

Chapter 24 Viability Testing



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Summary

Most forms of animals and plants that are both visible to the naked eye and healthy are relatively easy to recognise as living or dead: this is rarely true of stored seeds. External appearance is virtually no guide to a seed's viability, age or vigour! As a consequence, specific tests to assess physiological status and likely performance are a necessity. There are broadly two types of seed test which do this. The 'germination' test, as the name implies, measures the proportion of seeds that are capable of germinating. This is generally the favoured test because it measures the characteristic which most seed users wish to recreate. The alternative is one of a number of 'viability' tests which permit seeds to be classified as either alive (= viable) or dead. Viability tests are usually much more labour intensive, but are selected principally because they yield quicker results. It is vital to appreciate that viability tests are not a direct measure of the number of seeds capable of germinating and that 'viable' and 'germinable' are therefore not synonymous.

This chapter considers the theoretical and practical aspects of measuring seed germination and viability, and assesses the relative advantages and disadvantages of each type of test. In the absence of a widely accepted relationship which defines 'viable' seeds from a 'germination' test and enables them to be compared with 'viable' seeds determined by a 'viability' test, it concludes with the proposal:

$$\% \text{ 'viable' seeds (at the end of a germination or viability test) } \approx \\ \% \text{ normal seedlings} + \% \text{ abnormal seedlings} + \% \text{ fresh (or fresh, stained) seeds}$$

This is consistent with the concept that a 'viable' seed is one which appears to be 'alive'.

Introduction

Few authors differentiate as carefully as they should between 'viable' and 'germinable'. As a consequence, seed 'viability' has unfortunately become a widespread euphemism for seed 'germinability'. This usage has created the false impression that a 'viable' seed is synonymous with a 'germinable' seed. It also implies that 'viability tests' measure the same property as 'germination tests'. But as Copeland and McDonald (1995) point out, 'viable' seeds are not necessarily capable of germinating into 'normal' seedlings, and therefore 'viable' and 'germinable' are not the same.

In a chapter entitled 'viability testing', and against a background of such confusion, it is necessary to begin with a discussion of terminology. The chapter will go on to consider the theoretical and practical aspects of germination tests and some of the most common viability tests. Finally, it proposes an equation which will allow 'viable' seeds determined in a germination test to be related to 'viable' seeds identified in a viability test.

Terminology: ‘Germinability’ vs. ‘Viability’

The basic premise of this chapter is that there are two types of test available to assess the potential performance of individual seeds or seed populations (‘seeds’ will be used to mean fruits or seeds). A ‘germination’ test as the name implies, is designed principally to determine the proportion of seeds that are specifically capable of germinating into ‘normal seedlings’ i.e., it is a measure of actual performance. In contrast, a ‘viability’ test is any method which determines the proportion of seeds which are alive (= viable) or dead i.e., it is more a measure of likely physiological status. The important distinction is that ‘germination’ tests demonstrate seed ‘germinability’; ‘viability’ tests only reveal whether seeds are alive (= viable). Box 24.1 contains further discussion of the concepts behind ‘viable’ and ‘viability’. Table 24.1 summarises exactly how the terms are used in this chapter.

Table 24.1 Seed viability and germinability definitions adopted for this chapter

Property, term, expression	Definition
Viability test	Any technique used to determine whether individual fruits/seeds appear to be dead or alive, and which enables the proportion of live fruits/seeds in a population to be estimated.
Viable seed (s)	One which is considered to have been alive – when given a particular viability test. (Or the proportion of a population which is estimated as likely to be alive after the application of a viability test to a representative sample of the bulk.)
Seed viability	The state of a seed/seed population being alive – when given a specific viability test. N.B. Most viability tests are destructive; they destroy the property they set out to measure!
Germination test	The technique used to determine whether individual fruits/seeds are specifically capable of germinating into ‘normal’ seedlings, and which enables the proportion of ‘germinable’ fruits/seeds in a population to be estimated.
Germinable seed (s)	One which has germinated at the end of a germination test. (Or the proportion of a population which is estimated as likely to germinate following the earlier application of a germination test to a representative sample of the bulk.)
Seed germinability	The state of a seed/seed population being germinated, when given a germination test.

Box 24.1 How to define the concepts of ‘viable’ and ‘viability’

Dictionary definitions of ‘viable’ nearly always include the word ‘alive’, or a phrase such as ‘capable of living’. Therefore, as a first approximation:

a ‘viable’ seed is one that is ‘alive’.

Now, most forms of animals and plants that are both visible to the naked eye and healthy are relatively easy to recognise as living or dead – but external appearance is virtually no guide at all to whether a seed is alive or not. It is therefore necessary to identify a seed property which might indicate whether an individual seed is dead or alive, and then apply a technique which can be used to measure the property. Such a method is called a ‘viability test’. This is where the subject becomes more complex. There are several properties which can be used to recognise whether a seed is alive or not – for example, biochemical reactions and tissue, cellular or sub-cellular characteristics may all provide some indication. And there is often more than one way to measure each attribute. It should therefore be no surprise at all that different viability tests (which can measure different seed properties) can give rise to different estimates of the proportion of seeds in a seed-lot which are alive.

But this is just the first part of the confusion. Seed death is rarely an instantaneous event; it is usually the culmination of a series of processes. Since it is not really known where to draw the line in the processes of deterioration between seeds which are ‘alive’ and those which are ‘dead’, this is an additional reason why different viability tests can give rise to different estimates of viable seeds. Hence it is necessary to refine the above notion of a ‘viable’ seed to:

a ‘viable’ seed is one that is ‘alive’ – when given a particular ‘viability’ test.

Finally, it is important to appreciate that nearly all viability tests are destructive – they kill any live seeds they were trying to identify. This has two important consequences. Firstly, it means that strictly speaking a ‘viable’ seed is one which was alive – when given a particular viability test’. Secondly, it means that the only way to derive a measure of the likely viability percentage of a seed-lot is to apply a viability test to a sub-sample of the population and estimate the proportion of the population which might be alive. Hence a more accurate definition is:

a ‘viable’ seed is one which is considered to have been ‘alive’ – when given a particular ‘viability’ test. (Or viable seeds are that proportion of a population which is estimated as likely to be alive after the application of a viability test to a representative sample of the bulk.)

Choosing Between a Germination or Viability Test

Farmers, horticulturalists, nursery-managers, conservationists and seed scientists are generally most interested in wanting to know what percentage of seeds in a seed-lot will germinate. A germination test is the most direct and reliable method for determining this characteristic. However, the next two sections describe two seed characteristics (dormancy and recalcitrance) which can make a germination test impossible or impractical. Table 24.2 summarises the reasons why in these circumstances a viability test may have to be selected instead of a germination test.

Table 24.2 Seed characteristics which may favour selection of a rapid viability test

Seed characteristic	Reason for selecting rapid viability test
Dormant seed – incapable of germination unless pretreated	<ul style="list-style-type: none"> • Dormancy breakage pretreatment may be unknown • Pretreatment duration may exceed time available
Dormant seed – slow to germinate unless pretreated	<ul style="list-style-type: none"> • Optimum germination conditions may be unknown • Duration of germination test may exceed time available
Slow germinating seed	<ul style="list-style-type: none"> • Significant deterioration of seed-lot may occur over course of germination test on sample.

Dormant (and Slowly Germinating) Seeds

The seeds of many species can be incubated under apparently ideal conditions for germination, yet remain outwardly inactive. Even in the presence of ample water, good aeration and a suitable temperature for growth, the seeds either fail to germinate at all, or only germinate very slowly. Such seeds are said to exhibit seed dormancy [see reviews by Barton (1965a, b), Baskin and Baskin (1998), Bewley and Black (1994), Lang (1965), Nikoleava (1967a, b), Stokes (1965) and Vegis (1964)].

In nature, plants have evolved numerous forms of seed dormancy for ‘monitoring’ their environment and thereby increasing their chances of emerging in the most favourable season for seedling growth. Passage through a bird or animal gut, cycles of burial followed by re-exposure to light, and transitions between one (or more) cold winters and warmer springs are just

Table 24.3 Potential seed testing techniques to overcome seed dormancy

1. Incorporate germination stimulant into germination test.
 - a) Plant growth regulators, e.g., gibberellic acid, ethylene, cytokinins.
 - b) Chemical treatment, e.g., $\text{CS}(\text{N H}_2)_2$, H_2O_2 , KCN, KNO_3 , Na NO_2 , NaOCl ,
 - c) Special gases, e.g., NO_2
2. Apply pre-sowing, dormancy breakage treatment (pretreatment).
 - a) Chip, puncture, scarify, or abrade fruit/seed coat either physically, chemically or biologically (overcomes physical dormancy)
 - b) Wash fruits/seeds to remove inhibitors.
 - c) Incubate moist seed at $1-5^\circ\text{C}$ (Prechill)
 - d) Incubate moist seeds under alternate warm (ca $15-20^\circ\text{C}$) then cold (ca $1-5^\circ\text{C}$) conditions.

some of the stimuli used to bring about natural dormancy breakage which leads to subsequent germination. One seed testing skill is to identify and apply a germination stimulant during the germination phase, or find and apply a separate, artificial 'pretreatment' which aims to mimic natural conditions that overcome seed dormancy and stimulate subsequent germination under laboratory conditions. Table 24.3 lists some of the techniques that can be used. Unfortunately, none is universally effective, and effective pretreatments are not known for all species. In addition, for others, the pretreatment duration alone, or the combined pretreatment and germination duration, is much too long for the practical purpose of a laboratory germination test. In these instances, there may be no alternative but to select a more rapid viability test.

