From Forest Nursery Notes, Winter 2012

250. Standardizing seed dormancy research. Hilhorst, H. W. M. IN: Seed dormancy: methods and protocols, p. 43-52. A.R. Kermode, editor. Springer Science. 2011.

Chapter 3

Standardizing Seed Dormancy Research

Henk W.M. Hilhorst

Abstract

Seeds are very attractive and convenient for molecular genetic studies that challenge principal biological phenomena related to the initiation and suppression of growth (e.g., germination and dormancy, respectively). The number of reports in this field is rapidly expanding. Seed dormancy is a widely misinterpreted biological attribute. One of the main reasons is the general neglect of reliable dormancy assays; often, the sole criterion of current dormancy assays is the total germination of a seed population after a defined period of time. This is a very insensitive and inaccurate method, particularly when comparing dormancy levels of seeds from different genotypes, seeds subjected to different treatments, or seeds originating from different environments. Other seed parameters are far more useful. Furthermore, before undertaking comprehensive molecular and biochemical studies to elucidate the underlying mechanisms of dormancy of a given species, it is pivotal to determine the general types or categories of dormancy that are imposed and whether these are influenced by the external environment. Research strategies should be adjusted to this. In order to distinguish dead from dormant seeds, a viability test should be developed. This chapter addresses in a very general way these pitfalls in dormancy research with a focus on current plant model systems in molecular genetics, such as *Arabidopsis thaliana* and *Medicago truncatula*.

Key words: Seed dormancy, Dormancy classification, Embryo, Endosperm, Germination, Seed coat

1. Introduction

1.1. Definition and Types of Dormancy

Seed dormancy is very often regarded as the absence of germination. However, the absence of germination can have several causes: (1) the seed is nonviable; (2) the environment is nonoptimal for germination; or (3) the seed or dispersal unit is dormant. From this, a practical definition of dormancy can be formulated: "Dormancy is the absence of germination of a viable seed under conditions that are favorable to germination." The ecological relevance of seed dormancy can be defined as an effective delay of germination to

Allison R. Kermode (ed.), *Seed Dormancy: Methods and Protocols*, Methods in Molecular Biology, vol. 773,
DOI 10.1007/978-1-61779-231-1_3, © Springer Science+Business Media, LLC 2011

avoid germination and subsequent growth under unfavorable climatic conditions.

For meaningful genetic and molecular studies of seed dormancy, it is essential to know the type of dormancy of the species under study. The occurrence or absence of seed germination is usually the net result of two opposing forces, namely, the "thrust" force of the embryo (embryo growth potential) and the restraints exerted by tissues surrounding the embryo. A currently accepted dormancy classification (1) is based on the seed components that inhibit germination: embryo (endogenous) and seed coat (exogenous, including endosperm, perisperm, or megagametophyte, as well as fruit tissues that are part of the dispersal unit) (Table 1). In addition, different levels of intensity have been described for each of the dormancy classes, as well as combinations of dormancy classes, such as morphophysiological dormancy and combinational dormancy (a combination of physical and physiological dormancy) (1). An example of the latter can be found in the model species *Medicago truncatula*. In order to break dormancy of this species, a sulfuric acid treatment is required to break the physical dormancy component (hard seed coat); an additional moist-chilling treatment breaks the physiological dormancy component in the embryo (Fig. 1).

An additional classification of dormancy is based on the timing of its occurrence: primary dormancy indicates the type of dormancy that occurs prior to dispersal as part of the seed's developmental program and includes all the dormancy classes and their combinations. Secondary dormancy denotes the reacquisition of dormancy in a mature hydrated seed as a result of the lack of proper conditions for germination (3). Since secondary

Table 1
A simplified classification of dormancy

Class	Physiological dormancy	Morphological dormancy	Physical dormancy
Location of block	Metabolic block(s) in embryo	Immature embryo	Maternal tissues (seed coat), including perisperm, endosperm, megagametophyte, and fruit tissues
Physiological mechanism	Arrest of growth in apical and root meristems	Incompletely matured embryo in ripe seed	Inhibition of water uptake; mechanical restraint preventing embryo expansion; modification of gas exchange; prevention of leaching of inhibitors from embryo

