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HANDBOOKS FOR GENEBANKS. NO. 2

HANDBOOK OF SEED TECHNOLOGY FOR GENEBANKS

VOLUME I. PRINCIPLES AND METHODOLOGY

by

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* Volume II provides an Index to Genera, Tribes and Families in Volumes I and II.

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PREFACE

The purpose of this handbook is to provide a practical guide to those aspects of the management of seed stores for genetic conservation which require some understanding of seed physiology. The handbook assumes little previous knowledge of seed physiology, but aims to provide sufficient information on practical procedures either to avoid or to overcome potential problems in the day-to-day operation of the seed store and seed testing laboratory within a genetic resources centre. Most of the handbook deals with the assessment of seed viability when the seed first arrives and in subsequent monitoring tests during storage. Consequently a suitable sub-title for this handbook would be: the germination of dormant and non-dormant seeds from accessions maintained in medium- or long-term seed stores for genetic resources conservation. Advice on the preparation of seeds for storage (drying, cleaning, moisture content determination and packaging) is also included because it is important that these procedures do not cause additional problems in the subsequent assessments of seed viability, or reduce the potential storage life of accessions.

Although many of the problems are similar to those in other branches of seed technology, some gain added importance and some new problems are encountered when dealing with seeds for long-term conservation. For example in gene banks it is particularly important to ensure that initial processing does not unnecessarily reduce the quality of the seed since this affects potential longevity. Then again problems of seed dormancy interfering with viability testing are likely to be more severe in seed banks for several reasons: for example, in order to ensure maximum seed quality efforts will be made to store seeds as soon as possible after harvest and this is when dormancy is most pronounced, some accessions will represent wild progenitors of crops or primitive landraces and these are usually more dormant than modern cultivars; and the low temperatures used to preserve viability also tend to maintain seeds in a dormant condition. Finally the seeds in gene banks are precious and testing procedures are required which minimise the number of seeds destroyed. Because of these differences, the seed technology appropriate for commercial seed handling and testing is not always appropriate for gene banks.

To avoid any misunderstandings in the chapters which follow there are certain terms which must be defined and understood from the outset. As already stated, this handbook provides advice on the assessment of the viability of seeds from accessions of orthodox species in long-term storage; in most cases viability tests will be germination tests performed under conditions intended to overcome dormancy and thus result in the germination of all viable seeds; after an initial germination test subsequent tests will be carried out after each monitoring interval in order to determine whether the viability has fallen to the regeneration standard. When this happens arrangements will be made to regenerate the accession to produce a fresh stock of seeds or alternatively a sample of the same genetical constitution will be obtained from another source. The underlined terms are defined and explained below.

Accession. A sample of seeds representing a cultivar, a breeding line, or a collected field sample which is held in storage for conservation. It needs to contain sufficient seeds to represent the genetic variation of the population from which it was derived, to provide material for germination tests to monitor viability, and provide seeds for distribution and regeneration. Ideally IBPGR has recommended that an accession should consist of 4000 seeds for genetically homogeneous accessions and 12,000 seeds for genetically heterogeneous accessions.

Dormancy. As applied to seeds, the condition of a live seed which prevents germination when it is supplied with the conditions normally considered to be suitable for germination, viz. adequate moisture, a suitable temperature and adequate aeration. Unless appropriate techniques are adopted, a dormant seed may be assumed (wrongly) to be dead in a germination test.

Germination. Ultimately the production of a seedling from a seed; the emergence of the radicle from the seed is normally the first visible sign that germination has commenced, but germination begins from the first metabolic processes during imbibition.

Germination test. A laboratory test to estimate the proportion of seeds within an accession capable of germinating.

Long-term storage. The storage of seed accessions for long-term periods. Stores operated at sub-zero temperatures are generally classed as long-term seed stores, but the storage conditions recommended by IBPGR for long-term seed conservation are -18°C or less in air-tight containers at a seed moisture content of 5 ± 1%.

Monitoring interval. The period of storage between two viability tests to ascertain whether to continue storing the accession or to produce a fresh stock of seeds by regeneration or replacement from another source.

Orthodox seeds. Orthodox seeds can be dried to low moisture contents and stored at low temperatures without damage and their longevity in storage is increased by so doing. All arable crops and many small-seeded tree species produce orthodox seeds.

Recalcitrant seeds. Recalcitrant seeds cannot be dried without damage. Because they cannot be dried, recalcitrant seeds cannot be stored at sub-zero temperatures without freezing, and many tropical examples also show chilling injury (damage at about 10°C or less). Consequently recalcitrant species cannot be stored under the conditions recommended by IBPGR for long-term storage. No methods are presently available for the medium- or long-term storage of recalcitrant seeds (e.g. seeds of cocoa, rubber and many of the woody species which produce large seeds).

Regenerate. Grow a sample of seeds from an accession to produce plants from which new seeds can be harvested and provide a fresh sample of seeds of similar genetic constitution to the original accession and which therefore can be used to replace it in store.

Regeneration standard. The percentage viability of an accession, usually estimated by germination tests, which indicates that it is no longer suitable for genetic conservation and should be replaced by regeneration or obtaining similar seeds from another source. Loss of viability during storage results in genetic changes through associated mutation and, in the case of genetically heterogeneous accessions, through genetic erosion because of genetic selection against genotypes of shorter longevity. Accordingly the IBPGR Seed Storage Committee has recommended the regeneration standard adopted should be 85% for most species, though lower standards may have to be adopted for some.

Viability. The possession in a seed of those processes essential for a seed to germinate. Thus a viable seed is alive; but this does not ensure that the seed will germinate (for example, if the seed is dormant). Within an accession, percentage viability is the proportion of seeds which are viable; it is estimated from the result of a viability test.

Viability test. A test on a sample of seeds withdrawn from the accession designed to estimate the percentage viability of the accession. A germination test can only function as a viability test either if all the seeds are non-dormant, or if special treatments are applied to remove dormancy. An alternative viability test procedure which does not require special treatments to remove dormancy is the tetrazolium test; in forestry a viability test is often synonymous with a tetrazolium test.

