this knowledge gap and its implications motivated the development of a research agenda on seed-trait functional ecology published by Saatkamp *et al.* (2019). They defined more than 20 functional seed traits related to four major axes of plant regeneration functionality – dispersal (in space), persistence in soil (dispersal in time), seed germination timing, and seedling establishment. Additionally, we now recognise the importance of gametophyte traits, which determine successful seed production, especially in the face of climate change (Rosbakh & Poschlod, 2016; Rosbakh *et al.*, 2018; Tushabe and Rosbakh, 2024).

The role of functional traits in assisting plants to withstand challenges can be expressed as four questions:

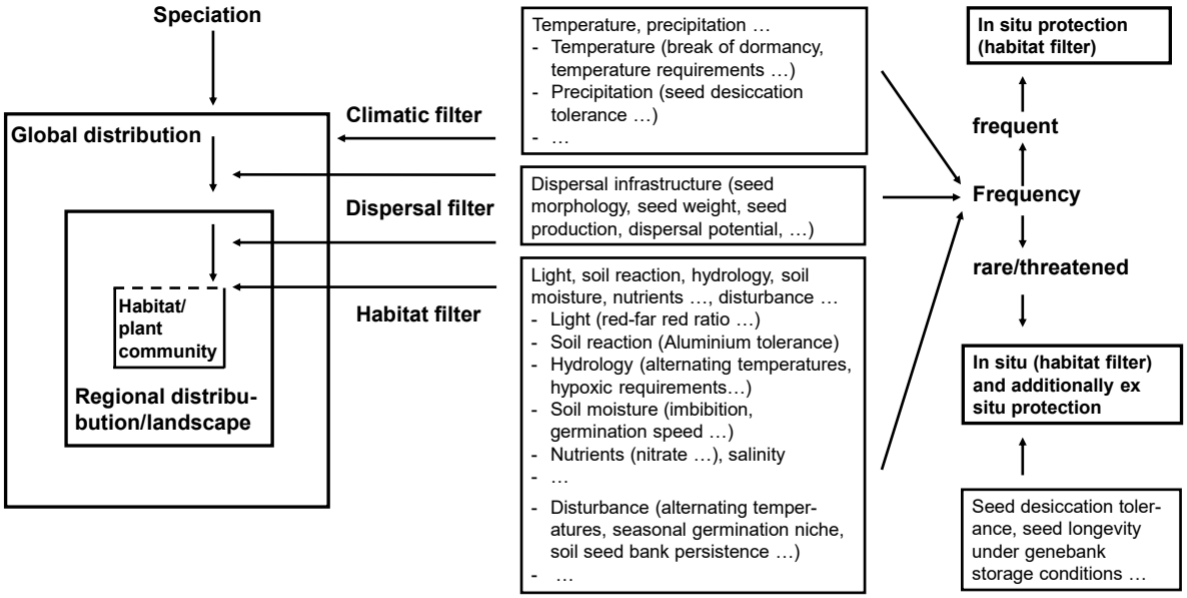
1. How do gametophyte traits interact with the environment to ensure successful fertilisation and, consequently, seed production?

2. How do plant traits and their dispersal units interact with biotic and abiotic dispersal vectors to allow seeds to reach favourable seedling establishment sites?

3. How do seed traits interact with daily, seasonal, and inter-annual variability in environmental factors to avoid, resist or survive temporally unfavourable conditions and sense favourable periods for seedling establishment conditions?

4. How do germinating seed and seedling traits interact with local habitat conditions, predators, pathogens, and competitors during seedling establishment?

Plants have to cope with the environmental conditions they encounter (Grubb, 1977) and today, with the rapid changes brought about by climate change, land use changes or other effects such as N deposition (Sala *et al.*, 2000). These environmental conditions and their changes filter the composition of plant communities, not only according to the requirements of the adult plant (Zobel, 1997) but also acting at the very earliest stages, beginning with the demands of the pollen whose sperm cells have to fertilise the egg cell to produce a seed (Rosbakh *et al.*, 2018) and second, the demands of the seed to arrive at a suitable site and to germinate (Poschlod *et al.*, 2013; Jiménez-Alfaro *et al.*, 2016; see Fig. 1).



158 **Fig. 1** Seed ecological traits affecting the distribution and the frequency of a plant
159 species and plant species assembly (from Poschlod, 2020).

160 This handbook covers traits related exclusively to plant regeneration, including
161 gametophytes (pollen and ovule), seed/fruit and seedling establishment traits. A
162 handbook on asexual regenerative traits such as clonal reproduction was recently
163 published (Klimešová *et al.*, 2019). We included sexual regenerative traits, which are, in
164 our opinion, the most relevant to answer the four questions above. We hope that this
165 handbook will spur the collection of standardised trait data that will provide new
166 insights into the mechanism by which plants and vegetation react and answer the
167 most challenging questions in the future related to changing land use, climate change
168 and tasks such as the *ex situ* conservation of plants in seed gene banks (Table 1).

Table 1 Functional gametophyte, fruit, seed and seedling traits and their relationship to environment and conservation. Resp. – response, cons. – conservation; int. dist. – intensity disturbance; fragm. – fragmentation; isol. – isolation; nutr. – nutrients; nat. enem. – natural enemies.

	Resp. climate change	Response to (changing) land use				Response to ecological parameters						Resp. ex situ cons.
		high int. dist.	low int. dist. (gaps)	aban-don-ment	fragm. and isol.	soil moisture/ drought	soil nutr.	fire	frost	floo-ding	nat. enem.	
1.1 Flowering and fertilization												
1.1.1 Flowering phenology	x					x	x	x	x		x	
1.1.2 Flower longevity	x	x			x	x	x		x			
1.1.3 Pollen dispersal vector/Pollination mode	x			x	x							
1.1.4 Pollen production per flower	x					x	x				x	
1.1.5 Ovule production per flower	x					x	x					
1.1.6 Pollen longevity	x				x	x	x					
1.1.7 Ovule longevity	x				x	x	x					
1.1.8 Stigma receptivity	x				x	x	x					
1.1.9 Self-incompatibility					x							
1.1.10 Pollen thermotolerance	x								x			
2.1 Attraction/defense (fruits)												
2.1.1 Fruit size	x	x			x	x	x					
2.1.2 Fruit crop size	x	x				x	x		x		x	
2.1.3 Dry pulp-dry seed mass-ratio	x					x	x					
2.1.4 Fruit colour	x											
2.1.5 Fruit scent	x					x	x		x			
2.1.5 Fruit chemical compounds	x						x					
3.1. Attraction/defense (seeds)												
3.1.1 Seed colour					x						x	

3.1.2 Seed surface					x							
3.1.3 Seed coat thickness					x			x			x	x
3.1.4 Seed coat chemical compounds				x	x						x	

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173 **Table 1** Continued

	Resp. climate change	Response to (changing) land use				Response to ecological parameters						Resp. ex situ cons.
		high int. dist.	low int. dist. (gaps)	aban-don-ment	fragm. and isol.	soil moisture/ drought	soil nutr.	fire	frost	floo-ding	nat. enem.	
3.2 Seed dispersal/dispersal potential												
3.2.1 Dispersule type/syndrome		x	x	x	x					x		
3.2.2 Dispersule structure				x	x							
3.2.3 Dispersule exposure		x			x							
3.2.4 Seed production	x	x	x	x	x	x	x				x	
3.2.5 Seed mass	x		x	x	x	x	x	x			x	
3.2.6 Seed size and shape	x			x	x	x	x					
3.2.7 Seasonality of seed release	x				x	x		x		x		
3.2.8 Dispersal vector	x	x									x	
3.2.9 Seed releasing height		x	x	x	x							
3.2.10 Terminal velocity					x							
3.2.11 Buoyancy					x					x		
3.2.12 Epizoochory					x							
3.2.13 Endozoochory					x							
3.3 Seed persistence												
3.3.1 Serotiny								x				
3.3.2 Seed longevity (in the lab)												x
3.3.3 Soil seed bank longevity	x	x	x	x	x	x	x	x		x		x
3.3.4 Seed coat water permeability						x		x			x	x

3.3.5 Desiccation tolerance	x					x						x
3.3.6 Seed metabolic rate	x					x	x					
3.3.7 Seed water content	x					x						x

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179 **Table 1** Continued

	Resp. climate change	Response to (changing) land use				Response to ecological parameters						Resp. ex situ cons.
		high int. dist.	low int. dist. (gaps)	aban-don-ment	fragm. and isol.	soil moisture/ drought	soil nutr.	fire	frost	floo-ding	nat. enem.	
3.4. Seed dormancy and germination												
3.4.1 Embryo-to-seed size ratio						x	x				x	
3.4.2 Post-dispersal embryo development	x				x	x	x	x	x		x	
3.4.3 Seed dormancy and dormancy-breaking cues	x					x	x	x	x	x	x	x
3.4.4 Seed viability	x					x					x	x
3.4.5 Seed light requirements for germination			x	x				x		x		x
3.4.6 Seed temperature requirements for germination	x											
3.4.7 Seed moisture requirements for germination						x						
3.4.8 Seed oxygen requirements for germination										x		
3.4.9 Response to heat	x					x		x				

3.4.10 Response to chemical cues			x			x	x	x		x
3.4.11 Safe site/gap detection			x							
3.4.12 Germination speed	x					x	x	x		
4.1. Seedling establishment										
4.1.1 Seed nutrient content	x					x	x			
4.1.2 Seedling morphology type						x	x			
4.1.3 Seedling emergence depth						x	x			x
4.1.4 Seedling growth rate	x	x	x	x		x	x	x		
4.1.5 Radicle/root elongation rate	x	x	x	x		x	x			
4.1.6 Seedling resistance to abiotic stress (e.g., frost, drought, salinity)	x					x		x	x	x

Principles of the handbook protocol

Guidelines for gametophyte, seed and fruit collection

First, check if collecting any plant material requires a permit from authorities because they may be internationally (e.g., Washington Convention on International Trade in Endangered Species – CITES, Annex A and B), nationally or regionally protected. If you collect plant material in a protected area, you also may need permission (Table 2).

Table 2 Issues to be clarified before fieldwork (according to Zippel & Stevens, 2014).

Land access	Inform authorities, landowners, and farmers of the area and, if necessary, make agreements
Target species protected	Apply for species protection certificate exemption/permit to the respective authority
Target areas in nature reserves and national parks	Apply to the respective authorities for exemption from the requirement to use paths in protected areas and for permission to collect parts of plants

The collection requirements for gametophytes (pollen and ovules) are different from those for fruits and seeds and are given in corresponding sections of the handbook (1.1.1 - 1.1.10).

Seeds and fruits should be collected during dispersal peak or a few days before. For seeds, natural dispersal usually comes when fruits open to release seeds or when closed and split fruits detach from the mother plant. The seeds within an infructescence may have different degrees of maturity. Only the parts of the inflorescence with ripe seeds are harvested. For species that flower and fruit over a long period or several times a year, collecting seeds several times a year to cover all phenological types is recommended. Gametophytes, seeds, and fruits should be collected in one site and the

habitat where the species has its ecological 'optimum'. Tree seed collection may require the use of rope and climbing techniques. For further guidance see Martyn Yenson *et al.* (2021), or for specific cases other resources can be consulted, e.g., Schmidt (2007) for forest trees.

For the particular seed trait or traits to be measured, around 100 to 2000 seeds per species should be collected. It could be more if there are specific questions, if measurements of multiple traits are planned, or if seeds are also required for restoration or ex-situ conservation. In the latter case, a careful workflow plan is recommended, as the same seeds can be used to measure multiple traits if these measurements are non-destructive. Whenever possible, obtaining measurements at the individual level is suggested. Still, researchers should be aware that many traits cannot be measured at the individual level (e.g. seed germination response to environmental cues), and seeds must be pooled from multiple individuals to run experiments. In any case, it is highly recommended to indicate whether measurements were made on individual seeds or a seed batch.

The collection should be made from at least 20 individuals. There are different recommendations, but we follow Leipold *et al.* (2020), who reported that about 14 sampled individuals are needed to cover 90% of the total genetic diversity and about 23 samples are needed to cover 95% of the total genetic diversity. According to Guerrant *et al.* (2014) and Menges *et al.* (2004), no more than 20% of the seeds available at harvest time should be collected (or this may be specified by national or regional collecting permits or licences). If a species is rare, or pollen, seed or fruit production is low, they could be collected at different sites and pooled together, but ensuring pooling is across collections from similar nearby habitats since different environments may result in different trait responses (see citations for the respective traits).

Guidelines for transport and cleaning

After collection, gametophytes should be transported as fast as possible to the laboratory for further measurements. Conditions for seed transport between the field and lab-

oratory can affect seed quality (Probert *et al.*, 2007; Royal Botanical Garden Kew, 2022b). Unprocessed seeds and moist or wet seeds or fruits should, therefore, be collected and transported in air-permeable cotton or paper bags and stored in a dry place after transport to avoid potential mould contamination. Manual or mechanical cleaning should be done as soon as possible to prevent the spread of insect pests. Cleaning techniques are described by Frischie *et al.* (2020).

Seeds of ripe fleshy fruit should be removed from the flesh as soon as possible, preferably on the day of collection or the following day, to prevent the fruit from becoming mouldy, beginning to ferment or rotting. Seeds from many fleshy-fruited species quickly lose water, which causes the loss of seed mass and alteration of the seed shape. Therefore, such seeds should be measured promptly after cleaning, with minimum storage. Fleshy fruits that are not yet entirely mature can be spread out and stored in well-aerated conditions until they are sufficiently ripe for cleaning.

Guidelines for storage

Long-lived pollen grains can be stored at 5 °C for a few days (Rosbakh & Poschlod, 2016). However, pollen of some species (e.g., Poaceae) might lose its viability within a few hours. Baskin & Baskin (2014) recommend storage of seeds prior to experiments at 4 °C (the specific storage time is species- and sample-specific). Importantly, seed germination behaviour may change during storage at low temperatures (e.g., dormancy-break or widening of temperature germination requirements). Thus, we recommend the start of experiments as soon as possible after collection and to keep track of the storage conditions (e.g. temperature and humidity) and time between collection and the start of the experiment.

Guidelines for germination experiments

There are several possibilities for how to germinate pollen (see section **1.1.10 Pollen thermotolerance**). For a specific recommendation, see the compendium of pollen *in vitro* germination media by Tushabe & Rosbakh (2021).

Before starting seed germination experiments, seeds have to be cleaned from the husk and then can be X-rayed (if available) so that only filled seeds are used (ISTA, 2023). Potential pathogens on the seed surface should be removed either by using a sieve under running water or alternatively using ethanol and sodium hypochlorite (NaOCl) solutions (Zalamea *et al*., 2021). Be aware that seed surface sterilisation may affect seed dormancy/germination patterns (e.g., Rosbakh *et al*., 2019).

Germination tests should preferably be done in Petri dishes (diameter adjusted to the seed size) on double-layered filter paper or agar. There are exceptions when seeds are large or will not germinate on watered filter paper (e.g., orchid seeds; in this case, agar may be used; see e.g., Zettler 1997; Mala *et al.*, 2017). Concerning larger seeds, one has to find appropriate pots or jars (e.g., Phartyal *et al.*, 2018) or germinate seeds on sand to provide enough contact with the moist media (Davies *et al.*, 2015). Different media are recommended for seeds that normally germinate only in the presence of symbiotic fungi, such as orchid seeds (Kauth *et al.*, 2008).

Although the International Seed Testing Association recommends four replicates of 100 seeds for a germination test (ISTA, 2023), testing different ecological conditions for wild plants is not always possible (see, e.g. Williams *et al.*, 1992). An overview of the literature gave various replicate approaches and the number of seeds per replicate (Sileshi, 2012). We recommend at least four replicates of 25 seeds. In cases where only a few seeds are available, one can decide to do five or four replicates of 10 or 20 seeds or even less (see recommendations in the single traits), especially in case of very specific treatments. The number of replicates should ideally not be lower than four in case pathogens spread over a Petri dish, which results in the complete loss of a replicate.

The filter paper should be kept wet throughout the experiment. Excess watering of the filter paper should be prevented unless seed germination response to hypoxic conditions is studied. Depending on the questions, counting of germinated seeds should be done once per week or for specific questions during the first week daily, if possible, and then after each third day. Germination experiments should run for at

least four weeks or until no further seed germination occurs over a period of two weeks. Seed viability testing before and after the germination experiment (see section **3.4.4 Seed viability**) is mandatory for proper interpretation of germination tests.

Layout of the protocols

The protocols for the selected traits follow a standard structure, beginning with a short trait definition and their detailed description, their functionality (traits may have multiple functions) and trade-offs, the sources of variability (e.g. intraspecific or interspecific), the methodology to measure them, and the units of measurements. In some cases, additional notes are added for clarity.

Traits are meant to be a measure of the organ or organism, but most germination and dormancy traits are descriptors of the seed environment (and hence the niche). We suggest considering dormancy and germination functional metrics as 'quasi' functional traits, which we think are helpful for understanding or predicting plant reactions and performance in different environments. Rather than organismal attributes, they are a proxy for the interaction between the organism's physiology and the environment. For a deepened functional ecological understanding of germination and dormancy, it might be helpful to carefully separate the environmental conditions necessary and organismic traits involved, such as seed coat permeability, embryo size and growth.

Statistical considerations

Statistical analyses are not proposed here since they depend on the specific research questions. We refer to the papers, textbooks and software on statistics (e.g., Sileshi, 2012; Ritz *et al.*, 2013; R Core Team, 2023). One important issue to be aware of is that data from germination experiments are not normally distributed.

Avenues of future research

Data quality and integrity

Standards for data reporting are beyond the scope of this handbook. However, there are proposals on how to report seed trait data (e.g., Poschlod *et al.*, 2003; Jackel *et al.*, 2006; Kleyer *et al.*, 2008) which have been taken up, e.g. into the global TRY database (Kattge *et al.*, 2011; 2020). There are also seed trait databases, e.g. the 'Seed Information Database' (SID; https://ser-sid.org/) and Seed Arc (Fernández-Pascual *et al.*, 2023). However, two major issues of TRY and SID are the lack of standardised measurements and standardised terminology. Therefore, they only allow very restricted analyses, although data entries in both databases are used to ask questions by probably more than a thousand researchers. They assume that the data quality is homogeneous, but this is not the case, which may result in incorrect interpretations. Therefore, as presented here, a new compilation of methods to standardise measurements and trait nomenclature (see Garnier *et al.*, 2017), is needed to overcome such hindrances. To avoid mistakes, data verification mechanisms should be developed.

To allow standardised analyses, data on the biome, vegetation type, etc. (Table 3) should be included. A similar format for seed germination data was recently proposed by SeedArc (Fernández-Pascual *et al.*, 2023).

Table 3 Additional requirements for each Individual Seed Trait Database entry.

ID	**Property**
Coordinates	Latitude, longitude, elevation
Biome	According to Breckle & Rafiqpoor (2022)
Vegetation type	According to Archibold (1995)
Country	country ISO code
Species	Verified accepted name according to GBIF (Telenius 2011)
Genus	Verified accepted name according to GBIF (Telenius 2011)
Family	Verified accepted name according to GBIF (Telenius 2011)

Towards a global database of seed functional traits (GDSFT)

The standardised measurements outlined in this handbook will finally allow the development of a global database of seed functional traits (GDSFT). Many products will arise from a GDSFT. First, it will facilitate communication among partners and allow broader questions to be asked compared to studies on biogeographic, macroecological and phylogenetic scales. In addition, the training of scientists is planned concerning the traits described in this handbook and the sharing of intellectual property around the globe, which is an immediate benefit to the scientific community (Fraser *et al.*, 2013). Second, if researchers want to sample a given trait not included in the seed trait handbook, a new protocol can be proposed and validated by a still-to-be-elected steering committee. The centralisation of protocols beyond the seed trait handbook at the GDSFT website will also facilitate future standardised studies (add-on studies), data inclusion in GDSFT, and continuous updates of the seed trait handbook. Third, a GDSFT data paper will be published once enough data are available. The data paper will be freely available to the scientific community. Fourth, a larger dataset of standardised measured traits will allow the validation of seed trait functionality.

1 Gametophyte

1.1 Flowering and fertilization

1.1.1 Flowering phenology

Trait description

Flowering phenology refers to the timing (onset, peak, and end) and duration of reproductive organ production events in vascular plants (flowers in angiosperms, cones in gymnosperms). It can also include additional phenological parameters describing flowering phenology in detail, such as sequence, intensity, synchrony, consistency, and flowering frequency (see Ollerton & Dafni (2005)). The major phenological events include the flower bud formation, the anthesis (timing of flower opening), flower pollination and flower wilting. These events can be observed at the individual plant, population, community, and landscape levels (Ollerton & Dafni, 2005; Shivanna & Tandon, 2014).

In a broad sense, flowering phenology also covers the time and duration of nectar secretion, anther dehiscence, stigma receptivity, pollen dispersal, pollen longevity, and pollination events. As these aspects require more elaborate studies, they are generally studied separately, and the protocols are described elsewhere (Ollerton & Dafni, 2005; Shivanna & Tandon, 2014).

Functionality and trade-offs

Flowering phenology is a key component of plant fitness because it determines the success of the whole reproductive process in plants and thus should take place under environmental conditions most suitable for pollination, fertilization, and seed maturation (Levin, 2006). Any deviations from the optimal timing and course of flowering will result in a limited capacity for seed production and/or low progeny quality (Elzinga *et al.,* 2007; Chuine, 2010; Munguía-Rosas *et al.,* 2011). For example, in seasonal climates, too early flowering may result in restrictions in pollen availability due to low pollinator activity or flower frost damage (Inouye, 2008). In contrast,

flowering that is too late often results in low seed production due to the shorter growing season (Kudo & Hirao, 2006).

The parameters of flowering phenology are strongly associated with (i) the physical environment (e.g. temperature, rainfall, day length, elevation, and latitude; van Schaik *et al.,* 1993; Fenner, 1998; Chuine, 2010; Ahmad *et al.*, 2023), (ii) the biotic environment (competition and facilitation by pollinators, florivory, antagonists (floral pathogens, pre-dispersal seed predation; Primack, 1985, 1987; van Schaik *et al.*, 1993; Fenner, 1998), (iii) phylogenetic identity (Levin, 2006; Willis *et al.,* 2008; Davis *et al.,* 2010) and (iv) an interplay thereof (Ollerton & Dafni, 2005). The trait has also been found to scale with other vegetative traits (e.g., plant height (Du & Qi, 2010), specific leaf area (König *et al.,* 2018), and regenerative traits (e.g., pollination mode (Jia *et al.,* 2011), seed size (Castro-Díez *et al.*, 2003; Du & Qi, 2010)).

Sources of variability

Individual plants, their single populations, and metapopulations demonstrate large spatial and temporal variation in their flowering phenology (Augspurger, 1983; Neil & Wu, 2006; Munguía-Rosas *et al.,* 2011), whereas the difference in the trait values among species is suggested to be a particularly conserved trait (Levin, 2006; Chuine, 2010). In the former case, the interannual, elevational and latitudinal variability in environmental conditions, particularly precipitation and temperature, are the main drivers of variation in timing and duration of flowering at individual and population levels (e.g., Phillips *et al.*, 1983; Shaver *et al.*, 1986; Cornelius *et al.,* 2013a).

Methodology

Data on flowering phenology can be obtained through field observation of the onset, peak, and end of flower budding and anthesis in tagged individuals, populations, and species. Flowering is considered to have initiated when about 10% of flowers in the inflorescence or 10% of the individuals in the population are open. The flowering is at its peak when more than half the flowers or individuals are in the same phase (i.e., open); the end of flowering is indicated when less than 10% of individuals have

remained in the phase (Meier, 2001). The flowering is considered to have been completed when no flowers or individuals are in the open phase (Shivanna & Tandon, 2014). The timing of the flower development is usually recorded as a Julian date, also known as a day of the year (e.g., 1st January – day 1, 1st February – day 32); the duration of flowering is calculated as the number of days between the onset and the end of the flowering (Morellato *et al.*, 2010).

We advise regularly carrying out phenological observations to cover plant phenological responses to possible interannual variation in environmental conditions, such as weather. In this case, we recommend publishing the data on flowering phenology together with weather data. For long-term observations, a sufficient number of single individuals or permanent plots can be labeled using various materials (Shivanna & Tandon, 2014).

Information on flowering phenology for some common species can be obtained from existing meteorological or phenological networks (van Vliet *et al.,* 2003; Schwartz *et al.*, 2012; Rosbakh *et al.*, 2021). Published plant identification books, floras (e.g., Synnott, 1985), databases (Klotz *et al.*, 2002) or observational databases (e.g. https://www.observation.org/) can provide data on flowering phenologies at larger spatial scales; herbarium specimens may serve as a valuable historical source of information on this trait as well (Primack *et al.,* 2004). However, such data should be used cautiously as they may not account for temporal and spatial variation in flowering times and duration (Ollerton & Dafni, 2005).

Flower bud formation and flowering are coded as stages 5 and 6, respectively, in the general plant phenology methodology developed for agricultural purposes ('BBCH-scale'; Meier *et al.*, 2009), but that could also be applied to natural plant populations (e.g., Cornelius *et al.,* 2013b).

Notes

Flowering phenology can be challenging to record for species with very short-lived flowers, e.g. *Papaver argemone* may open its flowers during late night and shed its

petals before 8 AM. In this case, flowering phenology can be estimated by the ratio of non-opened flower buds and wilted flowers or young fruits. Some plants (e.g., *Clypeola jonthlaspi*) never open flower buds (cleistogamy) and directly develop fruits. *Ficus* species hide flowers in closed syconia, so it is impossible to record flowering, and a surrogate stage needs to be assigned.

1.1.2 Flower longevity

Trait description

From a functional trait ecology perspective, flowers are reproductive organs of angiosperms, with various traits influencing plant fitness, such as pollinator attraction, pollen dispersal, fertilization success, and resource allocation. These traits include floral morphology (e.g., size, shape, and colour), phenology (timing of flowering) and physiology (e.g. longevity), all of which contribute to the plant's reproductive strategy and ecological niche. A widely used definition of flower longevity is the length of time a flower or a flowering unit remains open and functional (usually from flower opening (anthesis) to its senescence).

Functionality and trade-offs

Flower longevity is an important regeneration trait that directly and indirectly determines plant reproductive success and overall fitness because it reflects the balance between fitness consequences and maintenance costs. Fully functioning flowers require resources for respiration and pollinator attraction to contribute to plant fitness through ovule fertilization and pollen dissemination. Such floral maintenance costs may compete with future flower production or other plant functions if plant resources are limited (Ashman & Schoen, 1996; Castro *et al.*, 2008). Furthermore, floral longevity can positively influence the amount and quality of pollen a flower receives or disseminates (Primack, 1985; Rathcke, 2003). Additionally, it contributes to floral display size (the number of flowers open at any given time), the duration of floral display, and the total number of flowers per plant (Primack, 1985; Ashman & Schoen, 1996; Zhanag & Li, 2009).

Sources of variability

One of the most important factors affecting floral longevity is pollination success. Usually, pollen deposition on the stigma but not pollen removal considerably shortens floral longevity (Proctor & Harder, 1995; Castro *et al.*, 2008; Zhanag & Li, 2009).

At the species level, individual flowers' longevity varies greatly from just a few hours in, e.g., *Malvastrum coromandelianum,* to several weeks or months in orchids (Shivanna & Tandon, 2014). A part of this variation has been explained by the trade-offs with plant size and flower number (von Marilaun & Oliver, 1902), and a positive correlation with flower size and number of ovules per se (Stratton, 1989). Yet, the main source of the trait variation is the spatial and temporal variability in environmental factors, such as temperature or water availability, due to their direct effects on the costs of maintaining flowers (Ashman & Schoen, 1996; Yasaka *et al.*, 1998; Rathcke 2003). For example, upland species tend to flower longer, whereas flowers in warmer habitats, such as tropical forests, have a considerably shorter life span (Primack, 1985; Stratton, 1989; Blionis *et al.*, 2001; Fabbro & Körner, 2004; Ahmad *et al.*, 2023). Similarly, early-flowering species maintain their flowers longer than their late-flowering counterparts (Primack, 1985). Further, most species tend to produce longer-lasting flowers in climatically colder years (e.g., Vesprini & Pacini, 2005; Duan *et al.*, 2007). Ultimately, the degree of synchronicity within the population could also impact flower lifespan. For example, *Handroanthus guayacan* populations flower synchronously following rain events during the dry season in seasonally moist tropical forests, with flowers lasting only 1-2 days (Gentry 1976).

Methodology

Measuring floral longevity basically consists of recording the time of flower opening and senescence and counting the number of hours or days between these two events. Measurements should start immediately upon flower opening when the perianth appears fresh and end up on either corolla abscission or corolla or stamen wilting or discolouration (Roddy *et al*., 2021). The onset of flower senescence is indicated by turgidity loss in flower parts, drying and abscission of stigma and anthers, petal colour change or/and their wilting.

Labelling observed flowers with tags on an opening day will make observing longevity easy. A cheap and easy way to produce the tags is to cut heat shrink-tubes into rings of the desired size (tubes with different colours could be used to create rings for colour

coding). Make sure to observe floral longevity in at least ten individuals per population to account for possible intraspecific variability in this trait. We recommend observing several flowers per inflorescence in species with large inflorescences, such as *Apiaceae*.

To find out if pollination has any role in floral senescence, senescing flowers have to be excised and observed under the microscope for the presence of pollen on the stigma and correlated with pollination. Alternatively, one set of flowers can be manually pollinated at intervals and checked with reference to unpollinated flowers (Shivanna & Tandon, 2014). Finally, capping stigmas with straws or modelling clay can be experimentally performed to avoid pollen deposition (Roddy *et al.*, 2021).

1.1.3 Pollen dispersal vector/Pollination mode

Trait description

Pollination mode refers to a dispersing agent which helps pollen transfer from anthers to stigma.

The following types of pollination modes are distinguished: anemophily (pollen dispersed by wind), hydrophily (pollen dispersal by water), and zoophily (pollen dispersed by animals; Faegri & Van der Pijl, 2013). Depending on where pollination occurs, hydrophily can be classified into epihydrophily (pollination on the water surface) or hypohydrophily (in the water). Similarly, several specialised types of zoophily (entomophily, ornithophily, chiropterophily) can be distinguished based on the biotic pollen dispersal agent (insects, birds, bats, respectively). Typically, species possess one of these types, but a combination of pollination modes can occur (e.g., ambophily, the combination of anemophilous and entomophilous syndromes).

Functionality and trade-offs

Information on pollination mode is of key importance for studies on (i) the effectiveness of pollination, (ii) gene flow and plant population structure, (iii) the evolution of plant breeding systems, (iv) the evolution of floral traits (Kearns & Inouye, 1993; Shivanna & Tandon, 2014) and (v) conservation science (Vranckx *et al.*, 2011).

The trait is often coupled with other pre-fertilization traits, such as ovule and pollen size, pollen packaging, ovule and pollen production per flower, ovule and pollen longevity, and stigma receptivity (Faegri & Van der Pijl, 2013). Pollination mode also determines the structure and function of flowers, including their colour, scent, shape, and flowering phenology (Farré-Armengol *et al.,* 2015).

Sources of variability

Pollination mode is generally a fixed trait that shows variability only at the species level. Almost 90% of the flowering plants are pollinated by animals (insects, birds, bats), while the remaining 10% of the species use abiotic vectors, predominantly wind as a

pollination agent (Ollerton *et al.*, 2011). Hydrophily is rare and is limited to just about 30 genera of 11 families, largely monocots (Faegri & Van der Pijl, 2013).

At the community level, there is a declining trend in the proportion of animal-pollinated species along the latitudinal gradient from tropical forests to the temperate zone (Ollerton *et al.*, 2011; Shivanna & Tandon, 2014). Further research suggests non-random anemo- and zoophily distribution along elevational gradients (e.g., Pellissier *et al.*, 2010).

Methodology

Pollination mode is a categorical trait assessed through field observations (e.g., personal observations, camera traps). To determine the relative contributions of animals and wind to pollen transfer, one needs to bag flowers or look for evidence of pollen being carried by the wind. The bagging excludes insects, birds, and bats while allowing access to airborne pollen (Kearns & Inouye, 1993). The pollen carryover by wind can be detected by putting microscope slides coated with silicone grease at different distances from a potential pollen source and looking for the target pollen grains on the slides. A more quantitative method uses a volumetric pollen sampler (Kearns & Inouye, 1993).

At larger spatial scales, data on pollination mode can be collected based on flower and pollen morphology (Ackerman, 2000; Faegri & Van der Pijl, 2013; Shivanna & Tandon, 2014). Usually, anemophilous species produce non-showy flowers with reduced perianth, which lack colour, nectar, and odour. They produce many comparatively small pollen grains, whereas there are just a few ovules, often one (Pacini & Franchi, 2020). The pollen grains are dry and powdery with a smooth surface; the stigma is large and exposed. The flowers of hydrophilous species are similar to their anemophilous counterparts, including reduced perianth, lack of colour, nectar, and smell, and have a reduced number of ovules, usually just one. Yet, their pollen grains are covered with a coating of mucilage or oil, preventing them from wetting in water; many species may have filamentous pollen grains. In contrast to these two pollination modes, animal-

pollinated species tend to have comparatively large, colourful, and scented flowers with rewards (pollen and nectar) pollinators. The pollen grains are of variable size, sculptured, sticky, and, in extreme cases, tied together by thin viscin threads or in pollinia (Pacini & Franchi, 2020). Within animal-pollinated species, specialised types of pollinators (bees, birds, bats etc.) can be discerned based on flower morphological traits, such as spur length (Abrahamczyk *et al.*, 2017).

1.1.4 Pollen production per flower

Trait description

The trait is defined as the number of pollen grains produced per flower.

Functionality and trade-offs

Pollen counts are important for understanding male gametophyte fitness and its effects on seed production in plants (Ottaviano *et al.*, 1988; Shivanna & Tandon, 2014). Generally, higher pollen production positively influences seed quality and quantity, given that all pollen grains are deposited on receptive stigmas (Ter-Avanesian, 1978; Mulcahy, 1979; Allison, 1990; Brown & Kephart, 1999; Vara Prasad, 1999). In addition, the trait scales with the plant breeding system; the pollen production per flower increases with decreasing pollination assurance (Cruden, 1977; Cruden, 2009; Faegri & Van der Pijl, 2013). Furthermore, pollen production is often coupled with pollination type, with the highest counts in wind-pollinated species (Shivanna & Tandon, 2014). The results of a study by Kelly & Harvey (1978) suggest that different plant life forms may differ in their pollen production per flower due to differences in flower sizes.

The trait is known to be correlated with several other floral traits (Cruden, 2009): positively with anther size (De Vries, 1974; Molina *et al.*, 1996), ovule number, and flower size (Cruden, 2009) and negatively with pollen grain size (Reddi & Reddi, 1986; Vonhof & Harder, 1995) and stigma longevity (Cruden, 2009).

Sources of variability

In general, a stressful environment (extreme temperatures, water and nutrient shortage, herbivory, etc.) restricts plant growth and flower production resulting in a reduction of the number and quality of pollen produced (Stephenson *et al.*, 1992; Quesada *et al.*, 1995; Delph *et al.*, 1997; Rosbakh *et al.*, 2018). These negative effects on the trait have been mainly reported in experimental settings (Hall *et al.*, 1982; Etterson & Galloway, 2002) and rarely for natural plant populations (Markgraf, 2009). Pollen

production is also strongly subjected to seasonal variation in weather (Hill *et al.*, 1985; Latorre, 1999), resulting in great interannual variation (Shivanna & Tandon, 2014).

Methodology

Shortly before another dehiscence, collect at least ten flowers of a target species. Several typically developed and closed anthers per flower should be dissected from the flowers and put in separate plastic or glass vials. Count the number of anthers per flower.

Put fresh or dried anthers in a known volume of 50% ethanol and cut them open under a dissecting microscope. Ensure no pollen grains remain in the anther (a vortex or a sonicator could be used to release the pollen grains better). If pollen grains are clumped (e.g., viscin thread, sticky pollenkitt), surfactant, ethanol, hexane, or cyclohexane can be applied to remove the clumping material (Ollerton & Dafni, 2005). Drop a known volume of the pollen suspension on a haemacytometer and count the pollen grains in a surface unit. Calculate the total number of pollen grains. Electronic particle counters can automate the counting (and pollen size measurement) if necessary.

Note

It is important to remember that a comprehensive approach is needed for estimating male gametophyte fitness. Simply counting pollen grains is not enough. It must be combined with a thorough assessment of pollen viability (Ollerton & Dafni, 2005; Shivanna & Tandon, 2014). For the corresponding methods, see section **1.1.6 Pollen longevity**.

1.1.5 Ovule production per flower

Trait description

The trait is defined as the number of ovules produced per flower.

Functionality and trade-offs

Along with ovule size and volume, this trait estimates resource allocation to female function and, therefore, could be used as an indirect measurement for potential female fertility in plants. Moreover, ovule number is an important component of the pollen:ovule ratio, an indicator (to some extent) of the possible breeding system of the species (Cruden, 1977; see also section **1.1.4 Pollen production per flower**). Finally, there has been evidence that seed set can be correlated with ovule production (Allison, 1990).

The number of ovules per flower ranges over six orders of magnitude in angiosperms and may differ greatly even among closely related species (Stebbins, 1974). Sex allocation theory suggests that ovule numbers should increase in stochastic environments, where the probability of pollination by outcrossed pollen is comparatively low (Burd *et al.*, 2009). The core idea of that theory is simple: if plants undergo uncertain and highly disparate acquisition of pollen, they will generally make more seeds in total if every flower is stocked with enough ovules so that unpredictable windfalls of pollen receipt can be converted to large seed production. The more often such windfalls occur, and the greater their magnitude, the greater the ovule number that should be favoured by selection (Burd, 1994; Burd *et al.*, 2009). Indeed, a few studies have indicated that plants tend to produce more ovules in habitats where pollination is more uncertain (e.g., high elevations (Price *et al.*, 2005)) or climate is variable (Stone & Jenkins, 2008). Furthermore, animal-pollinated species were found to produce more ovules, whereas a great portion of wind-pollinated species has very few ovules, often one per flower. Moreover, the ovule number was found to be clearly higher in perennials compared to annual species (Jürgens *et al.*, 2002).

At least in some species, ovule numbers were found to be subject to a size-number trade-off; species with large ovules had a lower number of ovules (Schemske *et al.*, 1978; Greenway & Harder, 2007). Similarly, ovule numbers per flower were significantly influenced by flower size, with more ovules in larger flowers (Wetzstein *et al.*, 2013).

Sources of variability

Apart from the variation mentioned above in ovule number among species, this trait was found to vary within inflorescences (Diggle, 1995), as well as among individuals of one species (Thomson, 1985; Diggle, 1995) and different species (Shivanna & Tandon, 2014).

Methodology

For the trait measurement, fresh flowers or flowers preserved in a fixative, e.g., FAA (9 parts formalin, 1 part acetic acid, and 1 part absolute alcohol), can be used.

Under a dissecting microscope, cut open the flower parts containing ovaries (carpels) longitudinally and carefully scrape the ovules out. To prevent ovules from desiccating, put them in a drop of distilled water or glycerine, spread them in a thin layer and count. In the case of large ovules, the counting can be done with a magnifying glass or the naked eye. Measure ovule number in several ovaries per flower, several flowers per inflorescence (if present), and several individuals per species to account for possible variation in the trait values.

Pollen/ovule ratios can be calculated as an integrative, continuous trait linked to breeding systems (Cruden, 2009).

1.1.6 Pollen longevity

Trait description

Pollen longevity is the length of time a pollen grain remains able to complete all post-pollination events (germination, pollen tube growth, fusion with egg cell) on a compatible, receptive stigma and affect fertilization (Shivanna & Tandon, 2014).

Functionality and trade-offs

Pollen longevity is of key importance for successful fertilization. A decline in pollen longevity greatly diminishes the male gametophyte fitness and thus may directly influence reproduction output (Dafni & Firmage, 2000). Furthermore, the trait is also fundamental to the evaluation of dispersal and gene flow (Ollerton & Dafni, 2005), crop improvement and breeding programs (Ollerton & Dafni, 2005), and germplasm conservation (Hoekstra, 1995).

Sources of variability

Pollen longevity is one of the most variable pre-fertilization traits. It varies considerably at almost all plant organization levels, ranging from different samples from the same individuals to different species (Dafni & Firmage, 2000; Shivanna & Rangaswamy, 2012). The trait has also been shown to vary along spatial gradients, e.g., during flowering periods or at different times of the day (Shivanna & Rangaswamy, 2012). Reportedly, the average lifetime of a pollen grain can range from a few minutes to several months (Hoekstra, 1995; Dafni & Firmage, 2000; Shivanna & Tandon, 2014) and even years in some exceptional cases (Faegri & Van der Pijl, 2013). This variability has been shown to be associated with several internal (e.g., number of cells, water content, type of carbohydrate reserves) and external (pollination agents and pollen travel distance, temperature and precipitation) factors, and an interplay thereof (Faegri & Van der Pijl, 2013; Shivanna & Tandon, 2014). Other pre-fertilization traits, such as pollen competitive ability, stigma receptivity, and breeding system, have been reported to be related to pollen longevity (Dafni & Firmage, 2000).

Methodology

Measuring pollen longevity consists of evaluating pollen grain viability at regular intervals under given environmental conditions.

Pollen grain viability

Essentially, there are three most reliable approaches to test pollen viability: (i) capacity to effect seed set, (ii) pollen germination *in vitro,* and (iii) staining techniques (Kearns & Inouye, 1993; Shivanna & Tandon, 2014; Dafni *et al.*, 2005a). The first approach consists of depositing the pollen on receptive stigmas and determining whether seeds are produced. Because this approach is very labour-intensive and time-consuming, we recommend using it only when the other methods do not work. The pollen germination method is fast and relatively simple and correlates with the pollen's ability to sire seeds (Shivanna & Rangaswamy, 2012). Yet, the lack of a suitable pollen germination media to achieve satisfactory germination, especially in trinucleated pollen (Dafni *et al.*, 2005a), can limit the application of this test. For the protocol on *in vitro* germination, see section **1.1.10 Pollen thermotolerance**. The third approach is based on the correlation between the ability to fertilise an ovule and pollen grain's physical or physiological characteristics, such as viable vegetative cells and enzyme activity (Kearns & Inouye, 1993). Several staining techniques have been proposed to test pollen viability using acetocarmine, aniline blue, fluorochromes (FCR), tetrazolium tests (TTC) and the activity of peroxidases, esterases, dehydrogenases and galactosidases in pollen grains (Kearns & Inouye, 1993; Dafni *et al.*, 2005a; Shivanna & Rangaswamy, 2012). We recommend using vital dyes like FCR or TTC for testing pollen viability, as non-vital dyes (e.g., acetocarmine or aniline blue) do not accurately reflect the status of living cells and often yield misleading results by staining pollen that has already lost its viability. However, non-vital dyes can still be used for assessing pollen fertility, the ability of mother plants to produce well-developed pollen grains with dense cytoplasm, visible nuclei and intact cellular structures. Fertile pollen grains typically stain uniformly and intensively, resulting in a well-defined and consistent appearance.

Enzymatic activity test should be used with caution due to the fact pollen may still have active enzymes even though it has lost the ability to germinate (Ollerton & Dafni, 2005). Furthermore, the results of such tests can be species-specific due to different levels of enzyme activity in the pollen grains (Dafni & Firmage, 2000; Dafni *et al.*, 2005a).

Pollen longevity measurement

Because pollen longevity is strongly affected by various environmental parameters during presentation and dispersal, we advise measuring this trait under standardised conditions: 22^{o}C and 60% air humidity. This approach will allow for the correct estimation of intra- and inter- specific trait variability. Additionally, the effects of temperature and humidity stress on pollen longevity can be studied by exposing the pollen samples to corresponding environmental conditions (Bassani *et al.*, 1994; Shivanna & Tandon, 2014).

Pollen viability is measured at time intervals of 1, 6, 12, 24, 36, 48, 72, 96, 120, 144 and 168 hours. If the pollen is still viable after this time, its viability is measured twice a week. The time intervals for short-lived pollen (e.g., grasses) could be shorter, e.g., every 5-10 minutes. To express the pollen viability loss under standardised conditions, several arbitrary thresholds (10, 50 and 90%) can be calculated with the help of non-linear regression (Ritz *et al.*, 2015).

Working procedure

Collect healthy, well-developed single flowers, inflorescences, or flowering twigs from several individuals and bring them to the lab, keeping them in water until anthers begin to dehiscence. If the flowers have not opened, place them under a table lamp to encourage opening. Always use mature but unopened flowers for analysis of pollen productivity, viability, or fertility.

Collect fresh pollen from newly opened anthers, thoroughly mix it in a Petri dish, and place it under corresponding environmental conditions (e.g., lab bank or humidity chamber; Shivanna & Tandon, 2014). Alternatively, pick the ripe anthers from the flowers and let them dehiscence in a Petri dish under lab conditions or over silica gel in

a closed desiccator. Please consult Kearns & Inouye (1993), Dafni *et al.* (2000), Shivanna & Tandon (2014) for techniques on pollen collection.

At regular time intervals, remove a small amount of pollen from the lot and test for its viability: stain the pollen sample or let it germinate *in vitro*. The onset of the anthesis is time zero for pollen longevity measurements. Count the number of viable (stained or germinated) and non-viable pollen grains. A total of 300 (3 replicates x 100) should be counted in randomly selected fields in each sample examined.

1.1.7 Ovule longevity

Trait description

Ovule longevity denotes the period during which the ovule remains receptive to pollen tubes.

Functionality and trade-offs

The trait plays a decisive role in the effective pollination period, i.e., the number of days during which pollination is effective in producing a seed (Sanzol & Herrero, 2001; Williams, 1965); a short ovule life span limits the success of pollination and therefore affects seed production (Stösser & Anvari, 1982).

Ovule longevity has been reported to be affected by the temperature during flowering. Generally, low temperatures extend ovule longevity, whereas high temperatures shorten the time ovules can be fertilised (Postweiler *et al.*, 1985; Sanzol & Herrero, 2001). Furthermore, some experimental evidence shows that ovule longevity can be correlated with plant nutrient status (Sanzol & Herrero, 2001). For example, nitrogen fertilization positively affected ovule longevity (Williams, 1965). Lastly, several floral traits, such as age, size, colour, and location on a branch, related to flower vigour ('flower quality'; Williams, 1965) have been reported to affect ovule longevity (Sanzol & Herrero, 2001).

Sources of variability

Little is known about trait variability at individual plant and intraspecific levels. The findings of Cuevas *et al.* (1993) suggest that differences in longevity among ovules of the same flower were smaller than among ovules of different flowers.

Methodology

The primary method for estimating ovule longevity consists of hand-pollinating flowers at varying time intervals from anthesis and recording the seed set late in those flowers (Williams, 1970). Yet, this method is quite labour-intensive and time-consuming

since it requires hand-pollinating a large number of flowers and may include long waiting times until the seeds are fully ripe.

Alternatively, microscopic examination of ovules in flowers fixed in sequence after ripening is used as an easy and quick method to evaluate ovule longevity (Stösser & Anvari, 1982). It is because ovule degeneration is associated with the appearance of callose and its spread across the cells of the unpollinated ovules (Stösser & Anvari, 1982; Dumas & Knox, 1983; Cuevas *et al.*, 1993; Sanzol & Herrero, 2001). This phenomenon can be monitored with the decolourised aniline blue fluorescence method described below.

Ovule longevity should be studied in unpollinated flowers. Thus, enough flowers are emasculated and bagged to prevent pollen deposition on the stigma. After the onset of anthesis, at least 10 flowers should be collected at regular time intervals till the first signs of flower senescence are visible (petal wilting, anther fall, morphological changes on stigma surface, seed or fruit set visible) and fixed in a fixative (e.g., 70% ethanol).

The ovaries should be separated from the fixed flowers under the dissecting microscope, softened with 8N NaOH for at least 8 hours at room temperature, rinsed with water several times, and stained with 0.1% aniline blue overnight. The tissue softening can be sped up by autoclaving the ovaries in 50 g/l sodium sulphite (Na_2SO_3) for 10 min to 1 h at 121 ^{0}C (Dumas & Knox, 1983). The next day, the stained pistils are placed in a drop of water or glycerin on a microscope slide covered by a coverslip and gently squashed to make the ovules visible. The slides are observed under a fluorescent microscope; ovules with intensive fluorescence cannot be fertilised and, therefore, are considered non-viable (Postweiler *et al.*, 1985).

Currently, there is no rapid test for determining ovule viability or receptivity to fertilization. The semi-vivo or in-vivo pollination methods mentioned earlier are reliable but time-consuming. Testing ovule receptivity has been attempted in some systems by localizing micropylar exudates using Toluidine O dye, which stains sulphated and polysaccharidic exudates (Vikas et al., 2009). When possible, the

ambient temperature and humidity before and during flowering should be recorded and reported along with the ovule longevity data.

1.1.8 Stigma receptivity

Trait description

Stigma receptivity refers to the ability of stigma to support compatible pollen adhesion, germination and tube growth.

Functionality and trade-offs

Together with ovule and pollen longevity, stigma receptivity is fundamental to the efficacy of plant reproduction, through its decisive role in the effective pollination period (Sanzol & Herrero, 2001; Williams, 1965). Shorter periods when stigma remains receptive limits the success of pollination and directly affects production of seeds (Egea & Burgos, 1992; Sanzol & Herrero, 2001).

Sources of variability

There is high species-specific variability in the onset of stigma receptivity (before, during, or after anther dehiscence) and its duration (from a few minutes in grasses to a few weeks in orchids; Heslop-Harrison & Shivanna, 1977; Dafni *et al.*, 2000). Yet, the underlying ecological and evolutionary reasons for that variability have not been intensively studied.

Stigma receptivity has been shown to be affected by several factors, including flower age, flower longevity, flower nutritional status, pre-flowering temperatures, the time of the day, the presence or absence of stigmatic exudates (Arroyo *et al.*, 1985; Egea & Burgos, 1992; Nepi & Pacini, 1993; Dafni *et al.*, 2000; Sanzol & Herrero, 2001; Souza *et al.*, 2016).