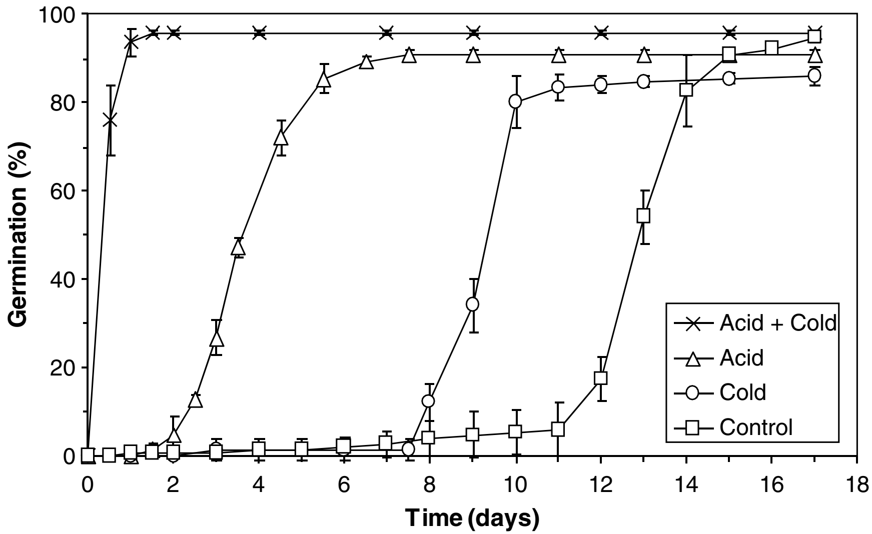

Fig. 1. Germination of *Medicago. truncatula* seeds at 20°C after various treatments for dormancy release: chemical scarification with sulfuric acid; cold imbibition (moist chilling) at 4°C for 36 h; chemical scarification plus moist chilling; and control. Each data point is the mean of three replications of 50 seeds. *Bars* represent standard deviation. Figure from Faria et al. (2) with permission from Oxford University Press.

dormancy may be repetitively broken and induced in the soil (see Chapter 5), it necessarily belongs to the class of physiological dormancy because of its reversible nature.

1.2. Seed Dormancy and the Environment of the Parent Plant

In addition to genetic factors, the environment may have a profound influence on the acquisition of dormancy during seed development. As yet, we cannot generalize as to which environmental factors result in consistent effects on dormancy over a range of species. In the literature, scattered reports can be found on a variety of environmental factors, including day length, light quality, mineral nutrition, competition, temperature, physiological age of plants, and position on the parent plant. A summary of these factors and their effects on dormancy can be found in Baskin and Baskin (4). Because of this, seed production for dormancy research is extremely demanding concerning precise control over the plant growth environment. For small-seeded species, such as *Arabidopsis*, the use of climate rooms may guarantee fairly constant environments but for larger species, such as tomato, seeds are usually produced in greenhouse environments, which are much more variable.

1.3. The Breaking of Seed Dormancy

As already exemplified above, different classes of dormancy require different methods of breaking dormancy. Under natural conditions, physical dormancy may be broken by factors such as fire, alternating high and low temperatures and humidity, or passage through an animal's digestive tract. In the lab, breaking of physical dormancy

usually requires fairly severe scarification, e.g., by mechanical means, such as abrasion or cutting, or by the controlled application of acid or boiling water. Physiological dormancy may be broken by dry after-ripening for often extended periods of time. After-ripening may be accelerated by elevating the storage temperature, but this also accelerates seed aging. A very common method to break dormancy is the exposure of imbibed seeds to cool temperatures between 2 and 10°C (cold stratification or moist chilling). In many species, including *Medicago*, *Arabidopsis*, and tomato, a few days are sufficient. However, seeds from species with a much deeper (physiological) dormancy may require several months of cold stratification to break dormancy (see Chapter 4). Seeds with morphophysiological dormancy may require a combination of moist-chilling and warm (>15°C) stratification to break physiological dormancy and promote late maturation or pregerminative (i.e., prior to radicle protrusion) growth of the embryo, respectively.