This handbook is divided into two volumes. The first volume provides a general introduction to the management of seed accessions within gene banks, seed physiology and seed testing with particular reference to the specific problems for gene banks operating long-term seed stores (though the majority of this handbook is also relevant to the operation of medium-term seed stores), and provides further, more detailed, information which is likely to be useful for the operation of seed stores for genetic resources conservation.

The second volume of this handbook* provides more detailed advice on the estimation of the viability of seed accessions of particular crop species, with the emphasis on treatments which promote the germination of dormant seeds. It is based mainly on a critical distillation of relevant literature together with additional advice based on the experiences of the authors and on some protocols developed by the staff of the Royal Botanic Gardens Kew Gene Bank at Wakehurst Place, Ardingly, West Sussex, England.

The technology of handling seeds for long-term conservation is relatively new. Moreover, in many species we have inadequate knowledge or experience. We have provided direct instructions for procedures to be followed where this is possible, but in many cases insufficient information is available at present for unequivocal advice to be given. Consequently this handbook is also intended to provide a guide to gene bank staff to help them find their own solutions. Many of the suggestions are tentative and it is expected that the techniques will be modified and expanded in the light of the experience of those in seed banks who are having to cope with these problems. Indeed it is hoped that this handbook will stimulate gene bank staff, and seed physiologists elsewhere, to solve many of the difficulties which are highlighted here. Accordingly we would be grateful for any suggestions which could help in the revision of future editions; these may be sent to either the authors or the Executive Secretary, IBPGR.

This handbook is intended to complement a previous IBPGR publication - The Design of Seed Storage Facilities for Genetic Conservation (full reference provided in Chapter 1). To avoid duplication that report is extensively referred to here as the 1982 Report; copies are available from the IBPGR Secretariat in Rome.

* Handbook of Seed Technology for Genebanks Volume II. Compendium of Specific Germination Information and Test Recommendations, IBPGR, Rome, 1985 (AGPG: IBPGR 85/85).

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In writing this handbook we have relied heavily on the publications and procedures of the International Seed Testing Association (ISTA), even though it has been necessary to suggest alternative approaches for gene banks. We are especially grateful for co-operation from the ISTA during the preparation of this handbook and in particular to Dr. S. Cooper for providing a draft copy of the 1985 ISTA Rules in advance of publication. We are also most grateful to the IBPGR Seed Storage Committee (Professor J.D. Bewley, Professor H.F. Chin, Dr. A.G. Gordon, Mrs. Erlinda Pili-Sevilla, Mr. R.D. Smith, Dr. P. Stanwood and Dr. J.T. Williams) for their helpful comments and suggestions, to the staff of Wakehurst Place (Royal Botanic Gardens Kew) for developing the algorithms presented in the second volume of this handbook and allowing us to publish these and for many other helpful suggestions, to Dr. B. Frankland for helpful suggestions with regard to light regimes for germination, to Dr. A.J. Murdoch for helpful comments throughout the preparation of this handbook, to Miss Diane Ellis for drawing several explanatory sketches, to Miss Anne Newlyn and her staff for their assistance in fulfilling a great number of inter-library loan requests, to Mrs. D.L. Roberts for providing the index to Volume I and helping to index Volume II, and to Mrs. Lizabeth Brenke and Mrs. Cherry East for their assistance in the preparation of the manuscript and photo-ready copy. It is not possible to acknowledge by name all those who have provided information or help during the preparation of this handbook, but we do thank them all for helpful and rapid replies to our questions. The final form of the handbook is the responsibility of the authors who apologise for any inaccuracies or inadequacies.

CHAPTER 1. MANAGING A LONG-TERM SEED STORE FOR GENETIC RESOURCES CONSERVATION: GETTING THE SEEDS INTO STORE

In essence the operation of a long-term seed store for the genetic resources conservation of orthodox species is simple: seed accessions are received, recorded, and maintained in store under cold, dry conditions and sub-samples withdrawn and despatched when required. However, the apparent similarity with the operation of, say, a warehouse containing manufactured goods is false. Whereas the latter are inanimate, seed accessions are or should be living material; if they are not then the accession serves no purpose. Many of the problems which have arisen in the maintenance of seed collections in gene banks have resulted from a failure to recognise fully that seeds are living material in a quiescent state. For this reason we begin this volume with some observations on the management of long-term seed stores with the aim of maintaining accessions containing a high proportion of live seeds.

RECOGNITION AND CLASSIFICATION OF SEED STORAGE CHARACTERISTICS

The use of long-term seed storage for the conservation of plant genetic resources is only feasible for those species which show orthodox seed characteristics (see Preface). Other techniques have to be used if a species produces recalcitrant seeds. Unfortunately several mistakes have been made in assigning species to these alternative categories - in particular several species have been reported as recalcitrant (and therefore unsuited to long-term seed storage) when they are in fact orthodox.

There are several reasons why mistakes can be made and these are dealt with in detail in a reference given at the end of this chapter. Often mistakes have arisen due to insufficient understanding of drying problems. For example, seeds have been killed by drying because the technique was inappropriate or because the seeds were immature. In other cases the drying technique was adequate but possible effects of drying on other aspects of seed physiology were not understood; e.g. in some species rehydration in germination tests is very slow after drying to low moisture contents and germination tests should be extended; sometimes hardseededness is induced on drying so that scarification is needed; and sometimes dormancy is induced which requires additional treatments. In other cases, after drying, the rehydration needs special care otherwise rapid water uptake results in imbibition injury. All these problems are dealt with in this manual. Here they are mentioned to emphasise the importance of understanding the problems if expensive mistakes are to be avoided.

Although it is not possible to store recalcitrant seeds for more than a few weeks or years (depending on species) alternative methods have to be used for genetic conservation of these species, e.g. regularly replanted orchards. Nevertheless seed technology is often an important part of the conservation process since germplasm is often collected and transported as seeds, and seeds are germinated in the course of regeneration and evaluation. Although the main emphasis in this manual is on orthodox seeds, it will be found that much of the information on germination and estimation of viability is also applicable to recalcitrant seeds. Furthermore because of the need for specific advice on the germination of recalcitrant seeds we have included several species in the second volume of this manual.