Methodology

There are three main tests to determine stigma receptivity. The first and easiest way to infer stigma receptivity is by observing morphological changes in the stigma. For example, in species with lobed stigma, the lobes are closed in the non-receptive stage but open out when the stigma becomes receptive (Kearns & Inouye, 1993; Shivanna &

Tandon, 2014). The presence of exudates in wet stigmas and colour changes may also indicate that stigma is in the receptive stage (Dafni *et al.*, 2000).

An alternative but more laborious approach is to determine stigmatic receptivity by staining or testing for enzymatic activity, particularly of esterases, oxidases, peroxidases, and phosphatases on the stigma surface (Dafni & Maues, 1998; Dafni *et al.*, 2000). Although this approach has been widely used, great care should be taken while interpreting the results, as the presence of enzymes does not necessarily indicate stigma receptivity. Please refer to Dafni *et al.* (2005b) for further details.

In the third and the most accurate but time-consuming test, the stigma receptivity is determined by hand pollination of flowers at different times, followed by estimating seed set after the pollination (Kearns & Inouye, 1993; Dafni *et al.*, 2005b). This approach assumes that conspecific, compatible pollen will germinate on receptive stigmas, and growing pollen tubes will fertilise ovules. It should be noted that, in practice, each method of assessing stigmatic receptivity must be calibrated for each plant species and, if possible, by comparison with *in vivo* pollen germination on the stigma (Dafni *et al.*, 2000; Shivanna & Rangaswamy, 2012).

1.1.9 Self-incompatibility

Trait description

The trait defined is the ability of plants to undertake (self-compatible) or prevent (self-incompatible) self-fertilisation in hermaphrodites by various mechanisms to promote outbreeding and maximise variability.

Functionality and trade-offs

Self-incompatibility (SI) is estimated to be present in at least half of all angiosperm species and is suggested to be one of the most effective pre-fertilisation barriers preventing inbreeding in flowering plants (Barrett, 1998; Sage *et al.*, 2005). It may result from a variety of mechanisms, including (i) lacking adherence of self-pollen to stigma, (ii) blocking of pollen germination on the stigma, (iii) arresting pollen tube growth in the style, and (iv) fruit abortion following fertilisation (Kahn & Morse, 1991; Kearns & Inouye, 1993; De Nettancourt, 1997).

The ability to sire seeds after being self-pollinated can provide reproductive assurance when pollinators are scarce or unreliable (i.e., selfed progeny are better than no progeny) or where populations are small and have a gene transmission advantage over outcrossing genotypes (Bond, 1994; Kalisz & Vogler, 2003; Moeller 2006). Therefore, self-incompatibility (SI) is crucial for plant population stability and resilience. Furthermore, selfing is an important energy- and resource- saving strategy. It often costs less to produce selfed seeds because fewer resources are expended to attract and reward pollinators (Good-Avila *et al.*, 2008). Nevertheless, these environmental advantages of selfing are potentially counterbalanced by its effects on the patterns of genetic transmission and the organisation of genetic variation in populations (Good-Avila *et al.*, 2008). Self-compatible plants have restricted heterozygosity due to inbreeding depression, and gene migration through pollen flow reduces the variation within the populations and increases the variation among populations. In contrast, SI promotes gene flow, reducing the likelihood of microgeographic differentiation and population substructuring (Kearns & Inouye, 1993).

The trait is often coupled with the species' colonisation ability; self-compatible species are more likely than self-incompatible species to establish new populations after long-distance dispersal (Baker, 1955; Hao *et al.*, 2011; Pannell *et al.*, 2015). Moreover, it is an important trait that may indicate species sensitivity to anthropogenic pressure, such as, fragmentation. A lower level of SI has been reported for disturbed habitats, probably due to lower pollinator abundance, smaller and sparser plant populations that attract fewer pollinators and contain fewer potential mates or some interaction between these two direct consequences of disturbance (Eckert *et al.*, 2010; Young *et al.*, 2012).

Sources of variability

Although SI is often assumed to be a binary trait, it is becoming increasingly clear that numerous intrinsic and extrinsic factors can influence the level of SI expression in a given species. At the individual plant level, these include: the types of S-alleles and their genetic backgrounds, naturally occurring rates of S-allele mutations, ambient temperature and humidity, floral age, the number of developing fruits on the plant, the physical age of the individual plant, and the presence of cross (mentor)-pollen (Sage *et al.*, 2005; Good-Avila *et al.*, 2008)

Within a species, SI has been found to be related to environmental favourability within a species' range. The loss of SI frequently occurs following population bottlenecks when the cost of inbreeding depression declines and reproductive assurance becomes critical to the persistence of small, isolated populations, e.g., at species range limits (Moeller, 2006; Darling *et al.*, 2008; Sutherland *et al.*, 2018). Pollinator density can also affect SI at the population level: populations that lack specialist pollinators tend to be selfing (Moeller, 2006). Population density has also been shown to affect the level of SI expression in plant populations (Murawski & Hamrick, 1991; Ward *et al.*, 2005). Together, these studies indicate that self-compatibility is, at least in some species, a plastic trait (Ferrer *et al.*, 2009; Sutherland *et al.*, 2018). Among species, SI follows the same ecological, geographical, and phylogenetic trends (Ward *et al.*, 2005; Allen & Hiscock, 2008).

Methodology

Usually, SI is assessed through pollination experiments conducted in the field or under controlled conditions (e.g., common garden experiment). The standard approach has been to estimate seed production without cross-pollen. This can be achieved by caging or bagging several flowers before anthesis (for detailed techniques, see Kearns & Inouye (1993); Dafni *et al.* (2005a)). It should be noted that some species demonstrate cryptic self-incompatibility, that is, lack of seed production when pollinated with pure self-pollen, but limited seed production when mixed pollen loads are applied (Kearns & Inouye, 1993).

Since SI is a plastic trait (see above), at least in some species, we recommend using the SI index, which is a continuous variable that is defined by the equation:

$$SIindex = \frac{SS_i}{SS_o},$$

where ss_i = the mean seed set after self-pollination in a plant and ss_o = the mean seed set after cross-pollination in the same plant (Butcher *et al.*, 2011). The level of SI expression could be identified based on the SI index groups, including (i) strongly self-incompatible (SI index = 0), (ii) self-incompatible (0 > SI index < 0.149), (iii) partially self-incompatible (0.15 ≤ SI index < 0.49) and (iv) self-compatible (SI index ≥ 0.5; Zapata & Arroyo, 1978).

An alternative but more laborious approach is to observe the difference during fertilisation (pollen germination, tube growth, and embryo development) in selfed vs outcrossed flowers (Sage *et al.*, 2005). Finally, information on self-incompatibility for some taxa or floras is available in the literature (East, 1940; Fryxell, 1957) or trait databases, such as BioFlor (Klotz *et al.*, 2002).

1.1.10 Pollen thermotolerance

Trait description

The trait refers to temperatures at which pollen germination (PG) and pollen tube growth (PTG) are initiated (minimal or basal temperature), their optima (maximum germination percentage for germinating pollen grains and longest pollen tubes for germinated pollen grains; optimal temperature) and is limited due to high-temperature stress (maximal temperature).

Functionality and trade-offs

Pollen, the male gametophyte, is the most temperature-sensitive part of the plant sexual reproduction cycle due to its comparatively small size, haploid set of chromosomes, lack of protective tissue, and direct exposure to the environment (Bedinger, 1992; Hedhly, 2011; Pacini & Dolferus, 2016). In general, temperature stress restricts both PG and PTG, reducing the number and quality of pollen produced (Rosbakh *et al*., 2018). Therefore, the trait reflects male gametophyte adaptations to ambient temperatures and its potential reaction to any deviation from the typical temperatures during its development.

Sources of variability

Variation in the thermal requirements for PG and PTG is strongly associated with the temperature conditions of a species' habitat. In general, species growing in warmer climates tend to have higher minimal and optimal temperatures of both pollen germination and pollen tube growth (Rosbakh & Poschlod, 2016; Wagner *et al.*, 2016). Based on this link, the trait has been suggested to be a good predictor of species occurrences along temperature gradients (latitude, elevation; Pigott & Huntley, 1981; Rosbakh & Poschlod, 2016).

Little is known about variability in pollen thermotolerance at the population level. The current status of pollen research is that there is a plastic component of the trait, but the range of this component of the phenotype may be limited within a given gametophyte

(Rosbakh *et al*., 2018). Species-specific responses of PG and PTG to temperatures have been widely reported (McKee & Richards, 1998; Harder *et al.*, 2016; Wagner *et al.*, 2016). The pollen thermotolerance varies widely from around 0 °C for alpine and nival plants (Steinacher & Wagner, 2012) to 70 °C in *Eucalyptus rhodantha*, an inhabitant of extremely hot climates (Heslop-Harrison & Heslop-Harrison, 1985).

Methodology

Because PG and PTG occur largely inside the pistil, ecophysiological investigations of these two processes *in vivo* are rather difficult. Therefore, *in vitro* germination techniques have been the most used technique in pollen research (Kearns & Inouye, 1993; Shivanna & Rangaswamy, 2012), providing a simple experimental method to study the pollen response to temperature stress and other physical and chemical factors.

The trait measurement workflow generally consists of fresh pollen collection, pollen hydration, pollen cultivation at the temperatures of interest, pollen fixation, and sample scoring. To obtain germinable pollen, fresh flower buds (1-3 days before anthesis) are collected from several random individuals growing at a distance from each other. After collection, the buds can be sterilised with 96% ethanol. The anthers are removed manually and left to dry for 2–3 d at room temperature in a desiccator filled with silica gel (relative humidity approx. 30 %) to ‘after ripen’. To extract the pollen grains, the dried anthers are subsequently crushed into small pieces and passed through a 200-µm sieve. Alternatively, individuals with closed flower buds at the same stage can be collected and kept in the lab until the onset of anthesis; the freshly shed pollen can be then used directly for further cultivation. This approach is particularly suited for species with short-lived pollen.

Several methods are available to study *in vitro* PG and PTG, including (i) hanging drop culture, (ii) sitting drop culture, (iii) suspension culture, (iv) surface culture, (v) cellophane membrane, (vi) alcain blue method (Shivanna & Rangaswamy, 2012). We particularly encourage the use of suspension and surface cultures due to their

simplicity and affordability. In both methods, pollen is cultivated in liquid aqueous (some gymnosperms) or sucrose solutions or solidified with gelatin, agar or phytogel sucrose solutions (see Tushabe & Rosbakh (2021) for further details on pollen germination media). Appropriate sucrose solutions range from 2% to 40% depending on the optimum for the species, which must be established empirically (Kearns & Inouye, 1993). Boron, calcium, potassium, and some other macro- and micro-elements are also required for pollen tube growth (Brewbaker & Kwack, 1963; Roberts *et al.*, 1983; Hodgkin & Lyon, 1986; Leduc, 1990; Musil, 1996). It should be noted that the cytology of pollen at the time of shedding (2 or 3 cells) has an important relationship with in vitro germination behaviour. Generally, 2-celled pollen has longer viability and can germinate better on a simple medium than 3-cell pollen (Dafni *et al.*, 2000; Shivanna & Tandon 2014). Please refer to Brewbaker (1967) for detailed information on pollen cytology at a family level.

To avoid the pollen grains bursting, a hydration procedure is used, exposing the pollen to the atmosphere with 80% air humidity at 5 °C for at least 30 minutes (Connor & Towill, 1993). After hydration, the pollen is either mixed with the appropriate pollen germination media or distributed over Petri dishes with solid media and maintained at the test temperatures, for example, on a thermogradient table or in germination chambers. Light is not necessary for PG and PTG. The pollen cultivation time should be defined in a preliminary test; it should be long enough to allow pollen tubes to grow (pollen of some species, especially at extremely low and high temperatures, might take several hours to germinate and grow). On the other hand, it should not be too long, as overlong pollen tubes are hard to measure (see below). We recommend cultivating the pollen in at least four replicates per each test temperature.

The pollen cultivation is terminated by pipetting of formalin acetic alcohol (9 parts 99% ethanol, 1 part formaldehyde, 1-part glacial acetic acid) into the test vessels; the ‘fixed’ samples can be stored in a fridge before measurement.

The responses of cultured pollen to the test temperature(s) are assessed as average pollen germination rate (the proportion of germinated pollen grains to the total

number of pollen grains) or average pollen tube length. The pollen grain is considered germinated when the length of its tube is more than two diameters of the pollen grain. PG is observed in a few randomly selected microscopic fields; scoring 200-300 pollen grains per replicate for each treatment should be sufficient. The length of pollen tubes can be measured directly with an ocular micrometre or by photographing the microscopic fields followed by image analysis, for example, with the help of free and open-source software 'ImageJ' (Abramoff *et al.*, 2004). Measuring 20-25 tubes per replicate will provide sufficient data.

To quantify minimum (T_{min}), optimum (T_{opt}), and maximum (T_{max}) temperatures of PG and PTG, the generalised plant growth model (Yin & Kropff, 1996) is fitted to PG rate and PTG length versus test temperatures. An iterative optimization approach implemented in MS Excel or R software is used to estimate the model parameters with residual sums of squares as the best model fit.

$$R = R_{max}\left[\left(\frac{T - T_{min}}{T_{opt} - T_{min}}\right)\left(\frac{T_{max} - T}{T_{max} - T_{opt}}\right)^{\left(\frac{T_{max} - T_{opt}}{T_{opt} - T_{min}}\right)}\right]^{a},$$

where T_{min}, T_{opt}, and T_{max} are the minimum, optimum, and maximum temperatures for PG rate or pollen tube length (R), T is the temperature at which germination and tube growth were studied, R_{max} is a maximum value of R at T_{opt}, and a is coefficient defining the curvature of the relationship.

2. Fruit

2.1 Fruit attraction/defence

2.1.1 Fruit size

Trait description

Fruit size is defined as the overall dimension of an individual fruit. Fruit size can be estimated in terms of mass to inform resource allocation to reproduction (Ågren, 1988) or length and width to evaluate the probability of seed ingestion by frugivores. Fruit size is an important component of morphological trait-matching in plant-frugivore networks (Bender *et al.*, 2018).

Functionality and trade-offs

Fruit size is an important trait in cultivated species for which knowledge is more advanced. In native systems, fruit size has long been shown to be an important trait shaping both the probability of fruit removal and that of frugivores will defecate, regurgitate or spit out seeds. For example, frugivorous birds with broad gapes consume larger fruits than narrow-gaped birds, but small fruits can be consumed by birds with a wide range of gape widths (Wheelwright, 1985). The probability that seeds are ingested or spat out also depends on fruit size in Neotropical monkeys (Fuzessy *et al.*, 2018). Because fruit size is related to removal probability, fruit size is strongly linked to plant fitness (Fontúrbel & Medel, 2017).

Sources of variability

Fruit size and weight are strongly influenced by multiple genetic factors (Pan *et al.*, 2020) and by selection imposed by frugivore assemblages (Lord, 2004; Lim *et al.*, 2020). In native species, fruit size varies among individuals, populations and years of fruit production (Wheelwright, 1993; Guerra *et al.*, 2017) and may not be constrained by phylogeny (Lord, 2004). As expected, fruit diameter and fruit mass are strongly correlated (Rojas *et al.*, 2022), but fruit size also correlates with fruit crop size, fruit

tannins and sugar content, total seed mass, seed number and seed size (Sadras, 2007; Fontúrbel & Medel, 2017; Ordano *et al.*, 2017; Rojas *et al.*, 2022).

Methodology

Sophisticated methods to estimate fruit size are widely used for crop plants (e.g. Gongal *et al.*, 2018), but a simple, low-cost and practical method is provided here that can be implemented under field conditions with inexpensive equipment. This method applies to both dry and fleshy fruits. First, only ripe fruits (fruits developed to the point of harvesting) should be collected, preferably during the fruiting peak of the study species. The number of individuals sampled depends on the study goals, but ten randomly chosen plants are suggested as the minimum sample size. Five randomly selected fruits should be collected for each individual to estimate fruit size and mass. Fruit diameter or fruit width is the relevant trait to be measured in the case of fleshy-fruited species, given that this trait determines the probability of ingestion by frugivores (Blendinger *et al.*, 2016). The maximum fruit equatorial diameter should be measured with a calliper to the nearest 0.1 mm in recently collected fruits (Lord, 2004; Rojas *et al.*, 2022). If storage is needed, fruits can be kept inside paper or plastic bags to prevent water loss for a maximum period of 12 hours. For measuring fruit dry mass, the fleshy pulp of each individual fruit is separated from the seed(s) and placed into paper bags. The content should be oven-dried for five days at 70°C and weighed.

Units

Fruit size can be expressed as millimetres when addressing length or mg when estimating dry mass.

2.1.2. Fruit crop size

Trait description

Fruit crop size is the total number of fruits produced by an individual plant during a fruiting event. Fruit crop size represents a conspicuous signal advertising the amount of reward to visually driven interacting partners (Palacio & Ordano, 2018).

Functionality and trade-offs

It has long been hypothesised that fruit crop size affects visitation rates by frugivores and fruit removal rates, the so-called fruit crop size hypothesis (Snow, 1971). Larger fruit crop sizes attract more dispersers compared to smaller fruit crop sizes, which is expected to result in higher fruit removal rates (Snow, 1971, McKey, 1975, Howe & Estabrook, 1977). Visitation rate and fruit removal rates are the two subcomponents of the quantitative component of seed dispersal effectiveness (Schupp *et al.*, 2010). The fruit crop size hypothesis can be decomposed to (i) the total number of fruits removed by frugivores and (ii) the proportion of fruits removed by frugivores. Fruit crop size is also related to the diversity of frugivores consuming fruits (Guerra *et al.*, 2017). The fruit crop size hypothesis has been generally accepted (Palacio & Ordano, 2018).

Sources of variability

Fruit crop size varies among individuals, populations and years (Ortiz-Pulido & Rico-Gray, 2000; Guerra *et al.*, 2017). Temporal variation is especially important in species with mast fruiting (Kelly & Sork, 2002). Fruit crop size correlates with plant height (Flörchinger *et al.*, 2010), fruit size (Ordano *et al.*, 2017), seed size (Sadras, 2007), dry seed weight, fruit sugar content, and plant fitness (Fontúrbel & Medel, 2017). A recent meta-analysis has shown that as crop size increases, a greater proportion of the available fruit remains on the plant, decreasing the relative plant fitness due to a reduction of its quantitative component (Fontúrbel & Medel, 2017).

A potential confounding factor is related to a trait named fruit neighbourhood (the number of fruits surrounding the parent plant), which may have stronger effects on

frugivore visitation rates (Saracco *et al.*, 2005) and resource specialization by frugivores (Guerra *et al.*, 2017) compared to fruit crop size (but see Blendinger & Villegas, 2011).

Methodology

Methods to estimate fruit crop size depend mainly on plant growth form and duration of fruiting phenology. For herbs and small shrubs, counting the total number of fruits may be feasible, but estimates of the total number of fruits produced are needed for larger trees. The number of individuals depends on the study goals, but we suggest a minimum of ten randomly selected individuals per population/species. The total number of fruits should be determined for each individual during the fruiting peak.

For large plants, it may be impractical to count each fruit. Therefore, one can select five infructescences (one per cardinal point and that from the apex), count the number of fruits per infructescence, and the number of infructescences per plant. Fruit crop size can be estimated as the average number of fruits per infructescence multiplied by the number of infructescences. Although this estimation method is practical, it assumes a linear relationship in fruit distribution within a plant (Palacio *et al.*, 2016). Alternatively, for tree species with very large fruit crops, one should count fruits in selected branches and then extrapolate the counting to the whole plant according to the number of equivalent branches or divide the canopy into quadrants, counting the number of fruits in one of them and extrapolating to the whole canopy.

For estimation of fruit removal, the number of fruits at the onset (the day when the first open ripe fruit was found) and end (the day when the last fruits were found) of the fruiting season should be counted (Ortiz-Pulido *et al.*, 2007) Seed traps should be installed beneath the parental plant to account for the fruits dropping off to the ground without being effectively removed (Christianini and Oliveira, 2010).

2.1.3 Dry pulp-dry seed mass ratio

Trait description

The ratio between the dry pulp mass and the total seed dry mass.

Functionality and trade-offs

Mutualistic interactions between fruits and frugivores often involve a balance of cost and benefits (Herrera & Pellmyr, 2002). From the plant perspective, a major benefit of having seeds dispersed by frugivores is spreading recruitment across time and space. The costs are related to allocating energy to the production of structures and rewards that attract frugivores that will consume the pulp and defecate or regurgitate the seeds. From the frugivore's perspective, the fruit pulp is the benefit derived from the interaction providing energy and nutrient intake. For frugivores, seeds represent the costs as they do not provide frugivores with energy or nutrients. Evidence supports the idea of co-evolution between fruits and frugivores (Fleming & Kress, 2011), so that each group may shape each other's traits in complex ways (Guimarães *et al.*, 2011).

Decreasing allocation to fruit pulp while keeping total seed mass constant tilts the pulp:seed mass ratio in favour of plants, maximising the benefits and reducing the costs. Conversely, increasing allocation to fruit pulp increases attraction and visitation rates but also increases the cost-benefit relationship. Therefore, from the plant perspective, selection should favour strategies that increase frugivore attractiveness through the lowest allocation of resources to fruit pulp. However, frugivores have evolved mechanisms to detect rewards in fruits and, in turn, are prone to select fruits that otherwise provide optimum resources from their perspective. Pulp:seed mass ratio informs allocation to dispersal-related structures. It may affect visitation rates and fruit consumption, key variables driving seed dispersal effectiveness (Schupp *et al.*, 2010).

Sources of variability

Fruits and diaspores have high morphological variation (see section **3.2.1 Dispersule type/syndrome**). This trait applies only to endozoochorously dispersed fleshy fruits and dry fruits with associated fleshy structures.

Methodology

For this trait, it is important to consider whether the dispersal unit comprises the fruit and other accessory structures (the diaspore). We recommend sampling at least five diaspores from 20 individuals. Ripe, healthy diaspores should be sampled, preferably at the fruiting peak. Individual diaspores are the unit of measurement, so each diaspore should be labelled and stored separately. They should be immediately taken to the laboratory for measurements to avoid rotting. If it is not possible to analyse the material immediately, then diaspores can be frozen at -20 °C.

Fruit pulp should be carefully separated from the seeds. In the case of multi-seeded fruits, all seeds within the fruit should be counted and washed in running water for 5 minutes to remove any residual pulp. The residual pulp should also be weighed. Fruit pulp and seeds should be dried separately in paper bags at 80 °C for at least 72 h (or until equilibrium mass in very large or hard-skinned seeds) and weighed.

The trait value is obtained by dividing the total pulp dry mass by the total seed dry mass. This trait can be expressed on a dry mass basis (mg/mg) or a number basis (mg/seeds). If it is not possible to express data on a dry mass basis, one can consider the pulp:seed ratio on a wet weight basis, which can be convenient under some circumstances.

2.1.4 Fruit colour

Trait description

Fruit colour is a characteristic of a vertebrates' visual perception. Colours can be described through colour categories or can be identified numerically along the colour space (a specific organization of colours).

Functionality and trade-offs

After more than a century of investigation, the adaptive value of fruit colour remains a contentious issue. Various aspects shape fruit colour (e.g. biotic pressures, abiotic conditions and genetic factors). Still, the main hypothesis on the evolution of fruit colouration is that the colour conspicuousness of fruits determines the attraction of frugivores and, thus, the fitness of plants (Renoult *et al.*, 2017). Fruit colour is particularly relevant for animal-dispersed seeds, particularly birds and mammals that disperse seeds internally (Valenta & Nevo, 2020). Fruit colour is a major communication channel between fruits and animals and is thought to have evolved as an honest signal to indicate nutrient rewards or to manipulate frugivore behaviour (Schaefer *et al.*, 2014; Stournaras *et al.*, 2015). Although fruits are intended to attract seed dispersers, seed predators may also rely on fruit colour as cues for finding resources (Ordano *et al.*, 2017). Colour change should be a proxy for when seeds are mature and ready to be dispersed. However, delays in visual signals may be adaptive, limiting the dispersal of unviable seeds (Cruz-Tejada *et al.*, 2018).

There is evidence that the contrast between fruit displays and their background is a more reliable visual cue for dispersers than fruit colour *per se* (de Camargo *et al.*, 2015; Nevo *et al.*, 2018b). Since fruit colour is determined by accumulating secondary metabolites (mainly phenolics), fruit colour is also related to the defence against microbial pathogens (Valenta *et al.*, 2018). Finally, in green fruits, colour is associated with the ability to photosynthesise when ripe (Cipollini & Levey, 1991) or protection against pathogens (de Camargo *et al.*, 2015).

Applied aspect

Fruit colours are frequently associated with different seed dispersers (Valenta *et al.*, 2018). Manipulating fruit colour using artificial fruits can be used to maximise outcomes in ecological restoration (Gagetti *et al.*, 2016).

Sources of variability

Fruit colour commonly changes along fruit ontogeny. Fruit colour is related to fruit scent (Korine *et al*., 2000; Valenta *et al.*, 2013), nutrient rewards (Valido *et al.*, 2011, Schaefer *et al.*, 2014), and fruit morphology (Valido *et al.*, 2011; see also section **2.1.3 Dry pulp-dry seed mass-ratio**). Fruit colour is constrained by the chemical properties of pigments, probably limiting fruit colour diversity (Stournaras *et al.*, 2013).

Methodology

There are multiple methods to measure fruit colour. We recommend measuring fruit colour in at least 20 fruits and the leaf background per species. Measurements are made using a spectrometer and a standardised light source coupled with a Deuterium-Halogen lamp mounted inside a matt black plastic tube to exclude ambient light. Reflectance is measured as the proportion of a standard white reference tile (Top Sensor Systems WS-2). The angle of illumination and reflection is fixed at 45° to minimise glare. Spectra are processed with SpectraWin software and calculated in 5-nm intervals from 300 to 730 nm (Schaefer *et al.*, 2006).

The measurement output is Munsell values of hue value and chroma (HVC) or values of red, green, and blue channels in the RGB system. Functions to convert data have been developed and are available in the R packages '*munsellinterpol*' and the soil package '*aqp*' using the '*munsell2rgb*' and related functions. RGB colours can also be used in multivariate analyses for comparative studies. Software packages are available to analyse spatial colour patterning (van den Berg *et al.*, 2020).

2.1.5 Fruit scent

Trait description

Fruit scent is the fragrance that arises from the accumulation of volatile compounds produced by the fruits that act as a signal to animals that consume fruits and/or seeds.

Functionality and trade-offs

The adaptive value of fruit scent is understood as olfactory signals that indicate fruit presence and ripeness. Fruit scent is relevant for attracting smell-oriented foraging animals, like bats and primates (Nevo & Ayasse, 2019), but also for a broad range of mammals (Herrera, 1989). Fruit scent plays a minor role in attracting visually-oriented seed dispersers such as birds (Nevo & Ayasse, 2019). Fruit scent is usually expressed as plant volatile organic compounds (VOCs), which are shaped by the accumulation of secondary metabolites (Crozier *et al.*, 2006; Rodríguez *et al.*, 2013), including terpenoids, fatty acid derivatives, aromatic compounds, and N- and S-containing compounds (Nevo & Ayasse, 2019). VOCs evolved to attract legitimate seed dispersers, but they also act as cues by seed predators and microbial pathogens and play roles in fruit defence (Rodríguez *et al.*, 2013; Nevo *et al.*, 2018a; Nevo & Ayasse, 2019).

Applied aspect

Fruit scent can be used to attract and capture fruit-eating bats inside forest remnants. This technique may potentially increase seed rain at specific locations, which is particularly promising for restoration projects (e.g., Bianconi *et al.*, 2007). Knowledge of fruit VOCs could be used in agriculture to generate attraction or repellency to pests and resistance to pathogens in fruits (Rodríguez *et al.*, 2013).

Sources of variability

Fruit scent evolved as a signal to indicate fruit ripeness. Thus, it is clear that fruit scent changes over fruit ontogeny, with a smell more pronounced when fruits are ripe. Fruit scent is one component of the seed dispersal syndrome. Thus, fruit traits, including

colour (Valenta *et al.*, 2013), nutrient content (Nevo *et al.*, 2019), and morphology (Herrera, 1989), usually co-vary.

Methodology

Ripe fruits should ideally be collected from at least five individuals and taken immediately to the laboratory inside completely sealed plastic bags. In the lab, fruits can be pooled together as a single sample. The scent is sampled using a semi-static headspace procedure (Nevo *et al*., 2018a). Fruits are stored in sampling bags tightly closed with a zip tie on one end and the other tightened around a Teflon tube on which a chromatoprobe scent trap is attached. The chromatoprobes contain 1.5 mg of Tenax, 1.5 mg of Carbotrap, and 1.5 mg of Carbosieve III trapped between layers of glass wool. Samples are left in the chamber for 30 min, after which the air in the bag is pumped for 1 minute onto the trap using a membrane pump at 200 ml/min. The scent is then left to build up for another 1.5 hours, after which the air in the bag is pumped onto the same probe for 10 min. The probe is then stored in a 2-ml glass vial sealed with a Teflon cap and stored at −20 °C. Control samples are collected by applying the same procedure with empty bags (Nevo *et al.*, 2018a). Samples are analyzed using gas chromatography (see details of chemical analyses in Nevo *et al.*, 2018a, 2019).

Fruit scent is expressed as the relative amounts of chemical compounds that allow species comparisons with fruits of different sizes because animals tend to perceive fragrance as mixtures rather than individual compounds (Nevo *et al.*, 2019).

2.1.6. Fruit chemical compounds

Trait description

Fruit pulp (the product of mesocarp development) content comprises the diversity and quantification of primary (proteins, carbohydrates, and lipids) and constitutive secondary metabolites (e.g. flavonoids, terpenoids) in the pulp of flesh fruits (Cipollini & Levey, 1991). Depending on the study goals, minerals may also be included (Levey & Martínez del Rio, 2001; Blendinger *et al.*, 2015).

Functionality and trade-offs

The main function of fruits is to attract primary and secondary seed dispersers. However, the nutritional rewards that attract beneficial consumers also attract consumers who kill seeds instead of dispersing them (Tewksbury *et al.*, 2008; Baldwin *et al.*, 2020). The relative proportion of different metabolites in fruit pulp content is associated with specific dispersal agents (Baker *et al.*, 1998). Primary metabolites (sugars, lipids, and proteins) are involved in the attraction and nutritional rewards of seed dispersers. In contrast, secondary metabolites (phenols, terpenes, alkaloids, and saponins) are involved in multiple functions, including disperser attraction (see section **2.1.4 Fruit colour**). Secondary metabolites in fruits serve multiple purposes, which are contingent on the study system (Cipollini & Levey, 1991; Tewksbury *et al.*, 2008). Secondary metabolites: (i) provide foraging cues (e.g., colours, odours, and flavours) that can be readily recognised and associated with rewards by frugivores; (ii) inhibit seed germination within fruits; (iii) induce frugivores to leave the fruiting plant early in a foraging bout, thus dispersing seeds away from the parent plant; (iv) act to alter seed passage rates, either permitting more rapid seed passage via laxative effects or slowing passage rates via constipating effects; (v) are potentially damaging to seed predators while remaining non-toxic to legitimate seed dispersers; and (vi) defend against microbial pathogens and invertebrates.

Applied aspect

Sugar- and water-rich fruits are usually produced by species with generalist dispersal systems, which attract a wide diversity of frugivores (McKey, 1975). From the metabolic perspective, these fruits are easier to digest for birds than lipid-rich fruits (Karasov & Martínez del Rio, 2007). Therefore, knowledge of fruit pulp content can be useful in prioritising species in ecological restoration programs to attract a high diversity of seed dispersers, which, in turn, would deliver more seeds to target restoration sites.

Sources of variability

Fruit chemistry changes during fruit ontogeny. While unripe fruits are chemically protected from pathogens and seed predators, ripe fruits accumulate more rewards (Cipollini & Levey, 1991). Therefore, the stage at which fruits should be sampled depends on the research question, but it should be clear that fruit pulp content of unripe fruits cannot be used to assess the fruit chemistry of ripe fruits and vice-versa.

Nutritional traits (e.g., lipids) can be positively correlated with some fruit colours (e.g., hue and chroma) and negatively correlated with fruit size (Valido *et al.*, 2011). Relevant trade-offs include the accumulation of sugars vs lipids (Mckey, 1975), defence against pathogens vs reduction in disperser preference (Maynard *et al.*, 2020), and carbohydrate concentration vs pulp/seed ratio (Janson *et al.*, 1986). Many nutritional traits are phylogenetically conserved (Jordano, 1995).

Methodology

Various analytical methods are available to sample phytochemicals (e.g., Karasov & Martínez del Rio, 2007), including commercial assay kits to measure fibre, starch, and sucrose contents. For simplicity, we provide classic examples of protocols widely used in plant science and ecology (see Schneider *et al*., 2021). These methods are relatively quick, inexpensive, and broadly accessible. However, we acknowledge that our list may not cover the needs of all studies. For example, we provide protocols for sampling lipids in fruit pulp but do not address issues related to determining each type of fatty acid. Please refer to Schneider *et al*. (2021) for non-targeted metabolomics approaches.

We suggest the sample material contain healthy fruits with no signs of pathogens or predators from at least ten individuals randomly selected in the population. At least 20 g (fresh mass) of each fruit species should be collected. Fruit pulp should be separated from seeds, which should not be included in the analysis. Depending on the metabolite of interest, fruits should be frozen, dried, or lyophilised.

To calculate dry pulp mass, fruit samples are dried at 60°C to constant weight for four days in a forced-air oven. Lipids are analysed following Bligh & Dyer (1959). Total proteins are estimated from total nitrogen determined using a digest method or elemental analyser and then converted into crude protein using an appropriate conversion factor (Mariotti *et al.*, 2008). Ash proportion is determined by incinerating the samples in a muffle furnace set at 550°C until the weight stabilises. Total carbohydrates are estimated by weight difference (Pizo & Oliveira, 2001; Valido *et al.*, 2011).

Total polyphenols are determined using Folin–Ciocalteu's reagent (Singleton *et al.*, 1999). The reaction mixture contained 20 µL of each preparation, 2 mL of distilled water, 200 µL of Folin–Ciocalteu reagent and 800 µL of sodium carbonate (15.9% w/v). Absorbance is measured at 765 nm (Blendinger *et al.*, 2015). For minerals, 0.20 g of the lyophilised samples are mixed with sub-boiling HNO3 (8 mL) in a quartz glass and maintained for 45 min in a microwave oven at 280 °C and 75 bar. Then, type 1 water is added until a volume of 25 mL is reached, and the disintegrated material is filtered through a 0.45-µm filter. The Na, K, Ca, Fe, Mg, and P levels of these solutions are determined by inductively coupled plasma (ICP) or inductively coupled plasma mass spectrometry (ICP-MS; Blendinger *et al.*, 2015).

Values for each metabolite are expressed on a dry mass basis. For minerals, values are expressed as milligrams per 100 g of dry weight.

3. Seed

3.1 Seed attraction/ defence

3.1.1. Seed colour

Trait description

Seed colour, including those beyond humans' visible perception, characterises how seeds are seen by dispersers and predators. Colours can be described using simple categories (brown, black, white, etc.) or numerically using a colour space system such as the widely used RGB.

Functionality and trade-offs

A major role of seed colour is to hide seeds from predators, a function known as crypsis and well documented experimentally, e.g., for *Acmispon wrangelianus* and *Pinus sylvestris* (Nystrand & Granström, 1997; Saracino *et al.*, 2004; Porter, 2013). However, contrasting colours, such as in bright red seeds of *Abrus precatorius,* also signal toxicity and contribute to predation avoidance (Galetti, 2002). Seed colours can also modify how the seed embryo and endosperm perceive light (Widell & Vogelmann, 1985). Black seeds (e.g., *Pancratium maritimum*) can also affect seed temperature compared to the surroundings by increasing light absorption.

Brightly coloured parts of seeds, such as arils, may also serve to attract primary or secondary seed dispersers (Nakashima *et al.*, 2008). In many cases, a single seed can exhibit several colours linked to, for example different functions of seed parts, such as black seeds and white elaiosomes in seeds of many *Acacia* species or by increasing the cryptic role in seeds of many species of *Fabaceae*.

Fruit and seed colouration patterns may therefore serve similar roles in attracting potential seed dispersers or mimicking toxic or edible fruits, seeds or other living objects (Midgley *et al.*, 2015; Myczko *et al.*, 2015; Pizo *et al.*, 2020). In these cases, we refer you to the section on fruit colour (see section **2.1.4 Fruit colour**). Convergence in

fruit and seed function is most evident in the case of fleshy seed integuments, observed in some gymnosperms and basal angiosperms, and the anatomical seed serves roles most often adopted by fruit structures.

Seed colouration and its contrast with soil colour has been shown to modify seed predation (Saracino *et al.*, 2004; Porter, 2013), and attract frugivores (Pizo *et al.*, 2020); in this context, data on soil colour and its variation is also needed. The accumulation of polyphenols mainly determines seed colour, so it may also correlate with protection against pathogens and longevity in the soil. Indirect effects of seed colour caused by phytomelanins have been found to protect against predation and desiccation in Asparagales and Asteraceae (Pandey & Dhakal, 2001).

Sources of variability

Important intra- and inter- specific differences in seed colour can be used to identify seeds of different species or even characterise new taxa (Bacchetta *et al.*, 2008; Dayrell et al., 2023b). Thus, seed colour appears to have a genetically fixed component that varies gradually between individuals, populations, and species. Seed colour can have a practical role in identifying seeds or separating unripe from ripe or dead from viable seeds. Still, heteromorphism has been documented in which seeds with different colours can have different properties (Gairola *et al.*, 2018). Seed colour may be correlated to variation of other seed traits such as mass, dormancy, and germination (Paolini *et al.*, 2001). The water content of the seed, seed ageing, and gut passage may also alter seed colour (Vázquez-Yanes & Oronco-Segovia, 1986). Since ripening processes and water content during collection vary over the years, it can be expected that there is some year-to-year variation in seed colours.

Methodology

Because of differences in light reflection from dry and wet surfaces, standard colour measurements are made on dry seeds (3% RH), but depending on context and questions, moist seeds might need to be measured. In any case, moisture state (wet or dry) or content should be reported.

Seed colour measurements need to be adapted to their purposes and may, therefore, differ in information content. Typically, the wavelengths of interest include colours visible to humans but may extend to UV or infrared light if colours visible to birds or insects need to be measured. Light emitted by seeds varies over the full spectrum of light wavelengths. Still, most studies use aggregated, simplified colour spaces such as the Munsell colour space, expressed as hue, value, and chroma (HVC; Grime *et al.*, 1981; Saracino *et al.*, 2004), the RGB system of red green blue channels or the CIELab system (CIE Colorimetry, 2004). Several colour systems may be used jointly (Bacchetta *et al.*, 2008; Lo Bianco *et al.*, 2017a; Lo Bianco *et al.*, 2017b). Colours can be transformed between HVC and RGB using the CIElab colour space, but transformation may result in some information loss. Simpler seed 'colour' definitions, such as a dark/clear dichotomy (Carta *et al.*, 2017; Vandelook *et al.*, 2018), common colour names, or grouped colour codes (González-Andrés & Ortiz, 1995) have been used in many cases.

Characterizing seed (and more typically soil) colour can be done by hand using a Munsell scale as a reference (Munsell, 1994). Colours can also be accessed using cameras (including flatbed scanners) under standard light sources to extract RGB and HVC values (Bacchetta *et al.*, 2008). RGB and HVC values are sensitive to the spectrum and intensity of the light source used for observation, and a standard light source of 4000K has been suggested for standardising Munsell colour assessments (Fan *et al.*, 2017), with no standard for light intensity to date. Detailed comparative works need to carefully mention and study the effects of observation light sources used.

For comparisons with previous studies, functions are available to convert HVC and RGB classification systems in the R packages '*munsellinterpol*' and '*aqp*' using the '*munsell2rgb*' and related functions. Recently, Dayrell *et al.* (2023b) introduced an automated phenotyping method with the software tool '*Traitor*' for measuring seed colours, enabling efficient and accurate analysis across taxa, including other visually measurable functional traits (shape, size) and for seed identification.

3.1.2. Seed surface

Trait description

Seed surfaces have multiple functions of interaction with dispersal vectors, predators, pathogens, other biotic interactions, soil, water and air, and they may have several subsequent layers such as the surface of the dispersal unit, the surface of the germination unit. This section deals primarily with the surface structures found at the dispersal unit, but in the case of fleshy fruited species also considers the surface of seeds contained within fruits after the natural disperser has removed the pulp since these surfaces more closely resemble those found in dry-fruited species. Seed surface structures consist of all morphological characteristics, including macrosurface structures such as wings, plumes, spines, hairs, hooks, and balloon-like structures, but also microsculpture such as in alveolar, verrucous, or smooth seeds. Given the many possibilities of seed surfaces, there is no single classification and no simple way to define continuous quantitative axes that would fit the multiple functions these structures might serve. Many species have some degree of heterocarpy concerning seed surface structures. This section focuses on visible morphological adaptations that, in many cases, contribute to the functions that might also be evaluated by dispersal performance traits such as 'Terminal velocity', 'Buoyancy', or 'Epizoochory' (see section **3.2 Seed dispersal/dispersal potential**). Seed surface is a categorical trait thus has no specific unit of measurement.

Functionality and trade-offs

The seed surface has crucial properties that define the seed's interaction with its immediate environment. Wings, hairs or smooth surfaces, and specific shapes interact with air to decrease or increase the terminal velocity (see section **3.2.10 Terminal velocity**) during flight. Plants that specifically depend on wind for dispersal, and bear wings or hairs, have longer dispersal distances (Tackenberg *et al.*, 2003a). Air-filled balloon-like or spongy appendages (e.g., *Carex, Cakile, Crithmum, Nypa, Cynometra*) increase buoyancy (see section **3.2.11 Buoyancy**) and hence dispersal distance in the

water. For external dispersal by animals, hooks and hairs increase the time seed attached to the animal (see section **3.2.12 Epizoochory**), while balloon-like structures decrease it (Römermann *et al.*, 2005c). When interacting with soil particles during burial by soil turbation created by rain, alveolar seeds -meaning seeds with many holes separated by ridges resembling a waffle, such as in *Silene*- bury faster than smooth seeds (Benvenuti, 2007).

Changes in seed size impose physical constraints on seed surface structures, e.g., very small seeds are easily transported by wind without any appendages. In contrast, medium-sized seeds rely on plumes whereas large seeds on wings (a situation that Reynolds numbers can characterise; Minami & Azuma, 2003). Size constraints also apply to the stability of structures; for example, spines and hooks need to be more prominent on large seeds than on small seeds for the same effectiveness. Given the considerable variation of seed size, it potentially imposes changes to all morphological surface structures.

More complex relationships with other traits result from common selective pressures on different dispersal syndromes, e.g., internal (endozoochorous) versus external (epizoochorous) animal dispersal imposes very different selective pressures on a number of traits, including seed surface structures, endozoochorous dispersed seeds have rather smooth surfaces while epizoochorous seeds might bear various appendages.

Applied aspect

The cleaning, packaging and counting of seeds for restoration projects, horticulture, and agriculture can be impaired by hooks and hairs in small seeds that make it virtually impossible to separate individual seeds. Hairs on *Rosa* and *Sterculia* seeds are highly irritative, and seeds must be treated with caution. Seeds with large spikes (e.g., *Ranunculus arvensis*) tend not to imbibe when placed on a smooth substrate in germination tests; sand or agar can be used as germination media.

Source of variability

Seed surface structures vary between seeds of the same mother plant in heterocarpous species. Individuals of the same populations also vary in seed surface structures. For example, *Ranunculus arvensis* with spiny and smooth seeds can co-occur in the same population or vary from population to population.

Methodology

Most seed surface structures can be classified by eye or with a dissecting microscope; microsculpture in very small seeds, e.g., Orchidaceae, Orobanchaceae, can be observed under a light microscope.

Characteristic dry seed surface structures also exist in fleshy fruit species once the pulp has been removed, e.g., the alveolar seeds of *Rubus*. How pulp is removed and seeds dry afterwards can impact seed surface structures; in this case, observe seeds after dispersal in the field.

The difficulty of designing a sound functional classification of seed surface structures lies in their many functions: dispersal by various vectors, burial in soil, and defence. According to the function in question, different features become relevant. Most features are probably adapted to several functions at the same time, e.g., hairs can increase wind dispersal attachment to animals and floatability.

There are two existing functional classification systems for seed surfaces. One classification is more advantageous for comparatively large seeds centred on animal-dispersed seeds (Römermann *et al*., 2005a), also used by the LEDA trait database (Kleyer *et al.*, 2008). Another, developed by Benvenuti (2007) for seed burial by rain for smaller seeds, focuses on seed-soil interactions. We stick to their definitions to make data gathered with future projects compatible with earlier records. Below, we delineate a list of classes that might be helpful when considering dispersal by several vectors or for describing seed interactions with water and soil particles.

We advocate here to subdivide the single trait ‘seed surface structure’ into a four-descriptor system with several classes: (i) size descriptor (none, appendages smaller than seed, larger than seed); (ii) form descriptor (none, fleshy, air-filled, flat,

elongated); (iii) specialised structure descriptor (none, hooks, twisted, pungent); (iv) microsculpture descriptor (smooth, alveolar, warty, spiny).

Macroscopic appendages

1. Fleshy appendages and nutrient rewards for dispersers

We refer to the fruit section for more detail, some of which are not necessarily perceived as fleshy. Here, we give a short list of appendages containing nutrient rewards.

Elaiosome is an appendage that is often oily and of lighter colour than the seed. It rewards ants that transport the diaspore and discard the seed after consuming or detaching the elaiosome.

Aril is a sometimes fleshy, sometimes dry or oily appendage that covers the seed only partly, as in *Acacia retinodes, Taxus baccata or Myristica fragrans.* It is often shiny red, yellow or white, contrasting with the less conspicuous and darker seed. Narrower definitions restrict the aril only to features that develop from the attachment of the seed to the ovary. In some cases, fleshy fruited species with a pulp that covers the seed are also termed arils since part of it may stem from seed to ovary attachment, as in litchee (*Litchi chinensis*). Arils can be oily or have various sugars, oils and protein components. Some arils can be sticky. Arils attach to single seeds, not several seeds at a time.

Pulp is a fleshy structure that completely surrounds a seed. It can envelope several to many seeds at a time and is often organised in a multi-seeded structure. It is covered in more detail in the sections **2.1.3 Dry pulp-dry seed mass ratio**.

Seeds with fleshy arils or pulp are grouped together in fleshy-fruited species, and details of pulp and endozoochorous fruits are treated separately (see section **2.1.6 Fruit chemical compounds**).

2. Air-filled appendages and floats

Balloon structures are composed of a thin coat or lamina that envelopes free air, either completely closed (e.g., *Carex*) or open (e.g., *Phalaris*). It can be further subdivided into open and closed balloon structures.

Spongy floats are composed of sponge or foam-like tissue that contains air in multiple, sometimes cellular structures. They can entirely surround the seed (*Cocos, Pancratium*) or only partly recover it (*Cakile*).

Both balloon structures and spongy floats increase floatability in water. They are derived from various structures, including bracts, calyx, stems, integuments, or carpel walls. There is an intergrading between spongy floats and fleshy appendages: spongy fruits with little sugar content and high floatability exist in species of *Syzygium*, also many oily fruits generally float well.

3. Flat appendages: thin and laminar

Flat appendages are attached to several medium to large sized-seeds. They are often thin, laminar and of an area larger than the seed itself. They are referred to as 'wings' such as in samaras of *Acer, Pterolobium* and *Swietenia.* Still, some are mere fringes of more or less flattened tissue without a specifically wing-like appearance (*Spergula, Ulmus, Tabebuia*). LEDA classifies this further into small and large flat appendages that are either smaller or larger on the surface than the seed itself. They are assumed to decrease terminal velocity and increase wind dispersal potential.

4. Elongated appendages: spikes, hooks, hairs and pappus

Elongated appendages that are not flat have several continuous characteristics: length, flexibility and various degrees of recurvature (hooks), and spininess. They can also be simple, come in multiple numbers, or be subdivided. Spirally twisted appendages that move with varying temperature, touch or humidity increase burial in soil or into animal fur (*Erodium, Aristida*; Peart & Clifford 1987). Earlier classifications (LEDA) suggested five subdivisions according to length and number, thus, a) one short elongated appendage, b) two or more short elongated appendages, c) one long elongated appendage, d) two or more long elongated appendages, plus e) a hooked

category.

5. Seeds without macroscopic appendages

Seeds without appendages are incorporated more easily in the soil (Chambers *et al.*, 1991), especially when they are small and have round shape. There are smooth seeds that have imprints on the surface or coloured traces of underlying structures that play a specific role, *e.g.,* for opening integuments of seed with impermeable seed coats such as lens and pleurogram in Fabaceae seeds (Rodrigues-Junior *et al*., 2019; 2021).

Microscopic seed surface: microsculpture

These structures are not readily visible to the naked eye, and best appear under 10-20x magnification. They have been shown to interact with soil particles during seed burial by natural bioturbation but probably also play a role in seed water relations and seed defense against small-sized seed predators and fungi.

Smooth surfaces do not show any microstructure under magnification: they can either be shiny (glossy, metallic, etc.) or dull (less reflecting). These features might hint at microscopic structures and are linked to seed colours and seed water interactions.

Alveolar surfaces exhibit a system of ridges that subdivides the surfaces into many hole-like fields. It is prominently developed in many medium to small, often rounded seeds (*Silene italica, Orobanche sp.*).

Warty surface shows many low, isolated, large protuberances “warts” on the seed surface (*Portulaca oleracea*).

Scaly seed surface bears numerous short laminar scales (*Consolida regalis*).

Spiny seed surfaces show many short spines (less than seed diameter) that are not visible to the naked eye (*Heliosperma pusilla, Spergula arvensis*) or large, macroscopical spines as in *Caryocar brasiliense*.

The unique feature of mucilage derives from smooth or seed surfaces, as in chia seed (*Salvia hispanica*), *Ocimum basilicum, Aethionema* or the spectacular *Scaphium*

scaphigerum. Seeds with mucilage are less transported than seeds without (García-Fayos *et al.*, 2010). Mucilage may also help maintain a moist environment around the seed during germination (Garwood, 1985) and plays a crucial ecophysiological function in dispersal-defence mechanism (*Dillenia indica;* Dasanavaka *et al.*, 2022).