Sampling

It should be assumed that every seed-lot is made up of a mixture of components such as pure seed, winged seed, seed of other species and inert matter. It should also be assumed that none of the components is mixed uniformly. As a prelude to seed germination or viability testing, it is therefore vital to mix the seed-lot as uniformly as possible, and then take a random sample. The detailed instructions on sampling contained in the International Rules for Seed Testing (ISTA, 1999) and Rules for testing seeds (AOSA, 1995) may be intended for commercial seed-lots with higher levels of contamination than most researchers and gene-bank staff will encounter, but the principle of obtaining a representative sample still applies. The overarching principle is that no matter how accurately the germination or viability test is carried out, it can only reflect the quality of the sample.

Methods of Measuring Seed Germination and Viability

Several methods can be used to test seed for germination and viability (Table 24.4). Germination and viability percentages are used to compare the quality of different seed-lots, and/or estimate the proportion of seeds which are most likely to yield plants under good growing conditions. This section will now consider the theoretical and practical aspects of measuring each.

Table 24.4 Methods which can be used to test fruits/seeds for germination and viability

General test type	Specific test type
Germination test	Germinate without pretreatment Germinate incorporating germination stimulant (see Table 24.3, section 1) Apply dormancy breakage pretreatment, then germinate (see Table 24.3, section 2)
Rapid viability test	Cut X-ray (including X-ray contrast technique*) Tetrazolium Excised embryo Electrical conductivity Vital stain
* not discussed in this chapter	

1. Germination Test

1.1. Objective(s)

The primary objective of the germination test is to determine the maximum potential of a seed sample (and by inference the seed-lot) to produce normal, healthy seedlings. The result is usually reported as a percentage of 'normal' germinants (or seedlings) and referred to as a 'maximum germination percentage' or 'germination capacity'.

A secondary objective may be to identify, classify and report the percentages of other categories of ungerminated seeds and malformed seedlings. A tertiary objective is sometimes to test for the presence or level of seed dormancy, or to confirm an absence of seed dormancy.

1.2. Principle(s)

The general principle of the germination test is to incubate seeds (usually a known number, but see ‘weighed replicate test’ below) under a single, standard (preferably optimal) set of environmental conditions, to achieve the quickest, most uniform and complete germination possible for the majority of seed-lots of a particular species.

The following must be considered when selecting a suitable germination method:

- Is seed dormancy suspected?
- Is the deterioration rate of the bulk comparable to the length of the germination test on the sample?
- Which environmental parameters are important for germination?
- What level of control is required for each?
- What size are the seeds to be tested?

All these features will influence the sophistication (and hence cost!) of the equipment required, the amount of space needed, the likely duration of the germination test, and whether a germination test is appropriate at all.

Where it is suspected that either significant deterioration of a seed-lot will take place during the period of a germination test, or dormancy will prevent a germination result from being obtained within an acceptable period of time, then various solutions are possible. Either a suitable germination stimulant must be identified and incorporated during the germination test (see Table 24.3, section 1) or a suitable dormancy breakage method will need to be found and applied as a pretreatment before the germination phase (see Table 24.3, section 2). If a germination test or combined pretreatment and germination test is likely to take more than about 6–8 weeks, then in commercial seed trade, a rapid viability test is almost always selected. However, seed bank germination tests (and especially those carried out on wild species) may be incubated for much longer before alternative tests are considered.

The number of environmental parameters which need to be controlled during a germination test are relatively few. There are really only three essential requirements for the germination of any live, non-dormant seed – **a suitable temperature, adequate moisture and sufficient aeration**. In this case, it is usually only necessary to provide illumination for the development of normal, healthy seedlings. In some cases, light and alternating temperatures may be required as additional germination factors. For dormant seed populations, specific dormancy breaking factors such as cold or warm stratification, seed coat scarification or the application of chemicals and growth regulators, may be necessary.

The exact level of control for each environmental parameter will partly reflect the accuracy demanded for the germination results, partly the sensitivity of the particular seeds to each parameter, and partly the germination facilities

available. For some test purposes, in certain parts of the world, outdoor conditions may be considered suitable. On other occasions, ambient office or laboratory conditions or a glasshouse or polythene tunnel, may be acceptable. In general, controlled-environment rooms and incubators provide the most accurate levels of control and certainly the most reproducible results.

Temperature and lighting

The control of temperature and lighting is frequently a built-in feature of the germination facility used. For example, controlled-environment rooms and relatively large, walk-in constructions frequently house their own lights and air-conditioning. The various sizes of germinator and incubator cabinet also incorporate their own refrigeration units, heaters and lights (and sometimes even humidifiers and dehumidifiers). It is important that temperature control is accurate and reliable, and is maintained as uniformly as possible throughout the facility, which therefore needs good air circulation and effective insulation.

The temperature at which the germination facility is run will depend upon the species under test. But although many species germinate optimally at specific constant temperatures, it is frequently beneficial to use an alternating temperature. This is because alternating temperatures often have the overall effect of increasing germination speed and final percentage. (There is some debate about whether this is due to alternating temperatures acting as a mild 'dormancy breakage' agent during the germination phase, thus overcoming any last remnants of dormancy, and hence maximising germination; or whether they act as a 'germination stimulant'.) Whatever the cause, the consequence is that the seed testing associations (ISTA, 1999; AOSA, 1995) most frequently prescribe a daily alternating temperature cycle of 30°C/20°C.

The provision of light during a germination test can have two effects on seeds. The main purpose is to avoid the growth of etiolated seedlings and promote the development of normal, healthy, chlorophyllous seedlings. Continuous illumination can be provided, but seeds are generally exposed to 8–16 h of light in any 24 h period. Where an alternating temperature regime is used, light is normally given during the warmer, day phase rather than the colder, night period. Secondly, in the same way that alternating temperatures may stimulate germination of weakly dormant seeds, light can also stimulate germination rate and final germination percentage. Light quality is especially important and sources of illumination rich in so-called 'red' light, such as fluorescent tubes, are generally most effective. As a rule, tungsten light bulbs should be avoided as these emit more 'far-red' light which tends to inhibit germination. The longer wavelengths in the emission spectrum of tungsten bulbs also mean that they generate more heat which interferes with temperature control.

Moisture and aeration – substrates and media

Seeds do not normally germinate well when either totally or partially immersed in water – probably because submersion restricts gaseous exchange. Hence, moisture and aeration can be closely linked. Seeds are therefore either incubated on, or in a material which is not only capable of providing adequate moisture but one that also allows respiration. Suitable materials include absorbent papers (such as filter paper, blotting paper or paper towels), agar-agar, or loose particles (such as sand, peat, peat-alternatives, soil, moss, vermiculite, perlite). When seeds are placed on the material, it is usually referred to as a *substrate*. When seeds are placed in the material, it is usually called a *medium*.

It is also important to provide a constant supply of moisture. Repeated wetting and drying are not conducive to optimal germination. Figure 24.1 shows a plastic germination box which not only provides a continuous supply of water but avoids the tedium of having to frequently ‘top-up’ Petri-dishes lined with filter paper leading to the possibility of causing intermittent waterlogging. This particular box is c. 175 × 113 × 45 mm and is initially filled with a reservoir of 150 ml which rarely needs topping up in 8 weeks, irrespective of temperature. The system is most useful for smaller-seeded species which are relatively quick to imbibe and germinate.

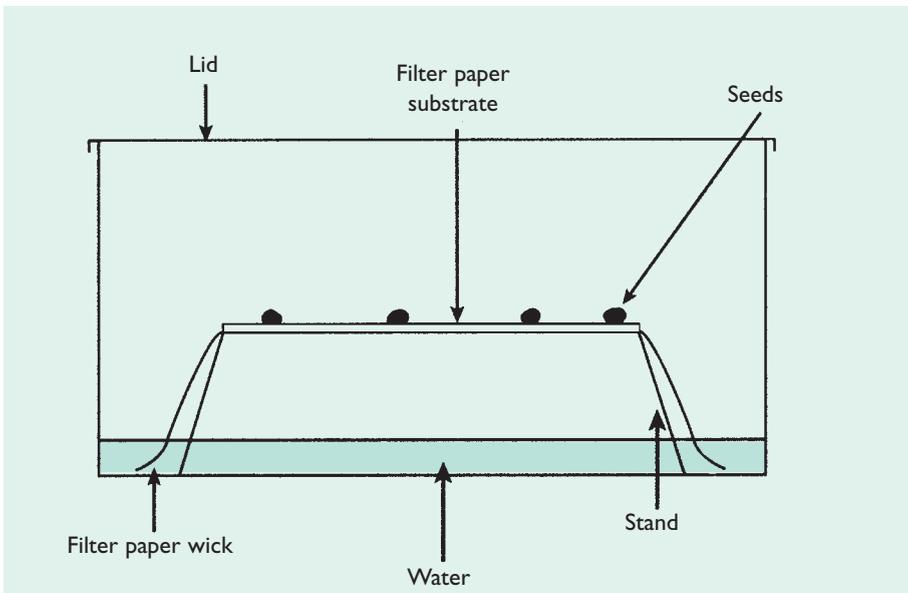


Figure 24.1 Plastic germination box showing reservoir and wick system, which provides a continuous supply of water to seeds.