TABLE 1.1 Gene bank Decision-making Management Table

QUESTION	DECISION	
	RBG Wakehurst Place	Other possibilities
Should collection be accepted for long-term storage?	Accept if (a) seed of wild material, (b) does not duplicate existing collection. (c) seed number acceptable, (d) germination above regeneration standard, and (e) the species shows orthodox seed storage characteristics.	e.g. is the material within the mandate of the seed bank? Does it unintentionally duplicate an existing collection? Even if the number of seeds is smaller than the standard accession size or below the regeneration standard it still may be decided to retain the accession if it is deemed important material.
What should the order of operation be when receiving seed collections?	e.g. Depulp, Dry, Thresh, Clean and Analyse Purity, Dry further, Determine moisture content, Pack, Store and Test Germination.	
What is the minimum acceptable number of seeds for long-term storage?	1,000	The standard accession size may vary with the type of material: e.g. IBPGR have recommended 4,000 seeds for base collections of genetically homogeneous accessions and 12,000 for heterogeneous accessions, but practical considerations may demand some flexibility.
Should seed moisture content be determined?	No, use standard drying times instead if species handled extensively before.	Moisture contents determined on some accessions only in order to monitor drying procedures.
If so, how?	Yes, use standard ISTA procedures, but with standard seed number (instead of standard weight).	Yes, use ISTA procedures, but using less weight of seed and more accurate weighing procedures.
Should accessions be bulk-stored or sub-divided into many containers?	Sub-divide.	Use a single container. The policy within a seed bank may be different for different species.
What form of storage containers are most appropriate?	Glass bottles with rubber seals.	Laminated foil packets. Metal cans.
Is seed health to be tested?	No.	Some health testing feasible, but comprehensive health testing impossible.
What are the appropriate germination test conditions?	Refer to algorithm (see Volume II).	Refer to prescriptions in the second volume. If no suitable prescriptions available, refer to Chapter 5 and the second volume.

Should a fixed sample size or a sequential germination test procedure be used?	Fixed sample size.	Fixed sample for initial germination test, sequential test for subsequent monitoring tests.
How many seeds per test?	100	400 for initial test, 40 for each group in sequential tests.
Should germination be evaluated according to the criterion of viability or the criterion of normal germination?	Viability (i.e. protrusion of radicle in germination test).	Normal germination (i.e. germination resulting in morphologically normal seedling). Exceptionally a tetrazolium test when pronounced dormancy cannot be overcome.
What is the maximum acceptable proportion of empty seeds?	5%	In some species e.g. some tropical grasses much higher values than this would have to be accepted.
What is to be done if proportion of empty seeds is greater than this?	Reclean.	Do not reclean but take number of empty seeds into account when assessing true seed viability.
What is the regeneration standard?	75% germination.	85% germination is more appropriate for most crop species in which high initial germination is expected, e.g. in cultivated cereals.
Should accessions which fail this standard be regenerated?	No. If possible recollect.	Yes, but hold sample of initial accession in store until regenerated seeds are obtained and shown to be of satisfactory viability.
How frequently should accession germination be monitored?	Every ten years.	Some sample accessions of seed might be tested sooner than this, particularly of species which store badly. The monitoring interval could then be reviewed in the light of experience.
Which categories of correspondent should be supplied with seeds on request?	All bonafide organisations which collaborate in the exchange and conservation of germplasm.	Working collections, when necessary.
How many seeds should be supplied to users of genetic resources?	50	The appropriate size of sample could vary in relation to the genetic heterogeneity of the accession.
What supporting information should be supplied?	Details of collection/characterization and recommended method for germination..	Passport data plus evaluation data where available.
Should repeat requests for an accession be supplied automatically?	Not within three years.	At any time if bank is satisfied with need.

Table 1.2 An outline of the sequence of operations required to incorporate an accession into the collection maintained by a gene bank. This example is for seeds of a grass species received as panicles.

ACTION	INFORMATION	PRECAUTIONS
RECEIPT	Allocate serial number, Date of receipt.	Insect infestation? Seed treated chemically? Do seeds and documentation match?
VERIFICATION	Despatch all relevant collection information together with herbarium specimens to taxonomist.	
DRYING	Location in drying room.	Open container to allow seeds to dry, but prevent mixing of separate accessions.
THRESHING	Cancel location in drying room, add date of threshing.	Keep discarded material for seed health analyses.
ESTIMATE MOISTURE CONTENT	Record moisture content.	If seeds too moist for long-term storage then return to drying room for further drying.
CLEANING	Provide estimate of number of seeds within accession.	Keep discarded material for seed health analyses.
PURITY	Provide estimate of empty seed fraction, reduce estimate of total seed number.	If too many empty seeds then return for further cleaning.
PACKAGING	No. of containers, type of container, batch no. of containers.	
STORAGE	Date of storage, location in store of all containers.	
ESTIMATE ACCESSION VIABILITY	Record all details of the test(s), Reduce estimate of total seed number by number of seeds removed to estimate viability. Estimate regeneration interval, decide date of first monitoring test.	Sample seeds at random, overcome seed dormancy in germination tests.
ADD TO SEED LIST	Allocate accession number, indicate distribution policy, no. of seeds available for distribution and monitoring and minimum no. of seeds required for regeneration.	Do not add to seed list if insufficient seeds, insufficient viability, verification of species not available, duplicates existing accession or outside remit of the collection.
(DESPATCH SAMPLE FOR DUPLICATION)	Record institute at which accession duplicated, despatch all information with duplicate sample.	

MANAGEMENT AND OPERATION SEQUENCE OF LONG-TERM SEED STORES FOR ORTHODOX SPECIES

Management, whether of a genetic resources centre or any other organisation, entails decision making. Three facets of management decision making are worth noting. First, the need for a decision should be identified; otherwise wrong decisions will often be made by default. Second, the decision is best specified in the form of an answer to a question. Finally, the solution should be clear and unambiguous.

Decision framework

A decision framework is very useful from the outset for managing a long-term seed storage facility. Of course as experience is gained the framework can be modified and developed. It will enable gene bank staff to be aware of potential problems (at least those anticipated by the manager) and to be clear on how to achieve their objectives.

The seed store operated by the Royal Botanic Gardens, Kew at Wakehurst Place, U.K. could not be used as a model of gene bank operation, because the material it handles is atypical of the majority of gene banks. Nevertheless an analysis of a decision framework with reference to the solutions adopted by the Wakehurst Place gene bank (Table 1.1) may help others in arriving at decisions appropriate to their own material and objectives. However the list of questions and solutions will often differ between gene banks.