3.1.3 Seed coat thickness

Trait description

Seed coat thickness (unit: µm) measures the width of the outer, usually darker and harder, layer of a seed. The seed coat excludes storage tissues, dispersal appendages, or fruit pulp. The classical definition of seed coat is restricted to the tissues formed by seed integuments with contribution from the chalaza (Werker, 1997), which in some cases needs a detailed study on the developmental origin of tissues. From the functional perspective, we suggest a pragmatic definition that focuses on the protective role, which includes the pericarp in cases of dry and indehiscent fruits.

Functionality and trade-offs

Seed coat thickness plays a prominent role in biotic interactions. It is one of the main seed defence traits; it is positively related to seed survival in the soil (Gardarin *et al.*, 2010), decreasing seed predation by invertebrates (Foffová *et al.*, 2020) and protecting seeds from heat shocks (Bell & Williams 1998). Seed coat thickness also acts as a barrier against soil-borne pathogens (Davis *et al.*, 2008). Coordination with chemical traits for defence against predators and pathogens is supported by some data (Dalling *et al.*, 2011; Davis *et al.*, 2016). Seed coat thickness is also linked to seed survival during gut passage in herbivores and contributes in this way to endozoochorous seed dispersal (Bruun & Poschlod, 2006).

The seed coat plays a role in water regulation by impermeable palisade layers, keeping seeds dry or wet depending on the ecological situation and the seed stage (McDonald *et al.*, 1988; Baskin & Baskin, 1998; Steinbrecher & Leubner-Metzger, 2017). While the impermeability of the seed coat is not necessarily dependent on a thick seed coat, since a thin layer of palisade cells is sufficient to provide impermeability, seed coat thickness is usually greater for seeds with impermeable coats (Baskin & Baskin, 1998). In contrast, recalcitrant seeds often have comparatively low seed coat thickness or even have seed coats that easily fall off, such as in avocado seed (e.g., *Persea americana*) or Olacaceae (Werker, 1997). Research by Fricke and Wright (2016) highlights the

relationship between seed toughness, seed size, and seed coat thickness. While larger seeds have greater absolute toughness, smaller seeds exhibit higher specific toughness (i.e., greater force required to break the seed coat per unit seed mass). Seed toughness can be measured using a mechanical force tester.

Sources of variability

Seed coat thickness varies within a single seed depending on seed surface sculpture and appendages, as outlined in the measurement section. Independent of these morpho-anatomical variations, it covaries strongly with seed size (Saatkamp *et al.*, 2014), and is variable within species, especially for heterocarpous seeds.

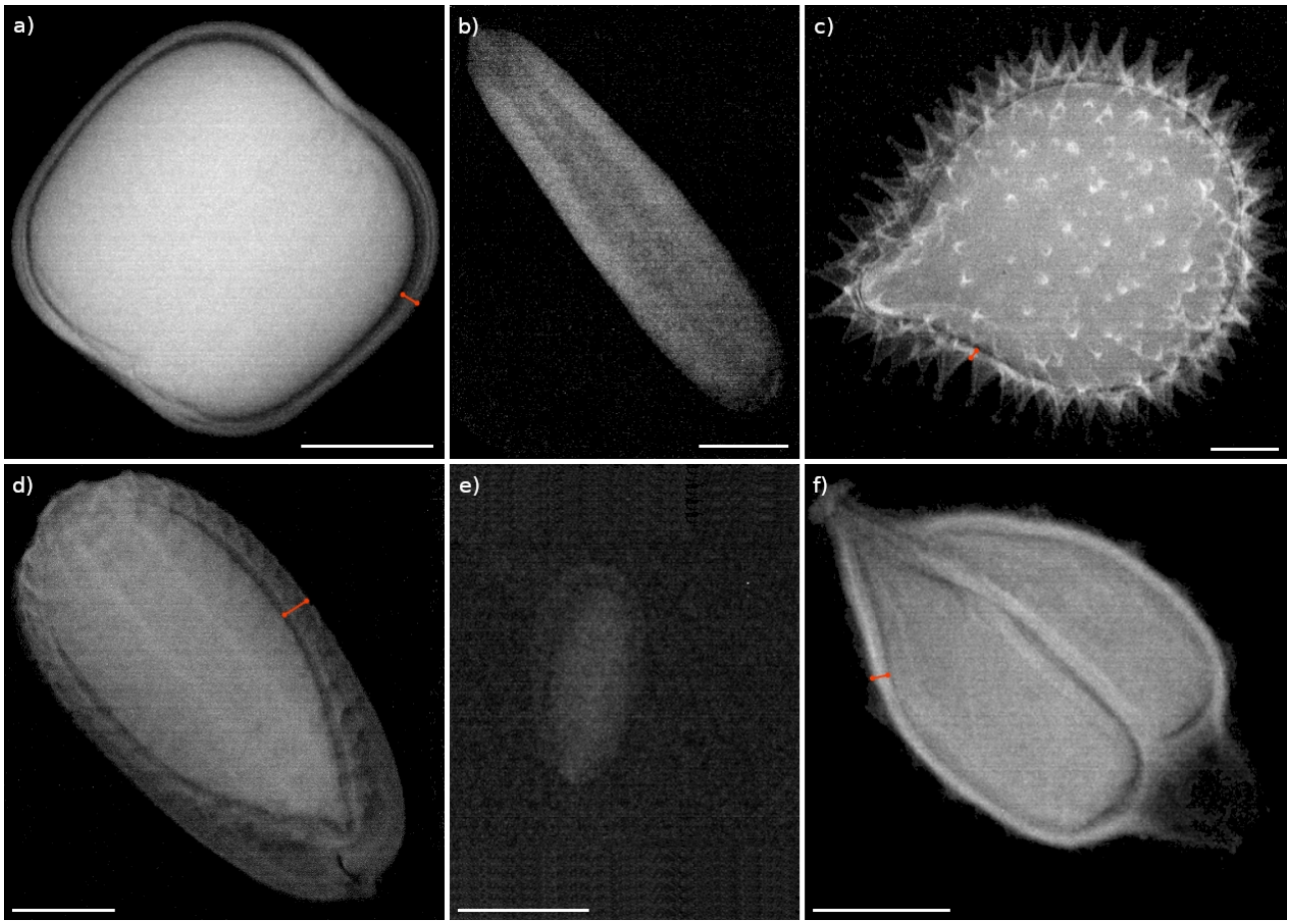
Methodology

Seed coat thickness can be either measured on intact seeds using X-ray images of known size and resolution using appropriate imaging software or on dissected seeds under a light microscope. On X-ray images, seed coats appear as clear outer areas, being denser than storage compartments (Gardarin *et al.*, 2010; Fig. 2). However, for seeds with very thin seed coats or for very small seeds, the information obtained from X-ray images is quite limited (Fig. 2b, e). For each X-ray measurement, ten to a hundred replicate seeds should be used.

When measured on thin transversal sections of seeds under a light microscope, variability of seed coat thickness within a seed and the contribution of different tissues to the seed coat (integuments, exo-, meso-, endocarp and outer fruit structures strongly adherent to the seed, e.g., Asteraceae, Apiaceae), might also be included in the measurement. Depending on the function studied, the tissues included should be noted. For example, in several cases, a dry pericarp plays a critical role in seed water relations and germination regulation (Steinbrecher & Leubner-Metzger, 2017). When measured under a light microscope, variance among seeds of the same species may be smaller when compared to X-ray techniques, since it enables direct manipulation during the measurement process and concomitant measurement of the embryo-seed

ratio. For tough seeds, soaking might not be sufficient to soften seed tissues before sectioning; therefore, embedding techniques may be necessary.

Fig. 2 Example of seeds observed with X-ray techniques: a) *Lathyrus vernus*, b) *Brachypodium sylvaticum*, c) *Cynoglossum germanicum*, d) *Carduus acanthoides*, e) *Campanula trachelium*, f) *Sanguisorba minor.* Red arrows indicate seed coat. Scale bars represent 1 mm.



Seed coat thickness can vary greatly in a single seed due to seed surface sculpture, e.g., *Silene, Cynoglossum* (Fig. 2c) and many Apiaceae. In this case, several measurements should be made, and minimum, mean and maximum thickness should be reported. In cases of prominent seed sculpturing, a more detailed comment might be needed to identify the section measured.

Special cases

In some species, several seeds might exist within one coat envelope (*Agrimonia, Sanguisorba;* Fig. 2f), or several embryos might exist within one seed (polyembryonic

seeds, *Pinus, Miconia*), making it necessary to measure the all-encompassing outer layer of these grouped embryos. Especially in recalcitrant tropical seeds, no functional seed coat might be left once dispersal structures are removed (e.g., *Persea americana,* Lauraceae).

3.1.4 Seed coat chemical compounds

Trait description

An array of secondary chemical metabolites, biotic defences, and morphological traits inhibit microbial infection of seeds and reduce seed consumption rates by invertebrate and vertebrate granivores. Chemical defences of seeds encompass a wide range of compounds with activity against a wide range of antagonists (Mayer, 2004). These include glucosinolates, alkaloids, terpenoids, saponins, phenolics, and cyanogenic glycosides. Seeds may also harbour polyphenol oxidase and other enzymes that can be activated in the presence of antagonists (Fuerst *et al.*, 2014). Biotic defences can include the seed-surface microbial community derived from the maternal environment or acquired after seed dispersal. Morphological traits that may have defensive properties include the thickness or hardness of seed-enclosing structures and the presence of mucus, spines, trichomes and wax layers on seed-enclosing structures (Dalling *et al.*, 2020).

Here, we focus on measurements of seed defensive chemistry based on (i) characterizing the diversity and concentration of soluble phenolic compounds, a widespread constitutive plant defence correlated with other seed traits (Gripenberg *et al.*, 2017), and (ii) the use of the brine shrimp as a toxicological bioassay. These measurements of potential defence constituents and activity are widely applicable across plant taxa and are amenable to replication for comparative studies. Brine shrimp assays can be carried out with minimal lab facilities, while the phenolic measurements described below require access to high-performance liquid chromatography (HPLC). However, samples for HPLC can be prepared in the same way as those used from the brine shrimp assay and transferred to a lab for later analysis. Seed phenolic compounds also appear to be quite stable, allowing analysis of dry-stored seeds (Hendry *et al.*, 1994). Alternatively, total phenolics can be measured colorimetrically using commercially available test kits. Colorimetric assays, however, do not distinguish among phenolic compounds that may differ in biological activity and cannot assess phenolic chemical diversity. The brine shrimp assay described here can

be augmented with inhibition tests against fungi (Appendix S1 in Zalamea *et al.*, 2018); however, no standard set 'test cultures' exist for this assay, so results are not comparable across studies. Chemical assays can also be complemented with measures of seed physical hardness, which measure the force necessary to rupture a seed (e.g., Davis *et al.*, 2016; Fricke & Wright, 2016) and require specialised force-testing equipment.

Functionality and trade-offs

Except for cyanogenic glycosides, which are not thought to be active against pathogens, all secondary metabolite classes appear to have broad activity against a range of herbivores/granivores and fungi (Mayer, 2004). Seed surface bacterial and fungal communities – the external seed microbiome – may also have antagonistic relationships with pathogens, potentially suppressing pathogenic infection at both pre- and post-emergence stages (Nelson, 2018). As yet, there is mixed evidence of direct trade-offs between seed chemical and physical defences (Moles *et al.*, 2013, Gripenberg *et al*., 2017). However, in general, large seeds tend to be more strongly chemically defended than small seeds (Wang *et al.*, 2018), potentially due to the reduced effectiveness of physical defences per unit seed mass as seed size increases (Fricke & Wright, 2016). In a study of 196 tree and liana species, seed polyphenol concentrations were found to be expressed in high concentrations in species with large seeds, short seed dormancy time, and low investment in seed mechanical defences (Gripenberg et al. 2017).

Sources of variability

Seed surface defences are highly variable among species, reflecting variation in seed size, dispersal mode, dormancy type and persistence in soil seed banks (Dalling *et al.*, 2020). Seed chemical defences can also vary in their distribution across plant and seed tissues (Hendry *et al.*, 1994; Whitehead *et al.*, 2013), and within seeds across populations (Zangerl & Berenbaum, 1997).

Phenolic compounds are the most widespread and common group of plant secondary metabolites and, therefore, the most frequently assayed constitutive plant chemical defence. They are defined as possessing an aromatic ring with one or more hydroxyl groups or their functional derivatives (Lattanzio *et al.*, 2006). Phenolics have an exceptionally broad range of biological activity, providing antifungal defences and deterring insect and mammal herbivory and granivory. Whereas most phenolic protocols are based on a measure of total phenolic content in plant tissue using the Folin-Ciocalteu reagent (e.g., Ainsworth & Gillespie, 2007), individual sub-classes or structures of phenolics may vary in their activity against individual natural enemies (Lattanzio *et al.*, 2006) suggesting approaches that quantify phenolic abundance, diversity and composition are more insightful for characterizing inter- and intra-specific variation in defences.

Methodology

Given the diversity of seed surface defences, measurements of single defence traits cannot adequately characterise the resistance of seeds to multiple natural enemies. Approaches are therefore needed to quantify continuous traits, such as secondary metabolite concentrations, in combination with discrete traits that describe metabolite diversity or indicate the presence of morphological adaptations. Bioassays that assess the activity of seed fractions or extracts against bacteria, fungi or invertebrates without explicit knowledge of the chemical constituents that confer toxicity may also be useful. Combinations of traits that can be summarised as principal components may be particularly useful for characterizing overall investment in defences in community-wide comparisons (e.g., Zalamea *et al.*, 2018).

Here, we provide the rationale and methods for two potential approaches to quantify seed surface defences attributable to seed surface metabolites.

1. **Characterization of phenolic concentration and diversity**

Characterization of seed surface phenolic compounds using high-performance liquid chromatography (HPLC) provides a relatively high-throughput analytical approach that

measures phenolic composition and diversity (Tiansawat *et al.*, 2014; Zalamea *et al.*, 2018). Initial analysis of HPLC data is based on peak retention time rather than compound identification and is coupled with a measure of abundance (based on the integrated peak area standardised by the mass of seed tissue used). Downstream statistical analysis, such as partial least squares regression or principal coordinates analysis, based on compilations of peak spectra from multiple species, can also be used to identify individual phenolic compounds (retention times) that are significantly associated with variation in other traits (e.g., seed persistence or toxicity in bioassays) and thus can be subsequently targeted for more detailed chemical characterization (a more cost-effective approach than chemically characterizing all phenolic components).

Protocols for seed phenolic analysis are published by Tiansawat *et al.* (2014) and Zalamea *et al.* (2018), adapted from Gallagher *et al.* (2010) by M. Berhow (US Department of Agriculture, National Center for Agriculture Utilization Research). In brief, the desired seed or seed structure is ground to fine homogenate and then 'de-fatted' by extracting in hexane. After the first extraction, a second extraction in methanol is used to separate soluble phenols. The supernatant is filtered through a filter syringe into scintillation vials for HPLC analysis.

Units

Individual absorbance peaks detected by HPLC represent potentially bioactive phenolic compounds. The number of absorbance distinct peaks represents a measure of phenolic diversity. By using phenolic standards across runs individual peaks can be numbered based on retention time and compared across samples to generate a phenolic dissimilarity matrix for downstream analysis (e.g., Principal Coordinate Analysis, Tiansawat *et al.* (2014).

A second measurement of phenolics derived from this assay is phenolic abundance, based on integrated peak areas obtained from the chromatogram. For each sample, the total mass-standardised peak area is obtained:

$$\frac{\textit{Raw peak area}}{} \times \textit{Total volume of extract}$$

$$\text{Mass-standardised peak area} = \frac{\text{peak} \times \text{Injection volume}}{\text{Sample mass}}$$

Mean peak areas are calculated for the three replicates per species.

Units for peak area are mV × minute.

Two measurements derived from this analysis: Total phenolic mass-standardised peak area, and phenolic diversity (number of peaks) can be used to characterise overall phenolic investment in seed tissue.

2. **Characterization of toxicity of seed extracts using the brine shrimp assay**

Brine shrimp (*Artemia franciscana*) is widely used as a rapid and inexpensive assay of the toxicity of biological extracts (Meyer *et al.*, 1982). The assay generates an index of the lethal concentration of a seed extract at which half the shrimp in the bioassay die (LC_{50}) and, therefore, a quantitative measure of toxicity that can be compared across samples. Sample preparation of the seed extract is the same as for the HPLC phenolic assay; see “Characterization of phenolic concentration and diversity” above, allowing further correlation between chemical composition and biological activity.

The protocol for the brine shrimp assay is published in Zalamea *et al.* (2018). Briefly, a dilution series of the initial methanol extract and extract-free controls is created and then added to tubes containing water and a known number of brine shrimp larvae (nauplii). After 24 hours, the numbers of surviving nauplii are counted.

Units and calculation of LC_{50}

Counts of surviving nauplii across the treatments are used to determine the lethal concentration (LC_{50}) of the seed extract in µg or mg/mL.

LC_{50} can be estimated in the R statistical software (R Core Team, 2023) by fitting a binomial errors logistic regression model of the proportion of dead nauplii against extract concentration and then using the dose.p() function of the package MASS (Venables & Ripley, 2002) to predict the concentration and standard error for 50%

nauplii mortality. An example is provided in chapter 16 in Crawley (2007). A wider variety of models for fitting dose-response curves is available using the R package drc (Ritz *et al.*, 2015).

3.2 Seed dispersal/dispersal potential

3.2.1 Dispersule type/syndrome

Trait description

Dispersal syndrome is categorised by associating morphological characteristics of the dispersule with potential dispersal agents or vectors. It is a simple binary assignment scheme classifying each species as either being (predominantly) dispersed by a certain dispersal vector or not. Although the dispersal of a species may occur via multiple vectors (Thomson *et al.*, 2010), and long-distance dispersal events are often via non-standard vectors (not according to the obvious morphological adaptation; Higgins *et al.*, 2003), the syndrome categorisation describes the most likely or legitimate dispersal agent. Dispersal syndrome is useful for understanding the distances dispersules of a species may cover, the routes they may travel and their likely final destination (Lososová *et al*., 2023). Therefore, the mere consideration of the dispersal syndrome may be strongly biased and lead to wrong assumptions/results (Tackenberg *et al*., 2003a; Green *et al.*, 2022).

The dispersule (or propagule) is the unit of seed or fruit as it is dispersed (Pérez-Harguindeguy *et al.*, 2013). In most cases, the dispersal unit corresponds to the seed. However, in many species, it is composed of the seed plus surrounding structures, i.e., various appendages or surface structures which are functionally relevant for the dispersal syndrome.

Functionality and trade-offs

A trade-off with dormancy has been suggested, i.e., seeds that can be dispersed greater distances may disperse in space rather than time and hence may be less dormant (Chen *et al.*, 2020a). For some syndromes, there are interactions with other traits such as plant height or release height (e.g., Tackenberg *et al.*, 2003a).

Further, plant lifespan is thought to be correlated with dispersal in space (dispersal syndrome, dispersal potential). Long-lived species often produce seeds or fruits with

low dispersal potential, in contrast to short-lived species, which often have a high dispersal potential (Pérez-Harguindeguy *et al.*, 2013). Poor dispersal capacity may be related to other traits, for example, clonal behaviour, which may be a means of short-distance migration when dispersal is poor (Pérez-Harguindeguy *et al.*, 2013).

Applied aspect

Dispersal is critical to conservation and restoration where, for example, it affects the ability of restored populations to become self-sustaining (e.g., Bakker *et al*., 1996) or allows threatened species to persist under changing conditions by moving to new sites (e.g., Poschlod *et al*., 2000). When dispersers are extirpated, e.g., through hunting pressure, the dispersal syndrome may also be valuable in predicting future population trends (Scabin & Peres, 2021). It is, therefore, relevant to conservation policy (e.g., Barton *et al.*, 2015).

Certain appendages or surface structures of the dispersule may affect not only the dispersal syndrome but also dormancy, germination or infection from pathogens during viability testing or propagation (Harper, 1977; McEvoy, 1984; Venable & Brown, 1988).

Sources of variability

Different dispersal syndromes of individuals within species (or within individuals) can occur due to heteromorphic dispersules (McEvoy, 1984). Some variation could also occur due to maternal effects and population divergence that may impact morphological characteristics, although this is unlikely to be so extreme that the syndrome changes (De Casas *et al.*, 2012).

Methodology

Dispersal syndrome is often categorised following variations of the Van der Pijl (1982) terminology (Table 4).

Table 4 Dispersal syndrome, the definition of the syndrome, and a list of morphological characters that are generally assigned to each syndrome.

Dispersal syndrome	**Definition**	**Morphological characteristics of the dispersule**	**Examples**
Unassisted	Seed falls passively beneath the parental plant	No obvious characteristics associated with dispersal	*Quercus* spp. (acorns), *Vellozia*
Anemochory	Dispersal by wind	Minute/tiny Pappus or long hairs Wings or flattened Spores Tumbleweeds of whole plant or infructescence	Orchidaceae, Orobanchaceae *Taraxacum* spp. *Acer* spp., *Betula* spp. Ferns, bryophytes *Eryngium campestre*, *Kochia scoparia*
Endozoochory	Dispersal by internal animal transport	Flesh/pulp, often brightly coloured, drupes, berries, large fruits Arillate seeds	Fleshy fruited species as *Prunus*., *Miconia*, *Solanum*, *Piper*, *Ficus*, *Punica granatum*
Exozoochory / epizoochory	Dispersal by external animal transport	Dispersules that get attached to fur, feathers, legs, bills, aided by: Hooks or burrs Barbs or awns	*Geum urbanum* *Aegilops* spp. *Plantago* spp.

		Sticky substances e.g., mucilage	
Hoarding	Dispersal by animals moving dispersules to a cache or hoard	Brown or green dispersules with thick indehiscent coats	*Corylus* spp., *Quercus* spp., Palms
Myrmechochorous	Dispersal by ants or related insects	Arils/elaiosomes Nectar/secretions	*Viola* spp., Euphorbiaceae, *Turnera ulmifolia*
Mellitochory	Dispersal by bees	Associated with fruits that produce resin as an attractant for bees (Wallace and Trueman 1995)	*Eucalyptus torelliana* *Coussapoa asperifolia*
Hydrochory	Dispersal by water (oceanic, freshwater, and rainfall)	Prolonged floating due to morphological and anatomical features that result in relatively low specific gravity: Corky tissue Balloon Air spaces	*Pachira officinalis* (Lopez 2001) *Carex* spp. *Menyanthes trifoliate*
Ballistochory	Dispersal by forceful	Capsules that launch seeds away from the	*Impatiens* spp.

	ejection or 'exploding' capsules. Sometimes known as 'self-dispersal'	parent plant, often due to osmotic pressure	*Hura crepitans*
Hygroscopic	Dispersal by hygroscopic bristle or awn contraction	Bristles or awns that expand and contract with wetting and drying or changes in humidity	*Avena sterilis*, *Trifolium squarrosum*
Deception	Dispersal by insects or other animals that have been deceived by the seed morphological characteristics and do not gain any reward	Mimicry of non-seeds (Midgley *et al.*, 2015)	*Ceratocaryum argenteum* *Zanthoxylum ekmannii* (Ruzi & Suarez, 2022)

Record potential syndromes (Table 4) that may be relevant for the dispersule's morphology in order of decreasing importance or likelihood (Vittoz & Engler, 2007; Tamme *et al.*, 2014). For similar potential contributions, prioritise the one with the assumed longer distance dispersal (Pérez-Harguindeguy *et al.*, 2013).

Dispersules may (occasionally) be transported by vectors even though they have no obvious adaptation for that vector. This is particularly the case for endozoochory and exozoochory (Pérez-Harguindeguy *et al.*, 2013). In general, the relationship between morphologically defined dispersal syndrome and long-distance dispersal (LDD) is poor, likely because LDD events are often associated with non-standard dispersal (Higgins *et al.*, 2003). Therefore, dispersal syndrome should only be used to infer the standard and most common dispersal agent for a species.

The validation, or combination of syndrome classification with quantitative measures of dispersal potential, is strongly recommended. This allows dispersal to be described along a continual range and can integrate the fact that many syndromes are scale and context-specific (Tackenberg *et al.*, 2003a). The method is best applied to fresh dispersules, but stored material can also be used, provided it has not had alterations to dispersule morphology, e.g., appendages removed.

3.2.2 Dispersule structure

Dispersule structure is the surrounding tissue or appendage that aids the dispersal of the dispersule (or propagule; i.e., the plant reproductive unit that gets dispersed; Pérez-Harguindeguy *et al.*, 2013). In many but not all cases, seed or fruit is accompanied by its dispersal structure.

Functionality and trade-offs

Dispersule structure is strongly correlated with dispersal syndrome (Table 4). Species with high biomass investment in dispersal structure disperse seeds further than species with low investment (Thomson *et al.*, 2018). In a binary presence/absence of dispersule structure, tall species are more likely to equip their seeds with a certain dispersule structure compared with short species (Thomson *et al.*, 2018). This increased resource allocation to dispersule structure could be an explanation for why large-seeded tall plants generally have increased seed dispersal abilities (Thomson *et al.*, 2011; Chen *et al.*, 2019b). Seeds or fruits with plumed appendages are transported with a higher probability and higher proportion over long distances by wind (Tackenberg *et al.*, 2003a). In contrast, those with hooked appendages or awns are transported with a higher proportion (but not with a higher probability) over long distances by animals (Fischer *et al.*, 1996).

Some dispersule structures (e.g. those adapted for dispersal by wind) interact with other traits, such as plant height or release height (Tackenberg *et al.*, 2003a). Producing dispersal structures costs resources, thus the size and/or number of dispersal structures may be reduced if resources are limited (Ellner & Shmida, 1981).

Certain dispersule structures (e.g. pulp) may affect dormancy, germination or pathogen infection during viability testing or propagation (Traveset, 1998). For example, in grasses, the hygroscopic movement of awns across the soil surface anchor the caryopses to microsites, which increases the germination speed (Peart, 1979; 1984).

The development of certain dispersule structures is often related to the occurrence in certain habitats. For example, there is a higher proportion of species with fleshy pulp towards the tropics where the habitat is wet, warm and with stable climates (Chen *et al.*, 2017). However, a detailed large-scale analysis across all types of dispersal structures is missing (Ronce & Clobert, 2012). Awn length in *Stipa purpurea* is correlated to the number of windy days and relative humidity (Li *et al.*, 2015). In the Siberian wildrye (*Elymus sibiricus*), awn length is negatively correlated to seed production. Meanwhile, awn length tends to increase dispersal distance and germination speed but decreases germination rate (Ntakirutimana *et al.*, 2019). In contrast, Johnson and Baruch (2014) reported that long awns improved seed germination and growth.

Sources of variability

Certain types of dispersule structures are found more often in certain families (Table 5). Within species, the mass of dispersule structure scales in an allometric manner with seed mass or other seed components and varies across environmental gradients (Guja *et al.*, 2014; Chen & Giladi, 2018). Among species, the mass of dispersal structure can be very variable and is affected by other traits or plant life-history strategies (Thomson *et al.*, 2018).

For a single seed, most species have a single dispersule structure, but a few species could have more than one type of structure that aids dispersal. These species disperse their seeds in separate phases known as diplochory or secondary dispersal (Wang & Smith, 2002; Vander Wall & Longland, 2004; Vander Wall *et al.*, 2005; Hämäläinen *et al.*, 2017; Zhu *et al.*, 2019). Correlations may exist among these structures. For example, with increasing seed mass in ballistic–ant diplochory, fruit coat mass scales at a lower rate than elaiosome mass (Chen *et al.*, 2019a).

At the species level, heteromorphic dispersule structures have evolved in response to highly variable environments such as deserts (Levins, 1968; Stebbins, 1974; Harper, 1977). However, there is also phylogenetic conservatism (Silvertown, 1984). Seed heteromorphism is commonly found in Chenopodiaceae and Asteraceae (Venable,

1985). For example, in the Asteraceae species *Gymnarrhena micrantha,* there are two types of achenes: aerial with pappus and subterranean without pappus. The first is dispersed by wind, the second remains at the mother plant (Koller & Roth, 1964; Gutterman, 1993). In another annual species of Asteraceae, *Geropogon hybridus*, achenes in the central whorls are non-dormant and equipped with plumed pappi functioning as parachutes in dispersal by wind, while achenes in the peripheral whorl are dormant and equipped with diminutive pappi of five short awns – this latter type is dispersed in the vicinity of the mother plant or rarely by epizoochory (Chen & Giladi, 2020)

Methodology

Dispersule structure can be categorised as in Table 5 (according to Poschlod *et al.*, 2003 and Römermann *et al.*, 2005a). A species can fall into several categories. Be aware of heteromorphic dispersules from the same plant individual or species. Dispersal structure can be weighed either fresh or dry, depending on the research purpose, in a similar way as seed mass. Dispersal investment ratio = dispersal structure mass/diaspore mass (Thomson *et al.*, 2018).

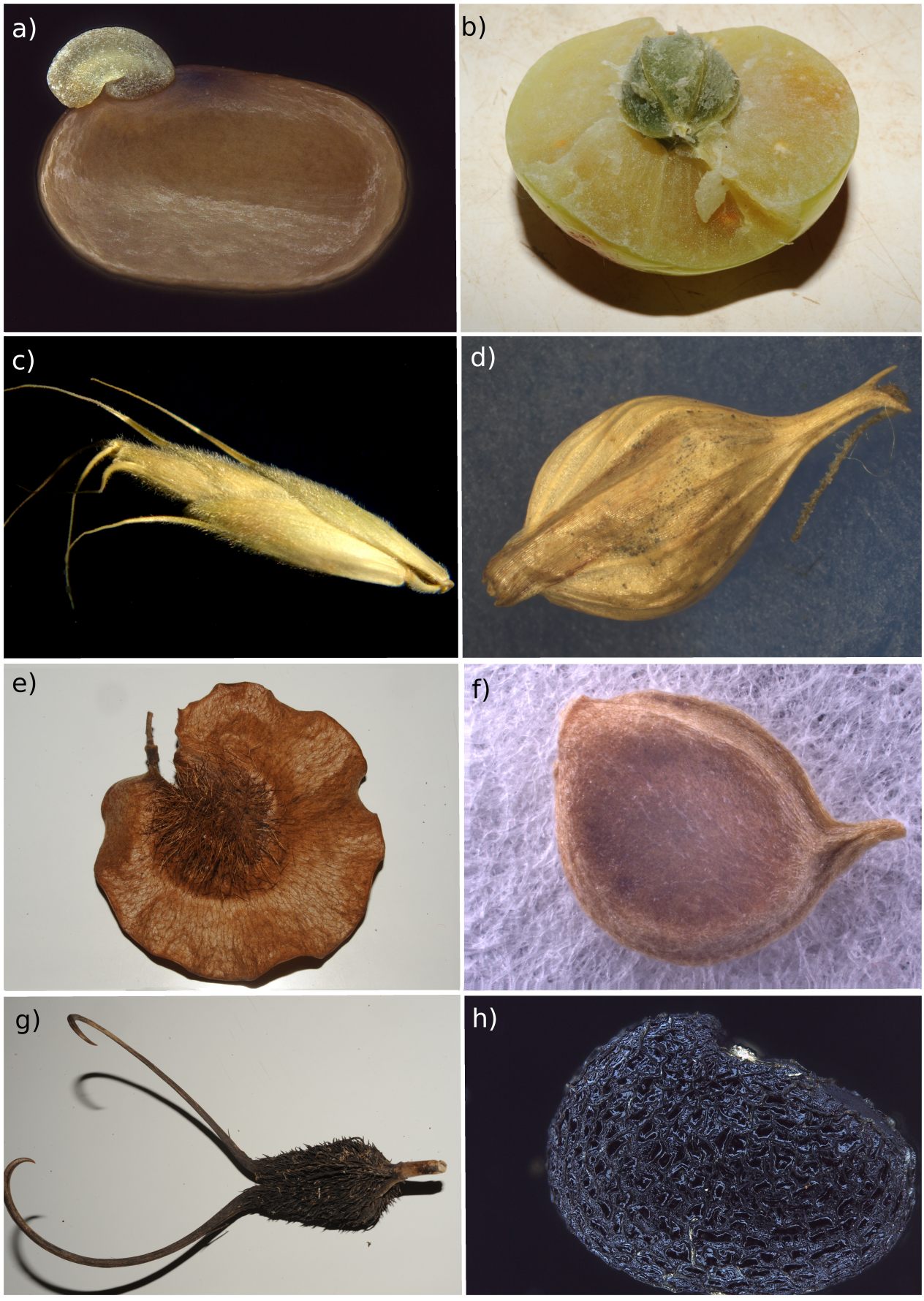
Table 5 Summary of the dispersule structure categories, sub-categories, and species examples according to Römermann *et al.* (2005a) and Dayrell *et al.* (2023a, submitted).

Main category	**Sub-category**	**Example**
1. Nutrient containing structures	1. Elaiosome	*Viola hirta, Bossiaea ornata* (Fig. 3a)
	1. Aril	*Taxus baccata*
	1. Pulp	*Prunus* spp. *Phyllanthus emblica* (Fig. 3b)
1. Balloon structures	1. Open structures	Glumes from the Poaceae (Fig. 3c)

	1. Closed structures	Utricles of *Carex* spp. (Fig. 3d)
1. Flat appendages	1. Small appendages	*Ranunculus acris*
	1. . Large appendages	*Acer* spp. *Pterocarpus spp.* (Fig. 3e)
1. Elongated appendages	1. One short appendage	*Ranunculus repens* (Fig. 3f)
	1. Two or more short appendages	Short hairs, *Scabiosa* spec.
	1. One long appendage	Awns, *Geum urbanum*
	1. Two or more long appendages	Long hairs, *Epilobium* spec. or long pappus, *Taraxacum* spp. (Fig. 3g)
Additional info	a. Spiral coiled elongated appendage a. Elongated bent a. Elongated hairy a. Elongated bristles a. Single hooked a. Multiple hooked	*Avena fatua* *Alopecurus myosuroides* *Erodium moschatum* *Bromus erectus* *Geum urbanum* *Agrimonia eupatoria*
1. No appendages	1 Mucilagenous surface	*Plantago* spp., *Linum* spp.
	1 Coarse surface	*Silene vulgaris, Aotus ericoides* (Fig. 3h)

	1 Smooth surface	*Lotus corniculatus*
1. Other specialisations		
1. Unknown		

Fig. 3 Examples of dispersules from different structure categories. a) *Bossiaea ornata* with nutrient containing structures (elaiosome); b) *Phyllanthus emblica* with nutrient containing structures (pulp); c) *Bromus secalinus* with balloon structures (open); d) *Carex rostrata* with balloon structures (closed); e) *Pterocarpus angolensis* with flat appendages (large); f) *Ranunculus repens* wiht Elongated appendages (one short); g) *Proboscidea fragrans* with elongated appendages (two or more long); h) *Aotus ericoides* with no appendages (coarse surface).


a)
b)
c)
d)
e)
f)
g)
h)

3.2.3 Dispersule exposure

Trait description

Dispersule exposure is how the dispersule or seed on the plant is exposed to the potential dispersal vector (Will *et al.*, 2007).

Functionality and trade-off

The exposure of dispersules is related to wind and external animal dispersal. For the latter, a strong correlation between the attachment potential to an animal coat and its surface structure (hairs, wool, feathers) was shown (Will *et al.*, 2007). Hooked dispersules or those with appendages such as awns or pappi have a much stronger attachment potential than dispersules without appendages (Will *et al.*, 2007).

Dispersule exposure may be related to a habitat's openness or its (land) use. In animal-grazed habitats, more species may have exposed dispersules, but this hypothesis has not been tested yet.

Applied aspect

Species with exposed dispersules are much better dispersed between fragmented or isolated habitats and, therefore, much more frequent in fragmented landscapes when animals acting as ‘moving corridors’ are present (Römermann *et al.*, 2008).

Methodology

According to Hintze *et al.*, (2013), three categories may be differentiated: ‘Exposed’, which are dispersules in an open outward-directed position on a fruit head or infructescence. ‘Partly covered’ which includes fruits that either become exposed when being touched or are already opened; the category also includes dispersules within infructescences where inner parts are not fully exposed, such as spikes and umbels, where, e.g. only the outer dispersules or seeds can attach to the surface of a passing animal. The third category is ‘Enclosed’, which includes dispersules or seeds locked within fruits such as capsules, pods or husks or which are fixed within a calyx or surrounding structures.

3.2.4 Seed production

Trait description

Seed production is the total number of seeds produced by an individual, a ramet of a clonal species, or the annual seed production per m^2 of canopy per year. Seed production may include viable and non-viable seeds. In many species, a large proportion of seeds may even be unfilled (without embryo or embryo with endosperm). In that case, it is helpful to estimate the viable fraction. Reproduction by seed may occur more than one time per year or in lower frequency episodes spaced by several years, depending on species, individuals, age or environmental conditions. Seed production is a continuous trait.

Functionality and trade-off

Seed production is strongly negatively related to seed mass, forming the well-known seed size-number trade-off, which is central to the reproductive ecology of plants (Leishman, 2001; Moles & Westoby, 2006). A high seed production increases the probability of dispersal in space and time. Bruun & Poschlod (2006) showed that the probability of being endozoochorously dispersed was strongly correlated to the seed production per area but not with seed size. The same was true for soil seed bank persistence. Saatkamp *et al.* (2009) showed that seed production was positively correlated to the soil seed bank longevity index. Larger seed production may also result in a higher chance that a germinated seed is also established (Poschlod & Biewer, 2005). In contrast, a lower number of seeds per fruit may decrease the competition between the offspring and increase the probability of successful establishment (Casper & Wiens, 1981).

Since there is considerable variability in the seed size-seed number relationship (e.g. Moles & Westoby, 2006), seed size and seed production can be alternative strategies to some degree, notably in long-living plants where survival might trade-off with seed production. There, habitat conditions and specific regenerative strategies (gaps, fire) might independently influence optimal values of seed size and seed production.

Seed production also depends strongly on a site's climate, hydrology and fertility. Seed production of *Phragmites australis* was strongly correlated with climatic conditions during flowering (McKee & Richards, 1996). The seed set was highest in years when rainfall in August was low but high in September and October and when the temperatures of these months were high. Precipitation timing also triggers seed production (Horn *et al.*, 2017, Souza *et al.*, 2019). Drought but also late frosts can result in ovule, seed or fruit abortion (Stephenson, 1981; Lee & Bazzaz, 1986; Nussbaumer *et al.*, 2020). Fire can launch massive flowering and fruiting in subsequent seasons for many resprouting plants in fire-prone ecosystems. In tropical plants, exceptional droughts, El Niño years and hurricanes can trigger flowering and fruit production. Flooding may prevent flowering and/or seed set (Kozlowski, 1997). Soil fertility, especially phosphorus availability, may increase seed production (Meyer & Root, 1993; Souza *et al.*, 2019). However, interspecific competition may decrease seed production (Brainard *et al.*, 2011). Seed production may also be strongly affected by seed predation (Ehrlén, 1996).

Applied aspect

Restoration management, e.g., establishment of a vegetation cover on inhospitable (bare) soils in mines or quarries, relies on species with high seed production (Giannini *et al.*, 2017). Many restoration projects use plants with high and frequent seed production.

Source of variability

Seed production may vary from year to year, especially in trees (masting years; Herrera *et al.*, 1998; Bogdziewicz, 2022) but also strongly depends on climate (Koenig & Knops, 2000; Kelly & Sork, 2002). Seed production also strongly varies depending on habitat conditions, as Salisbury (1942) showed for many species, especially annuals. There is strong intraspecific variability in seed production due to genetic and environmental components.

This variability is also found concerning the seed number per fruit in multi-seeded species. It may depend on climate, especially precipitation, during fruit sets and habitat quality, such as soil pH, soil moisture, and soil nutrients. Successful pollination, especially in insect-pollinated species, may also strongly affect the number of seeds (Zisovich *et al.*, 2012). Therefore, population size, fragmentation and isolation of populations may affect seed number per fruit (Steffan-Dewenter & Tscharntke, 1999).

Methodology

Seed production can be measured per individual (seed number per ramet; Kunzmann, 2005; Kleyer *et al.*, 2008) or unit area (Jackel & Poschlod, 1994; Šerá & Šerý, 2004). However, every measurement is climate and habitat-specific; therefore, data must be used carefully when compared across habitats, and seed production should be calculated for a species in its typical habitat. According to the suggestion of Jackel & Poschlod (1994) and Kunzmann (2005), a minimum of 10 infructescences per individual or ramet should be collected at the sample site or within a population before seed release. In case an individual or ramet produces several infructescences, the number of infructescences should be counted to calculate the total seed production per individual or ramet. In the case of rare species, we suggest following the recommendations of Ensconet that a maximum of 20% of the infructescences should be collected, which may be less than ten infructescences (Way, 2003; Royal Botanic Gardens, Kew 2022a; Universidad Politécnica de Madrid, 2009). In many cases, such as in Brassicaceae or Fabaceae, it might be useful to collect the fruits before their final ripening so as not to lose seeds when they have already started to open.

For species with tiny seeds, such as orchids, *Piper*, *Cecropia*, or Orobanchaceae, fruit can be cut in equal parts and seeds are counted to calculate the seed number for the entire fruit. Seed production can also be estimated by weighing the entire seed production and calculating the seed number per fruit or infructescence by dividing by the individual seed weight (see section **3.2.5 Seed mass**). A seed counting machine (e.g. Contador seed counter; https://www.pfeuffer.com/product/contador) may be useful for counting large numbers of seeds. In this case, the seed sample must be pure

without any contamination or debris. A seed X-ray or a cut test may be used to assess the proportion of filled or viable seeds.

Similarly, seed production of a unit area of vegetation (mainly $1m^2$, at least for grasslands) can be calculated by collecting all seeds, weighing them and dividing it through the seed mass of one seed (Šerá & Šerý, 2004).

For woody species such as trees, shrubs and lianas, an exact counting is often not possible, except for very large fruits (e.g., *Borassus*, *Durio*). Therefore, seed production has to be estimated by counting seeds for the infructescences of a single branch and then calculating according to the number and size of branches for the whole plant. In case seeds or fruits have fallen to the ground, the ground plot or quadrat method, i.e., the counting of the number of fallen seeds (or fruits) in ten 50 x 50cm quadrats under the canopy and calculating the amount either with the total canopy size or per m^2 has also been shown to give reasonable results (Touzot *et al.*, 2018; Tattoni *et al.*, 2021). Seed numbers in large trees can also be estimated by counting large fruits or infructescences on a section of a canopy photograph with a known area or by counting them on a portion of the canopy or the entire tree from a distance with binoculars as used in forestry.

3.2.5 Seed mass

Trait description

Seed mass is typically measured as the weight of the seed on a dry mass basis. While ‘seed size’ is often used interchangeably with ‘seed mass’, it is recommended to separate the two, as seed length is a more intuitive measure of size. Since many studies do not attempt to distinguish seed and diaspore, seed mass may also refer to diaspore mass for a practical purpose (Moles *et al.*, 2005a; Chen & Giladi, 2018). When taken in a broad sense, ‘seed’ may include diaspore or fruit structures and appendages. Hence, diaspore mass may also be reported as seed mass in the literature. The most commonly used measure is the oven-dried mass, by the definition of “biomass”. Oven-dried seed mass represents the allocation of dry matter in the seed, preferably to compromise among alternative measures and to maintain comparability with most existing data (Leishman *et al.*, 2000). However, in many cases, it is practical to use fresh or air-dried mass after an unspecified storage period. For example, seeds may be stored and desiccated in a room with 15% humidity. Air-dried seed mass could be more ecologically relevant, such as when seeds are dispersed or persist in the soil. The fresh mass may also be measured when seeds are newly harvested (Royal Botanic Gardens Kew, 2022a). While these weighing statuses are suggested to be explicitly reported, alternative measures are strongly correlated with each other across a wide range of species (Leishman *et al.*, 2000). If possible, we recommend measuring both air-dried and oven-dried seed mass so that the data could be reused according to the research context.

Functionality and trade-off

Seed mass is a fundamental plant trait, representing the amount of resources allocated and stored in the organ for the growth of the next generation (Westoby, 1998) and broadly informing regeneration strategies (Díaz et al., 2016). It plays a pivotal role in many plant life stages, including reproduction, dispersal, germination, seedling survival, and establishment (Leishman *et al.*, 2000). Seed mass is also correlated with

various life-history traits, such as plant height, lifespan, size of vegetation organs and seed bank persistence (Díaz *et al*., 2016; Saatkamp *et al*., 2019). The negative relationship between seed mass and seed number is a prevailing pattern in biological trade-offs across species, although it may vary or even vanish in certain taxa.

Source of variability

Seed mass varies at different levels of organisation (Herrera, 2017). Seed mass varies greatly among species across the plant tree of life by nearly 12 orders of magnitude, from dust-like orchid seeds (Orchidaceae) to double coconuts (*Lodoicea maldivica*; Moles *et al.*, 2005a). Many abiotic and biotic factors could select seed mass. On a global scale, seed mass increases towards the equator and is positively associated with net primary productivity, temperature, and precipitation (Moles *et al.*, 2005b). However, plant traits such as seed dispersal mode and plant growth form explain seed mass variation much more than environmental variables (Moles *et al.*, 2005b). For example, seeds are larger in woody species than in herbaceous species.

Compared to the interspecific variation, intraspecific variation in seed mass is small (Wang *et al.*, 2021). Intraspecific variations in seed mass might not necessarily reflect species filtering at the species level (Guo *et al.*, 2010). Based on 22 species collected in the Tibetan Plateau grasslands, species showed different elevational variations in seed mass, but no overall trend (Wang *et al.*, 2021). Intraspecific variations in seed mass caused intraspecific variations in seedling performance, evident in a study of two tropical tree species (Fricke *et al.*, 2019).

The two main components of a seed, seed coat and the embryo-cum-endosperm fraction (also known as seed reserve or seed kernel), are subject to different evolutionary selection forces and, therefore, show a broad range of variation in mass (Wu *et al.*, 2019). Their respective sizes and allometric scaling contribute to the variation in the mass of the whole seed (Chen *et al.*, 2020b).

Methodology

Seed mass needs to be measured according to the purpose and level (i.e., species, population, individual plant) of the study. No single measurement of seed mass is ideal for all purposes (Leishman *et al.*, 2000). We present the general protocol for species-level measurement, as other purposes could be adjusted accordingly.

When sampling, collect seeds from healthy adult plants and use mature and intact seeds around the point of natural dispersal. Seed quality should be assessed by observing seeds' external appearance using a cut-test or X-ray on collection sample. The number of seeds to be measured may also depend on the purpose and level of the study, as well as the accuracy of the balance. More seeds will be needed for species with tiny seeds. The ISTA Rules suggest using either the whole working sample (at least 2500 seeds) or eight replicates of 100 seeds (ISTA, 2023). However, while sample size can be achieved for seeds of crops, it is not possible in many cases for wild species. In the Millennium Seed Bank, the seed mass of a collection is weighed for five replicates of 50 seeds. It is also recommended that the collection needs to be from at least ten seeds from each of the ten individuals of a species (Pérez-Harguindeguy *et al.*, 2016).

It is best to measure seed mass as soon as possible after seed collection; otherwise, store seeds in a cool and dry environment. Dry the seeds until equilibrium mass (such as 80 °C for 48 h, or 60 °C for 72 h or 130 °C for 12 h; Chen & Moles, 2018). As seeds take up moisture from the air once taken from the oven, put the sample in a desiccator to cool down until weighing. Determine the mass of oven-dried seeds using an analytical balance. Note whether units are in milligrams (mg) or grams (g) and 'per seed' or 'per 1000 seeds' ('thousand grain weight').

3.2.6 Seed size and shape

Trait description

Seed size and shape represent morphological variation in seed characters. Seed size can be measured in three main dimensions: length, width, and height (Thompson *et al.*, 1993). Seed size is an interchangeable measure of seed mass in specific research contexts (e.g. Chen & Moles, 2015). Based on an analysis across 65 species, seed mass (mg) and seed volume (mm^3) show allometry (Moles *et al.*, 2005b):

$$Seedmass = 1.1 \times Seedvolume^{0.96}$$

Seed shape can be indexed based on the similarity to a given geometric object (Cervantes *et al.*, 2016).

Functionality and trade-off

Like seed mass, seed size represents an important ecological strategy axis and links many plant life stages (Westoby, 1998; Leishman *et al.*, 2000). Seed shape is hypothesised to be related to seed persistence, burial likelihood, dispersal ability and germination physiology (Thompson *et al.*, 1993; Funes *et al.*, 1999; Cerabolini *et al.*, 2003). Rounded seeds tend to be more persistent in soil than elongated, flattened or irregularly shaped seeds (but see Leishman & Westoby, 1998; Moles *et al.*, 2000; Peco *et al.*, 2003). However, in a study of 110 leguminous species, flattened seeds are an adaptation to prevent seed predation by bruchid beetles, thus could have higher survival (Szentesi & Jermy, 1995). Seed shape is also associated with agronomic traits, such as seed yield or quality (Adewale *et al.*, 2010; Williams *et al.*, 2013).

Source of variability

Like seed mass, seed size varies dramatically between species, from dust seeds of Orchidaceae to the massive seeds of double coconut (*Lodoicea maldivica*; Moles *et al.*, 2005b). The main dimensions – length, width, and height – can vary due to genetic and environmental factors resulting in the variability of seed shape. According to

Thompson *et al.* (1993), seed shape varies relatively little between individual seeds of the same species.

Methodology

Seed dimensions can be measured by callipers manually or through digital images and image analysis software (e.g. ImageJ, SmartGrain; Tanabata *et al.*, 2012). The former method is simple and depicts multiple dimensions but may not be applicable to minute seeds. The latter method provides data of more reproducibility and higher quality and has the potential to be automated.

After measuring the main dimensions, there are various ways to index seed shape. As proposed by Thompson *et al.,* (1993) and followed by many authors afterwards, seed shape can be analysed using the variance in diaspore dimensions after transforming all values to unite the length. In this way, perfectly spherical seeds have a variance of 0, while elongated or flattened seeds have variances of up to 0.33 or even larger.