For longer germination tests, a seed tray containing a mixture of peat and sand (2:1 vol.: vol. ratio), wrapped in a transparent 500 gauge polythene bag, is often best. Damp peat and sand provides ideal conditions of moisture and aeration and tends to better prevent fungal infection spreading from one seed to another than is the case with absorbent papers. Transparent polythene allows light to reach seedlings which break the surface of the medium.

Other environmental variables

Occasionally, it may be necessary to control other environmental variables to bring about dormancy breakage e.g., adjusting gaseous composition, or applying plant growth regulators or other germination stimulants.

Containers

Seed trays, flower pots, Petri-dishes, plastic boxes and almost any other vessel can be used as suitable containers for germination tests. The final choice is usually determined by a combination of what is available, seed size, what substrate or medium will be used, and how long the germination test will last.

Weighed replicate test

The weighed replicate test is an extremely valuable 'special-case' germination test. Above, it was stated that germination tests are usually performed on a specified number of seeds. However, there are occasions when this is either impossible or impractical. For example, in some genera e.g., *Eucalyptus*, the seeds may be so small that it is impossible to distinguish pure seed from inert matter. In other genera e.g., *Betula*, the small size of the seeds, their lightness and problems of static electricity, can make it too time-consuming to identify and count specified numbers of pure seed. In yet other cases, the effort of counting specified numbers of seeds may be unjustified because of very high percentages of empty seed. It is then extremely useful to weigh out seed samples that are likely to contain about 150–200 seeds and treat each as a germination test replicate. This is the so-called 'weighed replicate' germination test. In the case of weighed replicate tests, results cannot be reported as a germination percentage but only as 'germinable seeds per kg'.

1.3. Practice

Chapter 5 of the ISTA Rules (ISTA, 1999) and Chapter 4 of the AOSA Rules (AOSA, 1995) are two of the most detailed sources of information on laboratory germination testing. When any of the species included in either set of rules is being tested for official purposes, the methodology described must be strictly adhered to. However, for other species and purposes, the principles described above can be used in conjunction with the seed characteristics to determine the exact combinations of container, substrate or medium, temperature regime, etc. Ellis *et al.* (1985) is an extremely valuable source of specific pretreatment and germination information on a wide range of less commercially important species. It is important to stress that no single type of germination apparatus or method is ideal for the seeds of all species.

Box 24.2 summarises the bare essentials of how to do a germination test. Seeds and seedlings are visually inspected at regular intervals throughout the germination test, but it is vital to appreciate that no germination test is ever completed until not only the seedlings but also the ungerminated seeds have been classified. Box 24.2 concentrates especially on how to classify the appearance of seeds and seedlings at the end of the test. This is because there are a number of categories where the ISTA Rules are either unclear or inconsistent between the germination test and other tests. In these instances, proposals are made to provide additional guidance/clarification and also to provoke further thought on this important subject.

2. Cut-Test

2.1. Objective(s)

The objective of the cut-test is to determine the percentage of viable seeds in a seedlot by:

- 1) cutting open a sample;
- 2) examining and categorising the appearance of the tissues; and
- 3) interpreting whether individual seeds are dead or alive.

2.2. Principle(s)

The general principle behind the cut-test is that live fruits/seeds have a combination of three characteristics. Firstly, they are outwardly undamaged. Secondly, they contain all the tissues considered essential for germination. Thirdly, the tissues must look firm, fresh, healthy and apparently alive. The following fruits/seeds are not viable: which are either entirely empty; where a significant proportion of the storage tissues are damaged; or where the embryo is missing, under-developed, shrivelled, mouldy or insect damaged.

References to ‘cutting’ are liberally scattered throughout both the ISTA and AOSA Rules, but neither text devotes a chapter to the subject or provides an unambiguous description of how to classify the appearance of different seed tissues. Only the AOSA Rules provide any guidance at all on how to interpret which seeds are ‘viable’.

ISTA Rules 5.2.7, 5.2.7.A and 5.6.5, all refer to using ‘cutting’ to classify ‘ungerminated’ seeds at the end of a germination test. Rule 5.6.5.A.3, headed ‘Ungerminated seeds’ (implying a germination test has already been carried out) rather ambiguously states that ‘the following methods may be used before the germination test’, not a model of clarity!

In addition, few of the ‘definitions’ for different categories of ‘ungerminated’ seeds can be called precise. For example, ISTA Rule 5.6.5.A.3 para 3 states the following :-

“Dead seeds: obviously dead (soft, mouldy) seeds are counted and reported as such on the ISTA Certificate”

Box 24.2 How to do a germination test (an ISTA test)

Phase 1 – Assess likelihood of seed dormancy. If dormant, select and apply suitable pretreatment or aim to incorporate suitable stimulant in germination test.

Phase 2 – Take a random sample of a known number of fruits/seeds (or use a weighed replicate test).

Phase 3 – Select suitable medium/substrate to place fruits/seeds on or in and incubate at a suitable temperature, with ample water and good aeration for an appropriate duration.

Phase 4 – Inspect fruits/seeds and differentiate into categories described below.

Appearance	Category/ Sub category
ISTA rules 5.2.3, 5.2.4, and associated appendices provide several pages describing the characteristics of a normal seedling.	Normal seedling
ISTA rules 5.2.5 and associated appendices provide several paragraphs defining the characteristics of an abnormal seedling.	Abnormal seedling
ISTA rules 5.2.7 and associated appendices provide several paragraphs defining the different categories of 'ungerminated seeds'.	'Ungerminated seeds'
<p>ISTA rule 5.2.7 – 1 states: 'fruits/seeds which remain hard at the end of a germination test, because they have not absorbed water'.</p> <p><i>Propose – 'Filled fruits/seeds (see Box 24.3) with a rigid, impermeable coat, which has prevented imbibition by the end of a germination test. If there are 5% or more 'hard' seeds at the end of a germination test, then their hardseededness should be overcome (see Table 24.3, para 2 i), or their living status should be observed when cut open (see Box 24.3) or confirmed with a TZ test (see Box 24.6).'</i></p>	Hard
<p>ISTA rule 5.2.7 – 2 states: 'fruits/seeds, other than hard seeds, which have failed to germinate under the conditions of the germination test, but which remain clean and firm and have the potential to develop into a normal seedling'.</p> <p><i>Propose – 'Filled fruits/seeds (see Box 24.3) which are imbibed at the end of a germination test and appear clean, firm, fresh, healthy and apparently alive when cut open. If there are more than 5% 'fresh' seeds at the end of a germination test, their living status should be confirmed e.g., with a TZ test (see Box 24.6). If there is any doubt as to whether a seed is fresh or dead, then it must be classified as dead.'</i></p>	Fresh
<p>ISTA rule 5.2.7 – 3 states: 'fruits/seeds which at the end of the test period are neither hard nor fresh nor have produced any part of a seedling'.</p> <p><i>Propose – 'Fruits/seeds (which are neither hard nor fresh) but are discoloured, soft, rotting, unhealthy (or any combination of these) due to tissue decay, which may only be visible when cut open. (Similar to 'Perished' Box 24.2).'</i></p>	Dead
<p>ISTA rule 5.2.7A – 4 states: 'fruits/seeds which are completely empty or contain only some residual tissue.'</p> <p><i>Second part of sentence appears unquantifiable, and therefore propose: 'Fruit/seed cavity contains less than 50% of fruit/seed tissue' – See Box 24.3 for further details and explanation of why this category excludes 'insect-damaged' and 'mouldy'.</i></p>	Empty
ISTA Rule 5.2.7.A – 4 & see Box 24.3.	Embryo-less
ISTA Rule 5.2.7.A – 4 & see Box 24.3.	Insect damaged
ISTA Rule 5.2.7.A – 4 & see Box 24.3.	Mouldy

Box 24.3 How to do a cut-test. (Not clear from ISTA Rules, whether this is an ISTA test or not)

Phase 1 – Take a random sample of e.g., 100 fruits/seeds and cut open with a sharp knife, surgical scalpel or (if necessary) saw.

Phase 2 – Inspect contents and use appearance to differentiate between categories described below (at the same time consciously considering the ultimate interpretation of whether fruit/seed is more likely to be viable or dead).