Sequence of banking operations

In addition to anticipating matters on which decisions will be required, it is important to decide the sequence of operations each accession will experience from receipt at the gene bank onwards. Table 1.2 provides an example of operational sequences to incorporate an accession of a grass received in the form of panicles. This is in the form of a flowchart which includes not only the progress of seed materials into, through and out of the gene bank but also the vitally important information flow into, within and without the gene bank. Management information is also essential to ensure the maintenance of the accession. There are thus four categories of information associated with each accession: passport data - taxonomic status, common name and details of origin; characterization data - botanical and other easily observed heritable attributes; evaluation data - facts gathered by plant breeders or others during screening of the accession to determine its breeding potential; and accession management data. Accession management data includes: date of receipt; date of entry into the long-term store; store location; size of accession (weight or, preferably, the estimated number of seeds - ideally this also includes 1000 seed weight); initial viability; proportion of empty seeds (where relevant); dates, germination test conditions and results of all previous viability monitoring tests; and the date when the next monitoring test is due. Ideally the information would also include seed moisture content, the proportion of dormant seeds, the estimated storage life of the accession in the long-term store, observations on seed purity and seed health, a list of where, when and how many seeds from the accession have been distributed, and how many seeds remain in store. The management of the information required to conserve seed accessions within gene banks is not considered further here since this topic is covered by another IBPGR report cited at the end of this chapter.

From Table 1.2 we can see that the basic order of operations for an accession of a grass going into store is drying, threshing, moisture determination, cleaning and purity analysis, packaging, storage, and germination (viability) testing. This sequence will require alteration for other species. For example, an

accession received as fleshy fruits must have the seeds extracted before drying, but threshing will not be necessary.

Certain aspects of the order of operations shown for a grass in Table 1.2 need further explanation. Threshing occurs after preliminary drying. Thus seed heads will often be dried first as a whole unit. When dried the seed heads are easier to thresh. However, very dry seeds can be quite prone to mechanical damage (see later section on threshing) and threshing can involve a good deal of mechanical treatment of the seed. Consequently final drying down to between 4 and 6% moisture content for long-term storage is subsequent to threshing; hence the dotted line in Table 1.2.

Cleaning and purity analysis are each considered to be parts of the same operation since the result of the purity analysis may identify the need for further cleaning to reduce the empty seed fraction; again, as shown by the dotted line in Table 1.2. Germination testing is considered as the last operation within the sequence. There are a number of reasons for this. First there is little point in testing accessions with a high proportion of empty seeds for germination. Thus germination testing is subsequent to cleaning and purity analysis. Secondly the receipt of accessions at a gene bank will tend to be seasonal rather than regular throughout the year. It will be wise to concentrate the effort during these periods of peak input to enable the accessions to be placed in good storage conditions as soon as possible in order to avoid loss of quality. In which case germination testing can be carried out when time allows after the accession has been dried and placed in storage so that deterioration is arrested. Of course on occasion when this policy is adopted it may be found that some accessions with poorer germination than the regeneration standard are placed in storage. But this is of no consequence. Such accessions can remain in good storage conditions until it is known whether the accession can be replaced by another collection or until facilities are available for the regeneration of the accession - in which case a sample of the accession should remain in store anyway as a precaution in case of crop failure during regeneration.

The example given above shows the advantage of adopting a flexible approach to organising the work of a gene bank. It can be extended further by suggesting that, if the number of staff is limited, the seed testing operations could be minimised or even suspended during the peak input season. Another possibility is to organise the work of the seed testing laboratory seasonally with regard to the species being tested. For example, a higher throughput may be possible if only one species is tested in one part of the year: in the northern hemisphere autumn sown cereals might be tested in May/June so that facilities could be made available for sowing in the autumn if regeneration of any accessions is necessary; whereas spring sown cereals could be tested in October/November so that facilities could be made available for sowing in the spring if regeneration of any accessions is necessary. Organisation of monitoring tests in this way may increase throughput compared to testing accessions of any species throughout the year.

The main purpose of this manual is to provide advice, concerning methods of germinating seeds from accessions in store and, in particular, suggestions as to how to promote the germination of dormant seeds in order to provide for the germination of all viable seeds. Subsequent chapters provide detailed information on these aspects of the work of long-term seed stores for genetic resources conservation. The remainder of this chapter is mainly concerned with various aspects of the operation of gene banks which can affect the quality of seed accessions banked in the seed store.

EXTRACTING SEEDS FROM FRUITS

In very many species gene banks will in fact be storing seeds within dry fruits and other structures (e.g. a caryopsis within its attached lemma and palea, as in many grasses - see Chapter 3). Problems can

arise because in certain cases these fruits, though known agronomically as seeds, will not contain true seeds in which case they are known as 'empty seeds'. In other cases problems may arise because the dried fruit structure may contain more than one seed. Both types of problem are considered in a later section of this chapter; here we concentrate on the extraction of seeds from moist or fleshy fruits. In these cases the seeds must be removed from the fruits before long-term seed storage.

A general word of warning concerning extraction procedures in current usage is necessary by way of introduction. Many of the procedures widely used to extract seeds from fleshy fruits have been developed for the convenience of breeders who have often been more concerned with ease of extraction than with obtaining a high proportion of surviving seeds. It cannot be assumed that an extraction procedure described as satisfactory by a breeder does not damage the seeds and/or reduce subsequent storage life.

In a few species a small proportion of seeds may show a tendency to begin to germinate within the fruit as a result of insufficient dormancy. If this is likely then the seeds should be extracted from the fruits and dried as soon as possible. For the majority of species, however, dormancy is sufficient to prevent germination within the fruit. In dehydrated (or 'air-dry') seeds the higher the seed moisture content, the faster the loss in viability, but fully hydrated seeds are able to survive for quite long periods provided that they are prevented from germinating by dormancy. Thus hydrated, dormant seeds within fleshy fruits are not damaged by short periods of storage in the fruit. For example, in many orthodox species no damage appears to result from six weeks storage in moist fruits. Since oxygen is required for hydrated seeds to maintain viability it is essential that air should be able to circulate freely between the moist fruits and that the fruits be held in containers which provide no barrier to air. In general if fruits are to be stored for the short-term until seed extraction is possible it would seem preferable for the temperature of storage to be about 0° to 5°C and that the humidity should be sufficiently high to prevent excessive drying out of the fruits. This avoids both induction of secondary dormancy (see Chapter 5), which can occur at laboratory temperatures and above, and also decomposition of the fruits. Recalcitrant seeds of many tropical fruits may be killed by chilling injury - i.e. temperatures below 10° to 15°C - but such seeds cannot be dried and therefore will not be held in conventional long-term seed stores. Obviously, though, seed extraction should be undertaken as soon as possible after fruit harvest.