Gibberellins (GAs) are generally very effective in the breaking of dormancy. Application of GAs may often circumvent the need for "conventional" dormancy-breaking factors, such as moist chilling, light, and nitrate. However, it should be noted that seeds with deep physiological dormancy may not respond to GAs and require long periods of moist chilling (1).

1.4. The Assessment of Seed Dormancy

Although the assessment of dormancy of a batch of seeds seems straightforward, the results may lead to erroneous conclusions. It is common practice to subject seeds to a standard germination test at a certain temperature thought to be optimal for germination for a certain period of time, after which germination is counted. If germination is 100%, it may be concluded that the seeds are nondormant and at 0% germination, they are considered fully dormant. Any scores between these values are interpreted as "partially dormant." However, dormancy is very much defined in relation with the environment (5). For example, seeds with little dormancy have a much wider range of permissive temperatures for germination than seeds with deeper dormancy. Thus, seeds may appear dormant at one temperature and nondormant at another. When comparing different accessions, mutants, or cultivars of a species, a simple end-point germination test may not be sufficient to discriminate differences in dormancy (Fig. 2a). A more discriminative test assesses germination at several temperatures in order to estimate the width of the temperature window for germination. The generation of complete germination-over-time curves may be more discriminative, even at a single temperature. The more sensitive parameter to be determined is the time to 50% germination, t_{50} (Fig. 2b). In extreme cases, it can be seen that different seed batches that were originally assessed as nondormant (100% germination) are now displaying a range of dormancy levels (Fig. 2b).

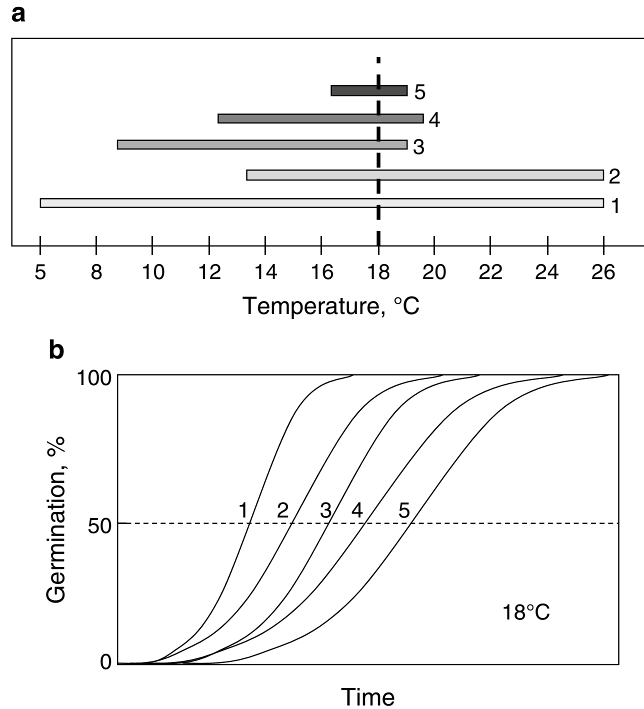

Fig. 2. A comparison of seed batches with different levels of dormancy. (**a**) Germination of five seed batches with different levels of dormancy. A wide thermal range is associated with a low dormancy level, and a narrow thermal range indicates deeper dormancy. Dormancy level increases with *gray scale* in figure. *Dotted line* indicates temperature at which all seed batches germinate, suggesting an absence of dormancy. (**b**) Germination-over-time curves of the five seed batches at 18°C, demonstrating differences in dormancy expressed in different t_{50} values, but not in final total germination.