Alternatively, seed shape can be determined by the ratio of seed length and width, known as the Eccentricity Index (EI):

$$EI = \frac{Length}{Width}$$

As digital images are two-dimensional, EI can be presented as the aspect ratio of the particle's fitted ellipse:

$$Aspect\ ratio = \frac{Majoraxis}{Minoraxis}$$

Seed shape also can be measured as roundness, ranging from 0 to 1 to show how closely an ellipse approaches a perfect circle:

$$Roundness = \frac{4 \times area}{\pi \times Majoraxis^2}$$

Similarly, a measure can also be used to express the extent to which seed shape differs from sphericity with data of three-dimensional sizes (Thompson *et al.*, 1993). A few

other descriptors of seed shape, such as the *J* index for cardioid figures, are comprehensively discussed in Cervantes *et al.* (2016).

The number of seeds to be measured depends on the study's purpose and organisation level. Thompson *et al.* (1993) measured five replicate seeds for each species, and other studies have measured ten replicates (Moles *et al.*, 2000) or a hundred replicates (Gardarin & Colbach, 2015).

3.2.7 Seasonality of seed release

Trait description

Seasonality of seed release is the period or time of the year (and, in some cases, the number of years) when seed release takes place. The length of the period may differ within and among species. The seed release period may be short and last only a few days or weeks, whereas, in certain species, it may last months or even years (i.e., aerial seed banks; see section **3.3.1 Serotiny**).

Functionality and trade-off

Seasonal seed release may strongly differ between species but also within species, as well as the length of the seed release period. Both strongly depend on climate (Seale & Nakayama, 2020) and the first also in many species to fire (Lamont *et al.*, 2019). Seasonal seed release is also often correlated with the availability of the dispersal vector.

In temperate spring geophytes, early flowering and seed release are concomitant with the seasonal peak of ant foraging (Oberrath & Böhning-Gaese, 2002; Guitián & Garrido, 2006; Boulay *et al.*, 2007; Warren *et al.*, 2011). Warren *et al*. (2011) showed that this relationship is temperature-dependent. Aquatic or semi-aquatic species that disperse most diaspores in spring and summer had a shorter seed release period than species whose seeds are dispersed in autumn and winter (Boedeltje *et al.*, 2004). The availability of the dispersal vector is also obvious in many arable weeds, which ripen at the same time as the cultivated plants. Many plant species could, in this case, migrate over large distances when the harvested seed contained both seeds of cultivated species and arable weeds. For example, many weeds from the Fertile Crescent or the Mediterranean region have migrated with such uncleaned seeds during the Neolithic Age and the Roman period to Central Europe and are now part of its flora, e.g. *Bromus arvensis, Agrostemma githago* and many others (Bonn & Poschlod, 1998; Poschlod & Bonn, 1998; Fuller & Allaby, 2009; Poschlod, 2015; 2017). Seed release of many species of the Cerrado in Brazil is triggered by the rainy season (Escobar *et al.*, 2018) or the end

of the dry season when most species have lost their leaves (Novaes *et al.*, 2020). Besides rainfall, wind speed is an important parameter for seed release. Seed release of wind-dispersed species may be favoured during dry seasons when atmospheric conditions favour uplift (Wright *et al.*, 2008; see also Tackenberg *et al.*, 2003b). For tree species with winged seeds and medium seed terminal velocity, Heydel *et al.* (2015) found a pronounced seasonal synchronization of seed release timing with high long-distance dispersal (LDD) by the wind. In many species of fire-prone ecosystems, seed release is strongly affected by fire, with fruits only opening with the fire or through the heat and smoke also breaking dormancy, increasing the probability of successful germination and establishment on newly open ground (Bond, 1984; Brown & van Staden, 1997; Keeley & Fotheringham, 2000; Pausas & Lamont, 2021). However, the advantage of the fire is only effective if fruits and seeds are ripe (Miller *et al.*, 2019). Seed release may also be related to daytime due to changes in atmospheric conditions like air humidity contributing to the opening or closure of fruits (Wright *et al.*, 2008). Seasonality of seed release is expected to maximise seedling establishment in seasonal ecosystems, where opportunities for establishment are not equally distributed over the course of the year.

The time and duration of seed release may strongly affect the dispersal potential (Wright *et al.*, 2008; Poschlod *et al.*, 2013). There may be a trade-off between dormancy and seed release period to fine-tune seedling establishment with optimum environmental conditions (Walck *et al.*, 2011; Escobar *et al.*, 2018). The time of seed release period may be extended via secondary seed dispersal and therefore contributes to higher dispersal distances (Kowarik & von der Lippe, 2011).

Applied aspect

When the seed release period and the suitable dispersal vectors may be disentangled, either by changing land use or climate change, species which may be concerned by these processes may become endangered (Poschlod & Bonn, 1998). Knowledge of seed dispersal season is important for planning and implementing seed-based restoration strategies (Buisson *et al.*, 2017).

Source of variability

The seasonality of seed release and the length of the seed release period may strongly vary due to the climate and habitat conditions. For example, populations on south-facing slopes or open habitats may release their seeds earlier than populations on north-facing slopes or in closed habitats such as forests. Fruiting phenology also varies among different years.

Methodology

The respective phenological observations should be made in the field and for all habitats or sites where a species occurs. Depending on the study goal, a minimum of ten individuals should be randomly tagged, and (whenever possible) we recommend weekly or biweekly observations of fruit ripening, the loss of seeds or fruit opening. Monitoring fruiting phenology in the tropics is usually done at monthly intervals, with observations occurring year-round. Observations should last until the moment when the last seed is released. The number or proportion of seeds released at any observation should be noted.

A special case is when seeds are only released at a certain time of day, which needs more intense observation during the day, such as every hour or so. Wright *et al*. (2008) even measured it every 20 minutes.

The beginning and end of seed release should be reported, as well as the length of the seed release period. The date or days after the 1st of January should be given (i.e., the day of the year), but data can also be expressed as mean angles in circular analyses (Morellato *et al.*, 2010). The minimum, maximum, and mean number of days should be given for the length of the seed release period.

3.2.8 Dispersal vector

Trait description

Dispersal vector is an agent that transports a seed away from its mother plant. It is either wind and water or may include a more detailed description of animal and human-mediated dispersal. This may be classified according to the animal species or any anthropogenic land use type and the related machines, vehicles, and other human activities, such as trade, which may disperse the seeds.

Functionality and trade-off

The potential dispersal vector of propagules depends on many parameters. Concerning animals, it may depend on the morphology (mean shoulder height) and the surface (type of fleece; Albert *et al.*, 2015a; Römermann *et al.*, 2005e; Tackenberg *et al.*, 2006), biology (time of seed retention in the fur or gut; Nathan *et al.*, 2008; Will & Tackenberg, 2008; Nield *et al.*, 2020), the feeding and movement behaviour as well as of the landscape and habitat an animal lives in (Will & Tackenberg, 2008; Nield *et al.*, 2020). Concerning human-mediated dispersal (until now, compilation of several hundred citations only exist for Europe; Bonn & Poschlod, 1998), it may depend on livestock kept by farmers, if the livestock is allowed to feed in the field during seed set or to feed from propagules containing hay, if manure or slurry is applied as a fertiliser and not mineral fertiliser (Poschlod & Bonn, 1998), and numerous other types of land use and the time of practising them (dispersal by a farm or forest vehicles: Veldman & Putz, 2010; Auffret & Cousins, 2013; combines: Petzoldt, 1959; haymaking machines: Strykstra *et al.*, 1996; 1997). Vehicles such as bikes or cars may disperse seeds (Clifford, 1959; von der Lippe & Kowarik, 2007; 2012; Taylor *et al.*, 2012; Ansong & Pickering, 2013; von der Lippe *et al.*, 2013; Rew *et al.*, 2018; Yang *et al.*, 2021) as well as hiking humans (Hodkinson & Thompson, 1997; Pickering & Mount, 2010).

Dispersal distances are strongly correlated with the dispersal vector. Each dispersal vector favours dispersules with a certain morphology or structure (see section **3.2.2**

Dispersule structure) and species that match the time when the dispersal vector is active (see section **3.2.7 Seasonality of seed release**).

Applied aspect

Information about the dispersal vector will allow the selection of suitable management of anthropogenous (human-created) habitats or the directed dispersal of species to restoration sites (Strykstra *et al.*, 1997; Poschlod *et al.*, 1998; Piqueray *et al.*, 2015). Vehicles and humans may also contribute to the spread of invasive species (von der Lippe & Kowarik, 2007; Veldman & Putz, 2010). This can be taken into account when trying to control species invasions.

Source of variability

Seed dispersal by individual animal or human-mediated dispersal vectors may vary due to the size of the seed and/or disperser, individual behaviour or functioning.

Methodology

There is no special methodology necessary. As already done in the studies from the 19th and the first half of the 20th century (Beal, 1898a; b; Ridley, 1930; Müller-Schneider, 1986; Poschlod *et al.*, 2003), single observations and the notation of the dispersal vector are sufficient.

Concerning animal dispersal, the common and scientific name of the animal should be given, as well as whether it was exo- or endozoochorous dispersal. Concerning human-mediated dispersal, any information on which type of land use process, machines or vehicles or human beings should be noted.

For all studies concerning the dispersal vectors, the date of the observation or study should be given as well as the habitat and location (site, country) where the study took place.

3.2.9 Seed releasing height

Trait description

Releasing height is the maximum height of a plant from which dispersules are released (Thompson & Kunzmann, 2005). It should not be confused with canopy height, which may be higher or lower than releasing height (e.g., rosette-forming herbs with long fruiting stems and trees fruiting on the trunk).

Functionality and trade-off

Releasing height, along with terminal velocity, is an important trait to determine seed dispersal distance that are dispersed by wind or gravity (Tackenberg *et al.*, 2003a; Nathan & Katul, 2005). Releasing height substantially influences seed dispersal distance more than seed mass (Thomson *et al.*, 2011). The higher from ground level a dispersule is released, the higher the probability of travelling long distances (Greene & Johnson, 1989; Carey & Watkinson, 1993). A high releasing height may also increase the probability of reaching upward winds, which are important for long-distance dispersal (Nathan *et al.*, 2002; Tackenberg, 2003; Tackenberg *et al.*, 2003b). Releasing height is also important for external animal (ectozoochorous) dispersal (Fischer *et al.*, 1996).

Given that growth form and plant height are related to releasing height (Tackenberg *et al.*, 2003a; Zhou *et al.*, 2019) and, therefore, to dispersal distances but also the proportion of long-distance dispersed seeds (Tackenberg *et al.*, 2003a). Woody and tall species generally have greater releasing heights than herbaceous and short species (Chen *et al.*, 2019b).

Sources of variability

Plant height, and hence release height may be very plastic and vary across habitats (environmental conditions) that the plant grows (Hiesey, 1953; Aronson *et al.*, 1992), management (Da Silveira Pontes *et al.*, 2010), and across years depending on the climatic variability (Leger, 2013). For woody plants, release height increases during their lifetime (Thomas, 2011).

Methodology

Measurement should be done during seed set or seed release. Releasing height is measured as the vertical distance between the highest dispersule and the ground. For shrubs and smaller trees, a telescopic stick with meter marks is recommended (Thompson & Kunzmann, 2005). For taller trees, releasing height should be measured using a laser rangefinder and trigonometric principles. It can be calculated by two methods:

(1) The 'tangent method': measure the horizontal distance from the person to the base of the tree (AB in Fig. 4) and the angle α between the horizon and the top infructescence (e.g. one tree length; Korning & Thomsen, 1994; Goodwin, 2004). The distance of the measuring point to the ground (AD) can be estimated from the height of the person on flat ground or on slopes by applying the sine method. AC is computed using trigonometric principles:

$$|AC| = \tan(\alpha) \times |AB|,$$

releasing height (CD) is then calculated as the sum of AD and AC.

(2) The 'sine method' (Larjavaara & Muller-Landau, 2013): measure the distance to the infructescence at the top of the tree (BC in Fig. 4) and the angle α between the horizon and the top infructescence (e.g. one tree length; Korning & Thomsen, 1994; Goodwin, 2004). The distance of the measuring point to the ground (AD) can be estimated from the height of the person on flat ground or on slopes by applying the sine method. AC is computed using trigonometric principles:

$$|AC| = \sin(\alpha) \times |BC|,$$

releasing height (CD) is then calculated as the sum of AD and AC.

Comparing both methods, Larjavaara & Muller-Landau (2013) recommend the application of the 'sine method', which was faster to learn and displayed less variation in heights among the measuring persons. Despite an underestimation of 20% on

average the error was much lower than in the ‘tangent method’. Commercially available laser range finders can calculate release height automatically.

A new method is to estimate tree height, which is often similar to the infructescence height, using airborne laser scanning techniques such as LIDAR (Holmgren *et al.*, 2003), but data are less precise compared with the conventional methods (Andersen *et al.*, 2006). Since releasing height is very variable, a measurement of 30 individuals per species or population is recommended. Sample size and values should be entered into the database with a note on whether the data are at the individual plant level or the species level (Thompson & Kunzmann, 2005).

Special cases

There are some special cases, such as epiphytes and aquatic plants. For epiphytes and epiphytic hemi-parasites, releasing height should be measured between the highest fruit and the base where the plant is attached. For aquatic plants, it is the distance between the highest fruit and the water surface (Thompson & Kunzmann, 2005).

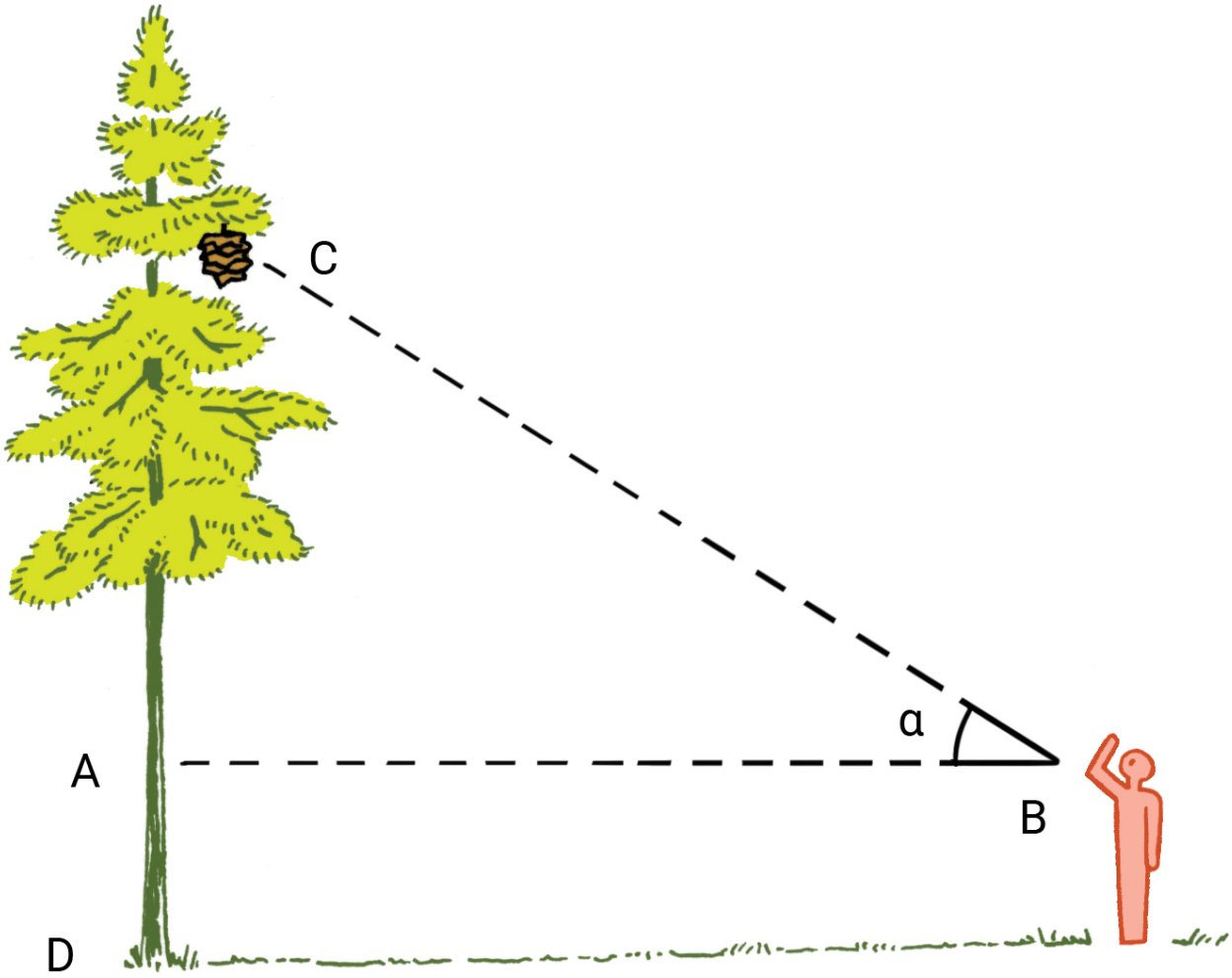


Fig. 4 Illustration of the tangent and sine methods of releasing height for taller tree.

3.2.10 Terminal velocity

Trait description

Terminal velocity is the maximum fall speed of a particle (dispersule or seed) in still air, which occurs when the effect of gravity is balanced by air resistance (Thompson, 2005).

Functionality and trade-off

Terminal velocity is strongly related to seed dispersal distance by wind (Tackenberg *et al.*, 2003a). Distances depend on air velocity or wind speed as well as its direction (upward, horizontal, and downward; Tackenberg *et al.*, 2003b; Savage *et al.*, 2014). Updrafts and horizontal winds are more effective than downdrafts (Greene & Quesada, 2011). Species with lower terminal velocity generally have longer dispersal distance and thus have smaller population declines or are less threatened with extinction than species with dispersal vectors other than wind (Ozinga *et al.*, 2009).

Dispersule or seed size, density, shape and surface, as well as the structure, especially of any appendages (e.g., wing, pappus, plume, membrane), may strongly affect terminal velocity (Tackenberg *et al.*, 2003b; Zhou *et al.*, 2019) and, therefore, dispersal distances by wind or the proportion of long-distance dispersed seeds or fruits (Tackenberg *et al.*, 2003b).

Applied aspect

A low terminal velocity is important for being dispersed over long distances by wind and, therefore, to escape competition with the mother plant and migrate between fragmented or isolated habitats (Tackenberg *et al.*, 2003a; Poschlod *et al.*, 2005). Species with lower terminal velocity tend to be more common in open habitats, e.g. grasslands, than in closed ones, e.g. forests (Willson *et al.*, 1990; Ozinga *et al.*, 2004). Recently, terminal velocity has also been shown to play an important role in the community assembly process (Rosbakh *et al.*, 2022).

Sources of variability

Variation in terminal velocity depends on the variation in dispersule or seed characters, especially size and weight, as well as the development of appendages (Sacchi, 1987; Andersen, 1992). Variation in these traits may be affected by the environment (e.g. air moisture affects the opening of the parachute shape of pappi) but also by the mother plant (maternal effects) occurring within seed lots or inflorescences (Sacchi, 1987; Gutterman, 2000; Chen & Giladi, 2020).

Methodology

Measurements should be made with freshly collected dispersules. Dispersule collection should not damage any appending structures such as pappi, wings, which are important for the fall velocity. Before measurements, dispersules should be screened to check if they are well developed and avoid embryoless seeds. Seed fill can be easily tested with an X-ray without destroying the structure.

Terminal velocity can be measured either by a dropping method or a method to float seeds in an upward air stream. Both methods give similar results when seeds are dropped from a respective height so that they may reach terminal velocity (Jongejans & Schippers, 1999). In the dropping method, measurements should be taken when the instant falling velocity has reached constant (usually at the last stage of falling). In the floating method, terminal velocity equals the upward wind flow velocity when the seed remains suspended in the wind tunnel.

Two useful techniques for measurements have been described. Askew *et al.* (1997) used an apparatus to determine the falling speed of seeds by detecting their passage through two fans of laser light. This apparatus is especially useful when measuring terminal velocity from small seeds. For larger seeds, camera recording is a more suitable method since it eliminates the effect of acceleration and allows for correcting seed terminal velocity. The method is less expensive and was described in detail by Liu *et al.* (2021; see also Chen & Giladi, 2020). Liu *et al.* (2021) also summarised several traditionally or commonly used methods measuring the terminal velocity of seeds.

For the measurements of one species or within a population, at least 10 intact dispersules are recommended (Thompson, 2005). It is important to note which kind of dispersule was measured (fruit, seed or vegetative dispersule). The square root of the loading of wind-facilitated appendage (seed mass divided by plume/wing area; Greene & Johnson, 1990) can be used as a surrogate for terminal velocity. The unit of terminal velocity should be $m \bullet s^{-1}$.

3.2.11 Buoyancy

Trait description

Buoyancy or floating capacity is the dispersal of dispersules or seeds by water. Dispersal by water is also called hydrochory or nautochory. Dispersal distance depends on how long dispersule or seeds can float on or drift by water (Coops & Van der Velde, 1995), but also if they may be deposited before they sink. Therefore, floating or drifting distances are affected by the wind on lakes, ponds or the sea, currents in the sea or flow velocity in rivers. Deposition may depend on structures at the water's edge, which may function as seed traps, and on flood and tidal ranges on marine coasts.

Functionality and trade-off

Buoyancy is related to the habitat and its connectivity (Moggridge *et al.*, 2009). In wetlands such as swamps and peatlands but also lakes, rivers and sea shores, hydrochory is an important dispersal mode connecting habitats (Middleton, 2000; Vogt *et al.*, 2004; Guja *et al.*, 2010; Kehr *et al.*, 2014). Hydrochory also structures riparian and wetland communities (van den Broek *et al.*, 2005; Nilsson *et al.*, 2010). Floods play an important role in this respect (flood pulse concept according to Junk *et al.*, 1989; see also Middleton, 2002; Boedeltje *et al.*, 2004).

Seed density (often related to air-filled seed coat or tissue) and volume:surface area ratio (Carthey *et al.*, 2016), as well as a hydrophobic surface (Cook, 1990), may strongly affect buoyancy. There may be a trade-off between buoyancy capacity and soil seed bank persistence. Species with long floating seeds have a higher proportion of transient seed banks than persistent ones (van den Broek *et al.*, 2005).

Applied aspect

Hydrochory is positively related to species richness in aquatic habitats (Jansson *et al.*, 2005) but is also an important vector for dispersing invasive species (Säumel & Kowarik, 2010; Schmiedel & Tackenberg, 2013). Hydrochory provides, however, a potential to contribute to wetland restoration when respective pathways for water

dispersal are available or restored, which was shown for riparian habitats (Helfield *et al.*, 2007) and tidal areas (Neff & Baldwin, 2005; Wolters *et al.*, 2005).

Sources of variability

Floating capacity varies depending on turbulence in the water caused by wind or currents but also sometimes on a variable surface structure of the dispersule and if the surface is more or less hydrophobic (Poschlod, 1990). In standing water, floating capacity may be longer than in running or turbulent water or vice versa (van den Broek *et al.*, 2005). Since dispersal only occurs when wind or running water causes seeds to drift, floating capacity should be measured, including the movement of water, which may be varied.

Methodology

There are several possibilities to assess buoyancy – the common and standardised way is to measure floating capacity (Danvind & Nilsson, 1997; van den Broek *et al.*, 2005). Alternatively, traits such as seed density and volume:surface area ratio may explain floating capacity, but they were only measured for a small set of species in the Australian flora (Carthey *et al.*, 2016). Floating capacity can also be measured under standardised conditions or in the field (Boedeltje *et al.*, 2004; Vogt *et al.*, 2004; van den Broek *et al.*, 2005). Distances covered may be measured by marking (e.g., water-proved colour) seeds.

According to Carthey *et al.* (2016), seed density (mg • mm^3) is calculated as mass divided by volume. Seed volume (mm^3) and surface area (mm^2) are calculated using volume and surface area formulae for a cylinder (seeds shaped like discs or rods), a sphere or an ellipsoid (blade-shaped seeds).

Measurements of floating capacity follow the method described in the LEDA handbook (Römermann *et al.* 2005b). Measurements should be made with ripe fruits and viable seeds. For heteromorphic species, floating capacity should be measured on all types. To measure floating capacity, if possible, two or more replicates of each 100 seeds per

species are recommended from plants growing in their typical habitats and from different individuals.

Floating capacity is given as the proportion of seeds floating after a defined time period. Floating capacity shows little intraspecific variability (Bill *et al.*, 1999). Seeds are put in glass beakers – Römermann *et al.* (2005b) recommended a standardised size of 10 cm width, 12 cm height and a potential volume of 600 ml – filled with 300 ml distilled water. Since seed dispersal may only occur when water is moving, we recommend the placement of the beakers on an orbital shaker (Phartyal *et al.,* 2020b). A movement with a frequency of 100/minute and an amplitude of about 1 cm is recommended. According to several studies (Bill *et al.*, 1999; Boedeltje *et al.*, 2004; van den Broek *et al.*, 2005), changes in floating capacity are largest during the first 24 hours. Differences observed between species usually stabilise after one week (Römermann et al. 2005b). Therefore, we recommend observing the proportion of seeds still floating at the intervals shown in Table 6.

Table 6 Floating time intervals according to Römermann *et al*. (2005b).

Interval:	Time step:
T0	Immediately
T1	5 min
T2	1 hour
T3	2 hours
T4	6 hours
T5	1 day
T6	1 week

Data entries should include the mean floating capacity (and median if more than two replicates are analysed), N (number of replicates), the standard deviation, the standard

error, the minimum and the maximum, the time step, and information about the examined dispersal unit. Floating capacity is the duration of time over which seeds float and should be given at least for two time steps (T_{50} or T_{90}: time `T´ when 50 % or 90 % of seeds have sunk).

To study seed drift on the water surface or in the water body, drift nets are applied (detailed description in Boedeltje *et al.* (2004); Vogt *et al.* (2004)) which have, however, the disadvantage that the proportion of invertebrates caught is much higher than that of seeds (Bill *et al.*, 1999). This means that drift nets can be applied only over a limited time period depending on the accumulation of drift material (10 minutes to 3 hours, own measurements). In 'wild' mountain rivers, seed drift is so low compared to drifting invertebrates (up to 18 million individuals per day in the upper part of the river Isar, Germany) that sediment baskets were used to collect drifting seeds (Bill *et al.*, 1999). Turf mats may be used to measure seed deposition during flooding (Vogt *et al.*, 2004; Wolters *et al.*, 2005; Moggridge *et al.*, 2009). We recommend a binary entry (yes or no) if a species was once proven to be dispersed by water (Poschlod *et al.*, 1998).

3.2.12 Epizoochory

Trait description

Epizoochory is the dispersal of diaspores on the surface of an animal, which may be the coat, fleece, fur, or feathers at any part of the animal, including feet and hooves. Vertebrates, mostly mammals and birds, facilitate external seed dispersal. Epizoochory is also called ectozoochory or external animal dispersal. Epizoochory consists of three processes – attachment (Will *et al.*, 2007), retention (Couvreur *et al.*, 2004; 2005; Römermann *et al.*, 2005c; Tackenberg *et al.*, 2006) and release, which should be measured separately but assessed together (Will *et al.*, 2007).

Functionality and trade-off

Epizoochory has been shown to be important in connecting habitats and maintaining viable population size. Dispersules of species with a low capacity of being externally dispersed by animals are rare today or declining (Römermann *et al.*, 2008). According to Ozinga *et al*. (2009), species that rely on epizoochory are threatened by a much higher proportion than species that rely on any other long-distance dispersal vector or process. Considering all long-distance dispersal possibilities, epizoochory belongs to the most effective ones (Manzano & Malo, 2006).

Diaspore size, shape, surface, and structure may strongly affect epizoochory (Fischer *et al.*, 1996; Römermann *et al.*, 2005c). Additionally, there is a trade-off to the diaspore exposure, including the releasing height and the surface type of the coat (Fischer *et al.*, 1996; Römermann *et al.*, 2005e; Hintze *et al.*, 2013; Albert *et al.*, 2015b).

Applied aspect

Epizoochory, especially through livestock herding, is an important management parameter to connect fragmented or isolated habitats (Poschlod *et al.*, 1996; Willerding & Poschlod, 2002; Auffret *et al.*, 2012; Rico *et al.*, 2013). Domestic livestock, especially sheep, have been shown by Fischer *et al*. (1996) to disperse many species in the fleece/fur or hooves and may, therefore, be used to restore specific habitats through

respective herding management (Piqueray *et al.*, 2015). External animal dispersal is the most important dispersal mode for extreme long-distance dispersal in terrestrial habitats (Manzano & Malo, 2006), including migrating birds (Viana *et al.*, 2016).

Sources of variability

Epizoochory depends on the size and surface structure of the animal. Therefore, the epizoochory of a species should be measured for each animal species separately (Couvreur *et al.*, 2004; Römermann *et al.*, 2005c; Horn *et al.*, 2013). Many parameters may vary from year to year, consequently influencing whether a diaspore is dispersed or not and the proportion of diaspores dispersed. These parameters may be releasing height, seed production, seed-releasing period, and diaspore morphological traits. However, there are no long-term studies in this respect.

Methodology

There are several possibilities to assess epizoochory – either as a (very) soft trait via diaspore traits (Couvreur *et al.*, 2004; Römermann *et al.*, 2005c), or as a hard trait at the living animal (Fischer *et al.*, 1996) or with a piece of coat of the respective animal (Couvreur *et al.*, 2004; 2005; Römermann *et al.*, 2005c; e). Measuring the full process implies addressing two separate processes: attachment and retention. The proportion (or percentage) of seeds that attach to the animal fur or feathers in an experiment is called attachment potential. Studies using living animals are time-consuming and depend on the willingness of the animal to cooperate. This is possible only with domestic or tamed (Fischer *et al.*, 1996; Stender *et al.*, 1997; Couvreur *et al.*, 2005) but not wild animals, except when they are captured by hunters (Vivian-Smith & Stiles, 1994). However, in the latter case, seeds can become attached after shooting. We recommend a binary entry if a species was once found on a certain animal of 1 and if not of 0 (Poschlod *et al.*, 1998).

Standardised quantitative measurements on living domestic or tamed animals can be done with diaspores marked with waterproof paint on the coat (Fischer *et al.*, 1996). The number of seeds remaining after certain time periods allows the calculation of the

proportion of diaspores covering certain distances depending on the animal's movement behaviour (Mouissie *et al.*, 2005; Bullock *et al.*, 2011)

Standardised measurements on the number of seeds getting attached (attachment potential) and retained (retention potential) can be easier done under standardised conditions in the lab. They can be done with a piece of coat (size depends on the animal size, e.g. for large herbivores, 30 x 50cm^2 is recommended) fixed on a vertical wooden board. Before the attachment and retention potential are measured, the coat should be 'homogenised' with a wooden comb (Römermann *et al.*, 2005c). This protocol can be adapted to other surfaces, such as feathers or feet.

For the attachment potential, the method slightly changed from Will *et al*. (2007) consists of the installation of one vertical coat board in a collection box. Ten replicate shoots with ripe diaspores are carefully sampled in the field and transported to the lab. Immediately after, the replicates are gently placed on the surface of the coat. To cover the variability of the coat, at least two different boards with a coat of the same animal species should be used, ideally five boards. After the placement, the total number of seeds attached and remaining in the infructescences are counted and the proportion of seeds attached is calculated. Since certain diaspores, especially mucilaginous seeds, show a different attachment but also retention behaviour when dry or wet or are even only attached when wet (e.g., on non-hairy reptile coats, bird feathers or feet; Yang *et al.*, 2012), measurements should be done for those diaspores under both conditions.

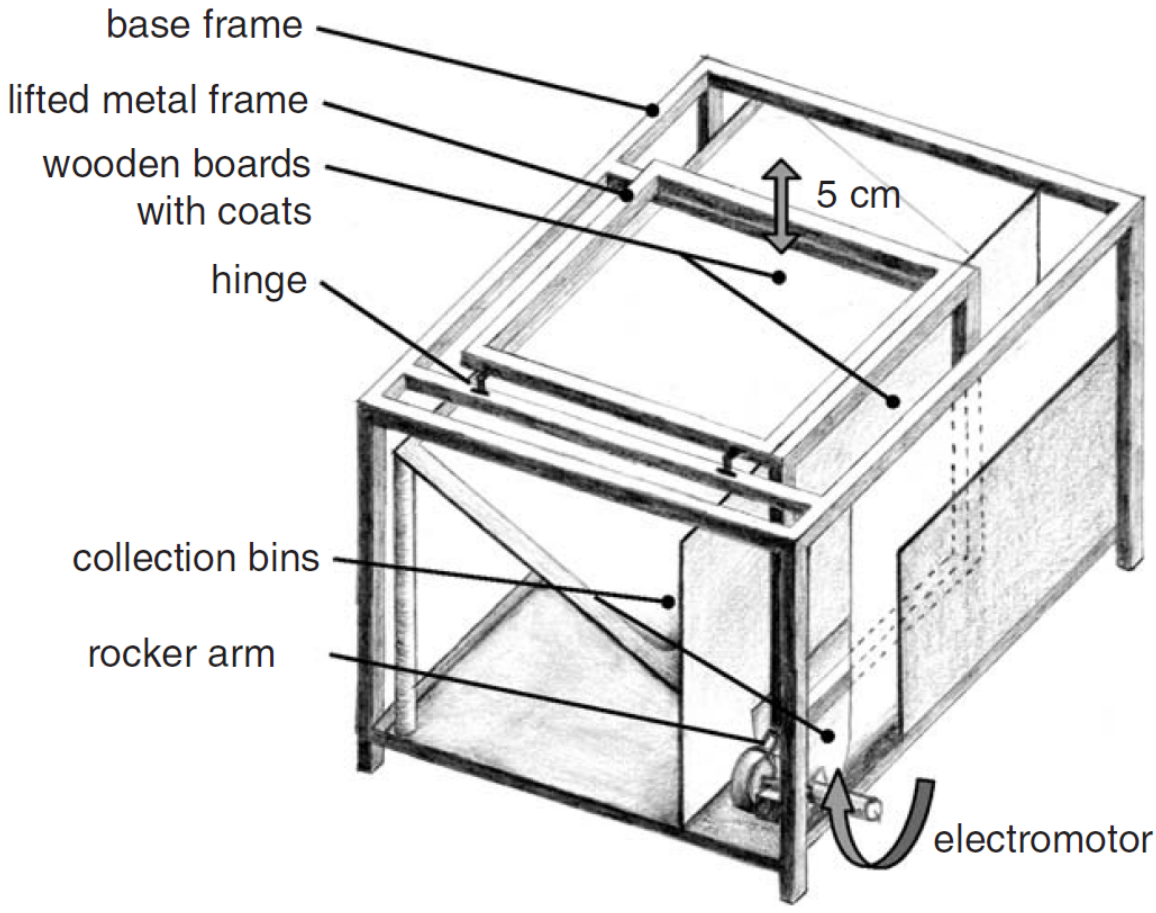
To calculate the attachment potential (ranging from 0 to 100%), algorithms for any coat type are presented. However, Couvreur *et al*. (2004) stated that although seed morphology is a good predictor for seed adhesivity on fur, less well-adhering seed types often still have relatively high adhesivity scores. Therefore, it is likely that nearly all species are, to some extent, able to disperse epizoochorously.

Retention, measured in hours, is when a seed remains attached after the animal starts moving. The method to measure retention time is slightly changed after Römermann *et al*. (2005c). Seeds are placed on the horizontal coat board and should be gently

pressed with another wooden board on the coat surface. Then, the board is erected vertically in the collection box to count the seeds which fall off immediately. Then, the vertical board with the coat is mounted in a 'shaking machine' (Fig. 5). Shaking movements should be adjusted according to the steps of a walking animal/minute. The proportion of retained seeds should be measured after distinct time intervals (e.g. 5, 10 min, 1, 2, 6, 12 hours, 1 day).

Exact entries in each experiment are recommended and a value (0 to 100%) which is calculated from the proportion of attached and retained seeds after a certain time interval (Hintze *et al.*, 2013). We propose one hour, during which most animals may potentially cover a distance of one kilometer.

Fig. 5 Example of a shaking machine to study epizoochory (from Tackenberg *et al.*, 2006).



3.2.13 Endozoochory

Trait description

Endozoochory is the dispersal of dispersules (seeds, fruits) by passing the digestive system of an animal (Römermann *et al.*, 2005d). Endozoochory is also called endozoochorous dispersal or internal animal dispersal. Endozoochory consists of two processes that define the qualitative subcomponents of seed dispersal effectiveness (Schupp *et al.*, 2010): quality of seed deposition and quality of treatment in mouth and gut. The treatment in vertebrate guts is a complex process. It encompasses a series of sequential steps that affect the probability of germination: (i) de-inhibition effect (removal of fruit pulp or seed cleaning), (ii) scarification effect (mechanical treatment of seeds by chewing and the chemical treatment by passing through the digestive system), and (iii) the fertilization effect – the effect of faeces on the probability of germination and early seedling establishment (Traveset *et al.*, 2007). All processes should be measured separately but assessed together (Bonn, 2004; Samuels & Levey, 2005; Robertson *et al.*, 2006).

Functionality and trade-off

Endozoochory is related to the connectivity of habitats between which animals move. According to Ozinga *et al.* (2009), a lower proportion of species that rely on endozoochory are threatened than species relying on epizoochory. Depending on the movement of an animal during the seed passage time, endozoochory can contribute to long-distance dispersal covering several kilometres. The passage through the gut amounts between 8 hours to 10 days. Transhumant shepherding covered up to 25 kilometres of distance per day when migrating between winter and summer pastures. The passage through the sheep gut amounts between 6 hours and 9 days (Bonn & Poschlod, 1998). Considering all long-distance dispersal possibilities, endozoochory belongs to the most effective ones, not only in terrestrial (Manzano & Malo, 2006) but also in aquatic habitats (Anderson *et al.*, 2011).

Endozoochory is strongly related to dispersule releasing height (Albert *et al.*, 2015b) and seed production (Bruun & Poschlod, 2006). However, both seed (size, shape, coat thickness) and animal traits (gut length, gut differentiation) interact to produce a complex pattern of survival during the passage through the digestive system (Traveset *et al*., 2008). Smaller and rounder seeds seem to pass better than large or elongated ones (Pakeman *et al.*, 2002). Furthermore, the survival rate of hard-coated seeds or those with an impermeable seed coat (physical dormancy) is higher compared to soft seeds (Janzen, 1983; Vellend *et al.*, 2003). Some seeds may germinate to a higher proportion only after gut passage (Traveset *et al.*, 2007; Fuzessy *et al.*, 2016) either by removing the pulp of fleshy fruits which may contain germination-inhibiting compounds (Mayer & Poljakoff-Mayber, 1982; Robertson *et al.*, 2006) or making a substantial impact to the seed coat (Barnea *et al.*, 1990). It has been hypothesised that seed transit time is a key driver of seed survival, as seeds passing faster through vertebrate guts have a lower probability of being damaged by mechanical and chemical scarification. However, attempts to correlate seed survival with transit times have failed to find a strong correlation between these variables, with strong species-specific effects (Traveset *et al.*, 2008; Messeder *et al.*, 2022).

Applied aspect

Endozoochory is an important parameter connecting fragmented or isolated habitats (Poschlod *et al.*, 1996; Willerding & Poschlod, 2002; Levey *et al.*, 2005; Lenz *et al*., 2011; Auffret *et al.*, 2012; Rico *et al.*, 2013; Albert *et al.*, 2015b; Emer *et al.*, 2018). In tropical rain forests, endozoochory is probably the most important dispersal vector (Howe & Smallwood, 1982; Levin *et al.*, 2003; Fleming & Kress, 2011; Fuzessy *et al.*, 2018). Endozoochory can also contribute to the depth distribution of seeds, e.g., via earthworms (Grant, 1983; Eisenhauer *et al.*, 2009). Endozoochory can also contribute considerably to community assembly processes (Rosbakh *et al.*, 2022).

Sources of variability

Seed size, which can strongly vary, may affect ingestion and survival rate after passing the gut (Pollux *et al.*, 2007). Internal dispersal depends on the mechanical treatment of the dispersule during chewing (mammals) or cracking (birds) and the chemical composition of the digestion system. Therefore, the optimal endozoochry of a species should be measured for each animal species separately. Seed traits related to endozoochory vary from year to year, such as seed production (Herrera *et al.*, 1998), seed releasing period (Malo & Suarez, 1995a; Du *et al.*, 2009) or even seed coat thickness (Schutte *et al.*, 2014).

According to Pakeman *et al.* (2002) and Kuiters & Huiskes (2010), dispersules with a low mass are rather endozoochorously or in comparatively larger quantities dispersed than those with a high mass. However, this result depends on the animals studied. In a study of endozoochory by primates in Neotropical forests, Fuzessy *et al.* (2018) showed that certain species preferentially disperse large and heavier seeds, whereas others disperse mainly small and light seeds. Whether a seed is ingested or spat out largely depends on seed size. There are also contradictory results for seed shape. Whereas rabbits and sheep internally disperse round rather than elongated seeds (Pakeman *et al.*, 2002), a meta-analysis of ungulates showed that elongated seeds were also well dispersed (Albert *et al.*, 2015b). One common result, however, limited to temperate regions, was that seeds with a long-term persistent seed bank also survive significantly better gut passage than those with a transient seed bank (Pakeman *et al.*, 2002).

Methodology

There are many processes affecting endozoochory. Here, we focus on the quality of treatment in vertebrate guts. This process encompasses a series of steps from ingestion, mechanical treatment by the teeth or beak, the chemical treatment (in the stomach and intestine, the first containing acid and enzymes, the latter enzymes) by passing through the gut (Bonn, 2004; Kleyheeg *et al.*, 2018), and finally possible germination in the faeces (Milotić & Hoffmann, 2016a). We propose standardisation of three processes, namely: (i) the de-inhibition effect (depulping or seed cleaning), (ii) the scarification effect (the effect of both chemical and mechanical scarification in the

guts), and (iii) the fertilization effect (the effect of vertebrate faeces on seedling emergence).

There are several approaches to assess internal seed dispersal - either as a (very) soft trait via dispersule traits or seed production (Pakeman *et al.*, 2002; Bruun & Poschlod, 2006; Kuiters & Huiskes, 2010), or as a hard trait by feeding experiments and analyzing the survival rate (Bonn, 2004), analyzing seeds germinating from dung or faeces samples (Malo & Suarez, 1995a; 1995b; Stender *et al.*, 1997; Cosyns & Hoffmann, 2005), or mimicking the above mentioned processes (Römermann *et al.*, 2005d; Milotić & Hoffmann, 2016b; Kleyheeg *et al.*, 2018).

Traveset (1998) emphasised that most studies did not test the effect of frugivores in separating the pulp from the seeds due to the lack of an 'intact fruit control' and also that the large majority of studies did not evaluate the viability of the seeds that failed to germinate. Almost two decades later, those issues remain the same (Fuzessy *et al.*, 2016). Therefore, we highly recommend the use of four treatments whenever possible: (i) seeds passed through a frugivore's gut, aiming to ascertain the effect of frugivory on the seed germination; (ii) hand-washed seeds (seeds removed from the fruit pulp and epicarp), aiming to ascertain the possible scarification effects; (iii) intact fruits, aiming to ascertain the cleaning effects; and (iv) seeds in faeces, aiming to ascertain the possible fertilization effect of the frugivore's faeces (Robertson *et al.*, 2006; Traveset *et al.*, 2001; 2007; Fuzessy *et al.*, 2016).

We suggest that the following guidelines would result in more accessible, complete and uniform reports on vertebrate gut passage effect on seed germination (Fuzessy *et al.*, 2016):

1) Fruit and seed sampling: Sample fruits from a representative number of individuals under natural conditions. The number of fruits and individuals depends on the number of seeds per fruit and population size. Fruits should be stored in paper or plastic bags and processed immediately after collection (ideally less than a week). No cold storage should be done. Seeds should be extracted manually from the fruit pulp and washed in

tap water for 5 minutes to remove the pulp. Seeds should be blot-dried under shade for at least 24 hours. Assure independence among replicates, using control replicates from different individual plants and different vertebrates on different days.

2) Establishing the controls: Defining the control treatment depends on the question asked. Ideally, the germination of gut-passed seeds should be compared to that of intact fruits and hand-extracted seeds (Samuels and Levey, 2005).

3) Gut passage treatment: Obtaining gut-passed seeds can be done through different methods. The most common approaches are capturing vertebrates, collecting seeds in traps or mist-nets, and offering seeds to captive animals in good health condition. The sample size is strongly determined by the availability of vertebrates. Studies should aim to incorporate variations in the number of captured or captive animals to ensure data independence. Therefore, we recommend that gut-passed seeds are obtained by at least four independent replicates of frugivores. Under natural conditions, faeces should be stored in paper or plastic bags and be processed as soon as possible, ideally in less than a week. If the material is to be used for the gut-passed treatment, then seeds should be rinsed in tap water for 5 min and blot-dried under shade for at least 24 hours. If the sample is used for fertilization treatment, no additional process is needed. In the case of captive birds, intact fruits should be offered preferably to one individual frugivore per cage. To determine transit times, we recommend timing the difference between the ingestion of the first fruit and the time of the first defecated seed. After a period in which seeds are no longer observed in the faeces, the experiment should be terminated, and the animal should return to its regular diet. The faeces-embedded seeds should be processed as described above. All ethical processes and licenses for using animals in research should be obtained prior to the experiments.

4) Experimental design: At least four replicates for each treatment should be used, but ideally, more than six replicates are recommended (Sileshi, 2012). The number of replicates and seeds per replicate should be the same across the treatments. The number of seeds set to germinate in each replicate varies with seed availability and seed size, but 25 seeds per replicate are recommended. Ensure that experimental (gut-

passed) and control seeds are placed randomly across germination plots (especially in greenhouse settings, where local variation in light or watering regime could affect germination speed).

5) Place of testing: The place where the experiments are conducted (lab, field or greenhouse) strongly affects the outcome of the germination tests (Robertson *et al.*, 2006). Ideally, field experiments are those with higher biological realism. However, field experiments may be difficult to run for small-sized seeds, which are amenable to experimentation only under lab or greenhouse conditions.

6) Germination conditions: Moisture conditions where seeds were placed to germinate (e.g., forest soil, sterilised soil, Petri dishes with filter paper, fungicide, watering frequency), environmental conditions (temperature and humidity) and seed conditions at the beginning of the experiment (i.e., a percentage of seed viability before ingestion) should be standardised for all treatments.

7) Length of experiment period: The length of the germination trial (after which time seeds were discarded or not monitored) should last at least 30 days. Allowing seeds to germinate for this period is important to provide information on seed dormancy and dormancy loss. Under field conditions, monitoring typically extends for the whole growing season.

8) What to measure: Minimum standards should provide estimates of initial seed viability (the number of seeds that are capable of germinating at the beginning) through a simple tetrazolium test (Delouche *et al.*, 1962), final seed viability (the number of seeds that are capable of germinating at the end of the experiment), germination percentage, and germination time. Such estimates should be obtained in a similar way across all treatments. More complete experiments can also assess seed transit times, seed coat permeability, and anatomical changes in the seed coat (see sections **3.1.3 Seed coat thickness** and **3.3.4 Seed coat water permeability**).

In the absence of frugivores to conduct more realistic assessments, alternative methods simulating gut passage can also be performed, i.e. a standardised method

would be to mimic the relevant processes, a dispersule is subjected during the endozoochorous dispersal process. The most detailed studies in this respect were done for livestock by Bonn (2004; see Römermann *et al.*, 2005d) and for ducks by Kleyheeg *et al*. (2018).

According to Bonn (2004; see Römermann *et al*., 2005d), for the simulation of the mechanical stress during ingestion by livestock, dispersules are placed on ‘plastic lids‘, which are attached to a wooden board. An iron stick (which has the same diameter as the ‘plastic lid‘ and is covered at the front with a thin technical fleece and masking tape) is pressed with a weight of around 70kg (a person) and rotated twice for 90 ° representing the chewing pressure (Fig. 6). To simulate the chemical stress representing the passage through the gut, the best results were achieved by placing the ‘chewed‘ dispersules for 8 hours into glass tubes with 0.1M HCl (Bonn, 2004). After this treatment, seeds are washed and germinated under standardised conditions in Petri dishes. The results correlated 80% and 79% to the relative survival of dispersules in a feeding experiment with sheep and cattle, respectively. Kleyheeg *et al*. (2018) simulated the mechanical stress during ingestion by a pressure test and scarification treatment and the chemical stress during digestion by incubating seeds in the first step in gastric juice and the second step in the intestinal contents of culled mallards. However, they did not consequently compare the results of the standardised treatment to feeding. This means that until now, a validated method is only available for sheep and cattle; other standardised methods wait to be developed.