The following notes are provided for specific categories of fruits.

Pomes e.g. apple and pears (*Malus* and *Pyrus* spp.). The fruits are easily bisected by knife and the seeds removed from the core by forceps. This is easier to accomplish with firm fruit.

Stonefruits e.g. *Prunus* spp. Maceration of the flesh by machine is often practised, but removal of the flesh with a sharp knife by hand is convenient for small quantities of seed. Immediately after extraction the stones should be washed copiously in water to remove all pulp and juice. The surface of the stones should then be blotted dry. If it is wished to extract seeds from stones then this is best accomplished after preliminary (one day) drying in the drying room. Split the stony endocarp of the dry seed with a vice or pliers by applying steadily increasing pressure at the broadest point of the longitudinal axis of the stone until the endocarp splits. Alternatively insert a strong blade into the crevice and twist.

Citrus fruits. Fruits can be bisected by knife and the seeds teased out with forceps or by squeezing the half fruit on a fruit juice squeezer. Seeds should be rinsed thoroughly in distilled water.

True berries e.g. Solanaceae, Vitis spp., Ribes spp. Certain fruits within the Solanaceae contain poisonous alkaloids (e.g. solanin in the potato) which can severely irritate the skin if the fruits are handled a lot. Light polyethylene gloves prevent this whilst not reducing dexterity.

The contents of the fruits are best removed either by cutting the fruit in half or by cutting off the distal end of the fruit and squeezing out the contents. Sometimes breeders homogenise fruits in blender/mixers for thirty seconds - the blades being protected by rubber - but in our opinion this procedure can damage seeds. The excised contents of fruits consist of seeds in a gelatinous coating. Six different procedures have been used to remove the gelatinous seed coating.

1. Natural fermentation where the gelatinous slurry is hermetically stored at about 20°-25°C for 3 days with occasional stirring, the seeds subsequently being washed out using tap water.

2. Enzyme digestion where a pectinase solution (0.1% weight/volume) is added to the gelatinous slurry in the ratio of 1:40, stirred for one hour and the seeds washed out using tap water.

3. Acid fermentation where 1 normal hydrochloric acid (917 ml of distilled water added to 83 ml of concentrated hydrochloric acid) is added to the gelatinous slurry in the ratio of 1:10, left for 15-30 minutes and the seeds washed out using tap water.

4. Enzyme digestion plus acid fermentation, either by acid fermentation (as above) followed by enzyme digestion (as above), or by mixing equal volumes of the gelatinous slurry and a 0.1 normal hydrochloric acid solution containing 0.1% pectinase, leaving for 24 hours, and then washing the seeds out using tap water.

5. Sodium carbonate digestion where a 10% sodium carbonate solution and the gelatinous slurry are mixed in the ratio 1:1, left for 18-24 hours, and the seeds washed out using tap water.

6. Washing seeds in running tap water under pressure for 15-20 minutes where the pressure of the water and the use of a spatula are sufficient to detach the gelatinous covering from the seed.

In most cases natural fermentation should be avoided since it can result in damage to the seed. Similarly acid digestion with 1 N hydrochloric acid can damage seeds - although it may only become obvious if germination is tested after a period of storage; treatment with 0.1 N hydrochloric acid has little effect on germination but treatment at this concentration fails to break down the gel and flesh and is therefore pointless - unless combined with a pectinase treatment. The sodium carbonate method also fails to break down large pieces of fruit flesh or fibre. Moreover since washing is successful in the majority of species we see no reason to use methods 1-5 inclusive. Washing should be done with the gelatinous slurry in sieves to prevent the seeds being washed away; a high flow rate of water is advantageous.

Cucurbits. In most cucurbits it is comparatively easy to cut open the fruit and then scrape out the seeds from the centres of the fruits. Wash the seeds in running water before drying.

Fleshy hips e.g. Rosa spp. A portion of the hip is cut away and the seeds gently squeezed or teased out.

Drupelets e.g. Raspberries. In these fruits the berries can be squashed between the hands and seeds washed out by washing.

Strawberries. In strawberry the seeds cover the surface of the fruit. This surface can be cut away from the bulk of the fruit, mashed between the fingers and the seeds separated out by washing.

After seeds extracted from fruits have been washed they should be blotted to remove excess moisture from the seeds. This is particularly important with very small seeds, otherwise on drying the seeds tend to cake together resulting in a very wet patch of seeds in the centre of the cake. After the removal of surface moisture the seed should be dried in the drying room (see next section) in very thin layers - preferably only one seed thick. The small amount of gelatinous material that may remain after washing is easily removed once the seeds are dried.

Fibrous drupes e.g. the palm fruits. The exocarp and mesocarp of the fruit enclose the endocarp - which is a hard thick shell. After the fruits have been harvested the fibrous mesocarp can be removed with a sharp knife, and the seeds can then be washed and surface dried by blotting.

Capsules e.g. Brazil nut (*Bertholletia excelsa* Humb. & Bonpl.) and Sapucaia nut (*Lecythis zabucajo* Aubl.). In these species the fruits are large woody structures which can only be cut with an axe. However, when the fruit has matured, there is a natural cap-like opening through which the dry seeds will fall out. The seeds are naturally clean and dry and do not require washing, but further drying may be required.

Cocoa pods. Cocoa is believed to be recalcitrant. The fruits are large, hard, woody pods consisting of a few compartments within which the seeds are embedded. The pods can be cracked open with a wooden mallet and the seeds extracted. The extracted seeds are covered in mucilage which is normally removed by mixing in sawdust. This can then be rubbed off, the seeds rinsed and their surface blotted dry.