2. Materials

2.1. Seed Production, Harvesting, and Storage

Required materials for seed production depend on the species. Here, only some general recommendations are given. For specific details, the reader is referred to the handbooks or common protocols for the species under study, e.g., *A. thaliana* (6) and *M. truncatula* (7).

1. Plant growth conditions should be as controlled as possible. This can be attained by using climate rooms for the smaller species or greenhouses for larger species. However, it is almost impossible to produce homogeneous plant populations at different points in time because even under controlled conditions, seasonal variation cannot be eliminated (see Note 1).
2. Besides the growing space, all materials, such as soil, pots, nutrition, watering, and irradiation, should be as constant as possible throughout the plant population (see Note 2).

3. Standard refrigerator-type incubator set at 20°C with a container on the bottom shelf containing a saturated solution of magnesium chloride, creating an atmosphere of 32% RH (see Note 3).
4. An oven with temperature set at 103°C for moisture content determination.

2.2. Assessment of Seed Dormancy

1. Several incubators to create a range of germination temperatures with a minimum of three, e.g., 10, 20, and 30°C. Light intensities and light regimes should be the same in each incubator. Alternatively, a thermogradient table may be used (see Note 4).
2. Gibberellic acid 4 + 7 (e.g., Berelex, ICI, UK).
3. Petri dishes of 50 or 90 mm in diameter or larger boxes, depending on seed size.
4. Filter paper (e.g., Whatman No. 1) with circle diameters of 50 and 90 mm, in larger sheets or in harmonica sheets of paper for large seeds.
5. Forceps, stereo microscope for small seeds.
6. Optional: Software to generate germination-over-time curves and parameters. For example, SeedCalculator (Plant Research International, Wageningen, The Netherlands).

2.3. Dissection of Seeds to Study the Contribution of Seed Components to Dormancy

1. Forceps, tweezers, scalpel, and razor blades to dissect seeds.
2. Dissection microscope.
3. Liquid nitrogen.

2.4. Determining Viability of a Seed Batch

1. Forceps, tweezers, scalpel, and razor blades to dissect seeds.
2. Dissection microscope.
3. 2,3,5-triphenyltetrazolium chloride (tetrazolium): 1% solution (v/v).

3. Methods

3.1. Seed Production, Harvesting, and Storage

1. Growing plants for seed production should be completely randomized. This means that plants within a climate room or greenhouse should be moved to another position within this space on a daily basis. Greatest spatial effects can be expected along edges of the plant population (see Note 5).
2. When comparing different genotypes, all of them should be grown in the same space at the same time to synchronize developmental periods. Yet, differences in flowering time may result

in (slightly) different periods of seed development and, hence, different levels of dormancy. Also, when seed harvesting is done from all plants of one genotype at the same time, seeds will not be equally mature. Therefore, plants/flowers should be tagged at the start of flowering (see Note 6).

3. After cleaning and initial rapid drying, e.g., under a forced stream of air, harvested seeds should be equilibrated at 32% RH for several days to reduce moisture content to approximately 5–7%, on a dry weight basis, before hermetic storage at –20°C or preferably –80°C. The drying time depends on seed size (see Note 7).
4. In order to avoid freezing damage, the seed moisture content should be low enough. Therefore, the seed moisture content must be determined. Preweighed seeds are placed in an oven at 103°C for 16 h and weighed again. The moisture content can then be calculated on a wet or dry weight basis.
5. It is essential that dormancy of the seed batches is determined at time zero (i.e., at harvest). This data serves as an important reference for comparison with results from other studies and for changes in dormancy during dry storage.

3.2. Assessment of Seed Dormancy

1. The type of dormancy can best be explored by applying a number of treatments that are known to break dormancy. For example:
   (a) Dry after-ripening, moist chilling, light, nitrate, or combinations of these factors may break *physiological dormancy*.
   (b) Whole or partial removal of seed coat may break *physical dormancy*.
   (c) A combination of (a) and (b) may break combinational dormancy.
   (d) A combination of moist-chilling and warm "stratification" (a period of warm temperature, such as 25°C, in which seeds are kept moist) may break *morphophysiological* dormancy.