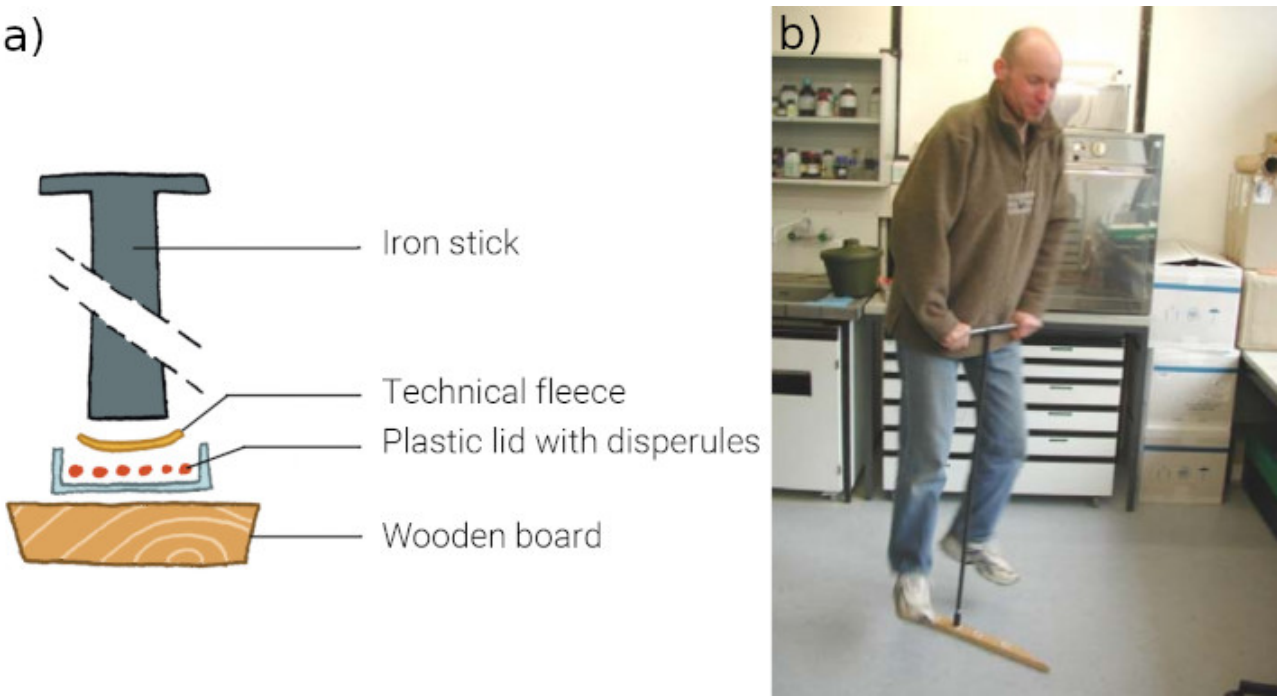


Fig. 6 Schema of tool used to simulate the mechanical stress during ingestion by livestock (a), simulation of chewing (b; from Bonn, 2004).

Since the development of these standardised methods is very time-consuming and nearly impossible for all potential seed disperser species, we recommend, in any case, studying the dispersules found in dung or faeces deposits in the first step. For this purpose, the dung of mammals is collected, and seeds either extracted by suspending it in water and spreading it out in a thin layer of about 0.5 cm in culture trays filled with sterile potting soil (if necessary mixed with sand). When appropriate, samples should be stratified before cultivation or cultivated in a field house over one winter period. Every seedling is identified, tagged and removed. Alternatively, seeds can also be separated from the dung and identified (Benthien *et al.*, 2016). However, in this case, a germination or tetrazolium test should be done to test for viability. This is more time-consuming and rarely done, if at all (Benthien *et al.*, 2016). Therefore, to allow data comparability, the first approach is recommended.

For birds, faeces can be studied accordingly. To collect the faeces, either buckets can be deposited under the nests (Kos, 2007) or artificial perching sticks are established in the vicinity of the nests (Guidetti *et al.*, 2016). We recommend either establishing large size seed traps (e.g. 1.2 x 1.2m with a nylon mesh) under the perch (Heelemann *et al.*, 2012)

or attaching a plastic funnel of respective size (e.g. Ø 25 cm) with a bag of nylon mesh attached under it to collect the seeds (Grunicke, 1996).

Units

For the database, entries on standardised measurements should include the number of dispersules tested and the proportion of seeds that have survived and germinated (0 to 100%) across all treatments. Transit times are to be reported in minutes.

3.3 Seed persistence

3.3.1 Serotiny

Trait description

The retention and subsequent delayed dispersal of mature seeds, held in cones or functionally similar structures, forming a canopy-stored seed bank. Seed release is often triggered by specific environmental cues, such as fire, drought, or senescence of branches or plants. The degree of serotiny can vary from strong (most seeds retained on live stems until release is triggered) to weak (most seeds released on maturation and few retained) to non-serotinous (Clarke *et al.*, 2010; Lamont, 2021). The terms bradyspory (used to describe seed retention; Whelan, 1995) and bradychory (specifically to mean the phenomenon of delayed dispersal; Thanos, 2004) are less common but sometimes used synonymously with serotiny.

Functionality and trade-offs

A strong degree of serotiny, where most seeds are retained until triggered to release by an environmental cue, provides a mechanism by which seeds can maintain a persistent seed bank and subsequently disperse into an environment *en masse*. One of the most common triggers is fire. In some fire-prone environments, many species maintain a canopy seed bank in woody capsules or cones. Seeds are protected from extreme heat during the fire event, and this same heat is required to melt the resin that seals seeds within the woody structures. Upon release post-fire, seeds are dispersed into conditions with high availability of resources such as key nutrients and lower levels of competition and predation (Whelan, 1995; Hernández-Serrano *et al.*, 2013). Weaker degrees of serotiny may provide a risk-spreading strategy by dispersing seeds gradually over time. This could be advantageous in environments where suitable sites for recruitment may be either temporally or spatially variable. A similar function has been proposed for strongly serotinous species that have secondary mechanisms for a gradual release of seeds from open follicles (Clarke *et al.*, 2010). In dry environments, serotiny can be associated with steppe-rollers (*Boophane, Seseli, Vaccaria*), where seeds

are retained in dry detached infructescences that serve as dispersal units, and the serotiny enables dispersal to places where these infructescences are driven to. Several dry-land annual plants (e.g. *Anastatica hierochuntica*) retain seeds on dead plants and release them over subsequent rainfall events.

Sources of variability

The degree of serotiny can vary between species, populations, and individuals, as well as with stand age (Lamont, 2021). High-frequency crown fire appears to provide selection pressure for greater degrees of serotiny (Hernández-Serrano *et al.*, 2013; Ladd *et al.*, 2022). There is also some evidence suggesting that synchronicity of release from open follicles could co-vary with different levels of the environmental trigger and/or other traits such as those related to dispersal (Clarke *et al.*, 2010).

Methodology

At its simplest, serotiny can be estimated from counts of closed versus open cones (or other structures) on individual plants at a single point in time, and the number of closed cones is divided by the total number of cones to calculate a percent per individual. The mean percentage can then be obtained from a sample of individuals within a population. This may be useful, for example, when understanding a species' life history is needed, and the potential for serotiny can be assigned. However, temporal elements of seed production and retention can significantly influence the calculation of the degree of serotiny (Whelan & Ayre, 2020; Lamont, 2021), and a more robust approach is needed if wanting to make comparisons with a higher resolution.

Methods to calculate the degree of serotiny can vary in complexity, mainly due to the resolution of the data required. The two methods below both incorporate a temporal element.

Many species with canopy-stored seeds develop whorls of branches annually and can be dated by counting internodes from the branch tips (youngest) (see examples for *Banksia* in Australia, Jenkins *et al*. 2005; *Pinus* in Europe, Hernández-Serrano *et al*., 2013). Cones can be dated via the branch they occur on. To measure serotiny, the age

of the oldest closed cone is assigned for each individual plant, and a mean age (expressed in years) can then be calculated for a population and used as an index of serotiny. Numerous examples of this approach have been reported, sometimes combining multiple serotiny metrics in comparative studies (e.g. Hernández-Serrano *et al*. 2013). In cases where the ageing of seed cohorts is difficult, qualitative estimates of the degree of serotiny can also be made. For example, Clarke *et al.* (2010) suggested weak, moderate and strong serotiny for the retention of seeds in cones for <2, 2-5 and >5 years, respectively.

A more labour-intensive but robust method, introduced by Lamont (1991, 2021), requires estimating the age of each cohort (as above) and then counting the number of open and closed cones (or even fruits in each cone). The number of closed cones (or fruit) is then divided by the total number of cones (or fruit) within each cohort, and a percent is calculated. By fitting a linear model to the percentage of closed cones using the data for progressively older cohorts - the inverse of the slope (m) can be calculated ($100/m$) and used as a degree of serotiny index. Values vary from 0 (non-serotinous) to ∞ (complete serotiny).

3.3.2 Seed longevity (in the lab)

Trait description

Longevity, or seed lifespan, is the period of time that a population or a sample of seeds remains viable (see section **3.4.4 Seed viability**) during storage under a defined set of conditions. Seed longevity encompasses the retention of the seeds' ability to germinate and produce healthy, vigorous, and normally developed seedlings.

Functionality

Seed longevity is a trait central to the storage of seeds for the conservation of plant biodiversity and genetic resources.

Applied aspect

Understanding seed longevity is necessary for the successful storage, management, and use of seed collections in gene banks. For example, the rate of viability loss of seeds under different storage conditions (e.g. differing degrees of drying and different storage temperatures, including sub-zero temperatures) allows for categorising seed storage behaviour (e.g. orthodox vs intermediate vs recalcitrant). Predicting seed longevity informs the curation of banked seed collections through the setting of viability monitoring schedules appropriate to the expected period of viability maintenance (e.g. one-third of the time predicted for viability to decline to 85% of initial viability; FAO, 2014). Longevity data can also assist in prioritising those seed lots expected to be short-lived for cleaning, drying and banking, and to inform the post-harvest drying and storage conditions (e.g., the use of cryostorage for short-lived seeds such as those of orchids) (Walters & Pence, 2021).

Sources of variability

Seed longevity is a complex trait (Walters, 2015; Nadarajan *et al.*, 2023). Significant variation in longevity can be observed between species and different seed lots of the same species – including between different varieties of agricultural species (Lee *et al.*, 2019). Longevity is partly under genetic control and can vary due to the maternal

environmental conditions during seed maturation, the seed maturity at the point of collection (Hay & Probert, 1995), seed desiccation tolerance and storage behaviour, post-harvest handling practices and the pre-storage environment(s), and the storage conditions (e.g., temperature, moisture and oxygen) (Hay & Probert, 2013; Nadarajan *et al.*, 2023).

Variation in seed longevity has been correlated to other seed traits, such as seed composition and mass, embryo size and the presence/absence of endosperm, storage syndrome (e.g. serotinous vs geosporous), as well as environmental conditions of the source plant populations (e.g. temperature, rainfall, altitude) (Probert *et al.*, 2009; Mondoni *et al.*, 2011; Merritt *et al.*, 2014b; Satyanti *et al.*, 2018). There is also a phylogenetic component to many of these trait associations.

Methodology

Seed longevity in the laboratory should be measured under controlled environmental conditions appropriate to the experimental hypotheses and/or context for which longevity is being determined. There is no single method that is universally applied to calculating seed longevity (Hay *et al.*, 2022). However, the primary variables that should be controlled are storage temperature and seed moisture content. Experiments to define the relationship between seed longevity, storage temperature, and seed moisture content have been conducted since the 1960s, initially on seeds of crop species. Modelling the viability decline of seeds stored across a range of temperatures (e.g. 25 °C – 60 °C) and seed moisture contents (e.g. 2% - >20%) led to the development of the seed viability equations (Ellis & Roberts, 1980) that describe the relationship between seed longevity and the storage environment and that can be used to predict longevity under a wide range of storage conditions (reviewed in Ellis, 2022).

It is difficult (and impractical for most purposes) to study seed longevity under storage conditions appropriate to seed banking (i.e. conditions of low temperature and low seed moisture content), as the time required for many desiccation tolerant seeds to age in real time can range from years to centuries. However, there are some long-term

studies of viability in cold storage over multi-decadal timescales (Walters *et al.*, 2005b; Chau *et al.*, 2019; Liu *et al.*, 2020).

To enable study on a more practical timescale, techniques of accelerated ageing or controlled deterioration, whereby seeds are stored at a high temperature either in an environment of high relative humidity, or hermetically sealed at elevated seed water content, have been used for many decades to study seed longevity of diverse crop and wild species (reviewed in Hay *et al.*, 2019).

Experimental storage conditions that have been widely adopted to determine comparative seed longevity under one set of conditions are detailed in the comparative longevity protocol developed at the Millennium Seed Bank (Probert *et al.*, 2009; Davies *et al.*, 2016; Newton *et al.*, 2022; http://brahmsonline.kew.org/Content/Projects/msbp/resources/Training/01-Comparative-longevity.pdf). In this protocol, seeds are aged at 45 °C and 60% RH, following an initial period of hydration at 47% RH and 20 °C. Samples of seeds are removed from these storage conditions (e.g. 50 seeds per retrieval time) at regular intervals, and their viability is tested, most commonly via a germination test. The percent viability is then plotted against storage time (days) to derive seed survival curves (e.g. refer to Newton *et al.*, 2022).

Seed longevity is calculated from the seed survival curves under a given set of conditions, as the time for viability to fall to a defined percentile. Most frequently the time for viability to fall to 50% (p_{50}) is used as the measure of longevity, but other percentiles may be calculated. Probit analysis of the seed survival curves is the preferred method to determine p_{50} via fitting the viability equation (Ellis & Roberts 1980). Still, other modelling methods, such as the Avrami equation (Walters *et al.*, 2005b), have also been used (reviewed in Hay *et al.*, 2014, 2019; Ellis, 2022).

The initial seed quality has a strong influence on the determination of seed longevity (i.e., the calculation of p_{50}). For studies examining seed longevity between different species under the same conditions, for example, to rank the relative longevity of

species or identify other seed or plant traits or environmental factors that may be associated with longevity, seed lots with high initial viability (i.e. ≥ 85%) should be used (Probert *et al.*, 2009).

Seed viability (see section **3.4.4 Seed viability**) in longevity experiments is most commonly determined via germination testing. In this respect, seed dormancy can potentially confound the viability test if the dormancy-break and germination test conditions are not optimised and careful evaluation of non-germinated seeds is required (e.g. via a cut-test or tetrazolium staining). Research continues to investigate other measures of seed deterioration as correlates of seed viability that are alternatives to (or more sensitive than) germination testing and that may be useful to identify the onset of viability decline earlier than is evident via germination testing alone (e.g. see reviews of Fu *et al.*, 2015; Nadarajan *et al.*, 2023).

Limitations

It is important to recognise that altering the storage conditions will change the longevity of a seed lot (Colville & Pritchard, 2019). Deriving a seed survival curve and a p_{50} value under a given set of storage conditions will only provide information on seed longevity under that set of storage conditions.

Due to the fundamentally different physiological processes that occur in seeds under different storage conditions, caution is advised in extrapolating comparative longevity derived from accelerated ageing to longevity in conventional seed banking conditions (Walters, 2005b; Pritchard & Dickie, 2003). For orthodox seeds, there is some evidence that relative longevity amongst species derived from the two storage conditions may be correlated (Colville & Pritchard, 2019; Davies *et al.*, 2020).

While life-span or seed longevity as a species characteristic influences the potential for seeds to survive in the soil, extrapolation or correlation of longevity (derived from any laboratory method) with survival in soil (see section **3.3.3 Soil seed bank longevity**) is not advised due to the numerous interacting factors that determine persistence in the soil (Long *et al.*, 2015).

Units

Preferred for comparative ability - The time (d) for viability of the seed population to decline to a defined percentile, commonly p_{50} (the time in days for viability to decline to 50%).

3.3.3 Soil seed bank longevity

Trait description

Soil seed bank longevity is the time a seed may remain viable in the soil before germinating or dying. This trait is also often called soil seed bank persistence. We differentiate transient, short-living, and persistent, more or less long-living soil seed banks (Bakker *et al.*, 1996; Thompson *et al.*, 1997; Poschlod & Rosbakh, 2018).

Functionality and trade-off

Soil seed bank longevity depends on the abiotic environment and its parameters, such as climate, soil type, soil moisture, soil chemistry. Shallow burial (light) and high temperatures may decrease soil seed bank longevity (Ooi *et al.*, 2009; Ooi, 2012; Ooi *et al.*, 2012; Mašková & Poschlod, 2022; Mašková *et al.*, 2022). Soil type or substrate also affects soil seed bank longevity; however, although soil seed bank longevity in dry soils is often reduced, the effect depends on which substrate the species originally grows (Abedi *et al.*, 2014; Mašková *et al.*, 2022). Soil moisture may increase but also decrease longevity depending on the species' ecological niche (Bekker *et al.*, 1998c). Higher soil nutrient contents may also deplete the soil seed bank of certain species (Bekker *et al.*, 1998b), which is often related to the effect of nitrate-breaking dormancy (Pons, 1989). Hypoxic conditions may increase the soil seed bank longevity of certain species (Villiers, 1973; Leck, 1989; Poschlod & Rosbakh, 2018). Species in frequently disturbed habitats often have a higher soil seed bank longevity than less disturbed ones, such as forests (Bekker *et al.*, 1998d; Poschlod *et al.*, 2013).

Soil seed bank longevity also depends on the biotic environment. Seed predators (Eisenhauer *et al.*, 2009) and pathogens decrease soil seed bank longevity (Schafer & Kotanen, 2003; Chee-Sanford *et al.*, 2006; O'Hanlon-Manners & Kotanen, 2006; Wagner & Mitschunas, 2008; Dalling *et al.*, 2011; Long *et al.*, 2015).

Soil seed bank longevity is also strongly related to seed desiccation tolerance. Desiccation-intolerant or recalcitrant seeds are always transient. Only desiccation-tolerant seeds can build up a persistent seed bank which also depends on other

parameters. Persistence in the soil and, therefore, soil seed bank longevity is also related to germination requirements. A prerequisite to persist in the soil is the light requirement (Grime, 1989; Mašková & Poschlod, 2022). Fluctuating temperature requirements also contribute to forming a persistent soil seed bank (Honda, 2008) but may not be a necessary prerequisite (Saatkamp *et al.*, 2011a).

There may be a trade-off with seed size and shape (Thompson *et al.*, 1993; Bekker *et al.*, 1998a) but also seed coat thickness (Gardarin *et al.*, 2010) and chemical compounds (Davis *et al.*, 2008). However, this trade-off is not consistent on a global scale, as shown for the seed size- and seed shape-seed longevity trade-off by Leishman & Westoby (1998) and Moles *et al*. (2000). Saatkamp *et al*. (2009) have shown a positive correlation between soil seed bank longevity with seed production. Although seed dormancy favours persistence (Long et al., 2015), it is not strictly a prerequisite for soil seed bank longevity (Thompson *et al.*, 2003; Honda, 2008). Nevertheless, it should still be tested with high-quality and larger datasets and for the different dormancy categories since persistence may be achieved by physiological dormancy or impermeable seed coats, which is physical dormancy (Saatkamp *et al.*, 2014; for physical dormancy see also Honda, 2008).

Applied aspect

Soil seed bank longevity is an important aspect of weed management, conservation and restoration management of threatened species or habitats (Bakker *et al.*, 1996; Long *et al.*, 2015).

Sources of variability

Soil seed bank longevity seems to be phylogenetically related (Gioria *et al.*, 2020). However, this study only differentiated into two categories, transient and persistent, and the transition of the two categories may be more nuanced. Soil seed bank longevity may also vary within an individual or even within fruits (Gutterman, 2000).

Methodology

Soil seed bank longevity in the field can be studied in different ways.

1) Burial experiments under natural or near-natural conditions are the most appropriate methods (Mašková & Poschlod, 2022). An example of artificial burial experiments is Dr. Beal's experiment in glass bottles with a standardised soil mixture and moisture (Telewski & Zeevaart, 2002). Under natural conditions, seeds are, therefore, buried in nylon bags (Bekker *et al.*, 1998b; Saatkamp *et al.*, 2009), which are resistant to decomposition (Garcia *et al*., 2017). Mesh size should allow soil organisms, such as fungi or microorganisms, to pass through but retain the seeds (e.g. 0.2 mm). Burial depth may affect soil seed bank longevity. If there is an interest in this effect, we suggest burial in 2.5 and 10 cm depth. If only longevity is interesting, burial at 10cm depth is recommended (but depending on soil depth at the study site). Control of longevity depends on the question – for short-term burial experiments, excavation and control are suggested during early spring and autumn in temperate regions, and for long-term experiments, only during the likely growing season. Excavated seeds should be controlled for germinability and viability (apply tetrazolium test to the non-germinated seeds). Seeds are scored as germinated, ungerminated or missing.

2) Soil seed bank studies along successional series (e.g., overgrown or afforested formerly open habitats; Poschlod, 1993b; Poschlod *et al.*, 1998) or in habitats with known last occurrence of a species (Poschlod, 1993a; Poschlod & Rosbakh, 2018) allow a good estimation of longevity. Depth distribution of germinable seeds in the soil, which was used to assess soil seed bank longevity by Thompson *et al*. (1997), has been shown not to be reliable for many species but may allow a first 'soft' assessment (Saatkamp *et al.*, 2009).

Sampling soil seed banks should take place during the time of the year when seeds of most of the species are non-dormant, e.g., in temperate climates after the end of wintertime during early spring. Soil samples should be taken with a corer which allows the differentiation of at least two soil layers (e.g. 0-5 and 5-10 cm). As a standard core diameter, 4 cm is recommended (Zwerger *et al.*, 1990). In case coring is not possible (e.g. in soft sediments), one litre of a sample can be taken (Poschlod & Rosbakh, 2018).

Taking into account the heterogenous distribution of seeds in the soil within a given plant community, ten cores per ten plots (in total, 100 cores) are recommended in open habitats (Mitlacher *et al.*, 2002). In forests, the density is often lower and thus requires a higher number of samples. In general, species with a low seed production but long-living seeds in the soil may not be detected even with a much higher number of samples (Saatkamp *et al.*, 2009).

Two approaches are applied to analyse the seeds in the soil: the emergence and the separation or extraction method (Bakker *et al.*, 1996). At the emergence method, soil samples are concentrated by sieving them through a sieve with a mesh size of 0.2 mm to reduce the bulk (Ter Heerdt *et al.*, 1996) and then spread in a thin layer on a sterile substrate and cultivated over a specific time period (six weeks recommended by Ter Heerdt *et al.* (1996) but according to Jackel & Poschlod (1994) better over 18 months since a large proportion of seeds only germinates after being exposed another winter period) or until second germination season (Walck *et al.*, 2005).

The emergence method is faster and takes place on its natural substrate, whereas the separation or extraction method is more time-consuming (Bernhardt *et al.*, 2008). This requires first identification of the seeds. However, seed identification keys are only available for a few floras. Even then, certain species cannot be identified without the help of a seed herbarium or cannot be differentiated at all because, within many genera, seeds are often very similar to each other (Lippok *et al.*, 2013; see also Arruda *et al.*, 2021). Second, viable and non-viable seeds are not differentiated. Only a few studies add a germination test after extraction, which is often done in Petri dishes. However, seedlings in Petri dishes cannot be clearly allocated to a certain species. In contrast, the emergence method allows the clear identification of species since seedlings can be cultivated until the identification is possible. The only advantage of the separation or extraction method is that it may result in higher amounts of individuals (Bernhardt *et al.*, 2008) and that species with strongly differing germination requirements are also detected. Especially in amphibious habitats (seasonally or long-term inundated wetlands), species with differing germination requirements occur,

which can only be germinated with specific water(ing) regimes (Ter Heerdt *et al.*, 1999; Valdez *et al.*, 2019).

3) In exceptional cases, the seed coat (pericarp, testa) of viable or germinated seeds may be dated by **the radiocarbon (C14) dating method** (McGraw *et al.*, 1991). With this method, Sallon *et al*. (2008; 2015) could date the hitherto most ancient and still germinable seeds of *Phoenix dactylifera* being around 2000 years old. Dalling & Brown (2009) applied accelerator mass spectrometry to carbon-date seeds of pioneer tree species extracted from undisturbed to date seeds which were up to 60 years old.

Unit

Soil seed bank longevity is measured in years (<1 year, 1, 2, 3... years) or the following classification is used: transient = <1 year – 1 year; short-term persistent = 2 – 5 years; long-term persistent = 6 – 20 years; very long-term persistent = 21 – 50 years; extremely long-term persistent = > 50 years; see Poschlod & Rosbakh, 2018).

3.3.4 Seed coat water permeability

Trait description

Seed coat water permeability is the property of the seed's external structure to permit water uptake between the environment and the seed's internal structure.

Functionality and trade-off

Seed coat permeability to water has important effects on seed germination and longevity. Species with permeable seed coats allow rapid imbibition when water is available, thus promoting germination when environmental and physiological conditions are suitable. However, seeds of many species are impermeable at dispersal because the seed coat has an anatomical structure that prevents water uptake (Werker 1997). These 'hard seeds' are generally assumed to exhibit physical dormancy, maintaining low water content in the seed until an external (mechanical, physical or chemical) factor makes the coat permeable. The evolutionary pathway of hard seeds has been traditionally linked to environmental cues, mostly fire regime (Pausas & Lamont, 2022) and endozoochory (Jaganathan *et al*., 2016), but alternative hypotheses point to predator avoidance and dispersal strategies based on a variation of seed coat impermeability from the same individual (Paulsen *et al*., 2013). Irrespective of the underlying evolutionary mechanisms, the impermeability of the coat permits hard seeds to persist for a long time in the soil by maintaining viability, thus increasing seed survival (Fenner & Thompson, 2005). Impermeable coats are generally formed by packed cells with no pores and diverse chemical substances (Bewley *et al.*, 2006). Seed coats may also be impermeable to gas exchange or act as a mechanical barrier to radicle emergence, further contributing to the regulation of seed germination. However, many ecological aspects of dormancy-breaking in hard seeds remain unknown (Jaganathan, 2022).

Applied aspect

Seed coat permeability has strong effects on the seed-water relationships that ultimately determine seed longevity, desiccation tolerance, and germination speed. In

agriculture, seed coat permeability may determine the absorption of chemical compounds used in seed treatments (Turner *et al*., 2013).

Sources of variability

The evolutionary imprints of seed development determine seed coat permeability and are associated with specific taxonomical lineages. For example, impermeable seed coats are typically found in species with physical dormancy from at least 20 angiosperm families, including *Fabaceae, Cistaceae, Convolvulaceae, Geraniaceae or Cucurbitaceae* (Baskin *et al*., 2000). However, species from the same family and genus may show both permeable and impermeable seed coats (Chen *et al.*, 2019c). During seed maturation and early dispersal, a single species may produce seeds with different degrees of permeability as an effect of seed water content and different status of the micropyle (Gama-Arachchige *et al.*, 2011; Jaganathan *et al.*, 2019). This variation depends on environmental factors, especially the seasonality of air humidity over the years (Jaganathan, 2016). Insect damage can also lead to the loss of physical dormancy, making seed coats permeable to water uptake without causing seed mortality (Tiansawat *et al*., 2017).

Methodology

Seed coat permeability to water can be estimated in the laboratory by calculating the increase in mass of seeds after imbibition in water, expressed as a percentage (Baskin *et al.*, 2004), using the formula:

$$Increase \in mass = \left[\left(\frac{weight\ of\ imbibed\ seeds - weight\ of\ dry\ seeds}{weight\ of\ dry\ seeds} \right) \times 100 \right].$$

For imbibition, seeds are moistened with distilled water in Petri dishes in laboratory room conditions. For large-seeded species, use wet sterilised quartz sand media in trays (instead of filter paper in Petri dishes) to increase the proportion of seed surface contact with moisture to facilitate uniform water imbibition. For small-seeded species (dwarf seeds or dust seeds), where weighing individual seeds may be difficult, it is

recommended to take four replicates of 50 to 100 seeds (or 1 gm seeds), depending on the size and availability of seeds.

In given time intervals, e.g., every 1 h for the first 6 h, 8 h, 16 h and 24 h, blot dry surface water, reweigh and return them to the moist substrate until they achieve a plateau in seed mass. No longer than 24 h are needed to test permeability to water, but relatively longer times for full imbibition will depend on the seed size and the chemical and physical structure of the seed coat.

The permeability of the seed integuments to chemical compounds can also be evaluated by using fluorescent dyes and fluorescence detectors (Salanenka *et al.*, 2011; Yang *et al.*, 2018), although these methods are mainly applied in agriculture and rarely used in seed ecology (but see Zalamea *et al.*, 2015; McCulloch *et al.*, 2024).

3.3.5 Desiccation tolerance

Trait description

Desiccation tolerance is the sensitivity of a seed to survive drought and revive from the air-dry state (Bewley 1979). Desiccation damage may result from the removal of water or from metabolic damage. Following Ellis *et al*. (1990), we differentiate three types of desiccation tolerance. Desiccation-tolerant or orthodox seeds can dry out without damage, even after getting imbibed several times. In contrast, desiccation-intolerant (desiccation-sensitive) or recalcitrant seeds do not survive drying below a certain moisture content. The third type, intermediate seeds, are desiccation-tolerant species for which tolerance is limited. Desiccation tolerance is, therefore, a continuous trait (Berjak & Pammenter, 2007), but for ecological questions, the differentiation into the two extremes - desiccation tolerant and desiccation intolerant - is useful (Tweddle *et al.*, 2003).

Functionality and trade-off

Desiccation tolerance is related to climate and habitat. On a global or regional scale, the proportion of desiccation-intolerant seeds is high in biomes with high humidity throughout the year, such as evergreen rainforests or habitats like forests and is low or not occurring in biomes with a dry season or in arctic or alpine ones or open habitats (Tweddle *et al.*, 2003). Desiccation-intolerant seeds are probably less frost-resistant (Hong *et al.*, 1998), but this has to be tested. Desiccation tolerance is also shaped by species phylogenetic affinities (Wyse & Dickie 2017).

Desiccation tolerance affects seed survival and is strongly related to soil seed bank persistence. Whereas desiccation-intolerant species are absent in soil seed banks, many desiccation-tolerant species form persistent soil seed banks (Thompson, 2000; Dickie & Pritchard, 2002).

Applied aspect

Desiccation-intolerant seeds cannot be stored under genebank conditions. They will lose their viability during drying (Hong *et al.*, 1998), which has important consequences for *ex situ* seed banking.

Sources of variability

The sensitivity of recalcitrant or intermediate seeds to drying may vary in the same species when grown in different environments. It may depend on the developmental status of the seeds and, therefore, may vary depending on the timing of seed collection. It may also vary according to the conditions under which the seeds dried (Pammenter & Berjak, 1999).

Desiccation tolerance may also affect soil seed bank longevity (see section **3.3.3 Soil seed bank longevity**). Exposure of orthodox seeds to different hydration-dehydration cycles or changes in soil water capacity may strongly affect their longevity (Kranner *et al.*, 2010; see also Abedi *et al.*, 2014; Mašková *et al.,* 2022).

Methodology

To find out if a species is orthodox or recalcitrant, the moisture content and viability of freshly collected seeds are analysed. To measure the moisture content, use a hygrometer sensor such as AW-DI0 from Rotronic Instruments (Hay *et al.*, 2008) or traditional lab oven dry method (see section **3.3.7 Seed water content**). Viability can be tested by applying a tetrazolium test or a respective germination test. Then, dry the seeds to 10 to 12% moisture content in an atmosphere which corresponds to a saturated LiCl solution (Hay *et al.*, 2008). When seeds have reached this moisture content, repeat the viability test. If most seeds have survived, they are orthodox; when most seeds have died, they are recalcitrant (Hong *et al.*, 1998).

To measure the critical water content (to which water content seeds can be dried), apply differently concentrated LiCl solutions (Table 7).

Table 7 Amounts of LiCl to add to 100 ml H_2O to produce certain relative humidities at 20 °C (according to Hay *et al.*, 2008).

Relative humidity (%)	Mass of LiCl to add per 100 ml H_2O (g)
11.2	Saturated (≥ 90)
15	74.1
20	64.0
30	52.0
40	43.5
50	36.4
60	30.0
70	23.7
80	17.1
90	9.4
95	4.8

3.3.6 Seed metabolic rate

Trait description

The sum of the total energy production of an organism, measured over time and expressed on a mass basis (IUPS 1987; Brown *et al.*, 2004).

Functionality and trade-offs

A seed must rely on its internal storage reserves (e.g. carbohydrates, proteins, lipids) to undertake activities that require energy, such as growth and repair. In seeds, metabolic rate is commonly measured as aerobic respiration, which has been linked to other functional traits of seeds. For example, higher respiration rates are associated with higher seed vigour and increased seed quality and viability (Bradford *et al.*, 2013; Bello & Bradford, 2016; Dalziell & Tomlinson, 2017).

Applied aspect

For orthodox seeds (i.e., seeds that are tolerant of desiccation and can survive low-temperature storage), storage under cool and dry conditions slows seed metabolism and the rate at which cellular reactions occur (Walters *et al.*, 2001). This significantly extends the lifespan of the seeds beyond which they would normally survive in nature. Conversely, recalcitrant seeds (desiccation sensitive) are highly metabolically active at the point of natural dispersal, and desiccation stress in these seeds is associated with metabolic imbalance during drying (Walters *et al.*, 2001). Respiration rates have been used to identify seed drying rates for cryostorage of recalcitrant seed embryos (Walters *et al.*, 2001).

Sources of variability

Aerobic respiration is dependent on seed hydration status and cellular/molecular mobility in the cytoplasm of cells. Seed respiration rate increases with seed moisture content (Dillahunty *et al.*, 2000; Bello & Bradford, 2016) as the seed progresses through the stages of imbibition, as well as with time since the commencement of imbibition (Bewley *et al.*, 2013). Temperature also affects respiration rate (Bello & Bradford, 2016),

with higher respiration rates at higher temperatures and a thermal peak for respiration (e.g., 70˚C for rice; Dillahunty *et al.*, 2000).

There is an allometric relationship between seed mass and respiration rate (Garwood & Lighton 1990; Huang *et al.*, 2020). However, this relationship does not scale isometrically and varies between studies (Dalziell & Tomlinson, 2017). The presence and type of seed dormancy may influence seed respiration rate, with studies indicating either no differences between respiration rates of dormant and non-dormant seeds (e.g., *Purshia tridentata*; Booth & Sowa, 2001), or a substantial increase in respiration rates of non-dormant seeds, compared to dormant seeds (e.g., rice; Footitt & Cohen, 1995).

Methodology

Seed respiration rate can be measured as a proxy for metabolic rate or converted to metabolic rate if measures of seed mass are taken. Seed respiration rate is dependent upon seed moisture content (water potential). The threshold water potential for aerobic metabolism in seeds is around -10 MPa (Vertucci & Leopold, 1984; Walters *et al.*, 2001). Changes in O_2 consumption or CO_2 production can be measured in freshly collected seeds if seeds are dispersed at high moisture contents (Garwood & Lighton, 1990), in (previously) dried seeds that are hydrated to the moisture content in equilibrium with *ca.* 95-98% RH (Garwood & Lighton, 1990; Dalziell & Tomlinson, 2017; Tomlinson *et al.*, 2018), or during the germination process itself for imbibed seeds (Bello & Bradford, 2016).

Seed respiration can be measured via changes in the gaseous environment (i.e. O_2 consumption and/or CO_2 production) surrounding the seed – either in a sealed system (i.e. closed respirometry) or in a system with continuous gas flow (i.e. open respirometry). A range of respirometry systems can be used to measure changes in O_2 and/or CO_2. More recent methods for measurement of CO_2 consumption in seeds include the use of a flow-through respirometer such as an infrared gas analyser (Dalziell & Tomlinson, 2017). Oxygen consumption can be measured via the

fluorometric quenching of a metal organic dye in a closed system (Bello & Bradford, 2016; Tomlinson *et al.*, 2018).

Alternatively, metabolic activity, including that due to respiration, can be measured via heat production (heat being a by-product of metabolic activity) using microcalorimetry (Edelstein *et al.*, 2001; Hay *et al.*, 2006). Metabolic heat production increases with increasing seed water content (Hay *et al.*, 2006) and during the early phases of the germination process (Edelstein *et al.*, 2001). Microcalorimetry and respirometry have been used together to calculate for *Cucumis melo* seeds the ratio between metabolic heat production and CO_2 evolution as an indicator of the substrate utilised for respiration (i.e. carbohydrates vs lipids) (Edelstein *et al.*, 2001).

Metabolic rate is temperature-dependent (Bello & Bradford, 2016; Tomlinson *et al.*, 2018), and measurement temperature(s) require consideration or optimisation for comparison across different species and environments. Measurement temperatures may include those at (or across) which germination is possible but may also exceed these limits. For example, to consider metabolic rate at a storage or ageing temperature of interest (Hay *et al.*, 2006).

Units

Metabolic rate can be calculated from measurements of respiration, whereby O_2 consumption or CO_2 production are expressed as a volume (i.e. uL) per unit of seed mass (e.g. mg, or g) per unit of time (e.g. s, min). Metabolic heat production is measured as heat flow (e.g. in joules) and expressed as a rate per unit of mass (e.g. µJ s^{-1} mg dry $mass^{-1}$).

3.3.7 Seed water content

Trait description

Seed water content (= seed moisture content) is the amount of water present in seeds at a given time and under specific environmental conditions. It is usually expressed as a percentage of the total seed mass on a dry (g H_2O/g dry weight) or fresh weight basis.

Functionality and trade-off

Measuring seed water content is important for understanding physiological processes like seed maturation, germination, and longevity. One of the most studied aspects of seed water content relates to the classification of fresh seeds into recalcitrant (desiccation sensitive) and orthodox (desiccation tolerant) seeds, with implications on ex situ storage conditions (Kermode & Finch-Savage, 2002). Recalcitrant seeds are sensitive to drying, mainly because maturation and germination occur only with high seed water content. Recalcitrant seeds remain metabolically active only in humid environments (e.g., tropical climates or temperate wetlands) and for a relatively short time. Thus, their persistence in natural habitats is limited to one or two years until they germinate (sometimes in the mother plant, i.e., viviparous germination). This makes it challenging to store recalcitrant seeds for a long time in ex situ seed banks, where they may survive for a few years only under high humidity and species-specific temperature conditions. In contrast, orthodox seeds experience a natural loss of water content during seed maturation (Kermode, 1990), allowing them to survive in dry environments (e.g., seasonal temperate climates). This ‘maturation drying’ blocks physiological mechanisms for post-dispersal germination until the seed is subjected to an environment with high relative humidity. At this point, seed water uptake and radicle growth depend on increased seed water content and the environmental cues regulating dormancy and germination. Orthodox seeds may germinate under different soil moisture conditions according to the gradient of water potential (ψ) between the seed and the soil. Water imbibition increases until seed water content equilibrates with the environment just before germination (Bradford, 1995). Although

all seeds are expected to germinate with water saturation (ψ = 0 MPa), soil water potential varies in time and space across habitats, and different species are adapted to germinate at slightly dry conditions, mainly between 0.05 and -1.5 MPa (Evans & Etherington, 1990). A key property of orthodox seeds is that they can remain viable for a long time with very little water content, favouring their ability to be dispersed and persist in the soil or to be stored in ex situ seed banks.

Sources of variability

At the species or population level, seed water content is influenced by the seed developmental stage (mature seeds generally have lower water content) and the morphological and chemical structure of the seed (determining seed coat permeability, seed section **3.3.4. Seed coat water permeability**). The seed content of oil and fatty acids may further regulate the quantity of water available in the seeds of different species (Levin, 1974), together with species-specific physiological traits. Besides those internal factors, seed water content strongly depends on environmental conditions. The seed environment moisture relationship is the difference between seed water content and the water in the air or substratum, which tends to equilibrate with time, given the hygroscopic nature of seeds. In natural conditions, seeds may be subjected to multiple climates and habitats, which ultimately determines the quantity of water available in the seed microhabitat.

Methodology

The most widely used method for calculating seed water content is weighing seeds before and after drying, thus calculating the water loss (gravimetric water content). Seed drying is conducted in a laboratory oven. Seed water content (*wc*) is then calculated as the % of fresh weight using the formula:

$$wc = \left[\frac{(fresh\,weight - dry\,weight)}{fresh\,weight} \right] \times 100.$$

Most species are dried at 120 ºC for 1 h, although seeds rich in oils and many tree species are dried at 100 ºC for 16 hours. After drying, the containers should be cooled for 30 min before weighing.

An alternative to multiple weighting is the use of a thermobalance, a device that can be programmed to weigh seeds repeatedly during heating.

In all cases, gravimetric water content is destructive because seeds will lose viability during drying. Seeds with physical dormancy (and very large seeds) must be cut, ground or crushed before weighing.

An indirect, non-destructive method for estimating seed water content is based on the water activity of hygroscopic substances by calculating the Equilibrium Relative Humidity (ERH) of the air around the seeds (Probert *et al.*, 2003). Using a sensor for water activity, the seeds are introduced into a small chamber and sealed with a probe to reach an equilibrium between the seeds and the air. After 20-30 min (depending on the species), the sensor provides the ERH as the relative humidity of air in equilibrium with seeds. The ERH can then be used to calculate seed water content using the known relationship for a given temperature (isotherm), which can be adjusted for every species (Copeland & McDonald, 2011).

A more advanced, non-destructive, but expensive method for calculating seed water content is based on nuclear magnetic resonance (NMR) spectroscopy. This technique offers a detailed pattern of the state and distribution of water in the seed (Fountain *et al.*, 1998), and it is generally used to compare the mobilization of water in germinating or non-viable seeds (Krishnan *et al.*, 2004).

Units

Seed water content is generally expressed as a percentage of the fresh weight. It can also be related to dry mass, such as g water / g dry mass (Bewley *et al.*, 2006). ERH varies from 0 to 100%. The seed viability equations website (Flynn & Turner, 2004) provides examples for calculating water content using ERH for a given temperature by considering the effect of seed oil content.

3.4 Seed dormancy and germination

3.4.1 Embryo-to-seed size ratio

Trait description

The embryo-to-seed size ratio is a measure of the relative amount of nutrient reserves available to the offspring stored inside the embryo and the size of the offspring relative to the seed as compared to nutrients stored in extra-embryonal nutritive tissues. Mature seeds contain an embryo and very often endosperm and/or perisperm. Endosperm tissue is triploid (3n) in angiosperms and the result of double fertilization, where two-thirds of the genome is of maternal origin. Perisperm occurs in fewer species and has a diploid (2n) maternal origin. Some authors wrongly use the term endosperm to cover all non-embryonic storage tissue, i.e. including perisperm. Proper naming of non-embryonic stage tissue is recommended. In the embryo-to-seed size ratio, seed size is quantified as the size of the embryo plus the endosperm and perisperm. In literature, the embryo-to-seed size ratio can be expressed either as the embryo-to-seed length ratio or the embryo-to-seed surface ratio.

Functionality and trade-offs

The embryo-to-seed size ratio has often been associated with morphological dormancy and germination speed. Numerous species with low embryo-to-seed size ratio display the growth of the embryo between dispersal and germination, a process that has been associated with delay of germination or morphological dormancy (Nikolaeva, 1977). A positive relation between embryo-to-seed length and germination speed has been observed in Umbellifers (Vandelook *et al.*, 2012). There was no relationship between embryo-to-seed surface ratio across the whole angiosperm phylogeny (Verdú, 2006). Evidence for the presence of large amounts of endosperm reducing predation damage is scarce, although it has been shown that the removal of up to 60% of the endosperm in two grasses did not affect the germination percentage (Zhang & Maun, 1989). The embryo-to-seed size ratio is usually negatively related to seed mass (Hodgson & Mackey, 1986; Vandelook *et al.*, 2012).

Sources of variability

The embryo-to-seed size ratio varies with the cutting edge, on which embryo and seed size are measured. Considerable variation in embryo-to-seed size ratio exists among species (Vandelook *et al.*, 2012), although most variation is situated at the family and order level (Martin, 1946). Variation within plants and within species is generally low, although considerable intraspecific variation in embryo-to-seed length ratio has, for example, been observed in *Daucus carota* (Vandelook, unpublished results).

Methodology

As seed internal tissues may shrink in a dried state, seeds should be measured in an imbibed state. Seeds that were in a dry state for a prolonged period of time should be placed in water for 24 h before measurements. For most seeds, embryo size and seed size can be measured when seeds are cut in half using a razor blade or scalpel along the sagittal plane (also known as the longitudinal plane) or the symmetry axis. In some families, e.g. Rubiaceae, the embryo is positioned asymmetrically inside the seed. In such cases, seed size is still determined based on the sagittal plane section, but the embryo needs to be cut out of the seed for proper measurement. From 10 to 20 seeds should be measured to cover variation within a sampling unit, although due to limited variation with seed batches, even smaller amounts of seeds will give a representative measure. Measurements can be made on photos taken with a regular light microscope and include a scale bar.

To determine the embryo-to-seed length ratio, the entire length of the embryo, which can be longer than the seed when curved, should be measured and divided by the largest seed length; this is the embryo plus storage tissues, excluding the seed coat and other covering tissues (Fig. 7).

To determine the embryo-to-seed surface ratio, the surface area should be measured and divided by the seed surface embryo; this is the embryo plus other storage tissues (Fig. 7). Measures of embryo and seed length and/or area should be reported as well, as they are informative data on their own.

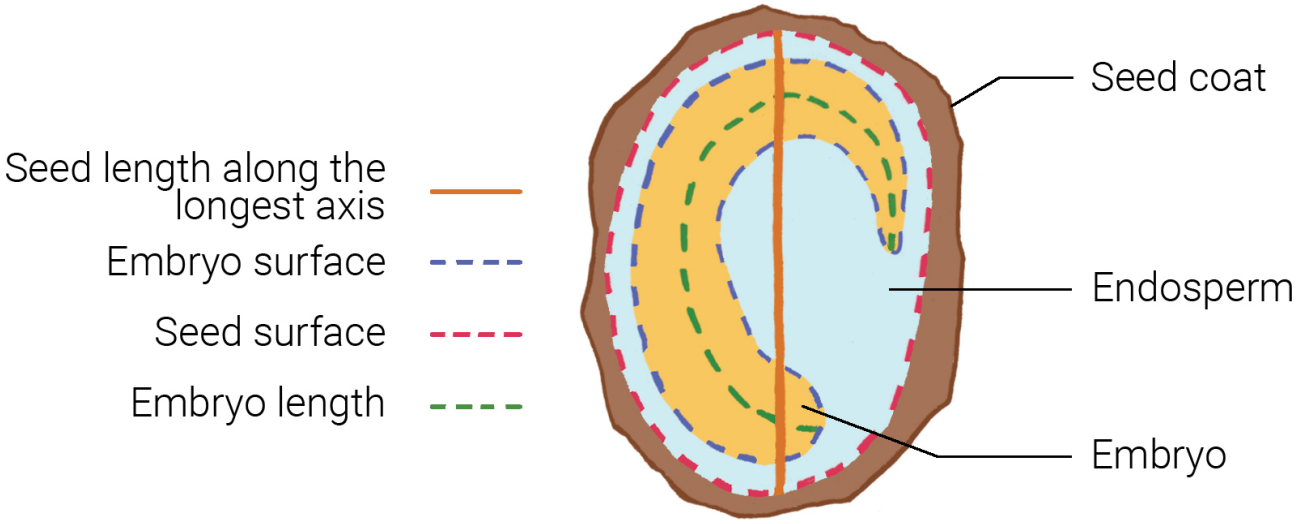


Fig. 7 Schematic drawing of a seed cut along the sagittal plane. From this picture, the embryo length (green dotted line) to seed length (yellow full line) ratio and the embryo surface (area with the red striped line) to seed surface (area with the blue striped line) ratio can be measured using e.g. ImageJ software.

3.4.2 Post-dispersal embryo development

Trait description

An embryo is a miniature plant that generally consists of a radicle (rudimentary root), hypocotyl (stem portion), an epicotyl (shoot) and one or more cotyledons (first leaves). A seed embryo may be fully developed or underdeveloped at the point of seed maturity. If an embryo occupies a relatively small (≤0.5) volume of the interior of the mature seed (Baskin & Baskin, 2007) due to limited anatomical and morphological differentiation or development at dispersal and must grow before radicle emergence (Fig. 8), it is referred to as an underdeveloped embryo (Crocker, 1916; Martin, 1946; Grushvitsky, 1967; Nikolaeva, 1999; Forbis & Diggle, 2001; Baskin & Baskin, 2014). However, not every small-sized embryo is classified as underdeveloped; some small types of embryos, such as broad embryos, do not grow prior to radicle emergence (Baskin & Baskin, 2007). The clear distinction is that a fully developed embryo does not grow inside the seed before radicle emergence (Nikolaeva, 1969, 1977). In general, an embryo is categorised as underdeveloped only if the embryo-to-seed length ratio increases considerably internally between imbibition and radicle emergence. Seeds with limited morphological development at dispersal have either a differentiated (radicle and cotyledon(s) present) but underdeveloped embryo or an undifferentiated underdeveloped embryo. Depending on the species, an underdeveloped embryo may be categorised as rudimentary, linear-underdeveloped or spatulate-underdeveloped (Baskin & Baskin 2007, 2014).

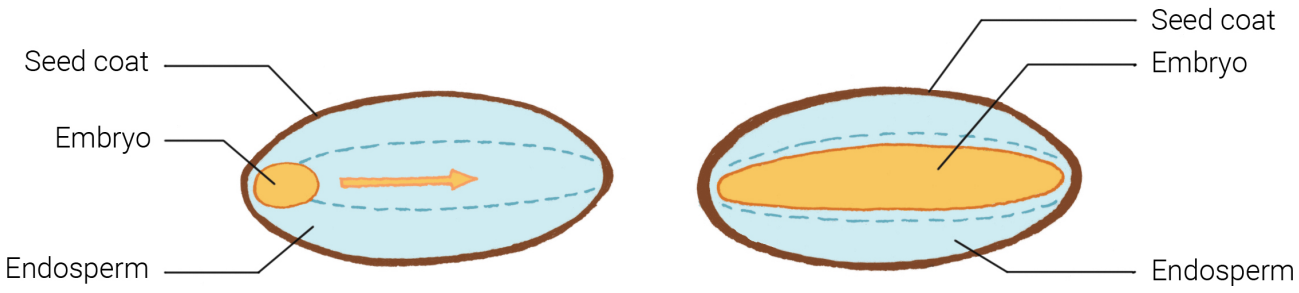


Fig. 8 Illustrations of an underdeveloped embryo at dispersal and a fully developed embryo just before radicle emergence. The green arrow indicates the direction of embryo growth.

Functionality and trade-off

An underdeveloped embryo requires a period of favourable environmental conditions for the resumption of growth to the point at which it occupies the full length of the seed and can break the seed coat (Forbis & Diggle, 2001; Forbis *et al.*, 2002; Baskin & Baskin, 2007) through radicle emergence. Embryo growth is associated with the morphological component of seed dormancy, i.e. morphological dormancy (MD) and morphophysiological dormancy (MPD) (Baskin & Baskin, 2014). For spring and summer germinating species in temperate climates, this trait can function 'as a starter kit' to initiate the germination process much in advance during the moist but cold and unfavourable winter season to synchronise seedling emergence only when environmental conditions are most conducive for their further growth and survival (Vandelook *et al.*, 2009). In contrast, if seeds take more than one growing season to germinate after dispersal (e.g., *Cardiocrinum spp.*), the embryo development process begins only at the onset of the first growing season and continues until the second or third growing season. Thus, this trait plays a crucial function in delaying dormancy break and seed germination during the harsh, unfavourable season likely encountered after seed dispersal (Kondo *et al.*, 2006; Phartyal *et al.*, 2012).