Pine cones. The ripe cones vary greatly in size and form between the various *Pinus* spp., but consist of brown to yellowish-brown overlapping woody scales; each of these scales, if fully developed, bears two seeds at the base. With drying the cones open up, and the seeds can be dislodged from the base of the scales by shaking. Ensure that the cones are harvested before the seeds have already been dispersed! It may be necessary to remove the wings from the seeds in order to reduce the seed bulk.

In the normal practice of seed production some fleshy fruits are picked and dried and only then are the seeds extracted, e.g. chillis, okra, gourds, tropical pumpkins and - sometimes - eggplant. Whilst this can be more convenient than extracting seeds whilst the fruits are moist it is inevitable that the seeds within the fruits will dry more slowly than they would if extracted from the moist fruits and dried separately. Seed quality is expected to be superior in the latter case and it is suggested that this procedure may be preferable to extracting seeds from dried fruits. Gene banks handling these species are recommended to compare the quality of seed produced by both procedures and act accordingly.

It was noted above that hydrated seeds can survive for quite long periods. It is important that once seeds are removed from fruits that they be dried quickly, not slowly, through the intermediate moisture contents (where loss in viability is most rapid) to the low moisture contents necessary for long-term storage. A long period of exposure to intermediate moisture contents will result in loss in viability. If there is likely to be a delay in drying the seeds then they should be maintained temporarily in the fruit. If this is not possible then the feasibility of temporarily maintaining the seeds imbibed with access to oxygen could be investigated.

DRYING SEEDS

The 1982 report recommends seeds are best dried in a drying room maintained at about 15°C and 10 to 15% relative humidity with good air recirculation. This requires the use of an air-dehumidifier (sorption type) with refrigeration to lower the temperature and remove the heat generated by the air dehumidifier. Large seeds require a substantial time to dry down to between 4 and 6% moisture content especially if the seeds are not force-ventilated. An alternative for large seeds (e.g. maize and beans) is to adopt a two-stage drying procedure. In the first stage a drying room is maintained at about 17°C with 40 to 45% relative humidity. In the second stage the seeds are placed in a self-contained drier with good air recirculation - again using sorption-type equipment - but with no refrigeration to provide an environment of about 30°C with 10 to 15% relative humidity.

The factors which are positively related to the rate of drying are temperature, vapour pressure differential (difference between vapour pressure of environment in equilibrium with seed at current moisture content and vapour pressure of drying environment), oil content, and airflow rate. Seed size is negatively related to the rate of drying. That is the larger the seed the longer it takes to dry. In addition certain features of the seed coat can affect the rate of drying - particularly the testa in legume seeds. The 1982 report considers the relationships between these factors in considerable detail. Here we consider a few practical details of the operation and performance of drying rooms in gene banks.

Drying room precautions

1. Clearly label each accession (or part of accession) being dried.

2. Although insects are generally reported to be inactive below about 30% relative humidity, there have been some reports that insects have reproduced and multiplied within seed drying rooms. Staff should inspect for such activity daily, and ensure that insects do not pass into the drying room from outside via the air-lock or via the dehumidifier. In addition obvious contaminants (e.g. larvae and galls) should be removed from accessions before drying - see the later section on cleaning. If insect activity is noted then clean and disinfect the drying room, and then check that the notional operating environment is being achieved.

3. Whilst seeds will dry much more quickly when exposed to the drying room environment in thin layers, it may be considered that the risk of mixing accessions (that is, contamination) is too great to allow this, in which case it would be preferable for the accessions to remain within bags. The exception to this is for very wet seeds (e.g. those extracted from fleshy fruits) which must be allowed to dry in very thin layers to avoid the seeds caking together. If seeds are dried within bags then the smaller the bag the better. For example, a large accession can be sub-divided into a number of smaller bags for drying rather than being left in a single large bag. The bags must, of course, allow air and moisture to pass through: cotton and hessian bags are recommended for small and large seeds respectively, with mesh sizes as large as can be tolerated for the seed in question.

4. Bags should not be piled upon and against each other, since this too will reduce the seed drying rate.

5. Finally bags should not be placed on the floor, or against the walls of the drying room, since - in addition to obstructing air-flow and thereby reducing the seed drying rate - the bags will act as a component of the drying room insulation, and seed temperature will be affected. (A temperature gradient

will operate from the side of the bag exposed to the room through the seed, through the wall or floor to the outside. In the case of a higher external temperature the seed would be heated. In the case of a lower external temperature the seed would be cooled, the humidity around the seed would rise and the seed might not dry in an extreme case.)

Several drying rooms have now been operated at the recommended conditions for many years. Not only have they proved simple, effective and safe to use, but - particularly when combined with a sensible collection policy where seeds are despatched to the gene bank as quickly as possible - they have also resulted in the banking of very high quality seeds. In contrast heated-air seed driers have several limitations which make them less appropriate for use in gene banks. These have been described in the 1982 report. Most of the problems reported when drying seeds stem either from the rapid rates at which damage can accumulate in seed at high moisture contents and high temperatures or from structural problems within the seed due to rapid changes in moisture content. The latter are described below.

Reduction of seed volume on drying

When seeds dry there is a decrease in both weight and volume. If large seeds dry too rapidly the outside of the seed will lose moisture more rapidly than the inside of the seed and will also reduce in volume more quickly. This can lead to cracking of the seed coat. Alternatively seed coats can fail to shrink sufficiently upon drying to continue to make contact with the embryo. These strained sections are brittle and can be easily broken when handled. Internal drying stresses also may increase susceptibility to mechanical injuries such as fractures to the radicle and within cotyledon attachment regions in legumes. In general these forms of damage are most noticeable when seeds have been dried very rapidly - as (for example) can occur in heated-air driers. Few such difficulties have been observed in seed dried more slowly in drying rooms operating at the recommended conditions.

Cleavage damage

However, considerable difficulties are encountered when soyabean (Glycine max) seeds are dried to very low levels - even when they are dried slowly in a gene bank drying room. When dried below about 8% moisture content soyabeans show what is known as cleavage damage. Here the seed coat cracks and the cotyledons may separate, particularly if the seeds are handled. To avoid this form of damage, soyabeans should either be dried in a drying room as specified above but removed when their moisture content has been reduced to 8% or be dried to equilibrium in environments with a relative humidity of not less than 40%.