Appearance	Category/ Sub category	Interpret
<p>Fruit or seed containing less than 50% fruit/seed tissue (Based on ISTA rules 5.2.7.A, 5.6.5.A.3 & 14.6.2).</p> <p><i>N.B.1 where 'emptiness' is due to significant underdevelopment (i.e., missing, stunted or deformed embryo or storage tissues).</i></p> <p><i>N.B.2 where 'emptiness' is due to:- significant shrivelling (i.e., complete fruit/seed contents present, but shrunk).</i></p> <p><i>N.B.3 where emptiness is due to significant insect attack (e.g., presence of insect larva(e), frass, or an entrance/exit hole).</i></p> <p><i>N.B.4 where emptiness is due to significant fungal/bacterial attack (e.g., presence of fungal hyphae).</i></p>	<p>Empty</p> <p>Underdeveloped</p> <p>Shrivelled</p> <p>Insect damaged</p> <p>Mouldy</p>	<p>Non-viable</p>
<p>Incomplete endospermic seed in which storage tissues are present but there is no embryo cavity or embryo (Based on ISTA rules 5.2.7.A & 5.6.5.A.3).</p> <p>Fruit/seed coat, sufficiently cracked, broken or damaged to jeopardise living status of seed (Based on ISTA rule 14.6.2).</p> <p>Fruit/seed with evidence of enough missing, damaged, discoloured, soft, rotting, unhealthy tissues to jeopardise living status (i.e., not clean, firm, fresh, healthy and apparently alive) (Based on ISTA rules 5.2.7, 5.2.7.A, 5.6.5.A.3 & 14.6.2 and opposite of 'Filled' below).</p> <p><i>Where 'death' is due to insect or fungal damage, this should be reported, since these are both sources of damage which can spread to live seeds.</i></p>	<p>Embryo-less</p> <p>Physically damaged</p> <p>Expired</p> <p>Insect damaged</p> <p>Mouldy</p>	
<p>Fruit/seed containing all tissues essential for germination (i.e., complete embryonic axis [+ endosperm where expected] appears intact, firm, fresh, healthy and apparently alive. No more than one third of the storage tissues appear unhealthy. If there is any doubt about a seed being 'filled' then it must be classified as 'dead' – non-viable) (Based on ISTA rule 14.6.2).</p> <p><i>Where unhealthy tissues are due to insect or fungal damage, this should be reported, since both sources of damage can spread to live seeds.</i></p>	<p>Filled</p> <p>Insect damaged</p> <p>Mouldy</p>	<p>Viable</p>

What about the seeds which are not ‘obviously dead’? Since ‘cutting’ is used to classify ungerminated seeds at the end of every germination test, and to eliminate ‘obviously’ dead fruits during the dissection stages of the tetrazolium test (TZ), the excised-embryo test (EE) and almost every other viability test, it is clear that cut-test instructions in the ISTA and AOSA Rules need significant improvement and clarification.

2.3. Practice

A procedure for ‘cutting’ seeds and assessing ‘cut’ seeds is described in several places within the ISTA rules (ISTA, 1999), for example, paras 5.2.7, 5.2.7.A, 5.6.5, 5.6.5.A.3 and 14.6.2. But unlike other ISTA ‘tests’, the technique does not have a chapter of its own. It is therefore difficult to know whether ISTA consider it to be a test in its own right. However, despite the ‘official’ uncertainty of whether cutting should only be applied to classify ‘ungerminated’ seeds at the end of a germination test, or whether it constitutes a test in its own right, it is unofficially the simplest, almost certainly the oldest, and probably the easiest method for assessing viability in the world. Unfortunately, many would say it is also the crudest technique! Box 24.3 provides guidance on how to carry out the test, classify seeds with different appearances and ultimately interpret whether they are likely to be dead or alive. It is based largely on the paragraphs above, and is divided into two main phases. But the text has also been modified so that it can be applied to seeds that are cut-open before or after a germination test. In addition, it attempts to remove some of the vagaries and ambiguity of the ISTA rules and make explicit links between the appearances of different seed categories and whether they are viable or not.

There is an important consideration of human perception when applying this evaluation in practice – it is generally much easier to identify seeds which are definitely ‘dead’ rather than those which are probably ‘viable’. Hence, consciously or sub-consciously the first seeds to be recognised in this test are those that have the distinct appearance of being dead. Although Box 24.3 reads from left to right and implies that the interpretation of whether seeds are dead or alive is the final stage, this may not be the case. In practice, the first stage may be the mental identification of dead seeds. The second stage may be the categorisation of these obviously dead seeds as mouldy, expired, etc. Thirdly, the most fully developed, completely intact, cleanest, firmest, freshest seeds may be categorised as ‘filled’. And only finally, by a process of elimination are these interpreted as most likely to be alive. The over-riding principle in this decision making is that if there is any doubt about a seed being ‘filled’ then it must be classified as ‘dead’ or, strictly speaking, ‘non-viable’.

Accepting the above as the most likely sequence of events in the classification of seeds in a cut-test is an important conceptual point. It means that the evaluation of a cut-test tends to be subjective. This has important consequences. The visual classification and interpretation of fruits and seeds is not only used in the cut-test, it is carried out as the final phase of classifying

ungerminated seeds at the end of every germination test, and as we will see later features in the preliminary stages of fruit/seed preparation for TZ, EE and almost every other ‘viability’ test. In other words, the accuracy of virtually all germination and viability tests relies on the somewhat subjective categories defined in the cut-test!

Another practical point to bear in mind during the assessment of the cut-test is to differentiate between endospermic and non-endospermic seeds. In endospermic seeds, the storage reserves are held in the tissues of the endosperm. Even in the complete absence of an embryo, endosperm can develop to fully occupy the fruit cavity. In such instances, although the seed is technically ‘empty’, the fruit appears full, therefore the term ‘embryo-less’ is probably more appropriate and certainly more informative. This term is borrowed from the ISTA Rules (paras 5.2.7.A and 5.6.5.A.3), but they do not make the distinction between ‘empty’ and ‘embryo-less’ as explicit.

The current ISTA Rules 5.2.7.A, 5.6.3.5.A.3 and 14.6.2 all imply (but importantly do not specify) that insect damaged seeds are dead. As a consequence, it is usually accepted that most percentages of ‘insect damaged’ seeds only include fatally-infested seeds. However, although some insects and fungi may have already caused seed death at the time of testing, others may have damaged, but not yet killed their host/victim. In this latter case, the harmful agent may go on to cause seed death, and in both instances the harmful agents may be capable of spreading to other seeds. The presence of insects clearly affects the quality of a seed-lot in more than one way. In this chapter, it is therefore considered important to record as separate percentages, lethal insect damage which has been responsible for emptiness or death; and non-lethal insect damage where at the time of testing, filled seeds merely have an insect presence which may subsequently prove fatal, or spread to other seeds. Box 24.3 describes sub-categories designed to give some guidance on this.

3. X-ray Test

3.1. Objective(s)

The objective of the X-ray test is to determine the percentage of ‘filled’ (= potentially viable) seeds in a seed-lot by:

- 1) X-raying a sample,
- 2) examining and categorising an X-radiograph of the seed contents.

3.2. Principle(s)

The general principle is to use X-rays generated within an apparatus such as that shown in Figure 24.2, to reveal the internal structure of seeds. In the simplest approach, seeds are merely X-rayed without undergoing any preliminary treatment, and the varying shades of light and dark on a photographic image enable filled, empty, and insect- and physically-damaged seed to be identified (Simak and Gustafsson, 1953) (Figure 24.3).



Figure 24.2 Introducing a seed sample, spread out on photographic paper into X-ray apparatus.

Alternatively seeds can be imbibed in solutions containing various heavy metal ions and then X-rayed – the so-called X-ray contrast technique. The principle behind this is that the heavy metal ions enter damaged, dying and dead tissues more easily than live tissues. Therefore when the soaked seeds are X-rayed, the dead tissues contain higher concentrations of heavy metal ions and are more X-ray opaque. The depth and pattern of shading in different areas is used as a means of distinguishing living from dead tissues. Hence, potentially viable versus non-viable seeds can be identified (Simak, 1957; 1991).

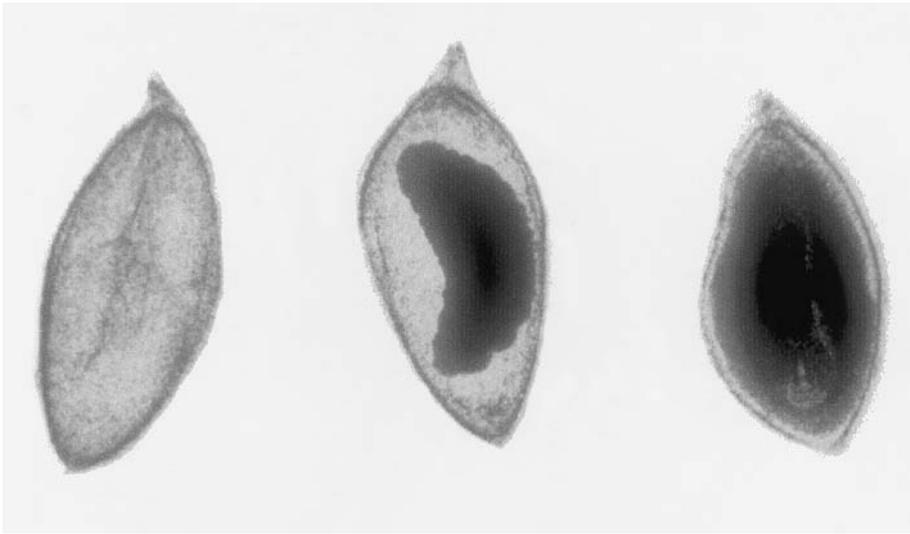


Figure 24.3 X-radiograph of Douglas fir (*Pseudotsuga menziesii*) seeds, showing (left to right) 'empty', 'insect-damaged' and 'filled' seed.