For autumn and winter germinating species of seasonally dry environments, such as Mediterranean-type climates, embryo growth commences only once sufficient soil moisture is available upon the onset of the wet season in late autumn, and germination of seeds with underdeveloped embryos can be delayed relative to seeds with fully developed embryos (Hidayati *et al.*, 2019). For monsoon-germinating species of seasonal subtropical and tropical climates, seeds disperse either several months before or during the monsoon (wet) season (Garwood, 1983; Singh *et al.*, 2017; Athugala *et al.*, 2016, 2018), and embryos begin to grow soon after the first spell of rain and radicle/epicotyl emergence is at the peak of the monsoon to ensure seedling

survival within a wet season, especially in seeds with epicotyl MPD (Athugala *et al.*, 2016, 2018). Embryo development consequently influences the timing of seedling emergence and contributes to temporal variation in plant establishment, both within and between species (Hidayati *et al.*, 2012, 2019). For example, in *Aristolochia* species, the first cohort of seeds in a population germinates early in the growing season (embryos grow at a relatively high rate), the second cohort germinates a little later in the growing season (embryos grow at a relatively slow rate). A remaining cohort does not germinate as embryos do not grow sufficiently for radicle emergence (Adams *et al.*, 2011). Underdeveloped embryos might be disadvantageous if the seeds fail to initiate the germination process quickly as they usually get a short spell of a favourable environment to germinate soon after soil or vegetation disturbance in dry habitats (Fenner & Thompson, 2005), or water drawdown in wet habitats (Poschlod & Rosbakh, 2018). Seeds with underdeveloped embryos are thus rarely found in arid environments of irregular rainfall and short periods of water availability (Rosbakh *et al.,* 2023).

Contrary to an underdeveloped embryo, a developed embryo does not require additional growth within the seed to initiate radicle emergence. Seeds will germinate when exposed to environmental cues required for breaking dormancy (if any) and subsequently experience appropriate environmental conditions for germination (Forbis *et al.*, 2002; Baskin & Baskin, 2014). A developed embryo is associated with non-dormancy (ND), physical dormancy (PY), physiological dormancy (PD), and combinational dormancy (PY+PD) (see Baskin & Baskin, 2014; 2021).

Source of variability

To initiate dormancy-break and germination, seeds require a moist substrate, suitable temperatures, and, in some species, a specific light/dark regime (Kondo *et al.*, 2011). Depending on the species, the rate of embryo development varies with variations in temperature (cool vs warm, constant vs alternative), irradiance (light vs dark) and oxygen (aerobic vs hypoxic) conditions experienced by seeds during the germination process. Embryo development rate also depends on the initial temperature regime in a move-along seasonal temperature sequence used to initiate dormancy break and seed

germination (Kondo *et al.*, 2006; Phartyal *et al.*, 2012; Baskin & Baskin, 2014). Further, different types and levels of MPD (Baskin & Baskin, 2014) (i.e., differing depths of dormancy within a seed population) may also cause variation in the rate of embryo development. For example, whilst it is common for the embryo to develop fully before radicle emergence, in seeds of some species with deep simple double MPD or with epicotyl MPD, embryo development may occur at a later stage after the radicle emergence (Phartyal *et al.*, 2014; Kondo *et al.*, 2015; Athugala *et al.*, 2018), or in even more specialised cases, root and shoot differentiation may occur only after the embryo emerges from the seed coat (Tuckett *et al.*, 2010).

Applied aspect

The loss of tolerance to seed desiccation is known to coincide with post-dispersal embryo development, like that seen in orthodox seeds upon radicle emergence (Ali *et al.,* 2007). Thus, knowledge of post-dispersal embryo development contributes to revealing which conditions are optimal for ex situ conservation or survival of seeds in the soil and for seed dormancy break and germination timing. Additionally, a species with desiccation-tolerant seeds stored in the genebank and likely to have an underdeveloped embryo may be weakly or transiently desiccation-tolerant and thus practically unsuitable for long-term ex situ conservation (Ali *et al.,* 2007).

Methodology

To determine embryo size, immediately after initial seed cleaning and processing, a minimum of ten seeds should be allowed to imbibe water overnight and then cut into thin sections, either manually with a surgical blade or automicrotome to measure the initial embryo dimensions (length and width) of fresh, dispersed seeds using a microscope equipped with a micrometre. The total length of the seed must also be measured and determined as the longitudinal length between the internal walls of the seed coat (see section **3.4.1 Embryo-to-seed size ratio**). Alternatively, capture a digital image of the dissected seed to determine the embryo and seed dimensions using appropriate image analysis software. The ratio of the embryo to seed length (E:S ratio)

can then be calculated. At this point, the developmental status of the embryo should also be determined, according to Baskin & Baskin (2007).

For seeds with undifferentiated or small (i.e. E:S ratio ≤ 0.5) embryos, it should then be determined if differentiation and/or growth of the embryo is required prior to radicle emergence. Depending on the kind, type, and level of the physiological component of dormancy (see Baskin & Baskin, 2014), embryo development may require either only a single temperature regime of warm (>15 °C; valid for tropical species) or cold (0-10 °C) for stratification (Baskin & Baskin, 1984a; Walck *et al.*, 2002), or a move-along sequence of temperature regime of warm + cold (Baskin & Baskin, 1984b), cold + warm (Walck *et al.*, 1999), and cold + warm + cold for stratification (Nikolaeva, 1977). Seeds may alternatively require a period of after-ripening under warm, dry conditions to initiate embryo growth once subsequently incubated at appropriate germination temperatures (Hidayati *et al.*, 2019). It is therefore recommended, on the same day, depending on seed availability, to place 4-5 replicates of 50-100 seeds on a moist substrate in each Petri dish for stratification at suitable temperatures to measure embryo development and growth at different stages of incubation. It is always appropriate to use temperature regimes for stratification and germination in which seeds are more likely to respond positively, i.e. a temperature regime seeds experience in their natural habitat after dispersal until the first growing season. After that, 2 to 4 stratified/incubated seeds must be retrieved at regular (weekly/fortnightly/monthly) intervals from each of the dishes haphazardly at random and used to measure embryo dimensions as previously described until protrusion of radicle tip and/or the emergence of epicotyl.

Optionally, it is also suggested to investigate the phenology of embryo development in outdoor natural habitats by placing several replicates of 10-20 seeds in fine-mesh polyester bags. First, flatten these bags in a metal net and then bury them at a soil depth of approximately 3 cm in a tray under near-natural outdoor environmental conditions, either inside a frame house or in the field, with protection against rodents and other predators. After that, at regular time intervals, retrieve one bag randomly

and measure embryo dimensions as described previously. More methodological details can be found in Phartyal *et al.* (2009, 2012, 2014) and Kondo *et al.* (2011, 2015). The extent to which the embryo must grow for germination can be calculated using the formula:

$$Embryodevelopment\,(\%) = \left[\frac{(E:Sratio\,(atpointofradicleemergence) - E:Sratio\,(freshseed))}{E:Sratio\,(freshseed)} \right] \times 100,$$

where the length of the embryo as a percent or proportion of the total length of the seed is expressed as the Embryo:Seed ratio.

3.4.3 Seed dormancy and dormancy-breaking cues

Trait description

Seed dormancy is a rather controversial trait, with manifold perspectives on its definition, origin, evolution and functionality (Baskin & Baskin 2014). For some, seed dormancy is simply the absence of germination due to the lack of a favourable environment. For others, it is due to extrinsic (physical) or intrinsic (morphophysiological) traits of the seed that prevents rapid germination over a wide range of environments (Baskin & Baskin, 2014). However, seed dormancy is best seen primarily as a seed trait rather than an environmental issue (Eira & Caldas, 2000). Thus, seed dormancy is defined as 'an inability of a viable seed to germinate (a sign of both radicle and epicotyl emergence) within a specified period (≤ four weeks) of time under any combination of the normal physical environment that otherwise seems to be favourable for seedling establishment (Baskin & Baskin, 2004, 2014). In contrast, if a seed with a fully developed embryo germinates rapidly within four weeks (usually in a few days) over a wide range of environmental conditions without any dormancy-breaking treatment, it is classified as a 'non-dormant' seed (Baskin & Baskin, 2004, 2014). The four-weeks threshold is, to some extent, arbitrary. Still, it allows non-dormant seeds to receive sufficient germination time but should be insufficient to receive cold (or warm) stratification for dormancy break (for more, see section 'Length of Germination Test Period' page 31 in Baskin and Baskin, 2014).

Several dormancy classification schemes are available. For example, Harper (1977) used developmental stages of dormancy as criteria and classified dormancy into three categories - innate (develop during maturation), enforced (non-dormant seeds fail to germinate due to the absence of one or two physical environmental factors), and induced (re-entrance of non-dormant seed to dormancy). However, this scheme is somewhat misleading and fails to accommodate and relate various kinds of dormancy to species' evolutionary position, lifeform, and biogeography (Baskin & Baskin, 2014). Later, Nikolaeva (1969, 1977) used the location of dormancy as a criterion and classified dormancy into two broad categories - exogenous (develops from outside of embryo in

the endosperm or seed coat as physical, mechanical and chemical dormancy) and endogenous (develops from inside in embryo as physiological, morphological and morphophysiological dormancy). However, applying these schemes in ecological studies, especially to disentangle how and when dormancy breaks and the seed germinates in nature, is a daunting task for seed ecologists.

Considering the above facts, Baskin & Baskin (2004, 2014) revisited Nikolaeva's scheme of seed dormancy and used both the physiology and phenology of seed germination as criteria to develop a modified hierarchical classification system. This new system recognised five classes of seed dormancy: morphological, physiological, morphophysiological, physical, and combinational dormancy (Table 8), which further categorised dormancy class into subclass, level, and type (for more details see page 40 in Baskin & Baskin, 2014 and Baskin & Baskin, 2021). This system suggested that not specifying the dormancy class in studies focusing on seed dormancy subjects may be somewhat analogous to not including the Latin name of the study organism in scientific articles (Baskin & Baskin 2004). Therefore, it is strongly recommended to identify dormancy class as one of the most relevant and crucial seed traits for plant regeneration (Saatkamp *et al.*, 2019).

Table 8 A broad overview of seed dormancy classes and their characteristics (adopted from Baskin & Baskin, 2014, 2021).

Dormancy class	**Characteristics**
Non Dormant (ND)	Seeds with water-permeable coats and a developed embryo that germinates (both radicle and epicotyl emerge) quickly within 4 weeks.
Morphological Dormancy (MD)	Seeds with water-permeable seed coat and an underdeveloped embryo (one that must grow inside the imbibed seed) that germinates (both radicle and epicotyl

	emerge) within 4 weeks.
Physiological Dormancy (PD)*	Seeds with water-permeable coats with a fully developed embryo but with low growth potential or push power to protrude radicle. This physiological inhibiting mechanism delays seed germination (both radicle and epicotyl emerge) beyond 4 weeks.
Morphophysiological Dormancy (MPD)	Seeds with water-permeable coats and underdeveloped and physiologically dormant embryo. The embryo in imbibed seed needs >4 weeks to grow and initiate seed germination.
Physical (PY)	Seeds with water-impermeable coats and a fully developed embryo. Scarified seed becomes fully imbibed within a day or two and germinates (both radicle and epicotyl emerge) within 4 weeks.
Combinational (CD; PY+PD)	Seeds with water-impermeable coats and a fully developed and physiologically dormant embryo. Scarified seed imbibe water readily but delay germination (both radicle and epicotyl emerge) beyond 4 weeks.

* Mechanical and chemical dormancy of Nikolaeva's scheme are derecognised and merged as an aspect of PD (Baskin & Baskin, 2004); therefore, it is suggested to avoid using mechanical and chemical dormancy as separate classes of dormancy.

Functionality and trade-off

Seed dormancy is a crucial trait that slows down the germination process when environmental conditions are favourable for germination but subsequent environments likely to be unfavourable for the survival of the seedling (Vleeshouwers *et al.*, 1995; Finch-Savage & Leubner-Metzger, 2006). Thus, the primary function of seed dormancy is to arrest germination and provide ecological advantages in adjusting germination to the favourable growth period for seedling establishment (Fenner & Thompson, 2005; Baskin & Baskin, 2014, Rubio de Casas *et al.*, 2017). Dormancy determines species' biology, ecology, geographical distribution, and habitat preference (Finch-Savage & Leubner-Metzger, 2006; Donohue *et al.*, 2010; Wagmann *et al.*, 2012; Rubio de Casas *et al.*, 2015, 2017). In unpredictable variable environments, it functions as a bet-hedging strategy (Cohen & Levin, 1991; Volis & Bohrer 2013; Pausas *et al*., 2022), while in a predicted invariable environment, where local competition between siblings is high due to limited seed dispersal, dormancy might function as an adaptation to reduce competition between siblings (Nilsson *et al.*, 1994) through the temporal distribution of seed germination.

Strong evidence of the ecological functions of PY has been documented by Jayasuriya *et al.* (2015). They argued that PY increases the fitness of species by helping seed to detect winter/summer temperatures (Van Assche & Vandelook, 2006; Jayasuriya *et al.*, 2008a; Ooi *et al.*, 2009; Gama-Arachchige *et al.*, 2012), canopy gaps (Vázquez-Yanes & Orozco-Segovia, 1994), and post-fire environments (Baskin & Baskin, 1997; Santana *et al.*, 2010; Moreira & Pausas, 2012, but see Rosbakh *et al*., 2023) to initiate germination process at the right time. They also highlighted the role of PY in endozoochorous (Janzen *et al.*, 1985; Michael *et al.*, 2006; Campos *et al.*, 2008) and hydrochorous (Guja *et al.*, 2010) seed dispersal to escape from predators (Paulsen *et al.*, 2013) and pathogens (Dalling *et al.*, 2011; Zalamea *et al.*, 2018), in maintaining sensitivity cycling of water gap (hilum) to control cyclic seed germination pattern (Jayasuriya *et al.*, 2008a, 2009), and to some extent in seed persistence in the soil seed bank (Leck, 1989; Thompson *et al.*, 1993; Gioria *et al.*, 2020).

The other four dormancy classes also play more or less similar ecological functions (Baskin & Baskin, 2014). For example, a substantial delay from radicle to epicotyl emergence (epicotyl dormancy- a subclass/level of PD/MPD, see Fig. 9) is hypothesised to be an adaptation that allows seedlings to have well-developed root system at the time of cotyledon expansion in early spring in the temperate region or to remain in the understory forest canopy until expose to suitable light intensity in the tropical forests (Baskin & Baskin, 1985; Jayasuriya *et al.*, 2012; Athugala *et al.*, 2016). Additionally, epicotyl dormancy is suggested to maintain the viability of desiccation-sensitive seeds during dry spells via water uptake (Athugala *et al.*, 2016, 2018; Jaganathan and Phartyal, 2024).

Sources of variability

Variation in seed dormancy occurs within and between individuals and populations of a species due to genotype, maternal environment during seed development, and environment experienced by seeds during postharvest and germination (Allen & Meyer, 2002; Baskin & Baskin 2014). Additionally, variation in degree (or depth) of dormancy (reflected by germination percentages of fresh seeds as no-dormancy vs dormancy or non-deep PD vs intermediate/deep PD) occurs in seed samples of some species from the same population collected at different times during a single seed-production season (Gutterman, 1994; Baskin & Baskin, 1995) or in different years (Baskin & Baskin, 1975a; Allen & Meyer, 2002; Petrů & Tielborger, 2008) or from the different populations (Jayasuriya & Phartyal, 2023). For example, seeds of *Lamium amplexicaule, L. confertum, L. hybridum* (Karlsson & Milberg, 2008) possess a higher degree of dormancy, whereas seeds of *Lotus tenuis* possess a lower degree of dormancy (Clua & Gimenez, 2003) if their seeds collected early (compared to late) in a seed-production season (Baskin & Baskin, 2014). Similarly, if PY seeds of *Ipomoea lacunosa* disperse at relatively high temperatures in early autumn, they show less sensitivity to dormancy-breaking (warm moist stratification for 2hr at 35 °C) conditions than those that disperse at relatively low temperatures in late autumn (Jayasuriya *et al.*, 2008b). In contrast, PD seeds of *Aesculus hippocastanum* disperse at relatively warm

autumn temperatures and show more sensitivity to dormancy-break (cold, moist stratification) conditions than those that disperse at relatively cool autumn temperatures (Pritchard *et al.*, 1999). These changes in the degree of dormancy might occur due to temporal differences in habitat characteristics such as diurnal photo- and thermo-periods, length of the growing season, moisture and nutrient status of the soil (Baskin & Baskin, 1973; Fenner, 1992a, b; Meyer *et al.,* 1995).

The position of the seed on the plant, the age of the mother plant (Fenner, 1991; Gutterman, 1992), and the production of heteromorphic diaspores/seeds (Venable, 1985; Ellner, 1986; Brändel, 2004; Baskin *et al.*, 2013, 2014) also cause variation in dormancy. Like the degree of dormancy, the class of dormancy also varies within a seed lot of a single species. Hidayati *et al.* (2000) reported approximately 50% of fresh mature seeds of *Lonicera maackii* and *L. morrowii* had MD, and the remaining seeds had MPD. At biogeographical levels, seed dormancy tends to decline towards the equator (an aseasonal environment) and remains predominant in seasonal environments towards temperate and desert habitats (Baskin & Baskin, 2014; Rubio de Casas, 2017; Rosbakh *et al.,* 2023). There is also a distinct variation in seed dormancy across families, e.g. PY dominates in 20 families, including Anacardiaceae and Fabaceae, while MD or MPD in Apiaceae, Liliaceae, Ranunculaceae and others (Baskin & Baskin 2014).

Applied aspect

The lack of knowledge about seed dormancy and dormancy-breaking treatment can severely hamper the effective utilization of viable seeds as a quality and cost-effective regeneration material in large-scale afforestation or restoration programs and even the conservation of rare and threatened species under ex situ conditions. Therefore, knowing the seed dormancy class, variation in the degree of dormancy and appropriate pre-treatments to alleviate dormancy can be critical for effective seed management techniques and ensuring greater plant establishment in restoration (Kildisheva *et al.*, 2019, 2020) and for ensuring synchronised germination during a routine viability test of ex situ banked seeds (Phartyal *et al.*, 2002).

Methodology

The methodology is divided into two sections; the first deals with determining dormancy class, and the second with what treatments (environmental conditions) require dormancy-break or acting as dormancy-breaking cues in nature.

1. *Determination of seed dormancy class*

To determine dormancy or non-dormancy in fresh mature seeds, incubate four replicates of 25 fresh, intact (non-treated) seeds on a moist substrate in Petri dishes or trays (depending on seed size). For incubation, use the most appropriate temperature and photoperiod regimes that seeds are likely to experience in their natural habitat during the growing season. For typical aquatic species, optionally use hypoxic conditions for seed incubation (more detail in Phartyal *et al.,* 2018, 2020a, b; Rosbakh *et al.,* 2020b). Monitor incubating seeds for germination (both radicle and epicotyl emergence) at 2-3 day intervals. If seeds imbibe water and germinate within 30 days, then categorise them as 'non-dormant'. However, if seeds fail to germinate or only the radicle emerges but there is no sign of epicotyl emergence within 30 days, then categorise them as 'dormant' (Baskin & Baskin, 2014). Simultaneously, set additional experiments to determine the seed dormancy class, using the steps outlined in the simplified protocol in Fig. 9.

1. *Determination of water permeability of fruit/seed coat*

Twenty families of angiosperm are known to produce hard and water-impermeable seed/fruit coat (Baskin & Baskin, 2014). However, not necessarily all hard seeds have water-impermeable seed coat. Therefore, it is always suggested to determine the water permeability of the seed/fruit coat as the first step to confirm or rule out PY. For this measure seed coat permeability by following the methodology from section **3.3.4 Seed coat water permeability**. Additionally, determine the proportion of permeable seeds (permeability %) in a seed sample based on the number of seeds with increased seed mass or size (swelling). This will help determine the dormancy depth in a seed sample based on the water-permeability of individual seeds (Fig. 10).

As a special case - if the fruit/seed coat is very hard and difficult to scarify due to fibre, such as seeds of *Terminalia spp.*, or in minute seeds (Ribeiro *et al*., 2015) use methylene blue or any other appropriate dyes, like a low molecular weight-fluorescent lucifer yellow dye (McCulloch *et al.*, 2024), for seed soaking to determine the permeability of the seed coat. Sometimes, only the outer fibrous tissues of the fruit/seed coat imbibe water, not the actual seed, which otherwise gives unreliable information about the water permeability of the seed coat. Observe the staining pattern around the embryo or endosperm of the soaked seeds after cracking/hammering to confirm the water permeability of the actual seed.

1. *Monitor embryo morphology, E:S ratio, and embryo development*

Seeds of Apiaceae, Aristolochiaceae, Caprifoliaceae, Liliaceae, Ranunculaceae and many more families are known to possess MD or MPD. However, in most papers on seed dormancy, experiments were not set up to monitor embryo development during seed germination. That eventually leads to misleading categorizations of MD as ND and MPD as PD seeds. Therefore, it is strongly suggested to monitor embryo morphology, E:S ratio, and embryo development during seed germination (before and after radicle emergence) to confirm or rule out MD or MPD. For more methodological details, see section **3.4.2 Post-dispersal embryo development**.

1. *Monitor time requires for radicle and epicotyl emergence*

A substantial delay (>30 days) occurs in radicle or epicotyl emergence in seeds with a physiological component of dormancy, depending on the subclass, level, and type of PD/MPD. Therefore, it is suggested to monitor the time the seeds require for radicle emergence or, in case of epicotyl dormancy, from radicle to epicotyl emergence (see Baskin & Baskin 2014). Depending on the biogeography of species (tropical/subtropical vs temperate/arctic zone) and seed dispersal season (spring/summer vs autumn/winter), radicle or epicotyl emerge at suitable incubation environments only after seeds were exposed to either warm (>15 °C) or cold (0-10 °C) moist stratification or to chemical growth hormones like gibberellic acid. To monitor delay in epicotyl

emergence, choose 10-25 fresh radicle-emerge seeds, place them either on moist filter paper in a laboratory beaker (Fig. 11) or bury them about 1 cm deep in the soil (Fig. 12) and then expose them to appropriate temperatures for stratification/incubation. Monitor them at 2-3 day intervals to determine the time taken from radicle to epicotyl emergence. If it takes >30 days, it confirms epicotyl PD or MPD, depending on embryo development (see Fig. 9). Optionally, it is suggested to investigate the phenology of radicle and epicotyl emergence in outdoor natural habitats. More methodological details about the phenology of radicle and epicotyl emergence can be found in Phartyal *et al.* (2009, 2012, 2014) and Kondo *et al.* (2011, 2015).

1. ***Treatments for alleviating seed dormancy***

The choice of the most appropriate dormancy-breaking treatments depends on dormancy class, seed dispersal season, phenology of seed germination in nature, and environment experienced by seeds from maturation to seedling emergence (Kildisheva *et al.*, 2020) as outlined in Table 9. To break the physiological component of dormancy, seeds may require either only a single temperature of warm (>15 °C) or cold (0-10 °C) (Baskin & Baskin 1984a; Walck *et al.*, 2002), or a move-along sequence of temperatures of warm + cold (Baskin & Baskin, 1984b), cold + warm (Walck *et al.*, 2000), and cold + warm + cold (Nikolaeva, 1977; Phartyal *et al.*, 2014) for stratification. Therefore, it is recommended to bet on one or two of the most suitable temperature regimes for stratification/incubation in which seeds are more likely to respond positively.

Table 9. An overview of common pre-treatments to break seed dormancy.

Dormancy class	**Description of treatment**
MD	● No specific treatment needed. ● Allow seeds to after-ripen at optimum incubation conditions for embryo development and germination.

PD	● Expose water-imbibed seeds either only to cold or warm temperature; or a combination of warm + cold; cold + warm; and/or cold + warm + cold temperature sequence to simulate near-natural habitat environmental conditions for stratification/incubation. ● Expose seeds to different growth promotors such as gibberellic acid, ethylene, Karrikinolide (smoke water), or potassium nitrate. ● Permit seed to dry after-ripening at the warm and humid environment.
MPD	● Expose water-imbibed seeds to dormancy-breaking treatments used for MD and PD. ● Since seed germination in several species requires >1 growing season, expose water-imbibed seeds to a move-along annual temperature sequence to simulate the post-dispersal environment, e.g., autumn (15/5 °C) → winter (0 °C) → spring (15/5 °C) → summer (25/15 °C) → autumn (15/5 °C) → winter (0 °C) → spring (15/5 °C). Seasonal temperature and duration can be adjusted as per study species.
PY	● Use scarification (manual, mechanical, chemical) treatments to make a scar on seed-coat to facilitate water imbibition. ● Expose seeds to high fluctuating summer or low winter temperatures to facilitate cracks on seed-coat. ● Expose seeds to the two-step sensitivity cycling model to facilitate the opening of water-gaps (see Fig. 13)
CD (PY+PD)	● Use scarification treatments to break PY. ● Expose water-imbibed seeds to cold or warm stratification

	treatment to break PD.

For more detail on sub-categories of seed dormancy classes and dormancy-breaking treatments, explore Sautu *et al.* (2007), Jayasuriya *et al.* (2009), Baskin & Baskin (2014, 2021), Erickson *et al.* (2016), and Kildisheva *et al.* (2020). For an alternative approach of seed dormancy and dormancy-release pathways, it is suggested to explore a recent publication by Lamont & Pausas (2023).

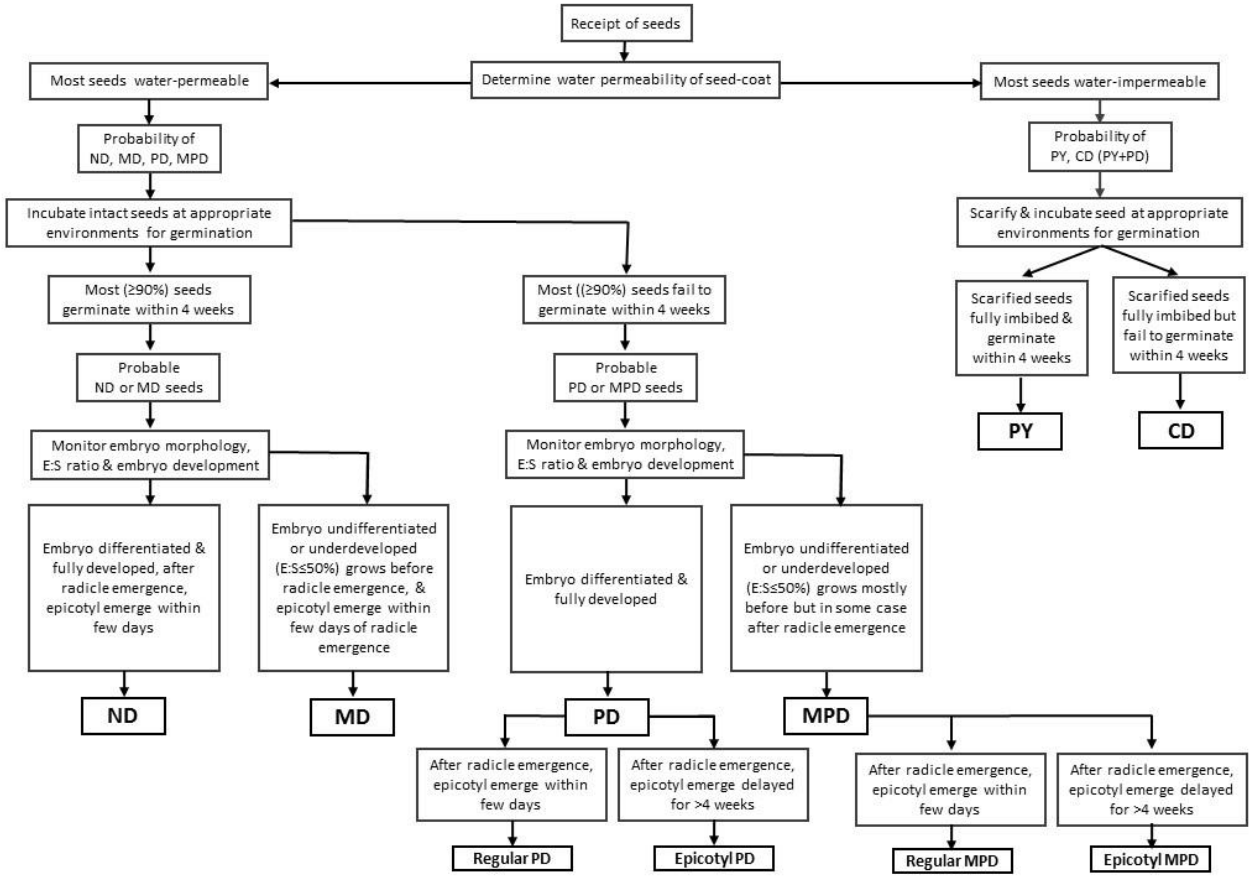


Fig. 9 Simplified outline of a protocol to identify seed dormancy classes in freshly dispersed seeds.

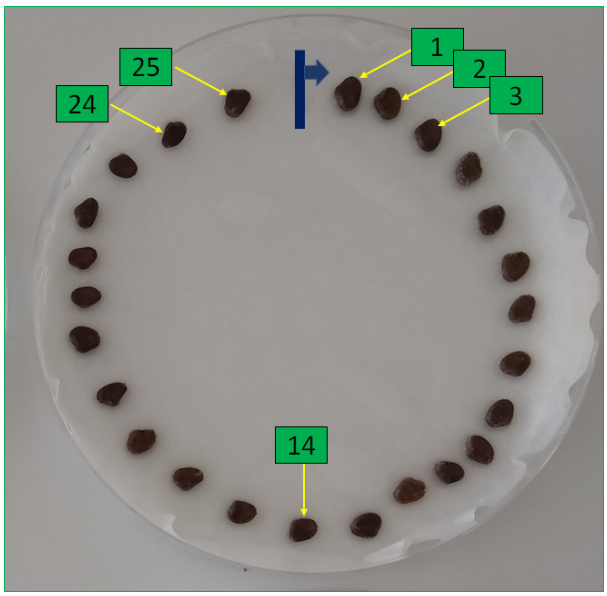


Fig. 10 Illustration to arranged individual seeds on moist substrate for the water-permeability test.

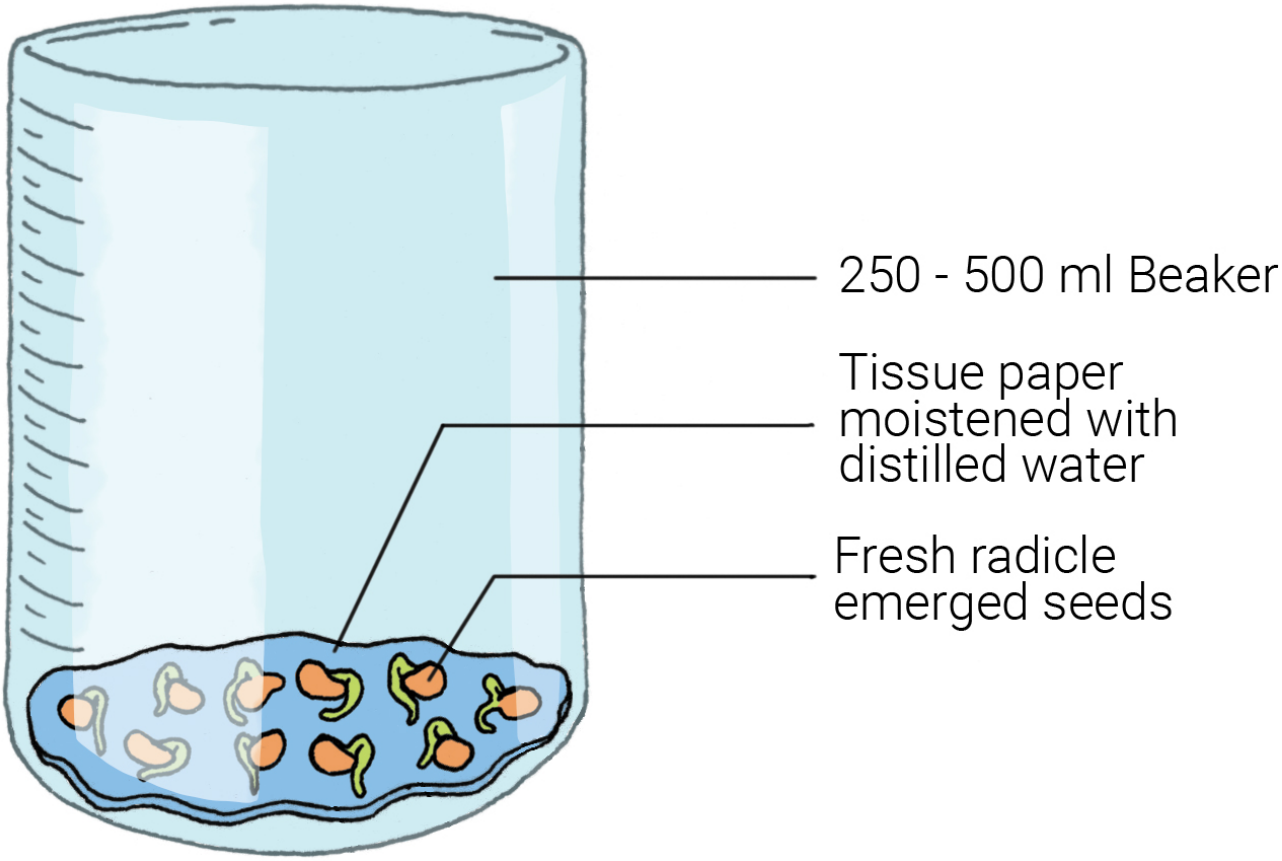


Fig. 11 Illustration of the use of beaker for stratification/incubation of radicle-emerge seeds to monitor time requires for epicotyl emergence.

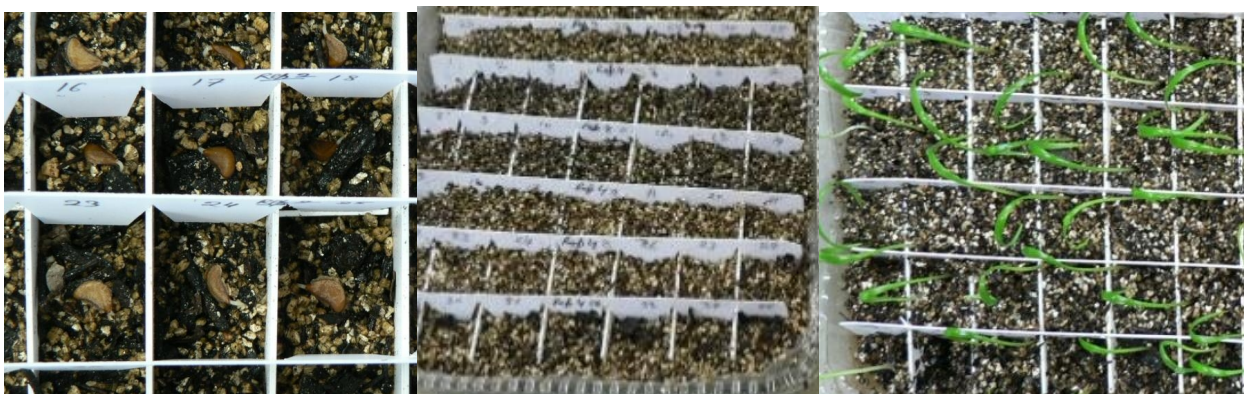


Fig. 12 Common food tray to bury radicle-emerge seeds in the soil for stratification/incubation to monitor epicotyl emergence.

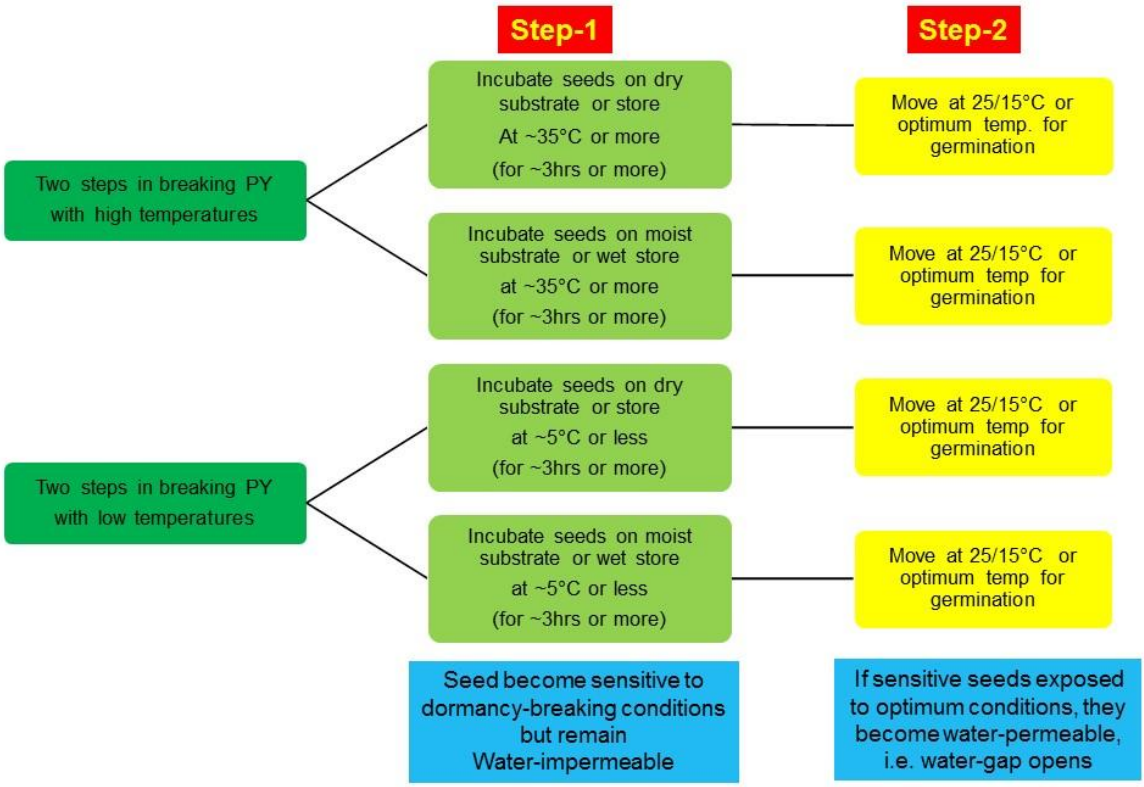


Fig. 13 A simplified outline of two-steps protocol in breaking PY using high or low temperature regimes. Depending on the habitat of the study species (tropical/sub-tropical vs cool/arctic) use either high- or low-temperature protocol (adopted and modified from Baskin & Baskin 2014).

3.4.4. Seed viability

Trait description

Viability is not strictly a functional trait but is a key characteristic important for measuring other seed traits. By analogy, whether a person is dead or alive (state) is not the same as whether he or she has blue or brown eyes (qualitative trait) or of a certain height or mass (quantitative trait).

Viability is the potential of a seed (or population, or sample of seeds) to germinate (resume embryo growth) and produce a healthy seedling, *i.e*. it is alive. Thus, a viable seed has an intact, living embryo and sufficient storage tissue to be capable of germinating once exposed to suitable environmental conditions (water, temperature, light) and any dormancy-breaking requirements (see section **3.4.3 Seed dormancy and dormancy-breaking cues**) have been satisfied. On the other hand, a non-viable seed is incapable of germination, given those conditions. Generally, a non-viable seed is dead but may not necessarily be dead. Rarely and under special circumstances is the rescue of embryos capable of growth from seeds otherwise incapable of germination possible (e.g., Ganguli & Sen-Mandi, 1995). Furthermore, there is evidence of successful germination of seeds developed from fragments of cotyledonary tissue in some large-seeded species with hypogeal germination (e.g., Dalling *et al.*, 1997; Edwards *et al*., 2001a; Harrington *et al*., 2005), the last of these describing to predation - induced embryogenesis.

'Dead' is a word that should be reserved for seeds that were once intact, alive and capable but subsequently have lost viability. It should not be used for empty seeds or those that have aborted or malformed embryos. Such seeds are obviously non-viable but never were viable and thus cannot be said to have died. However, it should be noted that some authors continue using the term non-viable to describe/include those unfilled, aborted or otherwise malformed entities that had failed to develop into functional, viable seeds.

Viability, along with vigour and integrity, are all elements of 'seed quality', itself described as a complex trait (Ligterink *et al.*, 2012), which also includes germination and dormancy characteristics, seed size uniformity, storability, normal embryo morphology and ability to develop into a normal plant. Seed quality is a term that perhaps has more significance in agronomy than in ecology. Vigour is related to viability but not synonymous with it. It probably has the most direct relevance in agriculture, where declining vigour can cause poor field emergence in crops. In the laboratory, it can be measured by a reduction in germination speed (e.g., Finch-Savage & Bassel, 2016) compared with a high vigour standard under sub-optimal conditions, usually low temperature, or by increased occurrence of deformed or defective seedlings. Loss of vigour appears to be a symptom of impending viability loss, and the two are correlated (Ellis & Roberts, 1981). It is also an indicator of potential longevity. Returning to the analogy above, like people, seeds age with time, decreasing in vigour before they finally succumb and die. There is evidence that loss of vigour can be reversed to an extent, through cellular repair processes that are promoted when seeds are at high moisture content (more or less fully imbibed) in an aerobic environment sufficiently supplied with oxygen (Butler *et al.*, 2009). This may be significant in extending soil seed survival (see section **3.3.3 Soil seed bank longevity**) in certain habitats, in which seeds in surface soil experience successive imbibition and drying through frequent rainfall events.

Functionality and trade-off

Viability is essential for germination and seedling emergence. Loss of viability is the ultimate outcome of seed ageing, probably mainly due to oxidative processes (Kranner, 2013), especially at relatively high moisture levels and temperature. Still, it can also result from cellular damage caused by drying, either of desiccation-sensitive seeds or of otherwise tolerant but immature seeds removed (harvested) from the mother plant prematurely or resulting from drought or other stress on the mother plant during seed maturation. Initial viability affects the longevity (survival) of a population or cohort of

seeds in both laboratory (Hay & Probert, 1995) and soil (see section **3.3.3 Soil seed bank longevity**). It potentially affects survival and, thus, dispersal in time.

A seed can also lose 'viability' through infestation by microorganisms and predation by insect larvae: some tissues may still be alive, but the seed is incapable of germinating. The microorganisms involved are mostly opportunistic/facultative on dead or dying/low-vigour seeds.

Environment (micro- and meso-climate) can affect the seed set itself (see section **1.1.10 Pollen thermotolerance**). Kochanek *et al.* (2010) have shown that even the pre-fertilization environment can ultimately affect the subsequent longevity of seeds (see also Whitehouse & Norton, 2022, for example). While there are examples where environmental factors (usually temperature and moisture) during seed development have been shown to affect seed longevity (maternal effects), it is mostly not clear whether that has been through effects on initial longevity (so-called *Ki* – see Ellis & Roberts, 1981), or 'vigour' of individual seeds or populations; or through some effect on subsequent rates of loss of viability; or both, following dispersal; or whether the effects are more upon proportion of competent seeds (% filled), rather than their degree of competence (viability). Jump & Woodward (2003) have shown significant variations in numbers between years of ripened, filled achenes of two UK *Cirsium spp* at the edges of their ranges, related to weather. In contrast, numbers did not vary in a related widespread species. Climate (temperature and moisture) can also affect viability and longevity post-harvest, and the effects of moisture and temperature on longevity under artificial conditions are extensively documented for around seventy diverse species (see SER-SID.org). However, the effects of temperature, moisture and other environmental factors *per se* on viability in the field are virtually un-studied. Natural (soil) vs artificial (*ex situ* seed bank) conditions (see section **3.3.2 Seed longevity (in the lab)**) differ, the latter usually being at more constant, low moisture content and temperature, and the underlying causes of loss of viability and death may be different in the two environments (see, for example, Walters *et al.*, 2005a).

There seems to be little advantage to a plant in filling and ripening seeds if they are not viable at some point, at least around dispersal and for some time after, so they could potentially germinate and develop into seedlings. It seems likely that any genetic or adverse environmental effects on the mother plant during seed set and early development would operate through abortion and reduced fertile seed numbers. In contrast, stress during late maturation, possibly drought, leading to early abscission, could result in reduced vigour and subsequent longevity. For *Cirsium acaulon* growing at the northern edge of its climatic distribution in UK, Pigott (1968) observed that cool, damp conditions during seed ripening reduced the number of ripe cypsellas borne per capitulum, compared to plants growing further south, with seedlings seen less frequently. However, it is not clear whether the few ripe, filled seeds also had lower vigour and/or potential longevity (see also Hay & Probert, 1995). Whether there are trade-offs between seed numbers, integrity, vigour, and viability is largely unknown for wild populations growing in the field but likely to be species and/or habitat dependent. However, there is some evidence from crops, such as maize, for trade-offs between numbers and quality under environmental stress during seed development (e.g., Wang *et al*., 2020).

Applied aspect

Most research on seed viability has been in the context of seed quality and its agronomic consequences, as well as the importance of high initial viability in determining the subsequent longevity of seeds, especially those stored *ex situ* in artificial seed banks for conservation (Hay & Probert, 1995, Probert *et al.*, 2007). However, while losses of viability through storage under controlled laboratory conditions (constant temperature and moisture level) have been relatively well studied (see section **3.3.2 Seed longevity (in the lab)**), there is much less specific evidence for the role of viability *per se* in survival in the soil (see section **3.3.3 Soil seed bank longevity**), compared with losses due to predation and pathogens (see Long *et al.*, 2015).

Sources of variability

The sources of variability have been listed in preceding sections (pollination, maternal environment, post-harvest/post-dispersal environment), with much research remaining to be done to elucidate the relative importance of genetic and environmental effects. Chambers (1989) reported both within and between year variability in seed viability among some North American alpine species. Genetic effects on initial seed competence and/or viability are likely to be especially important in small, isolated populations of plants due to inbreeding depression and the Allee effect.

Methodology

Four classes of tests are used to determine the level of viability in a sample from a population of seeds. They are not necessarily mutually exclusive and frequently used in combination. It is important to avoid bias by paying attention to an appropriate, random sampling of the population or individuals within it (positional or temporal effects), which may depend on the nature of the survey being conducted or the hypothesis being tested. For example, the focus of interest may be only on seeds (*sensu lato*) that are apparently fully formed, filled and healthy rather than the total output, where there is interest in the proportions of aborted embryos, abnormally small seeds, and predation. The flowering and fruiting of wild species is frequently protracted, and it may be necessary to sample viability on several occasions through the ripening period, when environmental effects on the mother plants, or predation, could vary with sampling time.

The germination test

A number of seeds are set to germinate on a suitable water supplying substrate (*e.g.,* filter paper, 1% water agar, moist sand) and incubated under conditions likely to break dormancy (pre-treatments – see section **3.4.3 Seed dormancy and dormancy-breaking cues**) and be optimal for germination (temperature, light). Strictly, this test is one of germinability. Germination is usually recorded after the emergence of a defined length of radicle, *e.g*., 2mm or greater than one third the length of the seed. However, simple radicle emergence does not always indicate that a normal healthy seedling will result, and where the capacity to produce healthy plants is important, normal

seedlings and abnormal seedlings are counted. A high proportion of the latter can indicate a loss of vigour (see Matthews *et al.*, 2012, and also for other methods to measure vigour; also FAO, 2014).

Tetrazolium test

Where there may be problems with dormancy, or a rapid assessment of seed viability is required, and a germination test may be otherwise protracted, vital stains can be used. The best known and most widely used of these is the Topographical Tetrazolium Test (TTZ or TZ test (see França-Neto & Krzyzanowski, 2022, for a recent review), which relies on a relatively subjective interpretation of red staining of living tissue in seeds incubated in an initially colourless dye (triphenyl tetrazolium chloride). Detailed procedures for a TZ test regarding seed preparation, optimum staining times, and interpretation of staining patterns are available through the International Seed Testing Association, but for most wild species, methodological development and verification of accuracy are required. Other stains, including fluorescein diacetate (FDA) and Evans Blue, are indicators of viability used in cell suspensions. These stains have been employed successfully with micro seeds, especially orchids (Wood & Pritchrd, 2004), but sometimes also with varying degrees of correlation to viability (Merritt *et al.*, 2014a). Mohammed *et al.* (2019) also used Resazurin partly successfully.

Physical integrity

The third class of tests involves examining the seed's physical integrity. For some authors, this is taken to be also covered by the word viability. However, they do not assess whether the seed or any part of it is viable (alive) or germinable (capable of growth). Instead, they establish whether it appears intact or filled, its internal morphology corresponding to that of a typical mature seed of that species, and *likely* to be capable of germinating. The most straightforward physical examination can be as simple as crushing using the fingers or an instrument but usually involves dissection (cut test), with or without a microscope. The cut test is frequently used at the end of a germination test to establish whether non-germinants are fresh, firm and apparently healthy (and thus probably dormant) rotten and obviously dead, or empty and likely to have been so at the start of the test. A cut test can be carried out on a representative

sample of seeds before any germination or viability test. Increasingly, an X-ray apparatus is used to reveal empty seeds, undeveloped embryos or insect damage results (see Rahman & Cho, 2016, for a recent review of X-ray and other non-destructive techniques for assessing seed quality).

Seed respiration

Direct measures of seed respiration (see section **3.3.6 Seed metabolic rate**), such as O_2 consumption or CO_2 production, are related to seed health and vigour and have been shown to be useful for determining seed viability (Xin *et al.*, 2013; Dalziell & Tomlinson, 2017). Respirometry is not yet commonly applied as a technique for determining seed viability, but such methods may be advantageous where dormancy precludes an accurate germination test and for predicting the onset of viability decline in storage (Bello & Bradford, 2016).