THRESHING

In some gene banks seeds will normally be received after threshing has been carried out. But in some cases it may be better to receive seed heads (inflorescences), unless this creates difficulty with quarantine services. The receipt of seed heads can aid taxonomic classification where this is in doubt. In addition the number of seeds recovered may be greater due to better technique, and it should be possible to identify and remove weed plants. Further, it should be possible to reduce the amount of mechanical damage that occurs to seeds as a result of repeated handling and threshing.

Seeds should be threshed when the moisture content is between about 12 and 16%. The reasons for this are: first, seeds within this range are easier to thresh than wetter seeds: the wetter the seed the

more likely will associated vegetation tend to block up any equipment; and, more importantly, the easier it becomes to detach seeds from seed heads as the attachment becomes more brittle as seeds are dried. Secondly, within this range mechanical damage to seeds is minimised.

Mechanical damage

Mechanical damage is the term used to describe those physical injuries to seeds which result from collision. The collision may either be between seeds or between a seed and other objects - such as the surfaces of seed-processing machinery. Obviously seed velocity at impact affects the extent and/or likelihood of mechanical damage. Thus seeds should not be dropped from great heights, although this is unlikely to occur in a gene bank. Another factor which affects the likelihood of mechanical injury is the plasticity or elasticity of the surface which seeds hit. For example, a rubber surface deforms on impact and thus cushions the effect of the collision for the seeds. Thus it is a good idea for metal surfaces in dividers etc. to be coated with rubber.

The suggested range of moisture contents for threshing is based on the fact that at either very high or very low levels of moisture content the likelihood of mechanical damage is increased. In moist seeds bruises result from impacts. The greater the impact the deeper the bruise. Bruising of the radicle is the most likely to prevent normal germination (see Chapters 4 and 10). Bruising of other areas can make the seed more prone to disease and/or imbibition injury (see Chapters 4 and 7). Bruising damage is the result of cells rupturing on impact and releasing destructive hydrolytic enzymes, e.g. on the surface of cotyledons in legumes. Apart from any effect on germination if tested immediately after impact, there is some evidence that bruised seeds lose viability more rapidly in storage - particularly when the seeds are stored very moist.

In dry seeds the usual form of damage is caused by fracturing since such seeds can be extremely brittle. Again the most prevalent area for injuries to occur is in the region of the radicle. In addition cracks in the seed coat can result, and these can increase the likelihood of damage to the seed occurring at imbibition (see Chapters 4 and 7). Seeds which are neither too wet for bruising to occur on impact, nor too dry for fracturing to occur on impact, can be considered to be at an optimum level of moisture for handling and threshing. Legume seeds are probably amongst the more prone to mechanical damage. The range of 12 to 16% moisture content is thought to be the optimum moisture content for handling and threshing seeds of legume species. For many other species the optimum range is wider than this. Nevertheless, for convenience, but also because not all species have been investigated in as great detail as the legume seeds, we suggest this range is adhered to, where possible, for all species.

Seed anatomy is also important. First, small seeds are far less prone to mechanical damage than large seeds - mainly because the former are more protected against impacts at harvest and threshing by the mass and nature of vegetation harvested with the seed. Secondly seed shape is important. Spherical seeds such as those of Brassica spp. are less prone to mechanical damage than flat seeds such as sesame (Sesamum indicum) or angular seeds such as onion (Allium cepa). Thirdly, the location of the embryo within the seed has an effect. In rye (Secale cereale), for example, the tip of the radicle protrudes somewhat from the line of the endosperm whereas in barley (Hordeum vulgare) the root tip is less exposed and protected by the seed covering structures; thus rye is far more prone to mechanical damage than barley. Finally, variation in seed coat characteristics influence susceptibility to mechanical damage - particularly in the Leguminosae - and this can be particularly noticeable within a species. Hard seeds (see Chapters 4 and 7) tend to have comparatively thick impermeable seed coats which give some protection

against impacts. Thus varieties with thinner seed coats may be more prone to mechanical damage. In addition the more tightly seed coats adhere to embryos the more resistant the seeds are to mechanical damage. There can be substantial varietal differences in the adherence of the seed coat to the embryo (e.g. in Phaseolus vulgaris) and consequently in susceptibility to mechanical damage.

Use of threshers

Seeds can either be threshed by hand or threshed mechanically. There are a number of points to consider if mechanical threshers are used. The threshing machine must be clean. This is essential to avoid seeds from one accession being contaminated with seeds of accessions previously threshed and to avoid diseases or insect pests being passed from one accession to another. Certain threshers do not require cleaning. However, others may need to be dismantled and thoroughly cleaned between accessions. Cleaning the thresher will involve the removal by brushing and blowing of seeds, stones, earth and other debris from the machine.

Mechanical threshers are rarely 100% effective in removing and separating seeds from seedheads and the associated vegetation. Frequently those seeds most firmly attached to the seed heads (e.g. in cereals those at the base of ears) are not dislodged during mechanical threshing. Also some seeds which are detached are caught in the associated vegetation. Thus a number of relatively precious seeds may be lost from the accession. In extreme cases there can be so much accompanying vegetation that seed heads are 'protected' from the thresher and virtually no seeds are recovered.

Finally even seeds within the range of 12 to 16% moisture content are nevertheless brittle, and mechanical threshing can result in broken seeds. If other considerations demand the use of threshers, steps should be taken to ensure that the process is closely controlled and damage minimised.

PURITY

In an accession in which all seeds are meant to be the same the definition of purity is simple. Every seed would be genetically identical, whole, undamaged and the accession should be free from pests, diseases and debris. We can consider this aim to be absolute purity. In other cases it will be intended that the accession is genetically heterogeneous but the other factors will still be relevant. However, each criterion is an ideal rather than a necessity.

Dispersal units

One point of difficulty concerns what exactly constitutes a seed? Botanically the seed is the ripened ovule containing the embryo, whilst the fruit is the fertilized and matured ovary of a flower (see Chapter 3). However, agriculturally the seed is described as the unit of dispersal or propagation. This can cause problems for in some species - particularly the grasses - it can be difficult to determine whether a seed (agricultural definition) does in fact contain a seed (botanical definition)! Hence the terms empty and full seeds.