3.3. Practice

X-radiography is especially useful for studying the seed-lots of many wild species which habitually produce large proportions of empty, insect-damaged and poorly-formed fruits and seeds.

Chapter 14 of the ISTA Rules (ISTA, 1999) includes details of the straightforward X-ray method, but not the contrast technique. Box 24.4 provides guidance on how to carry out a straightforward X-ray test, but for additional information on the technique, see Simak (1991).

4. Tetrazolium (TZ) Test

4.1. Objective(s)

The objective of the tetrazolium test is to determine the percentage of viable seeds in a seed-lot by:

- 1) preparing a sample of fruits/seeds (often carrying out a preliminary discard of the distinctly dead and empty embryos);
- 2) incubating the (often selected) seeds/embryos in TZ solution;
- 3) examining and categorising the colour, intensity and staining pattern of the tissues; and
- 4) interpreting whether individual seeds are dead or alive.

Box 24.4 How to do an X-ray test (an ISTA test)

ISTA definitions are given, and in addition further guidance/clarification is proposed.

Phase 1 – Take a random sample of say 100 fruits/seeds, and spread evenly on photographic film/paper.

Phase 2 – X-ray sample to reveal internal structure of fruits/seeds.

Phase 3 – Develop and examine photographic image (note this is only a 2 dimensional representation of a 3 dimensional object) and differentiate between categories described below (at the same time considering ultimate interpretation of whether fruit/seed is more likely to be viable or dead).

Appearance	Category	Interpret
'Fruit/seed cavity contains less than 50% fruit/seed tissue' (ISTA rule 14.6.2). See Box 24.3 for definitions of empty sub-categories 'underdeveloped', and 'shrivelled'. 'Mouldy' is unlikely to be discernible by X-ray. See Box 24.2. Outline of fruit/seed coat, cracked or broken (ISTA rule 14.6.4). (Propose: 'Outline of fruit/seed coat sufficiently cracked, broken or damaged to jeopardise living status of seed.)	Empty Insect damaged Physically damaged	Non-viable
Fruit/seed containing all tissues essential for germination present (ISTA rule 14.6.1). (Propose addition of: 'Complete embryonic axis appears intact; probably firm, fresh, healthy and apparently alive. No more than one third of the storage tissues appear unhealthy?')	Filled	Viable

4.2. Principle(s)

The tetrazolium test relies on the premise that only living cells have the respiratory enzymes (dehydrogenases) capable of converting the colourless, soluble compound (2,3,5 triphenyl tetrazolium chloride) into an insoluble red product (2,3,5 triphenyl formazan) (Lakon, 1942). Seeds are therefore soaked in a colourless solution of TZ which enters both living and dead cells, but only the living cells catalyse the formation of the red, formazan precipitate. There then follow several stages of interpretation. Stained and unstained areas are first used to differentiate between living and dead tissues. The size and position of the stained areas is then interpreted as an indication of live *versus* dead seeds. Finally, there are some seed testers who claim to make an additional level of interpretation and relate certain staining patterns to normal seedlings. Since it is the staining pattern which is so important, the test is more accurately described as a topographical tetrazolium test and therefore sometimes abbreviated to TTZ.

The tetrazolium test originated in Germany in the 1940s (Grabe, 1970), and is one of today's most widely known methods of viability testing. It is commonly applied to cereals and less commonly to other agricultural, vegetable and flower seeds. The method is especially suited to deeply dormant woody species – such as horticultural ones, and forest trees and shrubs – which require dormancy breakage pretreatments exceeding 6–8 weeks. In these latter instances, it may be the only practical technique for assessing the likely physiological status of a seed-lot within an acceptable timescale. It is also recommended for use at the end of any germination test where more than 5% of seeds remain ungerminated. However, it is not applicable to fruits/seeds which are too small to dissect, or others (such as some species of oak, *Quercus* spp.) where inhibitors can be present in the fruit which retard or even prevent the formation of the red, formazan precipitate in living seeds. In addition, it is worth adding a cautionary note. Although the test is extremely well known and in principle thoroughly attractive, in practice it is a complicated technique and the interpretation phase is exceedingly difficult.

4.3. Practice

The exact method used to carry out a TZ test depends to a very large extent upon the fruit/seed anatomy of the individual species under test. At the beginning of the test the fruit/seed must be prepared to facilitate the penetration of TZ solution; species with tough, impermeable exteriors often require the application of considerable ingenuity. Towards the end of the test, the tissues need to be opened up for visual inspection – care must be taken to avoid handling damage which can influence the staining pattern. Finally, the tissue soundness, staining colouration and pattern are evaluated in relation to the overall fruit structure; most species exhibit individual idiosyncrasies, and most seed-lots, unique features.

It is clear from the above that virtually every species requires its very own specific instructions for handling, preparation, imbibition, dissection (though not necessarily in that order!) and finally evaluation. As space does not permit detailed discussion of individual cases, the reader is directed towards Box 24.5 and the following references as helpful guidelines.

TZ test methodology for official seed testing purposes on temperate food crops and forest trees is contained in the International Rules for Seed Testing (ISTA, 1999) and Rules for Testing Seeds (AOSA, 1995). Moore (1985) and Grabe (1970) are two general sources of information on TZ. Enescu (1991) provides information with respect to tree seeds, and Bhodthipuks *et al* (1996) on tropical tree seeds. Elsewhere in this book, Terry *et al.* (2003 – Chapter 17) describe a TZ protocol that has been developed by the Millennium Seed Bank Project for conservation collections of wild plant species. As no official guidelines exist for the vast majority of species, the essence of this technique is to recognise three simple categories of seed in a sample: 'viable', 'dying' and 'dead'.

Box 24.5 How to do a tetrazolium test (an ISTA test)

See ISTA rules for specific procedures for different species and, Terry *et al.* (2003 – Chapter 17).

Phase 1 – Take a random sample of a known number of fruits/seeds. Apply any preparatory technique(s) that are necessary [e.g., i) open or partially dissect dry fruits/seeds; or ii) incubate intact or partially dissected fruits/seeds in water to assist subsequent dissection and/or staining. See ISTA rule 6.5.A *et seq.*]

Phase 2 – As each fruit is dissected, inspect contents and use appearance to differentiate between categories and sub-categories described in Box 24.3 Select only fully developed, completely intact, healthy and potentially living embryos for transfer to phase 3.

Phase 3 – Incubate intact fruits or seeds, or fully developed, completely intact, healthy and potentially living embryos in a 0.1–1.0 % colourless solution of 2,3,5 triphenyl tetrazolium chloride at 30°C.

Phase 4 – Examine the staining pattern of fruits/seeds or embryos and differentiate into categories described below (at the same time considering ultimate interpretation of whether fruit/seed is more likely to be viable or dead).

Appearance	Category	Interpret
Some unstained areas, but the 'essential' areas (see ISTA staining patterns for individual species) missing, flaccid, unhealthy, unstained or uncharacteristically coloured.	Specific unstained areas	Non-viable
Whole fruit/seed (or, if appropriate, embryo) unstained.	Completely unstained	
Whole fruit/seed (or, if appropriate, embryo) appears intact, firm, fresh, healthy and fully stained the characteristic rich, formazan red according to the ISTA staining patterns of each species.	Completely stained	Viable
Some unstained tissues, but the 'essential' areas (see ISTA staining patterns for individual species) intact, firm, fresh, healthy and fully stained with the characteristic rich, formazan red, or an acceptable plum or pink colour.	Specific stained areas	

Successful tetrazolium testing requires good vision, considerable manual dexterity, well-honed surgical skills, great care, meticulous attention to detail, natural aptitude and regular practice. However, even this long list excludes the most important element – a trainer who is experienced with the unique idiosyncrasies of the particular species under test. Unfortunately, it is teaching expertise which tends to be the most scarce resource.

5. Excised Embryo (EE) Test

5.1. Objective(s)

The objective of the excised embryo test is to determine the percentage of viable seeds in a seed-lot by:

- 1) preparing a sample of fruits/seeds (often carrying out a preliminary discard of the distinctly dead and empty embryos);
- 2) incubating the (often selected) excised embryos under favourable conditions for growth;
- 3) examining and categorising the embryo survival, growth or decay characteristics; and
- 4) interpreting whether individual seeds are dead or alive.

5.2. Principle(s)

The general principle is to excise embryos from fruits/seeds, incubate them on moist filter paper in axenic culture, and observe survival, growth, development or decay. Embryos which either remain firm and fresh or show evidence of growth (i.e., expansion, elongation or greening) or growth and differentiation (e.g., radicle and lateral root formation or epicotyl and first leaf formation) are considered viable. Embryos which show signs of decay are not viable.

The excised embryo (EE) test originated with work by Flemion (1934) on woody species such as peach, apple and hawthorn. Her aim was to find a way to short-cut the lengthy pretreatment requirements of these seeds and raise plants quickly. In particular, she hoped the removal of the outer fruit cases would eradicate any physical and/or chemical restraints which might prevent the germination of dormant, intact fruit. It is important to appreciate that although a small proportion of her excised embryos grew, they only grew very slowly, into quite abnormal seedlings and were very different to plants obtained from fully pretreated seeds. Subsequently, she (Flemion, 1938; 1948), Tukey and Barrett (1936) and Heit (1955) developed the technique more as a method for testing viability than propagating plants.