Unit

The viability of a seed population or sample is usually expressed as the proportion (or percentage) of individuals that germinate when tested under conditions appropriate for the species or seed lot, assuming there are no dormant individuals in the non-germination fraction. Alternatively, it is the proportion of individuals that stain positively in an alternative viability test, e.g. the tetrazolium test. Some authors point out the distinction between tests that measure germinability *per se* and alternative tests and reserve the term viability test for the latter (see also Gosling, 2002).

3.4.5 Seed light requirements for germination

Trait description

Seed light requirements for germination are the sensitivity/insensitivity of seeds to light (photoblasty or photoblastism) concerning germination. Seeds can require exposure to light for germination to proceed, or the germination is greater in light than in darkness (positively photoblastic). Light can also inhibit or prevent germination (negatively photoblastic; photoinhibition), or germination can be greater in darkness. Some seeds are light-neutral (indifferent to light), where light or dark does not affect germination (Górski *et al.*, 1977; Carta *et al.*, 2017).

Functionality and trade-offs

Light functions as one of the environmental signals to promote or delay seed germination depending on the dormancy state (dormant vs nondormant) and/or the nature of photoblasty. Positive photoblasty acts as a depth-sensing mechanism for seeds in the soil seed bank because light can only penetrate a few millimetres below the soil surface (Tester & Morris, 1987; Benvenuti, 1995). Seeds buried at a depth beyond which they can emerge may only germinate when light is detected, signalling they have moved close enough to the soil surface (e.g., through soil disturbance) to emerge successfully. In contrast, when conditions may not be suitable for seedling emergence/survival due to harsh environments such as drought or flooding in certain habitats, negative photoblasty acts as a physiological function to avoid germination on or near the soil surface (Carta *et al.*, 2017) or when seeds are superficially buried under leaf litter (Pearson *et al.*, 2003). For this reason, many small seeds require light to germinate (Milberg *et al.*, 2000; Pons, 2000), and photoinhibition is more common in larger seeds (Carta *et al.*, 2017). However, a recent study using a large dataset did not support linking diaspores size (mass, shape, volume) with photoblasty. It argued diaspore size is a loose predictor of the photoblastic nature of seeds (Dias *et al.*, 2020).

The most comprehensively studied light detection mechanism is the phytochrome-mediated response to red and far-red light wavelengths. Through phytochrome

photoreceptors, red light promotes germination, far-red light inhibits germination, and the germination response is reversible based on the sequence of exposure of seeds to red or far-red light (Seo *et al.*, 2009). These wavelengths, in particular, comprise a gap-detection mechanism for seeds, as red-light wavelengths increase at the soil surface with the removal of the plant canopy (Vázquez-Yanes & Smith, 1982; Vranckx & Vandelook, 2012). Other photoreceptors in seeds also exist that are sensitive to blue and green light wavelengths (Seo *et al.*, 2009; Goggin & Steadman, 2012). The gibberellins and abscisic acid are key signalling pathways for the light response. Therefore, photoblasty plays an important ecological role in detecting or avoiding canopy gaps in a natural ecosystem to synchronise seed germination and seedling establishment under optimal microclimatic conditions. For example, seeds of open grassy species are known to show strong photoinhibition under leafy canopy (Górski *et al.*, 1977; Baskin & Baskin, 2014), and in tropical forests, differences between species in the ratio of red:far-red light that initiates germination can contribute to niche partitioning with respect to the size of canopy gap required for germination, thereby promoting species co-existence (Daws *et al.*, 2002).

Applied aspect

Manipulation of soil burial depth or brief exposure of light-sensitive seeds from burial during ploughing can stimulate germination; thus, the photoblastic nature of seeds can be of relevance to weed control in agriculture (Scopel *et al.*, 1994). The light response of seeds of native plant species can be similarly important for the management or restoration of natural ecosystems. For example, grazing or mowing of calcareous grasslands can promote increased species richness by allowing light penetration into the soil (Jacquemyn *et al.*, 2011).

Sources of variability

The sensitivity of seeds to light can vary with populations, seasons (spring germinators vs summer/autumn germinators), level of hydration (dry vs moist), seed encapsulated or not by the fruit, kind of dormancy-breaking treatments (cold vs warm stratification),

incubation temperatures (constant vs alternating temperature), quality/intensity of light, presence/absence of germination stimulating chemicals in the substrate like nitrate, ethylene, karrikinolide (Baskin & Baskin, 2014, and several references cited therein). For example, depending on species, cold stratification may substitute light requirements (Shimono & Kudo, 2005), make seeds more sensitive to light (Batlla & Benech-Arnold, 2005) and / or fail to substitute light requirements for seed germination (Baskin & Baskin, 2003).

Similarly to dormancy-breaking conditions, the light conditions can also determine the efficacy of dormancy break and subsequent germination (Steadman, 2004) and become a source of variation. For example, dormancy release in *Lolium rigidum* is more pronounced during stratification in darkness than in light, and dark stratification increases the sensitivity of seeds to light (Steadman, 2004). Conversely, dry storage or warm-moist stratification of seeds in light can allow them to germinate in darkness, as observed in seeds of *Hygrophila auriculata* (Amritphale *et al.*, 1989), *Diamorpha cymosa* (Baskin & Baskin, 1972a) and *Draba verna* (Baskin & Baskin, 1972b). Seasonal changes in the dormancy state of the buried soil seed bank may also affect the light requirement (Derkx & Karssen, 1993; Milberg & Andersson, 1997).

In fire-prone ecosystems, smoke and smoke-derived chemicals are known to substitute the light requirement for seed germination (Merritt *et al.*, 2006). The phytochromes are located in the embryo of seed, and the optical properties of the dark seed coat are known to reduce light transmission to the embryo (Widell & Vogelmann, 1988). Thus, the sensitivity of seeds to light can also vary greatly with seed coat colour (light vs dark) depending on the geographical distribution (Carta *et al.*, 2017). Seeds exposed to variations in light and temperature during storage respond differently concerning dormancy and germination (Probert *et al.*, 1985).

Methodology

Once imbibed, seeds can detect and/or respond to very brief (seconds) exposure to light (Isikawa, 1954; Baskin & Baskin, 1975b), as well as wavelengths other than red or

far-red light (Goggin & Steadman, 2012). To evaluate the response to seed germination in darkness, imbibed seeds cannot be exposed to any period of any type of light. Note that exposure to light may extend to cleaning seeds from fleshy fruits, which is commonly performed through macerating the fruit pulp in water (e.g. Daws *et al.*, 2002). Seeds cleaned in this manner should be immediately dried in a dark room, with seeds exposed to far-red light to induce photo-reversion.

The photoperiod for seeds exposed to light should be a diurnal light/dark cycle, typically a 16/8 h, 14/10 h, 12/12 h, or 8/16 h light/dark cycle, depending on the day length of the region from which the seeds are sourced. Exposure of seeds to continuous light should be avoided to prevent the high irradiance response (Pons, 2000). Depending upon the purpose of the experiment, appropriate lighting sources should be included, like cool, white fluorescent lighting or LEDs that emit specific wavelengths. Commercially available light filters may also be used to test seed responses to specific wavelengths or red:far-red ratios (Goggin & Steadman, 2012; Tiansawat & Dalling, 2013). Incandescent lighting should be avoided as it emits lots of far-red light and heat (Steinbauer & Grigsby, 1957).

For dark treatments, light can be excluded by wrapping Petri dishes in a single- or preferably a double-layer of aluminium foil. Seeds of light-sensitive species (like *Lactuca sativa*) are known to respond to red light within 10 minutes of hydration (McArthur, 1978); therefore, Petri dishes need to be wrapped immediately after hydration of seeds for dark treatments. Seeds incubated in darkness should not be opened to check germination progress. It is recommended that seeds only be inspected at the end of the experiment and never exposed to any light (not even dim green light) during the incubation period (Baskin & Baskin, 2014). Methods should also be guided by the hypotheses being tested. For example, Milberg and Andersson (1997) showed that the duration of light treatment is important. For several weed species, seasonal changes in the light sensitivity of seeds in the soil seed bank were evident and associated with dormancy status, being only detected through a short-exposure treatment. Testing seeds only in light vs darkness may not identify changes in seed

sensitivity to light as seeds come out of dormancy, and seeds may be tested when freshly collected and at intervals during a dormancy-breaking treatment such as cold stratification (Baskin & Baskin, 2014).

The response to light sensitivity to seeds can be determined as relative light germination (RLG) expressed by Milberg *et al.* (2000) as follows:

$$RLG = \frac{Gl}{(Gd + Gl)},$$

where, Gl and Gd are the germination percentage in light and in darkness, respectively.

Unit

Percent or proportion of germination; germination speed measured by t_{50}, for example; light fluence rate (μM m^{-2} s^{-1}); light wavelength (nm); time of exposure to light or day length (mins, h). The RLG is a categorical trait and thus has no specific unit of measurement.

Special cases

In some species, a short green, safe light exposure (used for germination count) during dark treatments is known to stimulate germination, especially when seeds are exposed to dormancy-breaking treatment (cold moist stratification) as compared to seeds in control (non-stratified) treatments (Walck *et al.*, 2000). Thus, caution is required if using green light for a germination count to ensure seeds do not gain the ability to germinate in response to green light (Baskin & Baskin, 2014).

3.4.6. Seed temperature requirements for germination

Trait description

Temperature requirements for seed germination are closely linked to the release of dormancy (see section **3.4.3 Seed dormancy and dormancy-breaking cues**), and similar mechanisms to those in physiological dormant seeds apply to what happens during germination at non-optimal temperatures. Dormancy is a state of developmental arrest in a living seed that impedes its germination under environmental conditions that would permit germination if the seed was non-dormant. Once dormancy is released, under appropriate conditions, germination can start as an irreversible transition from seed to seedling, most often initiated by radicle extrusion. The whole process of dormancy release and germination is regulated by temperature as a key environmental driver, in combination with water (soil moisture). Combining both factors is often needed to fully understand the conditions necessary for dormancy release, dormancy induction and germination. To understand which temperature requirements are needed for seed germination, it is helpful to consider a sequence of three phases: (i) primary or secondary dormancy release, (ii) germination, and (iii) secondary dormancy induction (if germination is not achieved). Dormancy release widens the environmental conditions, including temperature, under which germination can occur. Dormancy induction reverses this process. Both dormancy release and induction often occur in environmental conditions that are different from optimal conditions for germination. All three processes have optimal temperature and moisture conditions where they occur fastest and also have specific lengths. These three processes vary between and within species, leading to fast or slow dormancy release, varying germination speeds, and simultaneous or dispersed germination.

Both dormancy and germination temperatures can be described with broad qualitative categories (e.g. ‘need for cold stratification’, ‘warm-cued germination’) or more precise quantitative metrics (e.g. thermal-time thresholds for dormancy release and germination; see methodology section). Data on thermal-time thresholds for germination are accumulating for wild species (Maleki *et al.*, 2022), but for the moment,

the temperature requirements for dormancy induction are only known for a few cases (Batlla & Agostinelli, 2017; Hawkins *et al.*, 2017). Studying the conditions for dormancy induction for a wider range of species is a challenge because of the time- and seed-consuming experimental settings.

Functionality and trade-offs

Dormancy and germination cycling allow matching germination timing with the start of favourable conditions for the seedling establishment or, in other words, to avoid germination during predictable unfavourable seasons ('best-bet', Pausas *et al.*, 2022). Dormancy can also function as a way to spread germination within and across seasons, thus increasing the probability of at least a fraction of the seed population regenerating in unpredictable environments ('bet-hedging', Gremer & Venable, 2014).

Thermal-time threshold values (e.g. base [T_b], optimal [T_o] and ceiling temperatures [T_c] for germination) are a formalization of the germination niche in the temperature dimension since they describe the performance of seeds along temperature gradients. They can be linked to conditions that affect seedling survival. High base temperatures in upland or boreal species have been interpreted as a mechanism to avoid late frosts. In contrast, low base temperatures, low ceiling temperatures and germination at low temperatures would avoid summer drought. Therefore, base temperature across species is positively related to seed size and is related to phylogenetically conserved thermal niches at a global scale (Arène *et al.*, 2017).

The range of temperatures between T_b and T_o is called the suboptimal range, whereas the range between T_o and T_c is called the supraoptimal range. These ranges may have ecological relevance in specific habitats when the temperatures in the population site vary mainly in the suboptimal or supraoptimal zones. For example, in the case of a summer annual plant, the temperatures above T_o may be well above the summer maximum temperatures. Then, the timing of germination will be driven by the interaction between T_b and increasing environmental temperatures during spring.

Sources of variability

The effect of temperature on dormancy and germination depends on moisture and other factors in the seed environment. Assuming sufficient water is available, germination may require that temperatures are presented in a diurnal range of alternating temperatures or accompanied by light. Most importantly, the range of germination temperatures (i.e. the germination temperature window) narrows and widens depending on the seed dormancy state.

In terms of thermal-time thresholds, the variability in dormancy and germination temperatures may change according to thermal-time parameters, which can be modified by the dormancy status of the seed (Chantre *et al.*, 2009). Conditions experienced by the mother plant (Luzuriaga, 2006; Tielbörger & Petrů, 2010) play a prominent role in determining interannual variation in dormancy and germination levels (also known as 'seed memory', Fernández-Pascual *et al.*, (2019)), thus contributing to spatiotemporal variation in germination phenology. Therefore, dormancy and germination temperature 'traits' should be interpreted and compared with care, especially in situations when small-scale environmental variability and different storing conditions come into play.

The variability within a seed lot can be described by the standard deviation of the thermal-time parameters (σ). Whether this variation is in the thermal threshold or the thermal time depends on the model chosen (see below), although theoretically, both parameters can vary among seeds. The parameters also vary across climatic regions, elevations and habitats, between summer and winter annual plants, and between plants with large or small seeds. Little is known about the relation between these values and thermal threshold values of other plant functions such as growth, photosynthesis, frost or heat damage.

Methodology

Temperature requirements for dormancy release

Characterizing the temperature requirements for dormancy release typically starts with a viable seed lot, collected during dispersal just after maturation, which is studied

within days (weeks) after collection. A first germination test is needed to establish if seeds are dormant, lasting at least four weeks (see the germination temperature section below for the selection of experimental durations and other experimental factors), and using temperature, moisture and light conditions that are suitable for germination. These suitable conditions can be informed by data on the local climate during the beginning of the vegetative period in seasonal climates.

A dormant seed lot can then be exposed to temperatures and moisture conditions that are hypothesised to release physiological dormancy (i.e., driven by environmental cues instead of coat properties like in physical dormancy). For seasonal climates, information about the season that precedes the onset of the vegetation period can be helpful. Two main types of dormancy-releasing conditions often apply: (i) warm and dry conditions, indicative of summer drought, may release dormancy, often termed 'after-ripening' and found typically in autumn germinating species; (ii) cold and moist conditions, indicative of winter, may release dormancy in a process called 'chilling' or 'cold stratification'. The length of exposure to these conditions needed to achieve dormancy release is species-specific. Beyond these two frequent types, any other combination of temperature and moisture can lead to dormancy release. In special cases, a longer sequence of several conditions or seasonal-like changes is needed (Baskin & Baskin, 2014 or see section **3.4.3 Seed dormancy and dormancy-breaking cues**).

Once the main type of dormancy-releasing conditions is known, temperature, moisture and exposure time can be varied experimentally, and the dormancy-releasing response to temperature can be characterised at two levels of detail: (i) by summarizing lab or field conditions that successfully broke dormancy and led to high germination percentages enabling to identify the conditions for dormancy release (Merritt *et al.*, 2007; Baskin & Baskin, 2014), or (ii) by modelling dormancy release based on temperature and moisture thresholds and hydro-thermal time constants (see germination section) enabling to predict response over all relevant conditions (Batlla & Benech-Arnold, 2003; Bair *et al.*, 2006; Allen *et al.*, 2007). Both approaches use a large

variety of descriptors, making it difficult to extract 'trait-like' parameters from these studies. Currently, the only standardised vocabulary on dormancy types is developed by Baskin & Baskin (2014), but there is no standardised way to describe the conditions for dormancy release.

Temperature requirements for germination

Germination experiments measure the effect of temperature on two aspects of seed germination: (a) the final germination proportion or percentage (i.e. the number of seeds germinated out of the total of viable seeds sown); and (b) the germination speed (i.e. the reciprocal of the time between the start of the experiment and germination, usually summarised as the time needed for the germination of a fraction of the seed lot such as the 50%). It is important to understand that these two aspects respond differently to temperature (Garcia-Huidobro *et al.*, 1982). The germination proportion usually remains high and stable over a wide range of temperatures and declines sharply at the extremes ('inverted U' shape; Fig. 14A). The germination speed responds linearly to temperature: in what is called the suboptimal thermal range, the rate increases linearly up to an optimum temperature; above the optimum lies the supraoptimal thermal range, in which the rate decreases with increasing temperature ('inverted V' shape; Fig. 14B).

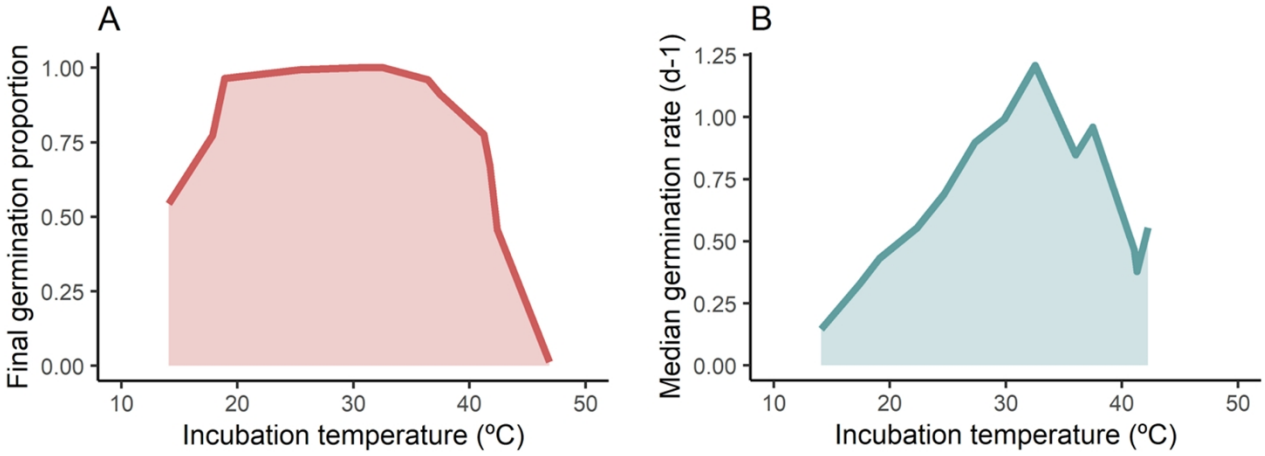


Fig. 14 Effect of incubation temperature on germination proportion (A) and rate (B). Data retrieved from Garcia-Huidobro *et al.* (1982), Fig. 4 (seed lot BK 560 of *Pennisetum typhoides*).

Experimental conditions

The germination response to temperature depends on the seed's degree of dormancy (Fernández-Pascual *et al.*, 2019). Therefore, if the aim is to characterise temperature requirements for germination in a standardised way, non-dormant seeds should be used. However, it is impossible to determine if a seed lot is fully non-dormant, and one should be aware that any 'residual' dormancy resulting from incomplete dormancy release or the beginning of dormancy induction potentially modifies the temperature requirements for germination. This can happen during prolonged storage and be influenced by different collection seasons, years or sites (Thompson, 1975; Bauer *et al.*, 1998; Benech-Arnold *et al.*, 2000; Batlla & Benech-Arnold, 2003; Chantre *et al.*, 2009; Wisnoski *et al.*, 2019). It can also happen during germination experiments, as dormancy levels can respond to some experimental temperatures, adding a further confounding effect (Batlla & Benech-Arnold, 2005).

Assuming that the seed lot is mostly non-dormant, germination can be studied under controlled conditions by using a temperature gradient that spans from cold to warm limits of germination. Ideally, these limits are known before planning the experiment. Otherwise, they can be inferred from the seasonal temperatures in the original habitat where the seed lot has been collected, but considering that either the suboptimal or the supraoptimal temperatures extend to values that may not be experienced in the field site (see functionality section). If the experiment aims to fit thermal time models (see below), the bare minimum to fit the suboptimal thermal model is three temperature points, the same for the supraoptimal. It is better to have more temperature conditions close to the limits as germination speed and percentages do not vary much around optimal conditions.

For the rest of the experimental conditions (e.g., photoperiod), the assumption of 'all else optimal' allows to obtain standardised responses. For example, the experiment should be conducted under light or darkness according to the species´ requirements. Again, this information is ideally known before setting up the experiment, and if not, it can be inferred from the species ecology, seed mass and phylogeny (Carta *et al.*, 2017).

If the data will be used to fit thermal time models, it is better to use constant temperatures, but some species may not germinate unless exposed to a diurnal thermal alternation. General principles to conduct ecologically meaningful germination studies (Baskin *et al.*, 2006; Baskin & Baskin, 2014) should be applied.

Germination tests should have at least 100 seeds per experimental treatment, although this number may depend on other factors, like the number of individuals sampled. When sowing the seeds, there is sometimes a tendency to choose the better-looking ones first. For this reason, preparation of different experimental treatments in sequence should be avoided to prevent each treatment from receiving specific fractions of the seed population. Seeds are usually sown on Petri dishes holding a germination substrate. Typical substrates are filter paper with distilled water and agar. While agar is more costly and time-consuming to set up, it can keep constant water availability for longer. In long experiments and at high temperatures, there is a risk of water loss in the dish. This loss can introduce noise in the germination records and should be prevented by sealing the dishes with parafilm and/or refilling them with distilled water. When working with large seeds, sterilised sand can be a useful alternative for the substrate. Natural soils sometimes give a different germination curve than lab media, a point worth noting, especially for modelling seedling emergence in the field.

Another key consideration for planning the experiments is the duration and frequency of germination scoring (Fig. 15A). As a rule of thumb, incubations should last for at least 4 weeks or until no new germinated seeds appear for a week. Most importantly, an ongoing germination curve should not be interrupted (Fig. 15B). Seeds are counted as germinated when white radicles extrude from the seed coat, and at this time, they are removed from the dish. Germination counting should be frequent enough to document 10%, 50% and 90% of seeds germinating and the endpoint of germination (Fig. 15C). Often, this means daily in the first week and twice weekly afterwards, but it varies from one species to another, and it can be a matter of hours in some. Again, prior knowledge of the germination timing of the study species is valuable to plan the

duration and frequency of germination scoring, but we must stress the importance of frequent scoring during the upward phase of the cumulative germination curve, especially if the aim is to fit thermal-time models. Each replicate might be watered on a separate day, yielding in a more complete set of control time intervals when germination controls cannot be scheduled every day (Cartereau *et al.*, Flora, accepted).

Once the experiments are terminated, non-germinated seeds should be inspected to record the causes of non-germination. This is usually done through a cut test or tetrazolium staining. Non-germinated seeds should be recorded as either viable (or normal), empty and mouldy. Only viable seeds should be used to calculate germination proportions and further statistical analyses, so it is highly recommended to test seed viability before the temperature trials.

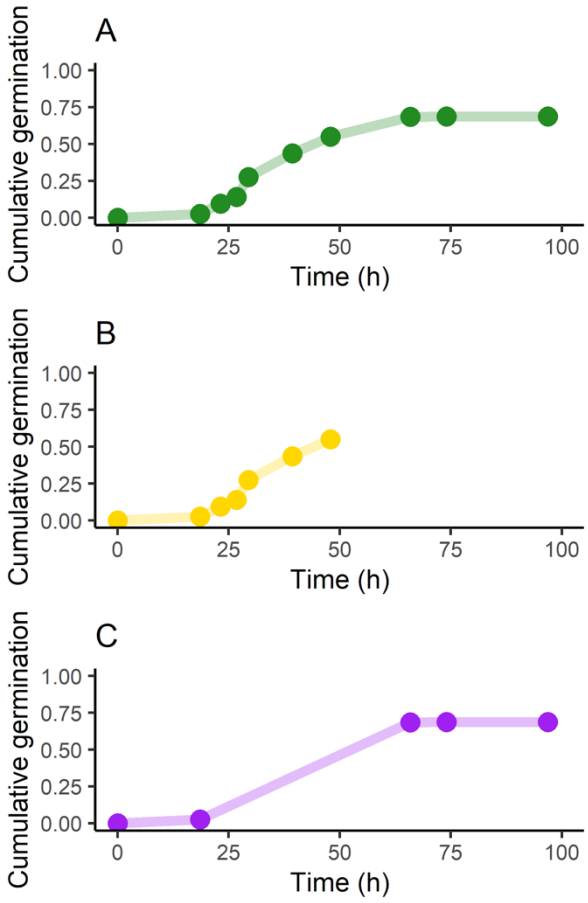


Fig. 15 Importance of the period and frequency of germination scoring. The same dataset is represented under three scoring scenarios. The circles represent scoring times. In plot A, both period and frequency are appropriate. In plot B, the experiment was terminated before time, cutting an ongoing germination curve: the final

germination proportion will not be known. In plot C, germination was not scored with enough frequency: the times at which different fractions achieved germination were missed. Data retrieved from Garcia-Huidobro *et al.* (1982), Fig. 3 (seed lot BK 560 of *Pennisetum typhoides* germinated at 42 ^{o}C).

Thermal time models

Data from a germination study can be used to fit thermal time germination models, which estimate several germination 'traits': the *thermal thresholds* and the *thermal time* for germination (Steinmaus *et al.*, 2000; Trudgill *et al*., 2000). An R package ('seedr', https://CRAN.R-project.org/package=seedr) has been developed to fit thermal-time models by performing the steps described in what follows.

The *thermals thresholds* are temperatures that delimit the range of temperatures in which germination can occur in a temperature gradient. There are two thermal thresholds: (a) the base temperature (T_b), which is the value above which germination can occur, and (b) the ceiling temperature (T_c), which is the value below which germination can occur. An optimal temperature (T_o) can be identified at the value where the germination speed stops increasing and starts to decrease with increasing temperatures, but often, germination speeds vary little around this value. The combination of T_b, T_c and T_o is referred to as the 'cardinal germination temperatures'.

There are two *thermal time* models: the suboptimal thermal time model works between T_b and T_o, and the supraoptimal thermal time model works between T_o and T_c. Therefore, identification of T_o is necessary to split the data for analysis, but this step is not always straightforward. Each of these two models has its own thermal time for germination (θ). The suboptimal thermal time (θ_1) is the sum of degrees above T_b that the seed needs to accumulate before germinating (when incubated at suboptimal temperatures). The supraoptimal thermal time (θ_2) is the sum of degrees below T_c that the seed needs to accumulate before germinating (when incubated at supraoptimal temperatures). The thermal time is usually measured in degrees-day (or hours-day). For example, imagine a seed with a T_b of 5 ^{o}C, a T_o of 20 ^{o}C and a θ_1 of 100 degrees-day,

which is incubated at 15 ºC. Each day, it will accumulate 10 ºC towards germination and germinate in 10 days. As we commented before, only one of the ranges (and of the models) usually has ecological significance for a species, depending on its habitat and phenology.

The two previous paragraphs describe the behaviour of a single seed, which we can imagine has its own values of T_b, T_c, T_o, θ_1 and θ_2. To model the behaviour of a seed lot, we need to extend the concepts to the population level. In principle, we can expect each seed in the population to have its own values for the threshold and the thermal time (and we know that they do not all have the same exact values for the two parameters, or otherwise, all seeds in a seed lot would germinate at the same exact moment, instead of the cumulative curves we see, Fig. 15). However, to achieve a solvable calculation of the model parameters, one of the two is assumed to be a population constant. For instance, in Garcia-Huidobro *et al.* (1982), the model assumes that T_b is a population constant and θ_1 is a function of the seed fraction (i.e., all seeds in the population have the same threshold value, and they vary in their thermal time values). In the hydrothermal model of Bradford (2002), the same is assumed for the suboptimal model, but the assumptions change for the supraoptimal: in that case, T_c is a function of the seed fraction and θ_2 is a population constant (i.e., all seeds in the population have the same thermal time value, and they vary in their thermal thresholds). In our opinion, the consideration of the threshold as the variable parameter makes more sense, as it allows for explaining the typical situation in which the final germination percentages vary between 0 and 100% at the cold and warm extremes of the gradient (Fig. 14A). If all seeds had the same threshold and varied in their thermal time, all experimental treatments should render either 0 or 100% germination, given enough time.

Once a decision has been made about which one of the two parameters is the population constant, a way to describe the variation of the other parameter within the population is needed. Many studies use a normal distribution to describe this; in this case, the parameter is summarised in the median value (the value for the 50th

4497 percentile) and the standard deviation (σ). In several cases, recent studies have shown
4498 that log-normal or other more flexible distributions are more appropriate to model
4499 germination response to temperature (Mesgaran *et al.*, 2013); however, the
4500 straightforward interpretation of parameters is lost.

4501 The chosen model can be used to relate the data obtained in the experiments, i.e., the
4502 cumulative germination percentage (G, probit transformed), germination time (t_g) and
4503 temperatures in the seed environment (T_{env}). In the suboptimal temperature range, the
4504 classical model of Garcia-Huidobro *et al.* (1982) is based on three parameters (T_b, θ_{150}
4505 and $\sigma_{\theta 1}$), and it can be used to model germination speed at any temperature in the
4506 suboptimal temperature range:

4507 $$probit(G) = \frac{(T_{env} - T_b)\, t_g - \theta_{Tb}}{\sigma_{\theta_{Tb}}}.$$

4508 In this model, T_b is assumed constant, and θ_1 follows a normal distribution described by
4509 its median and standard deviation. Parameters can be estimated by repeated
4510 regression varying T_b in small steps, retaining the T_b value of the highest R^2, and
4511 subsequently calculating θ_{Tb} and $\sigma_{\theta Tb}$ (Garcia-Huidobro, 1982; Bradford, 2002).
4512 Alternatively, all three parameters can be estimated simultaneously, e.g. using
4513 maximum likelihood (Hashoum *et al.*, 2020). Several alternative methods to identify the
4514 base temperature for germination are discussed in Steinmaus *et al.* (2000).

4515 Different models are used for the supraoptimal range. The simplest one understands
4516 T_c and θ_2 similarly to the hydrotime model: in this case, the ceiling temperature yielding
4517 50% germination (Tc_{50}) and its standard deviation (σ_{Tc}) are used. Both values, together
4518 with a constant characterizing germination time (supra-optimal thermal time constant,
4519 θ_{Tc}), can be found by using an equation that relates cumulative germination (probit
4520 transformed) to germination time (t_g) and temperature in the seed environment (T_{env})
4521 using T_c, σ_{Tc} and θ_{Tc} as parameters:

$$probit(G) = \frac{T_{c(g)} - \frac{\theta_{tc}}{t_g} + T_{env}}{\sigma_{tc}}.$$

More complete models describing the entire range of temperatures and water potentials (hydro-thermal time models) have been successfully parameterised (Rowse & Finch-Savage, 2003) and similar models have been developed for dormancy release (Batlla & Benech-Arnold, 2003; Bair *et al.*, 2006; Allen *et al.*, 2007), and dormancy and germination models have successfully been combined to model germination and seedling emergence in the field (Bauer *et al*., 1998; Chantre *et al.*, 2009; Gardarin *et al.*, 2012). More modelling approaches for seed germination exist as the field is still developing, and new methods integrating better experimental errors are under development (Onofri *et al.*, 2011, 2014).

Alternatives to thermal-time models

Other approaches use minimal, optimal and maximum temperature for germination in addition to the threshold parameters T_b, T_o, and T_c (Rosbakh & Poschlod, 2015). These values are identified by the experimental treatment that corresponds to the coldest temperature just permitting germination, the treatment with the highest or fastest germination, and the hottest experimental temperature that still permits germination. This method highly depends on the range and resolution of experimental temperatures used.

Units

The base temperature for germination (T_b), ceiling temperature (T_c) and optimum temperature for germination (T_o) are expressed in °C. The thermal time constants (θ) are measured in °C x d (other time units can be used depending on how fast the seeds germinate, but for standardisation purposes, we recommend always reporting degree days). The measurement unit of the standard deviation (σ) depends on which parameter it refers to - the thermal threshold (°C) or the thermal time (°C x d).

3.4.7 Seed moisture requirements for germination

Trait description

Seeds germinate under varying conditions of moisture and rainfall, and watering is generally considered one of the main triggers for the start of germination. Moisture in the seed environment is most often measured as water potential (Ψ), which is a negative pressure - imagine the force pressing out water from a wet sponge - measured in Megapascals (MPa). Seeds may germinate from -1.5 MPa (equivalent to the permanent wilting point) to 0 MPa (pure water). In the field, water potentials vary as a function of rainfall, evapotranspiration and soil particle size distribution but also on short distances with depths, microrelief, and reservoir size, creating a very high heterogeneity.

Functionality and trade-offs

Base water potential can be understood as a synthetic description of the germination niche in moisture gradients, and it is linked to the way in which seeds detect conditions too dry for seedlings to survive. In ecosystems that are strongly driven by water limitations, this may be the main factor controlling germination timing, making other germination cues of secondary importance. Since seedlings from large seeds reach deeper depth and might store more water within seeds or cotyledons, base water potential across species is negatively related to seed size (Daws *et al.*, 2008; Arène *et al.*, 2017). Base water potential can be used to model how many seeds germinate above a specific soil moisture content. Scattering base water potentials enable (mother) plants to disperse risk when moisture conditions are unpredictable. Modifying base water potentials is one of the ways plants can modify germination speed and final percentages during dormancy loss and induction, and very low base water potentials have been found for species from clay-rich and saline habitats (Allen *et al.*, 2000).

Sources of variability

The variability of base water potentials within a seed lot is described by an associated dispersion parameter ($\sigma_{\Psi b}$). Several factors can influence base water potential.

Removing or permeabilizing seed coats or alleviating physiological dormancy decreases base water potentials and increases final germination percentages (Windauer *et al*., 2012).

Methodology

Solutions of organic macromolecules that strongly bind water, such as polyethylene glycol (PEG) or mannose, can experimentally simulate conditions of low water availability or moderate drought stress. Many studies use the equation provided by Michel (1983) given below to calculate concentrations of PEG to obtain desired experimental water potentials at given temperatures:

$$[PEG] = \frac{\left(4 - \left(5.16 \times \Psi \times T - 560 \times \Psi + 16\right)^{0.5}\right)}{\left(2.58 \times T - 280\right)},$$

where [PEG] is the concentration of polyethylene glycol 8000 in g(PEG) /g(water), Ψ the desired water potential in bar (0.1 MPa), and T the temperature in °C under which germination tests are performed.

Seeds are then exposed to these solutions during the entire germination test, without adding water and by avoiding water loss by evaporation. Keeping a stable water potential in each treatment for the duration of the experiment is crucial to obtain robust results. If evaporation cannot be prevented, a refilling scheme should be implemented. The other conditions of the experiments (temperature, light, duration, replicates) should be chosen following the same principles outlined for the thermal time experiments in section **3.4.6 Seed temperature requirements for germination**.

A gradient of water potentials enables the identification of the dry limits of germination, quantified by the base water potential for germination (Ψ_b). Base water potential is the value above which germination can occur. Many studies use a normal distribution to take into account the variability of a seed lot in terms of moisture needed to trigger germination. In this case, the base water potential yielding 50% germination (Ψ_{b50}) together with a standard deviation ($\sigma_{\psi b}$) is used. Both values, together with a constant characterizing germination time (hydrotime constant, θ_H), can

be calculated by using an equation that relates cumulative germination (probit transformed) to germination time (t_g) and water potential in the seed environment (Ψ_{env}) using Ψ_b, $\sigma_{\psi b}$ and θ_H as parameters:

$$probit(G) = \frac{\frac{\Psi_{b(g)} - \theta_H}{t_g - \Psi_{env}}}{\sigma_{\Psi_b}}.$$

Parameters can be estimated by repeated regression varying θ_H in small steps and retaining the equation of highest R^2, and subsequently calculating Ψ_b and $\sigma_{\psi b}$ (Bradford, 1990); or directly using maximum likelihood (Hashoum/Saatkamp, under review). An R package ('seedr'; https://CRAN.R-project.org/package=seedr) has been developed to fit hydro-time models.

3.4.8 Seed oxygen requirements for germination

Trait description

The oxygen requirement for dormancy and germination can be defined as the oxygen level required for a seed to become non-dormant or to germinate.

Functionality and trade-offs

For non-dormant seeds, the majority of terrestrial species require oxygen for germination, emergence and early seedling establishment (Baskin *et al.*, 2014). Conversely, some aquatic and mudflat species will not germinate under normal atmospheric oxygen conditions (Baskin *et al.*, 2014; Rosbakh *et al.,* 2020b). For some dormant seeds, oxygen, or the lack thereof, is thought to play a role in the maintenance, induction or break of dormancy (Phartyal *et al.,* 2020a).

For seeds that require oxygen to germinate, oxygen in the soil acts as a depth-sensing mechanism. Seeds that are buried to a depth from which they cannot emerge may only germinate when oxygen is detected, indicating that the seed has moved closer to the soil surface or the soil has been disturbed, thereby allowing germination to proceed (Finch-Savage & Footitt, 2017). By contrast, a lack of oxygen in the soil may be caused by waterlogging or inundation. Anoxia/hypoxia caused by waterlogging inhibits germination in most species and can also induce secondary dormancy (Benvenuti & Macchia, 1995; Phartyal *et al.*, 2020a, b). However, anoxic or hypoxic conditions may also promote the germination of some aquatic or semi-aquatic species (Dalziell *et al.*, 2019; Rosbakh & Poschlod, 2019; Phartyal *et al.*, 2020a). For aquatic species inhabiting seasonally inundated wetlands which periodically dry back, a lack of oxygen may indicate the presence of water, which will support germination and subsequent seedling establishment. Oxygen is thought to play a role in the maintenance or induction of dormancy in seeds of some species via interaction with abscisic acid (ABA) (Finch-Savage & Footitt, 2017).

Applied aspect

Dormancy and/or germination can be stimulated or suppressed using oxygen under controlled conditions. Changes in oxygen concentration can impact total germination and the rate of germination. For example, elevated oxygen levels can overcome dormancy in *Arabidopsis* (Buijs *et al.*, 2018), and hypoxia can induce secondary dormancy in *Datura stramonium* (Benvenuti, 1995) and *Hypericum humifusum* (Phartyal *et al.*, 2020a). In contrast, prior exposure to a hypoxic environment broke physiological dormancy in *Lythrum hyssopifolia* seeds that otherwise required cold stratification (Phartyal *et al.*, 2020a). Germination speed in seeds of *Helianthus annuus* and seeds within indehiscent woody endocarps of *Astroloma xerophyllum* were increased with an increasing percentage of atmospheric oxygen (Gay *et al.*, 1991; Turner *et al.*, 2009a).

In certain scenarios, such as the conservation or restoration of wetland species, where waterlogging and anoxia are common, understanding species-specific oxygen requirements may be critical to enabling successful germination and subsequent seedling establishment.

Under seed banking conditions, the absence of oxygen may reduce the deleterious effects of reactive oxygen species (ROS), thereby decreasing the deterioration/viability loss rate in some seeds (Groot *et al.*, 2015). Conversely, elevated partial pressure of oxygen (EPPO) environments can be used to mimic and accelerate seed ageing (Buijs *et al.*, 2018).

Sources of variability

The morphology of the seed coat and the presence of covering structures or mucilage can contribute to differences oxygen diffusion capacity into the seed from the atmosphere or soil, which may influence dormancy break or germination (Baskin *et al.*, 2014). For example, the removal of the covering structures from seeds of *Helianthus annuus* and *Bromus rubens* results in higher total germination and reduces the time taken to germinate under oxygen-limited conditions compared with intact seeds (Gay *et al.*, 1991; Corbineau *et al.*, 1992).

Oxygen can also interact with other factors controlling dormancy and germination, such as temperature, water availability and light. For example, the negative impact of oxygen limitation may be reduced in some seeds at cooler temperatures (Corbineau & Côme, 1995; Corbineau *et al.*, 1995). Species- and cultivar-specific responses have been noted in response to changes in the partial pressure of oxygen (Corbineau & Côme, 1995; Corbineau *et al.*, 1995).

Methodology

To test the effect that oxygen has on dormancy (either induction or release) or germination, seeds may be exposed to normal atmospheric (normoxic), increased (hyperoxic), decreased (hypoxic), or absent (anoxic) oxygen conditions. Depending on the question being asked, this may be done on dry or imbibed seeds, e.g. seeds plated on Petri dishes containing agar. Atmospheric oxygen levels may be manipulated by placing seeds inside a gas-tight chamber and introducing gaseous oxygen or inert gasses such as nitrogen to reduce oxygen concentration, or introducing a commercially available oxygen absorber to the sealed container. Alternatively, imbibed seeds can be placed in a sealed container and left for a set period to allow for oxygen consumption within the container’s headspace. Seeds may then be assessed for germination or viability, or at a genetic level, for example, via qualitative trait loci analysis (e.g., Buijs *et al.*, 2018).

Unit

The percentage or proportion of germination and germination speed can be measured for experimental treatments where the oxygen concentration varies.

3.4.9 Response to heat

Trait description

The degree to which high temperature affect embryos' survival, germination, and dormancy. High temperatures may have positive (Keeley & Fotheringham, 2000; Auld & Ooi, 2009), negative, or neutral effects on germination (Daibes *et al.*, 2019). Seed responses to high temperatures are usually determined by applying heat shocks and observing embryo survival, changes in germination parameters, and changes in seed dormancy (Pausas & Lamont, 2022).

Functionality and trade-offs

High temperatures affect regeneration by influencing germination parameters, breaking physical dormancy or killing embryos. The effect of high temperatures is usually associated with fire passage. Still, temperatures as low as 35°C, which are not necessarily fire-related, may also break PY (Dayrell *et al.*, 2015) and can be used to detect the presence of canopy gaps in tropical pioneer species (Pearson *et al*., 2002). Similarly, temperatures up to 70°C can occur in fire-free vegetation types, such as arid dune systems (Ooi *et al.*, 2009). In fire-prone ecosystems, seeds in both soil and aerial (serotinous) seed banks are often exposed to fires, which may affect seed physiology in multiple ways. Fire effects on seed survival and germination can be categorised into physical (temperature) and chemical (e.g. sensitivity to karrikins; see section **3.4.10 Response to chemical cues**) effects. Seed responses to heat may be related to shifts in species relative abundance in communities. Increased germination and dormancy break in species with PY or PD dormancy is considered adaptive because it cues germination to environmental conditions when competition with neighbours is reduced (Pausas & Lamont, 2022).

Applied aspect

Heat shock is frequently used to overcome physical dormancy in many species useful for ecological restoration. Heat shock promotes the rupture of the seed coat, allowing imbibition, which results in greater, faster and more synchronous germination (Pausas

& Lamont, 2022). Heat shock can also alleviate physiological dormancy - in some species, bypassing the need for after-ripening (Turner *et al.,* 2009b). These traits are desired to produce seedlings in nurseries and to increase the chances of establishment after seed sowing (Turner *et al.*, 2013).

Sources of variability

Many species have temperature thresholds for dormancy break and mortality, and response to heat varies strongly between species, populations, individuals and dormancy classes (Auld & O'Connell, 1991; Livanage & Ooi, 2015). Some species also present dimorphic seed lots with hard (water-impermeable) and soft (water-permeable) coats (Paulsen *et al.*, 2013) and/or have seeds that lose impermeability or experience threshold reduction as they age (Liyanage & Ooi, 2017). Seed responses to heat tend to depend on seed mass and shape (Daibes *et al*., 2019) and vegetation type, though most studies focus on fire-prone ecosystems (Pausas & Lamont, 2022). Seed heat survival also depends upon seed moisture content during exposure (Tangney *et al.,* 2018). The experimental conditions strongly influence the outcome of heat shock tests. Wet heat shock is used to overcome dormancy for seedling production but does not reflect an ecological process. Therefore, for ecological and evolutionary inferences, dry heat shock is preferred.

Methodology

Laboratory experiments

To simulate fire effects as closely as possible, defining the duration and temperature of the heat shocks implemented should be guided by data on heat pulses within the upper soil profile (where the seed bank is maintained) obtained under field conditions. For example, very long duration (weeks) heat treatments can be applied to represent seasonal heating, whereas much shorter duration (minutes) would represent fire-generated temperatures. To account for variation both in seed response and temperatures experienced during fire, a range of heat shock temperatures should be applied at 20°C intervals. To avoid misinterpretation of results, particularly when

exploring questions around fire adaptation, we suggest that heat shock starting at 80°C represents temperatures that can only occur as a result of fire. Heat shock treatments at lower temperatures (~ 40°C to 60°C) can also represent natural soil temperature fluctuations. They should be interpreted in the context of vegetation type (e.g. closed vs open canopy), heating duration and background warm-season soil temperatures of the study region. We recommend including high temperatures between 100°C and 150°C to determine embryo mortality thresholds. Again, this temperature depends on the vegetation type and can be obtained using thermosensors placed on the soil surface during natural fires (Daibes *et al.*, 2017).

Seeds are placed in glass Petri dishes layered with a double sheet of filter paper. Dry heat shock is applied in a pre-heated muffle or laboratory oven with accurate temperature control. Seeds may alternatively be placed within pre-heated trays of sand. Each replicate experiences the heat shock separately to ensure independent samples (Morrison & Morris, 2000). Depending on the study goals, a factorial experimental design is needed to determine the effects of high temperatures, heat shock duration, and their interaction.

After heat shock treatment, seeds are tested for germination under optimal laboratory conditions. We suggested a minimum number of six replicates of 25 seeds per treatment. By the end of the trials, ungerminated seeds are scored dead or alive by viability tests. The number of hard seeds (remaining dormant) should be recorded in the case of PY seeds.

Field experiments

Disentangling fire cues (primarily heat and smoke) in field experiments is difficult. However, for species with a known heat shock response, such as PY species from fire-prone regions, it is possible to survey the density of seedlings that emerged in the post-fire environment and compare with those densities in unburnt plots. Comparisons can also be made between sites that have been burnt at different severities to explore questions surrounding fire severity, soil heating and dormancy-breaking heat

thresholds. A within-site comparison of post-fire seedling emergence can be used to examine the variation of soil heating by, for example, different fire types. It can include methods such as the measurement of graminoid scorch depth (Tozer & Auld, 2006) to explore soil heating x seedling emergence depth interactions.

Unit of measurement

Response to heat can be measured by any germination parameter of interest, such as germination percentage, time or synchrony, and statistically compared to the controls. Weibull survival regression analyses can be performed to determine lethal temperatures. Depending on the study goals, a useful way of measuring the effects of response to heat is to calculate the magnitude of the effects of the heat shock treatments relative to the controls. This can be obtained by subtracting the number of germinated seeds in each treatment from the number of seeds germinated in the control and using this to calculate effect size as a proportion of total viability:

$$\frac{Ngerm_{(treat)} - Ngerm_{(control)}}{Nviab_{(control)}},$$

where $Ngerm_{(treat)}$ is the germination percentage of heat-treated seeds, $Ngerm_{(control)}$ is the germination percentage of control seeds, and Nviab is the proportion of total seed viability.

This index can be positive or negative (i.e. with more or less germination in the treatment than in the control, respectively; Daibes *et al*., 2019).

3.4.10 Response to chemical cues

Trait description

The sensitivity/insensitivity of seeds to an externally applied chemical agent with respect to germination. The chemical agent may have a positive, negative, or no effect on germination. Both inorganic and organic chemicals can affect germination, including a suite of plant growth regulators. The chemicals may be naturally occurring, such as those produced by plants and microorganisms or endogenously within seeds, or the chemicals may be derived synthetically. Chemical agents that influence germination are very numerous (Baskin & Baskin, 2014); common chemicals include gibberellins, abscisic acid, ethylene, ethanol, strigolactones, salicylic acid, nitrates, karrikins, cyanide, and glyceronitrile (Hilhorst & Karssen, 1988; Hilhorst & Karssen, 2000; Finch-Savage & Leubner-Metzger, 2006; Yoneyama *et al.*, 2010; Flematti *et al.*, 2013; Cross *et al.*, 2014; Paparella *et al.*, 2015).

Functionality and trade-offs

Seeds in the soil seed bank can perceive and respond to exogenous chemical cues to regulate germination timing and extent. Chemicals such as nitrates and ethylene can vary in concentration in soils, depending on factors including soil temperature, moisture, microbial activity, and physical disturbance (Baskin & Baskin, 2014). Such variation in concentration can signal to seeds the presence of environmental conditions that are more favourable to seedling establishment. Chemical signals of a disturbance providing an opportunity for seedling establishment include smoke-derived chemicals signalling the passage of fire (Flematti *et al.*, 2013), or increased soil nitrate concentrations in bare soil indicating a gap in vegetation (Pons, 1989). Chemical cues may also signal suitable growing conditions for plants to complete their lifecycle, for example, ethylene signalling the presence of sufficient water for the growth of ephemeral wetland species (Cross *et al.*, 2014), helped to broaden their germination niche width (Phartyal *et al.*, 2022), or act as indicators of nearby host plants for parasitic weeds, as for the root exudates strigolactones (Cardoso *et al.*, 2011).

Applied aspect

Chemical agents can be used to elicit germination to test seed viability, to facilitate the growth of seedlings and plants in a nursery or for other applications, and as seed pre-treatments to enhance the chances of germination and seedling establishment in ecological restoration.

For many species, gibberellic acid, in particular, can be used as a pre-treatment to stimulate the germination of otherwise dormant seeds for propagation purposes without the need for other dormancy-breaking treatments (e.g. after-ripening or stratification). The response of freshly collected seeds to gibberellic acid is used to classify the type of physiological (i.e., deep, intermediate, or non-deep) or morphophysiological dormancy (Baskin & Baskin, 2004).

Seed priming with chemical agents can improve the speed and uniformity of germination, increase field emergence, and impart greater seedling stress tolerance. Much research has been done into different methods of priming that incorporate chemicals, including biopriming with beneficial microorganisms such as bacteria (Paparella *et al.*, 2015).