   It is impossible to give more detailed information because there is a wide variation in the dormancy-breaking requirements of different species. The above described strategy generally reveals the type of dormancy. However, the more intense a certain type of dormancy, the longer the treatments or the more factors required to break dormancy. For example, physiological dormancy may be subdivided into deep, intermediate, and nondeep physiological dormancy. These three levels require 3–4 months, 2–3 months, or 1 week of moist chilling, respectively, to break dormancy (ref. 1 and see Note 8).
2. Once the type of dormancy of a certain species is known, further analyses can determine differences in the depth of dormancy

among genotypes or the effect of certain environmental conditions. For this, it is important to test germination at different temperatures and generate complete germination-over-time curves (Fig. 2). Generating such a curve for a certain species or a genotype also reveals the minimum duration of the germination experiment. The experiment is not completed until maximum germination has been attained. For most of the model species, this duration is in the order of several days to some weeks, depending on the conditions. For example, germination at low temperature is generally slower than at higher temperature.

3.3. Dissection of Seeds to Study the Contribution of Seed Components to Dormancy

1. Dissect seeds rapidly and with a minimum damage into desired compartments. This means that seeds are subjected to as little stress as possible. Flash-freeze seed tissues in liquid nitrogen immediately after dissection. Seed material may then be stored frozen (−80°C) until further use (e.g., molecular analysis). It is recommended to practice seed dissection until results are satisfactory (see Note 9). It is possible to dissect even very small seeds, such as *Arabidopsis*, into embryo and endosperm and subject tissues to genomic analysis (8).

3.4. Determining Viability of a Seed Batch

It should be realized that dormancy research can be meaningful only if a reliable viability test is included. It is trivial, but evidently very important, to discriminate between dead seeds and nongerminating dormant seeds. Oddly enough, in many molecular studies of dormancy, this issue has been neglected.

1. A first inspection may already reveal whether nongerminated seeds are viable or not. Most viable seeds should resist the gentle pressure of forceps. If the seed is soft and can be mashed easily, it is probably dead. Viable embryos are usually white and firm.
2. A frequently used viability test is the application of gibberellins to induce germination. With the application of GAs, any requirement for dormancy-breaking compounds or treatments may be circumvented. However, deeply (physiologically) dormant seeds may not always respond to GAs (4). Furthermore, seeds with hard and/or thick seed coats may not respond if GAs cannot be taken up. GA_{4+7} is approximately 1,000 times more effective than GA_3, which is the most widely used gibberellin (9). An (prolonged) incubation of seeds in 100 μM GA_{4+7} or 1,000 μM GA_3 at the optimal germination temperature should be effective. It may be necessary to scarify the seeds to facilitate the uptake of GAs.
3. A vitality stain, such as tetrazolium, may be a very reliable, semiquantitative, viability test. Overnight staining of (scarified) seeds in a 0.1–1.0% tetrazolium solution at elevated

temperature (25–30°C) is usually sufficient for optimal staining. Viable seed tissues are stained brightly red, whereas dead tissues are not stained at all. Depending on the species, staining of only parts of the embryo may be acceptable, since embryos with some dead tissues can still develop into normal seedlings (10).