Like the TZ test, the EE method is especially suited to deeply dormant woody species requiring pretreatment exceeding 6–8 weeks. But it has also been successfully used on numerous less dormant seeds such as shrubs and vines, and even non-dormant woody plants, flowers and vegetables where especially quick results have been needed. However, it is not applicable to fruits/seeds which are too small to dissect or others (such as some walnut species e.g., *Juglans nigra*) where the fruit case is so tough, and the embryo anatomy so convoluted, that extraction of an intact embryo is virtually impossible.

5.3. Practice

In the same way that the exact procedures used to carry out a TZ test depend upon the individual species, the same is true for the EE test. In the case of the EE test, the ultimate aim is to extract fully developed, completely intact, healthy and potentially living embryos for transfer to the moist filter paper. Again, it is the initial preparation and excision phases which both require considerable ingenuity. Multi-layered fruits and hard-coated species may require the dry fruits or seeds to be cracked open with anything ranging from a hammer, through nutcrackers to a vice. Whereas for others, more subtle pressure applied along a suture line is enough to assist extraction. Some may need preliminary cracks, nicks, holes or slices made in their outer layers prior to one or more soak phases to allow embryo imbibition before extraction.

After the embryos have been removed, by whatever technique, the next phase is to incubate the excised embryos on moist filter paper. The usual incubation temperature is 20°C, the duration 14 d and the medium is generally water. This is suitable for the majority of species. However, many conifer embryos (despite their relatively shallow dormancy in comparison to the majority of broadleaved trees) appear unable to survive for longer than 7d under these conditions. EE tests on conifers are therefore either terminated at 7d, or nutrients are added to the incubation medium. The addition of nutrients, not only allows embryos to survive for longer, but it also appears to stimulate growth (Carpita, *et al.*, 1983).

The final phase is evaluation of the survival, growth and sometimes differentiation of the embryos. Further guidance is summarised in Box 24.6.

6. Other Viability Tests

There are numerous other viability tests which have been used at different times, for different species and for different specific purposes.

Conductivity methods are based on the premise that the cell membranes of dead and dying seeds are more permeable and leak larger quantities of solutes than the membranes of live seeds. Hence, soaking single seeds, or a sample of seeds, in water and measuring the electrical conductivity of the surrounding solution can indicate the viability of an individual seed (or seed-lot).

Many chemical methods rely on the differential uptake of a stain by living versus dead tissues. Hence indigo carmine and other aniline dyes have been used as 'vital stains' because they penetrate dead tissues and stain these areas, whereas live tissues remain impenetrable and unstained.

Box 24.6 How to do an excised-embryo test (an ISTA test)

See ISTA rules for specific procedures for different species.

Phase 1 – Take a random sample of a known number of fruits/seeds. If possible, open and excise embryos from dry tissue: if impractical, soak to assist dissection and embryo removal.

Phase 2 – As each fruit is dissected, inspect contents and use appearance to differentiate between categories and sub-categories described in Box 24.3. Select only fully developed, completely intact, healthy and potentially living embryos for transfer to phase 3.

Phase 3 – Incubate embryos for an appropriate duration (7–14 d) at a suitable temperature (e.g., 20°C) with ample water and good aeration.

Phase 4 – Inspect embryos for survival, growth, development or decay and differentiate into categories described below (at the same time considering ultimate interpretation of whether fruit/seed is more likely to be viable or dead).

Appearance	Category	Interpret
Any quiescent, growing or differentiating embryo which has died or been killed by infection and needs removal from the test substrate.	Decayed	Non-viable
Healthy embryo, similar in appearance to when excised, i.e., firm, slightly enlarged (due to imbibition) and either white, yellow or green (depending upon species).	Dormant	Viable
Healthy embryo exhibiting quantitative changes in shape such as radicle elongation and expansion/greening of cotyledons.	Growing	
Healthy (usually 'growing') embryo exhibiting qualitative changes in shape and structure such as development of root hairs, lateral roots, epicotyl, first leaves, and secondary, tertiary, etc internodes.	Differentiating	

In the same way that the tetrazolium test differentiates between living and dead tissues by revealing the presence or absence of dehydrogenase enzymes and hence enables the identification of viable versus non-viable seeds, many other enzymes such as oxidases and hydrolases can also be used. Copeland and MacDonald (1995) review a number of these enzyme methods and Wood *et al.* (2003 – Chapter 26) describes the fluorescein diacetate (FDA) test which can be used effectively to test the viability of microscopic seeds.

Identifying Viable Seeds

Lakon (1918, reprinted 1952) was the first to discuss the theoretical concept of 'inherent' or 'inborn' 'germination ability' ('innewohnende keimfähigkeit') and 'potential germination' ('keimpotenz') – the forerunners of today's 'viable' seed. At a time when there was only the equivalent of a 'cut-test', he emphasised that this property should be considered completely independently of a germination test, that it did not require any substantiation by a germination test, and that a germination test should not act as a reference standard, rather it was a totally separate assessment of likely seed performance. However, he also appreciated that there ought to be a relationship between a 'viable' seed and the results from a germination test. He therefore suggested that at the end of a germination test, dormant seeds, hard seeds and infected seeds (all of which were clearly separate from germinating seeds) could be considered 'viable'. Unfortunately, there was some scepticism about his inclusion of 'infected seeds', and he did not make it clear that germinated seeds should also be considered viable!

Over the intervening decades the ISTA Rules have incorporated several viability tests such as X-ray, TZ and EE. However, throughout their development and revision, the Rules have consistently avoided defining any relationship between germination and viability results – even though unfortunate inferences have crept in. For example, today's Rules (ISTA, 1999) have evolved some surprisingly circular statements. There are three rules which contribute to the confusion. In the 'Germination' chapter, rule 5.2.2 states that "The percentage germination indicates ... seeds which have produced 'normal' seedlings ..." and rule 5.6.5.A.3 "If 'fresh' seeds are to be reported at 5% or more, it must be verified that they have the potential to produce a 'normal' seedling ... with a tetrazolium test or other appropriate method." In the 'Tetrazolium' chapter, rule 6.5.2.A.4 states "Viable seeds relate to those that are capable of producing 'normal' seedlings in a germination test .." Putting these three rules together in sequence we have the absurd situation that – **at the end of a germination test, if more than 5% of seeds have remained 'fresh' (i.e., failed to produce a 'normal' seedling), then it is necessary to apply a viability test to the ungerminated seeds to determine whether they have the potential to produce a 'normal seedling'!** This is obviously completely circular and in need of clarification and, preferably, resolution.

The most recent discussion of these anomalies was published by Steiner *et al.* (1999), and specifically investigated whether tetrazolium viability results could/should be compared to germination test results. In their words, it was misleading to relate 'viability' and 'germination' since it 'ignores the distinctiveness of each result', and 'could imply that germination testing was

attributed priority or even dominance over viability testing.’ Therefore their overall conclusion was that ‘relating viability to germination ... is unhelpful’. Unfortunately, this does not resolve the central dilemma. On the one hand, there are numerous people who use seed viability as a euphemism for seed germinability. And on the other, there are a significant minority who correctly point out that this should be avoided because it is inaccurate (Copeland and MacDonald, 1995; Schmidt, 2000) and confusing (Hampton, 1995). Perhaps what is now required is a universal recognition that it is impractical to expect people to avoid drawing comparisons between germination and viability tests. Therefore, an acceptable (if approximate) relationship between the two is required.

Another aspect of the problem is what is really meant by authors who write about ‘equating’ or ‘relating ... viability with germination’? It is rarely clear whether it is a specific question of comparing ‘% viable’ from a viability test with the percentage of ‘normal seedlings’ in a germination test; or the more general question of whether viability test results should or should not be compared to germination test results. In my opinion, it would be best to return to the position of Lakon (1918, 1952) and ask: ‘Can a germination test identify *viable* seeds as well as *germinable*?’ If so, ‘What categories of seeds at the end of a germination test should be considered *viable*?’ And finally, ‘Can the *viable* seeds identified in a germination test be meaningfully compared with *viable* seeds from a *viability test*?’

Figure 24.4 shows the appearance of three seed categories which ISTA recognise at the end of a germination test: ‘normal-’ and ‘abnormal-’ seedlings plus ‘ungerminated’ seeds. The criterion used throughout this chapter for a ‘viable’ seed is that it is one which is ‘alive’ according to whatever test technique has been used. In the germination test, there can be no doubt at all that a ‘normal’ seedling must have germinated from a live seed – a normal seedling is therefore ‘viable’. Similarly, any seed which germinated into an ‘abnormal’ seedling must also have been alive before germinating – therefore it too is ‘viable’. But what about ungerminated seeds? According to ISTA Rule 5.6.5.A.3, ungerminated seeds can be cut open to determine whether they are ‘fresh’ or not. I would suggest that seeds which appear ‘fresh’ (see proposal in Box 24.2) also fulfil the definition of being ‘viable’. And finally, if there are 5% or more ‘fresh’ seeds and it becomes necessary to invoke the further requirement of ISTA Rule 5.6.5.A.3 and use a more refined method of viability test such as the TZ, I propose that any ‘fresh’ seeds which stain positively with TZ are also viable.