Sources of variability

The magnitude of the response of a seed population to chemicals may be influenced by factors including light and temperature conditions in the germination environment, dormancy status of the seed population, and the seed lot (i.e., maternal environmental effects and storage history effects). There is an interaction between light and the efficacy of some chemicals, and several chemicals (e.g. gibberellic acid, karrikins) can promote light-requiring seeds to germinate in darkness (Merritt *et al.*, 2006). Alternatively, for seeds to respond to some chemicals, particularly nitrates, they must also be exposed to light (Baskin & Baskin, 2014).

The depth of dormancy of the seed population, as influenced by seasonal changes in temperature and moisture conditions in the soil seed bank, affects the response of seeds to chemical cues such as nitrates, smoke, ethylene, and strigolactones, and the

sensitivity of seeds can increase as dormancy is relieved (e.g. during cold stratification or after-ripening) or decrease (e.g. as secondary dormancy is induced; Bouwmeester *et al.*, 1994; Baker *et al.*, 2005; Cardoso *et al*., 2011; Phartyal *et al.*, 2022).

Methodology

A solution of known concentration of the chemical agent may be added directly to the sowing medium (e.g. Petri dishes containing filter paper irrigated with the chemical solution, or the chemical solution incorporated within solidified agar added to the Petri dish or pots with sown seeds can be watered with a chemical solution). Alternatively, seeds can be treated with the chemical agent through imbibing in a solution of known concentration (e.g. for 12 – 24 h, ideally informed by prior knowledge of the time required for seeds to imbibe fully) or through exposure of seeds to a gaseous agent in sealed vials (e.g. ethylene; Cross *et al.*, 2014; Phartyal *et al.,* 2022), prior to sowing in an appropriate medium that is kept moist with pure (e.g. deionised) water.

Seeds should be incubated for germination at an appropriate temperature regime. Constant and alternating temperature regimes may be used, as in some seeds, chemicals promote germination in alternating temperatures but not in constant temperatures (Baskin & Baskin, 2014). Seeds should be placed in both an alternating light/dark regime and in constant darkness to quantify any requirement for, or interaction with, the light environment in the chemical response. Dormancy-breaking treatments such as stratification or after-ripening and the potential for changes in seed sensitivity to chemicals during exposure to such dormancy-breaking treatments should be considered to fully characterise the response to chemicals (Bouwmeester *et al.*, 1994).

Units

Concentration (M) of the chemical agent, percent or proportion of germination, germination speed as measured by t_{50}.

3.4.11 Safe site/gap detection

Trait description

Gap detection denotes processes at the seed level that promote germination upon forming a gap in the canopy of the surrounding vegetation. This includes increased rate (speed) or percentage germination in response to increased diurnal temperature fluctuations compared to more constant ones (Thompson & Grime, 1983), very high dormancy-breaking temperatures generated by solar radiation warming bare ground (Ooi *et al.*, 2014), increased exposure to light levels (Milberg *et al.*, 2000), or high red:far-red light ratios compared to low ones (Kruk *et al.*, 2006). Sometimes, gap detection is used as a synonym specifically for increased germination under daily fluctuating temperatures as a trait-like response of seeds.

Safe-site detection adaptations are those that increase germination in or on the safe site (Harper *et al.*, 1965), including increased moisture requirements for germination, photoinhibition of germination (Carta *et al.*, 2017; Vandelook *et al.*, 2018) or increased germination at constant temperatures compared to diurnally fluctuating (Saatkamp *et al.*, 2011a).

Functionality and trade-off

By definition, safe-site and gap detection include the interaction between physiological response and adaptation of seeds and habitat conditions to increase seedlings' fitness after germination. Gaps and safe sites are important for the regeneration by the seed of many plant species. Gaps are understood as openings in otherwise continuous herbaceous or dense woody vegetation (Thompson & Grime, 1983; Daws *et al.*, 2008) that increase light levels, diurnally fluctuating temperatures and red:far-red light ratios but decrease moisture. Safe sites are microenvironments that increase the survival of seedlings or juvenile plants (Harper *et al.*, 1965); this is a more general term and might include small variations in the litter cover and soil microtopography, such as dips, holes, small mounds, and the microenvironments surrounding stones. Rock surfaces, accumulated woody debris, tree trunks, and other similar structures might also modify

the temperature, increase moisture and decrease seed or seedling predation to increase seedlings' fitness (Harper *et al.*, 1965; Grubb, 1977).

Germination response to diurnally fluctuating temperatures can also increase the survival of seeds in the soil (Saatkamp *et al.*, 2011b) and contribute to how depth of burial is detected by seeds (Saatkamp *et al.*, 2011a), to optimise the trade-off between access to soil moisture and distance reachable with the seed reserves (Bond *et al.*, 1999). Gaps and safe sites have a temporal component both on a seasonal scale (Grubb, 1977) or with variable frequency in successional series, forest and disturbance dynamics.

Gap and safe-site detection mechanisms are related to seed size since the level of reserves modifies the level of moisture needed for germination and seedling establishment (Daws *et al.*, 2008; Arène *et al.*, 2017). The seed size-related competition-colonisation trade-off is understood as the main axis of trait variation for seeds (Muller-Landau, 2010). In this way, gap and safe-site detection mechanisms are related to various germination requirements regarding temperature, moisture, light, and chemical or biotic cues for germination. Gap and safe-site-related chemical cues include nitrates, oxygen, smoke-derived substances, and fire temperatures; these trait-like seed responses are discussed in the relevant sections.

Applied aspect

Knowing seed responses to diurnally fluctuating temperatures might help to predict optimal conservation management in terms of vegetation clearing when the aim is to increase the regeneration by seeds of decreasing populations of wild plants. In heterogeneous habitats with high disturbance frequencies, gap and safe-site detection might be a very common feature explaining regeneration by germination. In fire-prone ecosystems, understanding gap-detecting mechanisms can help identify species can persist in long unburnt habitats.

Sources of variability

Seed responses to temperature and light cues vary as a function of the seed's physical or physiological state, especially during seasonal dormancy cycles. Thus, the date and pre-exposition or treatment of seeds seems important to note.

Methodology

Response to diurnally fluctuating temperatures (DFT) can be measured as a proportional increase of germination in response to DFT compared to constant temperatures by performing two germination experiments, one under constant and another under fluctuating temperatures. Temperatures should match typical temperatures at the soil surface during the germination season, and seeds should be non-dormant, i.e. stratified or scarified prior to the germination experiment. For each experimental condition, at least three replicates of at least 20 viable seeds should be used. A thermogradient bar has been used to effectively study temperature effects on seed germination (Thompson 1970, Thompson & Whatley 1984). The experiment might combine with a light and a darkness treatment, depending on the question and whether seeds are rather buried in soil or at the soil surface in the ecosystem considered. Germinations should be scored regularly until no further germinations occur for several days. The final germination count can be then used to calculate an index of relative germination under diurnally fluctuating temperatures, **ΔG**DFT (Saatkamp *et al.*, 2011b), for the relative germination in diurnally fluctuating compared to constant temperatures, being positive when germination is higher under diurnally fluctuating than at constant temperatures and negative when germination is higher under constant temperatures relative to diurnally fluctuating:

$$\Delta GDFT = \frac{G_{fluctuating} - G_{constant}}{G_{fluctuating} - G_{constant}} \times 100.$$

Using final germination counts under fluctuating temperature ($G_{fluctuating}$) and the number of germinated seeds under constant temperature ($G_{constant}$), relative to the sum of seeds germinated in these two experimental conditions ($G_{fluctuating}$ + $G_{constant}$) in the germination experiment.

There are seed germination responses to other environmental cues that are linked to vegetation gaps, e.g. high temperatures or red:far-red light ratios. High temperatures generated in gaps can be identified by testing threshold temperatures required to break dormancy primarily for physically dormant seeds (see Ooi *et al.*, 2014 for methods and concepts). Response to light is explained in section **3.4.5 Seed light requirements for germination**, and the measurement of germination requirements of R/FR ratios is discussed in Tiansawat & Dalling (2013) and Kruk *et al.* (2006).

3.4.12 Germination speed

Trait description

Germination speed is the time period from the beginning of imbibition until the radicle starts to protrude (Soltani *et al.*, 2015). It is sometimes called germination rate, but the latter could misleadingly refer to other attributes, such as the percentage of germinated seeds. Thus, we recommend using germination speed.

Functionality and trade-off

Germination speed is determined genetically but also depends strongly on macroclimate and the local ecological conditions. Fast germination enables species to tolerate or avoid water stress (Gutterman, 1993). For example, species in the open matrix between trees in a South African savannah germinated much faster than those under the canopy (Kos & Poschlod, 2010; Poschlod *et al.*, 2013). According to Grubb (1977), species with rapid germination gain a competitive advantage if subsequent conditions remain favourable. In contrast, species with slow germination are favoured when species with rapid germination are killed due to subsequent unsuitable conditions such as drought. Dormancy-breaking stratification treatment (e.g. cold stratification) may significantly accelerate germination speed (Barnett & McLemore, 1984). Several studies have reported that arable weeds germinate faster in light (Jensen, 1995; Milberg, 1997; Colbach *et al.*, 2002; Batlla & Benech-Arnold, 2005).

A meta-analysis shows that early emergence positively affects seedling growth and fecundity but does not affect seedling survival (Verdú & Traveset, 2005). They also show that perennials germinated faster than annuals, suggesting that faster seedling emergence allowed perennials to place seedlings into the best conditions each year. In contrast, the annuals have to spread their emergence risk over time. Dormancy level, seed lipid content and seed area to mass ratio are strongly and positively correlated with the germination speed in 25 arable weed species (Gardarin *et al.*, 2011). Germination speed has also been faster in species with a high base temperature for germination (Gardarin *et al.*, 2011). Finally, germination speed is also related to the

amount of nutrients stored in the embryo compared to endosperm or perisperm, with species dispersed with smaller embryo-to-seed size ratio germinating slower (Vandelook *et al*., 2012, 2021).

Applied aspect

Slow or delayed germination may be a problem in agriculture, forestry and horticulture. Therefore, treatments to accelerate germination are described for many species (Bonner *et al.*, 1974). Fast-germinating species may be useful in restoration since the establishment rate may be higher, especially in dry and unpredictable environments (Wagner *et al.*, 2011; Pedrini *et al.*, 2019).

Source of variability

Germination speed may vary depending on environmental conditions, especially temperature and soil moisture (see above). Germination speed is slower at lower temperatures and drier conditions (Balkaya, 2004). Also, seeds germinate more slowly after artificial ageing experiments (Soltani *et al.*, 2015).

Methodology

Germination speed measurements should be done under controlled conditions in the lab. It is measured when the radicle has protruded. Since germination is temperature-dependent, it is useful to calculate germination speed for all species within one experiment at the same temperature regime (e.g., for temperate flora at 22 °C during the day and 14 °C at night). It can be measured for different temperature regimes to show the variability of this trait when one or a few species are used. Since germination can occur very quickly, the germinated seedlings should be counted for the first three days every six hours, then daily (or even two times per day) during the first three weeks. Later, time steps of two days or one week can be chosen. Calculation is done for certain proportions of seeds compared to the total germination speed (see below units of measurement).

The seed germination data can be further used to calculate various indices of seed germination speed (e.g. mean germination time, germination speed; Ranal & de Santana, 2006), also using the R statistical software (e.g. GerminaR package; Lozano-Isla *et al.*, 2019)

4. Seedling

4.1. Seedling establishment

4.1.1 Seed nutrient content

Trait description

The seed nutrient content is a complex trait. It can refer to the amount of (i) major storage components such as oils, carbohydrates, and proteins (or more precisely, different types of fatty acids, carbohydrates, or amino acids) and (ii) macro- (such as nitrogen, phosphorus, potassium) and micro- (such as iron, manganese, zinc) nutrients stored in the embryo and endosperm or perisperm by the mother plant during seed maturation. For ecological questions, the content of non-structural carbon (C; mainly oils and carbohydrates), nitrogen (N), and phosphorus (P) is most relevant (Sterner & Elser, 2002).

Functionality and trade-off

The seed nutrient content reflects the nutrient availability during seed maturation and shows significant phylogenetic constraint among species (Kerkhoff *et al.*, 2006; Bu *et al.*, 2018). The storage tissue of the seed serves as a source of energy and a source of nutrients during seedling establishment (Milberg & Lamont, 1997). Some aspects of seed nutrient content can be confounded with seed nutrient quantity, usually expressed as seed size, at both the intraspecific (Obeso, 2012; Vaughton & Ramsey, 2001) and interspecific levels (Levin, 1974; Lee & Fenner, 1989; Mašková & Herben, 2021). A negative relationship has, for example, been observed between seed mass and N, P, and Mg for tropical rainforest species (Grubb & Coomes, 1997) and between seed mass and P for temperate herbaceous species (Mašková & Herben, 2021). In contrast to seed mass, it is assumed that seed oil content does not increase along a latitudinal gradient from temperate regions to the tropics (Levin, 1974). On a global or regional scale, the seed nutrient content is believed to be rather fine-tuned to

environmental conditions for supporting seedling survival and establishment (Bu *et al.*, 2018; Mašková & Herben, 2021). As such, seed nutrient provisioning is considered especially important in plants growing in nutrient-poor soils, although evidence is rare and inconsistent (Kuo *et al.*, 1982; Jurado & Westoby, 1992; Denton *et al.*, 2007). Seed oil content does tend to increase with woodiness and habitat shadiness, but evidence beyond legumes is missing (Levin, 1974).

The seed nutrient content could also be related to biotic factors, such as dispersal and predation, due to the attractiveness of seeds to granivores (Gong *et al.*, 2015; Yadok *et al.*, 2020). However, no relation was found between seed predation and seed protein content of temperate fleshy fruited species (Kollmann *et al.*, 1998). The relation of seed nutrient content to other functional traits remains unexplored at many levels (Franco *et al.*, 2023), and further investigation is necessary.

Applied aspects

Seed nutrients are an essential part of the food chain because seeds are an important food source for many animal species and humans. Legumes are for example considered as important source of proteins in low income countiers and a crucial component in the ongoing protein shift (Semba et al., 2021). Investigating seed nutrient content has vast potential for finding new sources of nutrition and support rewildling by providing resources for the animal community.

Sources of variability

The major storage components vary widely within genera, among genera of the same family, and among families (Levin, 1974; Mašková & Herben 2021). The oil content is stable at the species level, typically showing high heritability (e.g. Baker & McKenzie, 1972). Individual components of seed nutrient content may vary intraspecifically according to seed mass (Vaughton & Ramsey 2001; Obeso, 2012; but see Levin 1974); provisioning by maternal tissues (Bedi *et al.*, 2009); concentration of atmospheric CO_2 (Steinger *et al.*, 2000; Saha *et al.*, 2015); and can vary between populations from different latitudes (De Frenne *et al.*, 2011; Sun *et al.*, 2012). The impact of different

environmental conditions, such as maternal effects on intraspecific variability in seed nutrient content, is unclear (Nishizawa and Aarssen, 2014), and further investigation is needed.

Methodology

To measure seed nutrient content, fully matured seeds (i.e., it is possible to separate seeds from the mother plant with a light touch) should be collected. Randomly selecting harvested seeds and inflorescences is necessary to obtain the broadest possible natural spectrum of diaspores and avoid maternal effects on the seed nutrient content. We recommend working with easily separable units; in a case where the fruit-derived part of the dispersule is inseparable (e.g., achene) to include it as a seed component because it plays the same ecological role as seed testa (Hintze *et al.*, 2013). We recommend using freshly collected seeds when possible. To measure P content, use flow injection analysis after perchloric acid mineralization (Wieczorek *et al.*, 2022). To measure N content, use flow injection analysis after Kjeldahl mineralization (Kjeldahl, 1883). Non-structural carbon is represented by carbohydrates (fructans, starch) and oils. Before summing up these parts, correction for C content is needed. The estimation of the C-weighted fraction is 44.45% C for carbohydrates and 77.2% C for oils (Rasor & Duncan, 2014; Orsavova *et al.*, 2015). For measurement of carbohydrates, use the enzymatic procedure Megazyme (McCleary *et al.*, 1994) and for measurement of oils, use Soxhlet extraction (ISO, 2009). See section **2.1.6 Fruit chemical compounds** for further details.

4.1.2 Seedling morphology type

Trait description

Seedling morphology type is not a single trait but a complex of several morphological features that can vary considerably with phylogeny and stage of development during this relatively transitory stage in the seed plant life cycle. The 'classic' definition of a seedling as a juvenile plant from germination to development of the first true leaf notwithstanding, the word 'seedling' is difficult to define precisely, as it forms part of the growth and development continuum immediately following germination. While it is intuitive to propose that the seedling stage begins with the protrusion of the radicle from the seed coat, various authors have proposed alternatives (Leck & Outred, 2008; see pp 17-18). The end of the seedling stage and transition to juvenile is less clear and occurs at a mostly indeterminate and almost arbitrary stage (Bell & Bryan, 2008).

The seedling consists of an axis with a primary root (radicle) at one end and the shoot apical meristem at the other (plumule). There is a transition zone is where the root and shoots join, which is often indistinct and unrecognisable without microscopic examination. Borne on the axis are one or two (depending on species – monocotyledonous or dicotyledonous; sometimes more, e.g., gymnosperms) cotyledons. These specialised organs are interpreted as modified first leaves and have variously absorptive, photosynthetic and nutrient storage functions. The hypocotyl is the axis between the cotyledonary node and the transition zone; the epicotyl is immediately above the cotyledons.

Functionality and trade-offs

Functionally, seedlings are frequently classified dichotomously based on their mode of germination, which can be either epigeal, where the cotyledons are borne above the soil surface on the emerging axis (Fig. 16a, b, e), or hypogeal, when the cotyledons remain at or below the soil surface (Fig. 16c, d, f, g). A more precise description is possible by applying a further binary classification, which uses the terms: cryptocotylar, when the cotyledons of the germinated seed remain enclosed in the seed coat (Fig.

16b), and phanerocotylar, when the cotyledons are released from the seed coat or pericarp (Fig. 16a). This classification does not altogether accommodate whether cotyledons have a storage or assimilatory (fleshy or foliar) role. In many cases, this seems unlikely to be a clear binary, more somewhere on a form-function continuum, although cotyledons that remain below ground cannot be assimilatory. In some groups of monocotyledons, the single cotyledon extends to push the seedling away from the seed coat and food store (Fig. 16f). This is known as remote germination, for example, in palms, in contrast to non-remote germination, when the cotyledon remains within the dispersule, e.g. in the caryopses of Poaceae (Fig. 16g). Indeed, monocotyledons appear to show much greater variation in seedling morphology than do most dicotyledons (e.g., Tillich, 2007). Likewise, seedling morphological diversity appears greater among tropical tree species than among other life forms and vegetation types. Garwood (1996) has produced a comprehensive classification of tropical seedling morphological types set in a functional context. There has been little or no published research into the functional significance of the variation in seedling morphology types. However, Gardarin *et al.* (2016) concluded that as well as germination speed being faster for species with a high base temperature, they were also higher for species with seed reserves located principally in the embryo (rather than the endosperm or perisperm).

Sources of variability

Variability among species has a strong genetic component and relies heavily on phylogeny. Seedling morphology is likely to be associated with species' embryology and the morphology of the embryo (see section **3.4.2. Post-dispersal embryo development**) within the seed, but Niklas (2008) warns against confusing such associations with evolutionary trajectory.

Plasticity, maternal effects on developing seeds, or micro-environmental conditions at the germination site are all possible sources of variation within species and are subject to change during seedling ontogeny (see Leck & Outred, 2008).

Methodology

The seedling morphology type is recorded using the usual method of morphological study, careful observation and measurement, as appropriate. Refer to published and other literature to confirm any classifications applied. Seedlings germinated under 'natural' (soil) conditions should be the subjects for morphological trait observation/determination, as substrates such as agar may not promote 'normal' onward development. Likewise, because of the possibility of ontogenetic change, some measures of seedling age – days from germination – should be recorded (Table 10).

Table 10. Morphological trait states for the five seedling components (abstracted from Appendix 2.1 in Leck & Outred (2008).

Cotyledons	Number	usually 0-2, varies from 0-13, and some variation within species (>2 mostly in gymnosperms)
	Shape	narrow; petiolate; sessile; lobed; fuses
	Phyllotaxy	opposite; whorled; alternate
	Similarity	similar or dissimilar
	Length	variable; usually mm or cm, but up to 1-4m in cotyledon stalk of *Lodoicea maldivica* (3-4 years old)
	Longevity	not persistent or persistent

	Axillary buds	absent or present; if present can be swollen, or develop into rhizomes or creeping stems
	Colour	green; red; green above white below
Roots	Radicle or primary root	long or short
	Fibrous	long with laterals; single root with fibrous adventitious roots
	Prop roots from stem internode	
	Radicle	persistent long-lived; non-persistent or lacking
	Colour	orange-red; yellow with red laterals; bright red; pink or flesh coloured; brown hairs; red root tips
	Nodules	present or absent
	Coralloid roots	present or absent
	Adventitious roots	from cotyledon node; from leaf base; from hypocotyl base; from

		stem node
Stems	Entirely stem	
	Hypocotyl	undeveloped; developed; length; thickness (can be up to 1.75 cm in Durio); woody; hollow; below ground only; adventitious buds
	Colour	green; colourless; red or purple; yellow/straw; flesh-coloured
Leaves	Present at germination	yes or no
	Scale leaves present	many hypogeous spp.
	Simple or compound	
	Dimorphic	
	Phyllotaxy	opposite; alternate; whorled – may change with age/development
	Heteroblasty	

	Heterophylly	mainly aquatic plants
	Colour	green (most spp.); red (on new leaves); lacking in chlorophyll (parasites or albinos)
Seedling architecture	Monopodial or sympodial	

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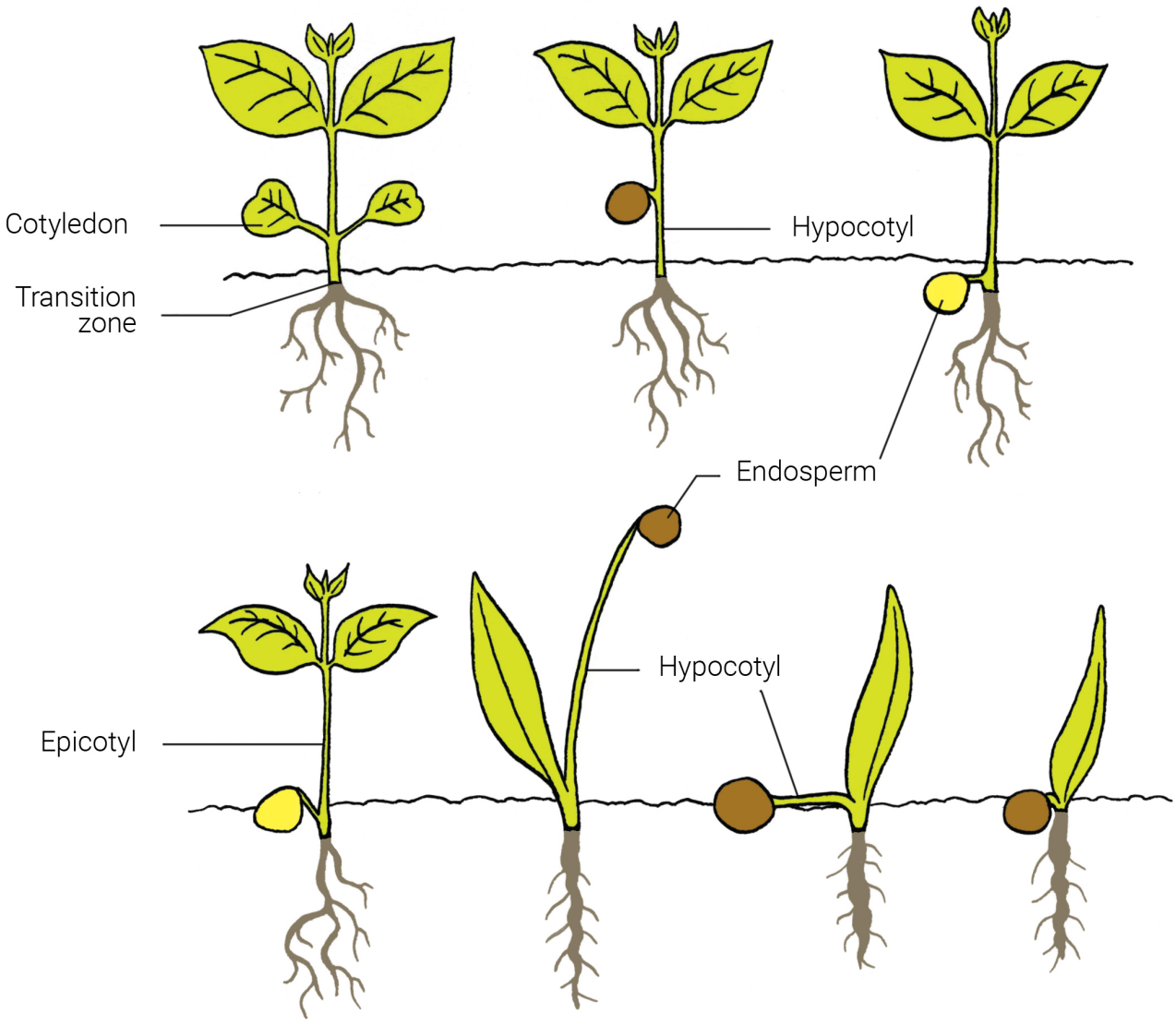


Fig. 16 Seedling morphology types - a) epigeal phanerocotylar germination in dicotyledons, b) epigeal cryptocotylar germination in dicotyledons, c) hypogeal germination in dicotyledons, d) hypogeal remote germination in dicotyledons, e) epigeal germination in monocotyledons, f) hypogeal remote germination in monocotyledons, g) hypogeal non-remote germination in monocotyledons.

4.1.3 Seedling emergence depth

Trait description

Seedling emergence depth is the depth in a soil profile from which a seed can reach the soil surface via a germinated seedling. Emergence depth as a trait may be measured as the maximum depth from which seedling emergence is possible (preferential data) or the optimal depth from which total seedling emergence is greatest (secondary data). Ideally, studies should document the depth distribution through experiments that test different emergence depths in an opaque substrate that prevents photosynthesis. The first parts of the seedling to emerge from beneath the soil surface may be the coleoptile, cotyledons, or hypocotyl.

For aquatic species, the trait should refer to the depths in the sediment. The maximum water depth from which aquatic species might emerge to the water surface is a distinct trait in our understanding, since growth based on photosynthetic activity can occur for many submerged plants. Maximum emergence depth may be viewed as an outcome of the interaction between seed traits (e.g., seed mass, root:shoot allocation) and soil characteristics (texture, compaction, rock content, moisture availability).

Functionality and trade-offs

Maximum emergence depth depends on seed reserves, making seed size a primary determinant of maximum emergence depth (Bond *et al.*, 1999). Mechanisms for seeds to detect burial depth are important as deeply buried seedlings might deplete all their reserves during growth within the soil profile since they have no access to light and eventually die by exhaustion, sometimes termed ‘fatal’ or ‘suicide’ germination (Gardarin *et al.*, 2012). It can be hypothesised that dependence on light or oxygen for germination prevents this type of seedling mortality. This situation might well explain which selective forces drive the higher light requirements for the germination of small seeds (Milberg *et al.*, 2000; Saatkamp *et al.*, 2011a). Altogether, gradients with soil depth of environmental variables such as light, moisture and temperature fluctuation play a major role in determining germination within the soil profile.

In many environments, most seeds reside within the top few centimetres (0 - 5 cm) of the soil seed bank in natural (i.e., non-cultivated soils). For example, 99% of seeds in the study of Mediterranean grasslands emerged from within the first centimetre (Traba *et al.*, 2004), and 75% of seedlings of European alkaline grasslands emerged from within the top 0 - 5 cm (Tóth *et al.*, 2022). However, a study of semi-natural grassland in Sweden found an even distribution of seeds between upper (0 - 4 cm) and lower (0 - 8 cm) layers (Milberg, 1995). In some habitats and soil types, seeds can also be deeply buried. For example, seeds of three species of a salt marsh with the highest densities at both 0 - 4 cm and 12 - 16 cm as seeds fell into deep cracks during the dry season (Espinar *et al.*, 2005). Seed burial depth can vary naturally due to soil disturbance, rainfall (Benvenuti, 2003), movement by invertebrates (Forey *et al.*, 2011), birds, and other animals (Saatkamp *et al.*, 2014), and through self-burial, e.g. through hygroscopic awns. Seedling emergence declines with burial depth for all species (Pearson *et al.*, 2002; Traba *et al.*, 2004). However, variation between species in maximum seedling emergence depth is an important component of niche differentiation. The ability of seedlings to emerge from greater depths protects seeds from the higher temperatures, desiccation, and risks of predation present on the soil surface. The upper and lower limits of soil temperature and moisture conditions, and their degree of seasonal and diurnal fluctuation, vary with depth. The light quality and oxygen conditions also vary with depth. Through variations in these abiotic factors, the vertical position of seeds in the soil profile influences seed persistence, dormancy release, and the timing and extent of germination. Sensing of burial depth can occur in seeds via light cues and/or dormancy release since both light and temperature fluctuation are greater at or near the soil surface (Saatkamp *et al.*, 2011a). Soil depth sensing is related to gap detection. Small seeds close to the surface or within the litter layer rely on light and diurnal temperature fluctuations to identify a canopy gap.

The higher soil moisture availability in greater soil depth favours seedling establishment. Longer periods of favourable soil moisture conditions at greater depth can advantage seedling emergence in dry environments (Saatkamp *et al.*, 2011a), and

form a key consideration for the sowing depth of crops, which can be planted and emerge from depths of up to 20 cm (Mohan *et al.*, 2013), much deeper than seeds of most wild species (Bond *et al.*, 1999; Benvenuti *et al.*, 2001).

Burial depth influences the temperatures to which seeds are exposed through the passage of fire. Near the soil surface, within the top 0.5 – 1 cm, soil temperatures can reach more than 100 – 250^{o}C during the fire and exceed those at which seed survival is possible (Auld & O'Connell, 1991; Tangney *et al.*, 2018; 2020). However, as seed burial depth increases, so does survivability, as soil temperatures decrease markedly with depth due to the insulating properties of soil (Tangney *et al.*, 2020). Therefore, seeds that can emerge from greater depth have greater protection from fire. Insulating effects of soil also influence seed dormancy-break, particularly of species with physical dormancy, as well as PD. There might be a trade-off between tolerance to heat and emergence depth (Tangney *et al.*, 2020).

The time needed for seedling growth to emerge at the surface is a variable that must be considered when germination timing in the field is compared to relevant laboratory data on germination speed since deeply buried seeds are slower to emerge.

Applied aspect

Seed sowing depth is a major factor in seedling emergence and establishment both in agricultural and ecological restoration settings. Depth of sowing and final seed position in soil profiles can impact crop development (Kirby, 1993) and the success of restoration techniques such as mulch or soil seed bank transfer (Rokich & Dixon, 2007). Manipulation of the soil seed bank by mimicking tilling to bring seeds closer to the soil surface depth is used for weed control in agriculture. Knowing the optimal sowing depth is also crucial for population translocation in nature conservation contexts.

Sources of variability

Soil physical properties such as compaction, crusting, rock content, texture, and moisture and oxygen availability influence optimal emergence depth. Pre-emergent loss of seeds or seedlings due to predation or microbial-induced decay might bias

measures of maximum emergence depth, reflecting mortality rather than depth, a potential confounder for seeds buried in the field. Using sterilised growth media in pots or trays in a nursery can help to avoid this.

Methodology

Sow seeds ready to germinate (viable, non-dormant) at a gradient of known depths either by covering them with a measured substrate depth or by digging holes of appropriate depth. This kind of experiment should be done at seasons and moisture levels that are optimal for the germination of the considered species. They are most accurate in nursery or glasshouse settings, where this can be done in pots or trays of sufficient depth to test the desired sowing depth. Place a layer of substrate (e.g. soil or sand) in the base of pots or trays of sufficient depth to test the desired sowing depth. It is either (i) possible to sow seeds on the same base level substrate and then add a measured amount of substrate above the seed, up to the desired sowing depth or (ii) to add varying amounts of sand to the base, and then sow and cover, to bring all pots up to equal level. The latter might be preferable since it will bring all pots to the same final soil level, preventing shading by deep pot sides – but means differing amounts of soil below the seeds. Pots of varying depths can avoid this, such as cutting plastic pipes to differing lengths, thereby allowing for a constant depth of soil below the seeds and a varying level of soil above to fill the pot to level (Kirby, 1993).

Caution should be paid to the fact that pots or trays behave differently regarding oxygen and temperature fluctuations than natural soil profiles. Experiments to evaluate limiting life stages in soil (i.e., seed germination vs seedling emergence) can include seeds buried in mesh bags, typically nylon mesh, which can be retrieve to assess the proportion of seeds that germinate at a given depth.

Units

Depth below the soil surface in cm

Data capture can include the proportion of seeds that emerge from a given depth, the timing of emergence (number of days to first emergence or a defined percentile such as 50% of the sown seeds), and the entire distribution of seedling depth distribution.

Special cases

Saprophytic, hemi-saprophytic (orchids…) and parasitic seedlings might follow very different functional relationships in relation to soil depth since they do not necessarily depend on photosynthesis.

4.1.4 Seedling growth rate

Trait description

The relative growth rate is a concept introduced to describe the growth phase of plants by Blackman (1919). It is the most useful and widely used index to quantify the speed of plant growth. It shows the rate of accumulation of new dry biomass per unit of existing dry biomass. The relative growth rate is considered the main determinant of plant competitiveness and, as such, is seen as a central element of plant species' ecological strategy and its potential for success (Hunt & Cornelissen, 1997).

Functionality and trade-off

Relative growth rates differ substantially between species and are associated with environmental nutrient availability and plant growth strategy. On a global or regional scale, slower-growing species tend to be adapted to resource-limited habitats, such as shaded habitats and habitats with low-phosphorus soil (Chapin, 1980; Wright *et al.*, 2010; Reich, 2014; Poorter *et al.*, 2019). Seedling growth rate also strongly depends on temperature (Carroll *et al.*, 2021) and precipitation (Lai *et al.*, 2019) regime.

The relative growth rate is mostly negatively correlated with seed size (Fenner, 1992; but see Turnbull *et al*., 2012 for other possible confounding effects). It is related to initial seedling weight and other morphological parameters, particularly specific leaf area (Maranon & Grubb, 1993; Antúnez *et al.*, 2001).

Applied aspect

Relative growth rate refers to a species' potential success in competition. A higher relative growth rate for invasive species than their native counterparts has been documented (James & Drenovsky, 2007). Therefore, the relative growth rate may predict potential species invasiveness. Further, differences in relative growth rate according to different environmental conditions could help to find the most effective procedure during seed-based restoration.

Sources of variability

The relative growth rate is variable within species. It is connected to both above- and below-ground resource availability. It could also be connected with other environmental conditions, such as temperature (De Swart *et al.*, 2006) and intraspecific differences in seed mass (Meerts & Garnier, 1996). The relative growth rate is highly connected with growth form, decreasing from herbs to woody perennials (Galmés *et al.*, 2005).

Methodology

To measure the relative growth rate across the interval of seedling development, dry biomass of the whole seedling from the beginning and end of this interval is needed. This measurement is, therefore, always destructive.

Cultivating plants in controlled conditions is recommended. A clear report of growing conditions such as temperature, soil moisture regime, and light intensity is necessary for disentangling if differences in relative growth rate are based on functionality or environment. The relative growth rate is affected by the size of plants. Therefore, it is necessary to choose plants for each harvest randomly to minimise the effect of the initial size of individuals. The relative growth rate decreases with plant size increases, more harvests across seedling stage is recommended to understand the growth dynamic better. The first harvest should be immediately after seedling emergence. The interval of consequent harvest depends on the plant species and may vary from shorter than 1 week for fast-growing herbaceous species to longer than several months for slow-growing woody species.

During plant harvest, remove the plant carefully from the substrate and wash the root system precisely to obtain whole plant biomass. Let the plant oven-dried to the constant weight at 70 °C and weigh it.

To calculate the mean of relative growth rate (R) across the harvest interval t_1 to t_2, use the formula:

$$R = \frac{\left(\ln W_2 - \ln W_1\right)}{\left(t_2 - t_1\right)},$$

where W_1 and W_2 are the weights of dry plant biomass at times t_1 and t_2, respectively.

The concept of the relative growth rate of whole plant biomass could be extended to other attributes. Similarly, for instance, the relative root or shoot elongation rate could be measured (see section **4.1.5 Radicle/root elongation rate**; Larson *et al.*, 2016).

4.1.5 Radicle/root elongation rate

Trait description

The radicle elongation rate (often also called root elongation rate) describes the radicle or root growth rate in a specific time span.

Functionality and trade-off

The radicle elongation rate is associated with the success of the seedling's establishment. It is strongly influenced by habitat and soil conditions, for example, soil nutrients (Ogawa *et al.*, 2014), soil strength (penetration resistance) and soil humidity (Azam *et al.*, 2012). Soil strength and water potential may be the most important soil parameters to understanding radicle elongation rate (Materechera *et al.*, 1991; Bengough *et al.*, 2011). Drought generally decreases the elongation rate (Larson & Funk, 2016). However, a fast radicle elongation rate is pivotal under dry conditions. Therefore, species in regions of low rainfall or dry habitat conditions have a higher radicle elongation rate (Nicotra *et al.*, 2002; Kos & Poschlod, 2010). Climate, such as temperature and water availability, may also affect the radicle elongation rate (Teskey & Hinckley, 1981).

Root elongation rate may be related to several seed traits, such as seed mass. Smaller seeds have slower germination speeds and slower radicle elongation rates than larger seeds (Daws *et al.*, 2007). Fast germinating species may have a higher radicle elongation rate than slow germinating ones (Kos & Poschlod, 2010; Poschlod *et al.*, 2013).

Applied aspect

Studies have shown that a higher radicle or root elongation rate in trees is related to higher survival against drought (Horton & Clark, 2001; Stella & Battles, 2010). Species with a high radicle or root elongation rate may be promising for restoring drylands or bare soil (Garbowski *et al.*, 2020).

Sources of variability

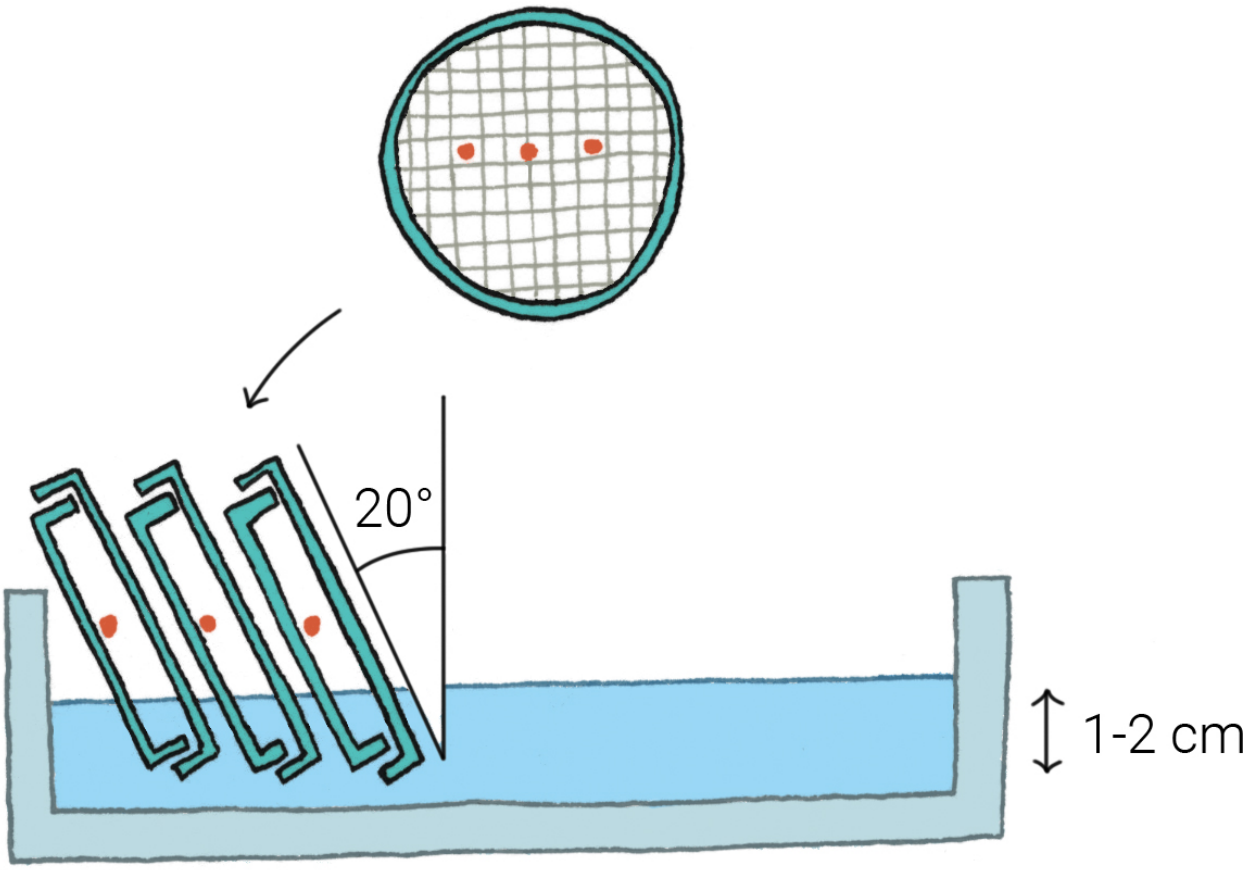
Variability of the initial radicle elongation rate of germinated seeds is, until now, only described for varying environmental conditions (e.g. Ogawa *et al.*, 2014). Any intraspecific variation independent from environmental conditions has not yet been described but may vary in seeds of different quality (see above).

Methodology

Radicle or root elongation rate should be ideally measured on the substrate with the same penetration resistance and water potential (Pagès *et al.*, 2010).

Since measurements in soil *per se* is already a challenge (Pagès *et al.*, 2010), we recommend measuring the seed's capacity for the rate of radicle or root elongation in a climate chamber under standardised temperature regimes in a Petri dish (Kos & Poschlod, 2010). Place seeds on a horizontal line on a wet filter paper disc placed in the lid of a Petri dish. To allow direct reading of root length, place a transparent disc with a laser-printed millimetre grid under the filter paper. Set upside down the lid of the dish Petri dishes at a ca. 20° angle from the vertical in a glass basin filled with 1–2 cm water to ensure constant moisture supplied to the seeds (Fig. 17). Check seeds at a 12-hour interval until the radicle becomes visible. Begin to measure radicle/root length immediately and then after another 24 hours. The elongation rate in mm per day or cm per day is calculated as the difference between these two values (e.g. Larson & Funk, 2016).

Alternatively, a rolled towel-based growth assay can be used (Draves et al. 2022) for some large-seeded species. Field-grown soil conditions can also be simulated in flat rhizoboxes with a transparent front side.



5395 **Fig. 17** Experimental setting of Petri dishes for root elongation measurement. Blue line
5396 indicates water level to ensure constant moisture supplied to the seeds.

4.1.6 Seedling resistance to abiotic stress (e.g., frost, drought, salinity)

Trait description

Seedling resistance to abiotic stress is defined here as the ability of a seedling to survive abiotic stress (i.e., distress, temperature extremes, extreme or prolonged water deficit/drought, and extreme salinity) while minimizing reductions in growth and, ultimately, fitness (Kranner *et al.*, 2010).

The definition of the 'seedling' varies among the field of research and ranges from 'true' seedlings (young plants with cotyledons but not true leaves; Rosbakh *et al.*, 2020a) to juvenile plants up to several years old (Zurbriggen *et al.*, 2013).

Functionality and trade-offs

Following germination, seedling recruitment represents a major bottleneck to plant recruitment (Grubb, 1977; Fenner & Thompson, 2005). Due to the low accumulation of dry matter in the roots, which is necessary to recuperate after damage, seedlings have low survival rates when exposed to stressful conditions and demonstrate lower resistance as compared to adult individuals (Körner, 1999; Marcante *et al.*, 2012; Sierra-Almeida & Cavieres, 2012). Therefore, quantifying seedling resistance to temperature, water, and salinity stress is important for understanding seedling recruitment potential under stress. Recruitment under stress may have implications for plant community assembly, species distribution patterns at different scales (Zedler *et al.*, 1990; Splunder *et al.*, 1996; Engelbrecht & Kursar, 2003) and plant responses to global change (Edwards *et al.*, 2001b).

Sources of variability

Seed reserves determine seedling performance; in general, seedlings germinated from larger seeds can tolerate better abiotic stress (Castro, 1999; Engelbrecht & Kursar, 2003; Paz & Martínez-Ramos, 2003; Kennedy *et al.*, 2004; Leiblein-Wild *et al.*, 2014; Lazarus *et al.*, 2018). Furthermore, the trait is strongly associated with the growth stage; during emergence, the plants exhibit maximum sensitivity during the period of

rapid elongation. The most sensitive phase in dicotyledons is during the unfolding of the cotyledons and primary leaves, whereas in graminoids, it is at the beginning of coleoptile elongation (Sakai, 1987; Marcante *et al.*, 2012). Additionally, the trait is influenced by a complex of ecological factors, including duration and severity of abiotic stress, cultivating conditions, acclimation/deacclimation cycles and other associated factors (Meyer & Badaruddin, 2001; Zurbriggen *et al.*, 2013).

Methodology

There are two approaches to measuring seedling resistance to abiotic stress: field experiments and trait measurement under controlled conditions (e.g., Marcante *et al.*, 2012; Rosbakh *et al.*, 2020a). Although the former approach provides information on seedling stress resistance under natural conditions, we advise using the latter because of the possibility of separating the effects of the factor of interest from other interacting or random factors.

The trait measurement consists of three steps: (i) subjecting seedlings to abiotic stress (distress, e.g. extreme temperatures, extreme or prolonged water deficit/drought, or extreme salinity), (ii) evaluating the effect of the treatment, and (iii) evaluating the ability to recover from the stress. Low and high-temperature stress can be simulated by seedling incubation in chambers of different models or temperature-controlled glasshouses or greenhouses. Drought is created by discontinuing the watering of pots with seedlings, seedling cultivation in solutions with different water potential (e.g. polyethylenglycol, mannitol) or wet-blotting with, for example, filter paper. Salinity stress is simulated by exposing seedlings either to seawater or pure salt solutions (e.g. NaCl, MgSO4) of different concentrations (e.g., from saline water > 1,000 ppm to seawater approximately 35,000 ppm).

Regardless of the type of stress, the standard procedure for the testing is (i) randomly select a sample of seedlings from the population of interest, (ii) place them into a testing system (e.g. freeze chamber, temperature controlled greenhouse, a substrate with different salt concentrations), (iii) keep/cultivate the seedlings at different levels of

abiotic stress for a given time period (or multiple durations) and monitor the decline in function or death, (iv) return the seedlings to the starting conditions, and (v) estimate seedling survival rates, fitness and/or viability.

Several aspects of this procedure warrant attention. First, to avoid shock reactions at step 2, the seedlings should be exposed to the test level of stress gradually (i.e., ramping; Geange *et al*., 2021). For example, in frost-resistance research, it is recommended to lower the temperature at a rate of -2K/h down to target temperatures (Neuner *et al.*, 2020). In some cases, seedling acclimation before and after exposure to the abiotic stress is recommended to allow the seedling to develop resistance, which is otherwise not activated (Geange *et al.*, 2021). Second, the the stress and duration level of step 3 should correspond to the conditions the seedlings experience in the field (e.g., 4-hour night frost; Sakai, 1987; Neuner *et al.*, 2013), because longer exposures normally increase damage. Third, the effects of other factors, which could also negatively affect seedling resistance, should be accounted for. For example, while testing seedling frost resistance, ensure that seedlings are regularly watered to prevent drought stress that can cause seedling mortality. Studies that investigate both upper and lower extremes (e.g., both heat and cold effects) are recommended where possible (Geange *et al.*, 2021).

After exposure to abiotic stress, the seedlings are cultivated under optimal conditions for their growth to allow the damage to develop. Seedling viability can be further determined by visual inspection or the topographic tetrazolium test (Cottrell, 1947). In the former case, seedlings with undamaged roots and vivid green-coloured, healthy-looking cotyledons that are able to continue growing are considered viable. In the latter case, seedlings with dominating red colouring are rated as viable (Marcante *et al.*, 2012). Alternatively, seedling damage can be estimated with the help of the membrane electrolyte leakage method (Gurvich *et al.*, 2002).

Seedling resistance to abiotic stress can be numerically expressed as lethal thresholds, i.e. the minimum level of the stress factor at which a certain percentage of a random seedling population will survive or sustain a given level of damage. For example, the

term LT50 (lethal temperature for 50% of a population) is commonly used to define the (seedling) frost resistance level (Rosbakh *et al.*, 2020a). To achieve that, some functions (e.g., logistic or Weibull functions; Ritz & Streibig, 2005) are fitted to the seedling damage/survival data and lethal thresholds are calculated.

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Author contributions

PP initiated the handbook project. TM coordinated writing and edited all sections. SR wrote section 1.1.1 – 1.1.10 and 4.1.6 with input from LG for sections 1.1.9 and 4.1.6. FAOS wrote section 2.1.1. – 2.1.6. PP wrote sections 3.2.1 – 3.2.4, 3.2.7 – 3.2.13, 3.3.3, 3.3.5, 3.4.12 and 4.1.5 with input from SC, LG, AS, FAOS and JBS. AS wrote sections 3.1.1 – 3.1.3 with input from JD, JBD and PP, sections 3.4.6 and 3.4.7 with input from BJA and EFP and section 3.4.11 with input from MO. JD wrote section 3.1.4. SC wrote sections 3.2.5 and 3.2.6 with input from JBD. MO wrote section 3.3.1. DJM wrote sections 3.3.2, 3.3.6, 3.4.5, 3.4.8, 3.4.10 and 4.1.3. with input from ED, LG, SSP and AS. BJA wrote section 3.3.4 and 3.3.7. FV wrote section 3.4.1. SSP wrote sections 3.4.2 and 3.4.3 with input from DJM and JBD. JBD wrote section 3.4.4 with input from DJM and section 4.1.2. FAOS, MO and DJM wrote section 3.4.9. TM wrote section 4.1.1 with input from FV and section 4.1.4.

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