4. Notes

1. Rather than attempting to standardize growth conditions for seed production to compensate for seasonal variations, a standardized dormancy assay for a certain species is much more useful when comparing results from different locations and times. It is important to monitor conditions during seed production to find possible relations between the environment and dormancy of the mature seeds.
2. Attention should be given to possible temporal and spatial variations in light, nutrition, and watering. Even with a rigid randomization schedule, these variations may have an impact on dormancy, particularly when the duration of seed development is relatively short.
3. This is a very effective and inexpensive solution as compared to the purchase of incubators that have a controlled RH. Tables for equilibrium relative humidities of saturated salt solutions at different temperatures can be found in Sun (11).
4. Thermogradient tables need constant attention during germination experiments. Particularly at higher temperatures (>25°C), seed samples may dry out. Independent monitoring of temperature by placing T-probes near the seeds on the thermal plate is essential.
5. In order to eliminate edge effects, control plants may be placed along the edges of the plant population.
6. It may suffice to record the time of first flowering of each genotype and adjust the time of seed harvest accordingly.
7. There are reports of differences in seed quality as a result of the rate of drying (12). Thus, possible effects should be determined by comparing two drying rates, e.g., at different relative humidities.
8. This further subdivision into dormancy levels is solely based on practical considerations (1).
9. No matter which method is used, dissection of seeds always imposes stress to the tissues and, hence, may influence the molecular properties of the tissues, including gene expression.

Thus, dissection methods should be aimed at reducing stress. The applied stress is not only mechanical; long dissection procedures may also lead to (partial) dehydration of the tissues. It is, therefore, important to dissect seeds under high humidity, e.g., in a glove box at 100% RH.

References

1. Baskin, C. C., and Baskin, J. M. (2004) A classification system for seed dormancy. *Seed Sci. Res.* **14**, 1–16.
2. Faria, R., J.M., van Lammeren, A.A.M., and Hilhorst, H.W.M. (2005) Changes in DNA and microtubules during loss and re-establishment of desiccation-tolerance in germinating *Medicago truncatula* seeds. *J Exp Bot* **56**, 2119–30.
3. Amen, R.D. (1968) A model of seed dormancy. *Botanical Rev.* **34**, 1–31.
4. Baskin, C. C., and Baskin, J. M. (1998) Seeds: Ecology, biogeography and evolution of dormancy and germination. Academic Press, San Diego.
5. Vleeshouwers, L. M., Bouwmeester, H. J., and Karssen, C. M. (1995) Redefining seed dormancy: an attempt to integrate physiology and ecology. *J. Ecol.* **83**, 1031–7.
6. Rivero-Lepinckas, L., Crist, D., and Scholl, R. (2006) Growth of plants and preservation of seeds. In: Salinas, J., and Sanchez-Serrano, J.J., (eds) Arabidopsis protocols. Methods in Molecular Biology 323, 2nd edn. Humana Press, New York, pp. 3–12.
7. Barker, D.G., Pfaff, T., Moreau, D., Groves, E., Ruffel, S., Lepetit, M., Whitehand, S., Maillet, F., Nair, R.M., and Journet, E.-P. (2006) Growing *M. truncatula*: choice of substrates and growth conditions. In: Mathesius, U., Journet, E.P., and Sumner, L.W. (eds) The *Medicago truncatula* handbook. http://www.noble.org/MedicagoHandbook/
8. Penfield, S., Li, Y., Gilday, A.D., Graham, S., and Graham, I.A. (2006) Arabidopsis ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. *Plant Cell* **18**, 1887–99.
9. Groot, S.P.C., and Karssen, C.M. (1987) Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants. *Planta* **171**, 525–31.
10. Leist, N., and Kramer, S. (2003) ISTA working sheets on tetrazolium testing, Volume I – Agricultural, Vegetable and Horticultural Species; Volume II – Tree and Shrub Species. Volumes I & II, 1st Edition, 2003. ISTA, Switzerland.
11. Sun, W.Q. (2002) Methods for the study of water relations under desiccation stress. In: Black, M., and Pritchard, H.W. (eds) Desiccation and survival in plants. CABI Publishing, Wallingford, pp. 47–91.
12. Soeda, Y., Konings, M.C.J.M., Vorst, O., van Houwelingen, A.M.M.L., Stoopen, G.M.C.A., Maliepaard, C., Kodde, J., Bino, R.J., Groot, S.P.C., and van der Geest, A.H.M. (2005) Gene expression programs during *Brassica oleracea* seed maturation, osmopriming and germination are indicators of progression of the germination process and the stress tolerance level *Plant Physiol.* **137**, 354–68.