Hence we can propose the equation:

$$\begin{aligned} \% \text{ ‘viable’ seeds (at the end of a germination test)} &= \\ & \% \text{ normal seedlings} + \% \text{ abnormal seedlings} + \% \text{ fresh} \\ & \text{(or fresh, stained) seeds} \end{aligned}$$

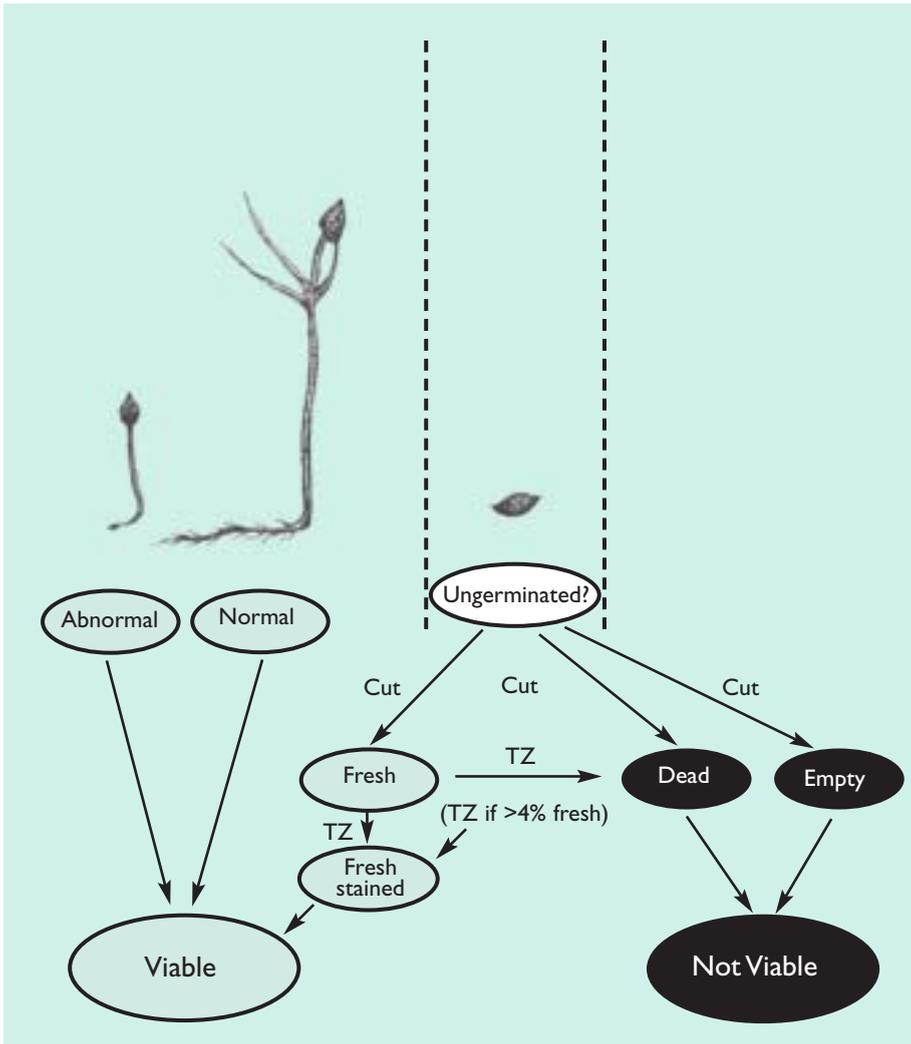


Figure 24.4 Appearance and classification of seedlings and ungerminated seeds as either viable or not viable at the end of a germination test.

This equation avoids any implication that germination tests are a reference standard for the other viability tests since it relies on using other viability tests to identify ‘viable’ seeds within the germination test itself. It is also similar to other viability tests in that it recognises more than one category of seed within the germination test as contributing to the total viable percentage. But above all it is consistent with the concept that a viable seed is one which appears alive.

In fact, it is completely consistent with this concept and we can view the equation equally well from the perspective of a viability test:

$$\begin{aligned} \% \text{ 'viable' seeds (at the end of a viability test)} = \\ \% \text{ normal seedlings} + \% \text{ abnormal seedlings} + \% \text{ fresh} \\ \text{(or fresh, stained) seeds} \end{aligned}$$

Let us take each of the categories in turn. Firstly, every viability test should recognise 'normal' seedlings as being alive. Secondly, although it is generally acknowledged that viability tests cannot discriminate between seeds with the potential to either germinate normally or abnormally (Schubert, 1961), it is equally widely accepted that embryos which are likely to become 'abnormal' seedlings will be recognised as alive. MacKay (1972) has even shown that those seeds which would germinate abnormally because they have suffered from phytotoxic chemicals will stain positively with TZ and are therefore deemed viable. Finally, there are the categories of fresh, and fresh, stained. It is a widely held belief that these ungerminated seeds are dormant (Grabe, 1970; Steiner *et al*, 1999). In other words, they all appear 'viable'.

There are however three issues which require further discussion. One is whether the existence of fresh (or fresh, stained) ungerminated seeds at the end of a germination test is a totally accurate indicator of dormant seeds. The second is what are the advantages and disadvantages of different germination/viability tests. And the third is the different methods for reporting germination and viability results. These topics are covered in the next three sections.

Identifying Dormant Seeds in Germination Tests

Germination tests on most species assume that seeds are 'non-dormant' unless there is evidence to the contrary. Hence most germination tests are carried out on untreated seeds, and dormancy is only suspected if a 'cut-test' at the end of the incubation period reveals a significant number of 'fresh' seeds remaining. Under these circumstances, the most common action is merely to report the percentage of 'fresh' (or sometimes 'fresh, stained') seeds as an additional category, and attribute their presence to dormancy. Occasionally, a second sample may have been taken at the outset, or more often a new sample might be taken after a significant percentage of fresh seeds have been detected, and a second germination test carried out on pretreated seeds. If subsequent germination of the pretreated seeds is significantly higher, this not only confirms that the original seeds were dormant, but also demonstrates that a satisfactory pretreatment has been identified. It follows on from this that ISTA, AOSA and most seed scientists perceive 'fresh' seeds at the end of a germination test as the main (and often the only) evidence necessary to

Table 24.5 A ‘double’ or ‘paired’ germination test revealing ‘dormancy’ due to ‘fresh’ seed differences: results from germination tests on Sitka spruce, *Picea sitchensis* (Gosling, unpublished)

	Seedlings		Ungerminated seeds	
	Normal	Abnormal	Fresh	Dead
Untreated	70	–	30	–
Pretreated	100	–	–	–

identify dormancy. Table 24.5 shows the results from a typical ‘double’ or ‘paired’ germination test illustrating these characteristics. It shows that 30% of seeds remained ‘fresh’ at the end of an untreated germination test. When a suitable pretreatment was applied to another sample (prior to germination), then the proportion of the population which previously remained dormant and fresh, were stimulated to germinate. The almost universal conclusion is that the ungerminated, fresh seeds must have been dormant.

Unfortunately, this simple observation with an apparently neat explanation is not the complete story. Table 24.6 shows data for another ‘double’ germination test. In the top row, at the end of the germination test, untreated seeds either germinated into normal seedlings or were dead. At first sight, the automatic conclusion, is that ‘dead’ seeds cannot be viable, therefore how could these results possibly reflect dormancy? The most likely explanation is that they probably are dead. However, the second row shows that in this particular case, if a similar sample of the same seed-lot were given the correct pretreatment, then the percentage of normal seedlings rose from 70 to 95%! This is not a supernatural ‘resurrection’ of dead seeds. The explanation is that at least 25% of the untreated seeds which were alive at the outset of the germination phase, were unable to germinate under the conditions provided and died during the course of incubation. However, when the same population of seeds were pretreated prior to incubation, their dormancy was broken, and they were able to germinate under the same incubation conditions.

Table 24.6 A ‘double’ or ‘paired’ germination test revealing ‘dormancy’ due to ‘dead’ seed differences: results from germination tests on Grand fir, *Abies grandis* (Gosling, unpublished)

	Seedlings		Ungerminated seeds	
	Normal	Abnormal	Fresh	Dead
Untreated	70	–	–	30
Pretreated	95	–	–	5

This is an exceptionally important observation. It means that the survival of 'fresh' seeds at the end of a single germination test on untreated seeds is not the only indicator of a dormant seed-lot. It must be appreciated that in some circumstances, dormant seeds can die during the course of a germination test and therefore not reveal their presence as fresh, viable seeds at the end of the germination test. This phenomenon is especially likely where there is a combination of dormancy, and low-vigour and when the duration of germination tests is extended, perhaps to allow more time for the germination of dormant and slowly germinating seeds.

The message from the above section is that ungerminated seeds which are proven to be dead at the end of a germination test may not necessarily rule out seed dormancy. In the case of particularly valuable seed-lots, it may therefore be important in some circumstances to carry out 'double' or 'paired' germination tests as a matter of routine. The only other alternative is to confirm the result with a different viability test, applied to a separate sample, straight from the seed bank.

Different Germination/Viability Tests:

Advantages and Disadvantages

The greatest advantage of the germination test is that it provides an estimate of the proportion of the population which will germinate, and this is a direct measure of the characteristic most people wish to reproduce. There are three great disadvantages of the germination test. Firstly, it may be impossible. Secondly, it can take longer than the time available. Thirdly, it may underestimate 'viability'. It may be impossible, because an effective dormancy breakage pretreatment, or suitable germination conditions, may be unknown. It may take 'too long' because the bulk of the seed-lot of a 'recalcitrant' species may deteriorate or die while the sample undergoes its germination test; or in other cases, seed dormancy might be so deep, that the combined duration of a suitable pretreatment plus germination phase might exceed the time available. Although there is no universally accepted maximum duration for a germination test (because germination results are used for so many different purposes), as a rule of thumb, the limit for tests on agricultural, flower and vegetable tests is about 2–3 weeks and for woody trees and shrubs about 8 weeks. If a germination test, or combined pretreatment and germination test is likely to exceed this duration then a more rapid viability test is usually selected. However, for conservation collections of wild plant species, it is not unusual for germination tests to last for many weeks.

The greatest advantage all viability tests have over a germination test is their speed. Their biggest disadvantage is that they are only an indirect measure of whether a population of seeds has the ability to germinate. However, they also all suffer from the following limitations:

- They are unable to detect phytotoxic effects of some seed dressings (see e.g., MacKay, 1972);
- They are unable to detect abnormal germinants (Schubert, 1961); and
- They are unable to differentiate between dormant and non-dormant seeds.

Each of the viability tests described above also has its own, sometimes unique combination of benefits and drawbacks. For example, the cut-test is particularly quick and requires very little specialised equipment. But it can be criticised for relying exclusively on a rather subjective interpretation of whether tissues appear dead or alive. The X-ray test has two great advantages – it is non-destructive and provides a permanent photographic record of the tests results. But the disadvantages are that it requires an expensive piece of equipment, it only reveals missing or damaged tissues (unless combined with contrast agents e.g., heavy metal ions) and the image is just a two dimensional representation of a three dimensional object. Tetrazolium testing is probably the world's most widely known method of viability testing. One unique advantage of the test is that it is the only method available for assessing some hard-coated, deeply dormant woody fruits/seeds (e.g., *Cornus*, *Euonymus*, *Juglans*, *Rosa*, *Viburnum*). In these species, germination tests are usually precluded due to pretreatment durations, and it is impossible/impractical to excise an intact embryo for EE testing. The main disadvantages of TZ testing are that it requires an experienced trainer who is often difficult to find, an extensive training programme and considerable practice and patience. Although the TZ test is the most widely known and probably most frequently used of all the viability tests, it is also perhaps the most difficult to interpret – especially correctly! Excised embryo testing is rarely used on seeds other than those of forest and fruit trees. Its great advantage is that it is especially useful for the many deeply dormant woody species which exist. However, it suffers the disadvantage that the integrity of the excised embryo is so important that it is even more time consuming and difficult to perform than the TZ test, although interpretation is easier.

A synopsis of these and other advantages and disadvantages is given in Table 24.7.

Table 24.7 Advantages/disadvantages of different germination/viability tests

Test	Advantage(s)	Disadvantage(s)
Germination	<ul style="list-style-type: none"> • Direct measure of germination 	<ul style="list-style-type: none"> • Duration may exceed time available • Duration may exceed longevity of some recalcitrant fruits/seeds
Cut	<ul style="list-style-type: none"> • Quick result (hours) • Cheap equipment • Especially useful for checking maturity and quality before collection and during processing, also ungerminated seeds at end of a germination test 	<ul style="list-style-type: none"> • Indirect measure of germination • Subjective interpretation
X-ray	<ul style="list-style-type: none"> • Quick result (hours) • <u>Non-destructive</u> • <u>Permanent record (photograph/negative)</u> • Especially suited to many wild species which habitually produce large proportions of empty, insect-damaged and poorly formed fruits and seeds 	<ul style="list-style-type: none"> • Indirect measure of germination • Expensive equipment • Only reveals missing/damaged tissues. • Does not reveal whether tissues are dead or alive (unless combined with contrast agents e.g., heavy metal ions) • <u>Two dimensional representation of 3D material</u>
Tetrazolium	<ul style="list-style-type: none"> • Quick result (1–3 d) • Only method of assessing some hard-coated, deeply-dormant woody fruits/seeds (e.g., <i>Cornus</i>, <i>Euonymus</i>, <i>Juglans</i>, <i>Rosa</i>, <i>Viburnum</i>) where germination tests are often precluded due to pretreatment durations, and it is impossible/impractical to excise an intact embryo for EE testing • Especially suited to most other deeply dormant species requiring > 6 weeks to pretreat and germinate 	<ul style="list-style-type: none"> • Indirect measure of germination • Very labour intensive • Fairly dextrous surgical skills required • Skilled interpretation of staining patterns, colours and intensities necessary • Unable to detect phytotoxic effects of some seed dressings (MacKay, 1972) • Unable to detect abnormal germinants (Schubert, 1961) • Unable to differentiate between dormant and non-dormant • Not suited to very small seeds • Not suited to some fruits with inhibitors which prevent enzyme reaction e.g., <i>Quercus</i>
Excised embryo	<ul style="list-style-type: none"> • Quick result (7–14 d) • Especially suited to most deeply dormant species requiring > 6 weeks to pretreat and germinate 	<ul style="list-style-type: none"> • Indirect measure of germination • Exceptionally labour intensive • Exceptionally dextrous surgical skills required • Fairly skilled interpretation necessary • Not suited to very small seeds • Not suited to some fruits with a very tough fruit case and convoluted seed preventing extraction of intact embryo (e.g., <i>Juglans</i>)

Underlined text indicates highly significant feature

The Results of Germination and Viability Tests

Boxes 24.2–24.6 describe a wide range of different categories and sub-categories into which seeds and seedlings can be classified following different germination and viability tests. There are many ways in which the final results can be reported.

The most straightforward concept is that seeds are either ‘dead’ or ‘alive’, but in many instances this is too simplistic. For example, in commercial seed testing, the ISTA have clear instructions on the reporting of germination results. Certificates usually report results in five categories:-

1. ‘Normal seedlings’; 2. ‘Abnormal seedlings’; 3. ‘Fresh seeds’; 4. ‘Dead seeds’; and 5. ‘Empty seeds’. In the case of species of the *Fabaceae*, it is mandatory to report a sixth category ‘Hard seeds’.

The over-riding ISTA principle is that “the sum of the percentages of ‘normal’ + ‘abnormal’ seedlings + ‘ungerminated seeds’ must be 100% (ISTA Rule 5.8.A). Unfortunately, the consequence of this approach is that completely empty, and therefore incompetent, seeds are regarded as if they are part of the seed population. Thus a seedlot giving 50% germination but with 50% empty seeds would be regarded as 50% viable. Commercial seed companies usually distribute seed by weight and therefore this convention gives users the simplest means of calculating sowing rates.

Many seed banks however use a different reporting procedure in which the empty seeds are ignored. The results of germination tests are corrected to eliminate the proportion of empty seeds. Thus a seed collection that gave 50% germination with the remaining 50% being empty would be regarded as 100% viable. For seed banks it is important to be able to monitor the decline in viability of the seed population through time where the loss in viability would be due to ageing. Since empty seeds were never viable it is not appropriate to consider them as part of the seed population.

Unlike commercial seed companies, seed banks usually distribute relatively small samples of seeds by number. At the time of distribution, numbers are corrected on the basis of the most recent viability test to take account of both empty and non-viable seeds so that users can expect to get a given number of seedlings.

The convention of expressing viability percentages as a percentage of ‘filled’ seeds appears to have been adopted at the first meeting of an International Board for Plant Genetic Resources (IBPGR) *ad hoc* Advisory Committee on Seed Storage in September 1981.

Conclusions

This chapter summarises a few of the methods used to assess seed germination and ‘viability’. It emphasises that more precision is required when defining and using the term ‘seed viability’, and to bring about greater consistency in the interpretation of seed viability, it suggests slight modifications to several other terms which are widely used in seed testing such as ‘fresh’, ‘dead’, ‘empty’, ‘insect-damaged’ and ‘embryo-less’.

The chapter firmly supports the judgements of Copeland and MacDonald (1995), Hampton (1995) and Schmidt (2000) that ‘viable’ and ‘germinable’ must not be used synonymously. At the same time it acknowledges that it is impractical to avoid drawing comparisons between the results of germination and viability tests. An acceptable (even if approximate) relationship which allows a comparison between the two types of test is required.

A case is made to adopt the principle that ‘viable’ seeds are those which are alive by whatever test has been used to determine such a physiological status.

From the perspective of any viability test, whether a seed is dormant or non-dormant; whether it has the potential to germinate normally or abnormally; whether it is dormant and strong enough to survive until the end of a germination test as fresh (or fresh, stained); or whether it is alive but so weak that it might die during the course of a germination test – all must be considered ‘viable’.

From the perspective of a germination test, ‘normal seedlings’ plus ‘abnormal seedlings’ plus ‘fresh’ (or ‘fresh, stained’) ungerminated seeds – again, all exhibit living characteristics and must therefore be considered ‘viable’.

In other words, a ‘viable’ seed is one which is considered to be alive, but not necessarily ‘germinable’.

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