Other difficulties may arise when the dispersal unit being stored consists of dried fruits which contain more than one seed. For example this is the case in Beta spp. where the dried fruit is called a cluster. The cluster is the unit of propagation: in multigerm lines each cluster may contain as many as four or five seeds; in monogerm lines each cluster normally contains only one or two seeds. In agronomic practice

clusters of multigerm lines are often 'rubbed' - that is, a substantial part of the fruit is eroded by friction - to produce clusters containing only one or two seeds. The reason for this is to avoid as many as four or five plants growing at one spot after sowing, all but one of which in farming have then to be removed ('singled'). There is no problem in storing dried clusters of Beta spp. and thus no immediate reason for gene banks to attempt either to reduce the number of seeds within clusters or to extract seeds from clusters. Moreover the process of 'rubbing' is to be avoided by gene banks since it considerably reduces the number of seeds available and incidentally increases the risk of loss of genetic heterogeneity. There are circumstances in which gene banks may wish to extract seeds from fruits containing more than one seed, for example when regenerating or multiplying an accession if the number of fruits is limited, or if a topographical tetrazolium test for viability is required. In the example of Beta spp. extraction is quite easy. Each seed is contained loosely within a cellular capsule. Each capsule has a flat plate-like lid on the exterior of the cluster. This can be cut away or persuaded to open and the true seed allowed to drop out. Particular care should be taken when imbibing these excised dry seeds (Chapters 4 and 7).

Debris and other seeds

Debris can consist of a wide range of plant and soil-derived matter which has failed to be separated from seeds during harvesting and threshing. Examples of soil-derived debris include soil, sand and stones. Examples of plant-derived debris include chaff, stems, leaves, flowers, nematode galls, fungus bodies and seedlike structures where the embryo (and endosperm if originally present) have been replaced by insect larvae. Indeed seed-borne insect pests can be a serious potential problem and it is a useful precaution to open containers containing seed materials sent to gene banks in laminar-flow cabinets with the air direction reversed. (That is air from the laboratory is drawn into the cabinet and filtered before being exhausted.) This avoids insects being introduced into the gene bank and possibly escaping. In the earlier section on drying it was advised that, to avoid this, accessions should be opened and given a preliminary cleaning at receipt. The most problematic form of plant debris are broken seeds, pieces of seed covering structure and empty seeds. In general - in line with ISTA rules - where half or more of the true, botanical, seed is present it is considered to be a seed, not a piece of debris since the part of the seed present may be capable of producing a plant. Seeds of weeds and other crop species can usually be detected by eye.

Seed cleaning

In most gene banks it will be necessary to improve the purity of some accessions by cleaning. Commercially the cleaning of large bulks of seeds is usually achieved by mechanised means - passing the seeds through rotating drums or across vibrating screens of various apertures. This often has the (commercial) advantage of producing a uniformly sized seed lot - very small and very large seeds being excluded from the seed lot. Clearly this would not be helpful in gene banks for such a policy could lead to selection within genetically heterogeneous accessions. Moreover, the equipment itself requires rigorous cleaning and often careful adjustment between accessions. Consequently gene banks are strongly advised to clean accessions by hand. Hand sieves with graded mesh sizes can be used to remove large and fine debris provided, particularly when the accession is meant to be genetically heterogeneous, care is taken to return small, shrivelled and immature seeds to the accession bulk.

Small accessions can also be fairly easily cleaned by hand - particularly if this is done on an opaque glass sheet illuminated from below. Finally in [illegible] species the seed can be blown in a ventilated air column - this enables lower density materials - leaves, awns, etc. - to be separated. In most gene banks a combination of blowing, sieving and visual inspection will provide the most suitable cleaning procedures.

It should be noted that those concerned with seed health testing have urged that material discarded in the cleaning process could be used for preliminary seed health tests if these are undertaken, because such material often contains a high level of pests and diseases and because this can reduce the demands of seed health testers for seeds sampled from the accession itself.

Assessing purity

Having cleaned the accession it will probably be necessary to assess purity. In gene banks, because of the low seed numbers involved, it should be possible to remove all seeds of weed and other crop species and most of the debris by cleaning. However in many species the possibility exists that some of the pure seeds remaining are empty; that is the seed-like structure lacks an embryo. The unrecognised presence of empty seeds will make the estimation of percentage germination - and thus comparison with the regeneration standard - impossible. Consequently some method of determining the presence of empty seeds is required.

Three methods of determining the empty seed fraction are available: the International method; the Irish method; and X-ray methods. Of these X-ray methods are the simplest, quickest procedures and their use is recommended in gene banks. The only drawback is the initial cost of the equipment. Methods of determining the empty seed fraction are discussed in Chapter 8. To avoid confusion - particularly for those to whom samples of accessions are distributed - it is recommended that a target is set for the proportion of full seeds in accessions, e.g. 95%, and that accessions failing to meet this target be re-cleaned as many times as is necessary to reach this standard. However, in some grass species, for example, there are no satisfactory methods for removing empty seeds and the proportion of full seeds will be much less than this; it is nevertheless important to know what this proportion is.

SEED NUMBER

Having ensured that the proportion of seed-like structures which contain a true seed is known, it is then necessary to determine the number of seeds present within the accession. This is required in order to determine whether there are sufficient seeds available for conservation and, if so, the number of seeds available for distribution, evaluation, viability monitoring and regeneration. Moreover, when seeds are removed from the accession for any of the above purposes it is vital to ensure that the record of seed number is reduced accordingly; otherwise the accession may be lost through seed depletion without sufficient seeds for regeneration.

Seed number can be determined directly (the whole accession being counted) or estimated indirectly (the whole accession is weighed, a sub-sample from the accession counted and weighed, and seed number estimated by dividing the total weight of the accession by the estimated mean seed weight. Further details and various alternative procedures for determining/estimating the seed number of accessions are provided in Chapter 2.

SEED HEALTH

Fungi, bacteria, viruses and nematodes can all be seed-borne (and also debris-borne). One problem which arises from this is that quarantine services can delay and even destroy seed material before receipt or after despatch from gene banks. Another common problem is the misuse of disease-eradicants which are toxic to seeds (see also Chapter 9). Since there is evidence that some chemicals used to treat seeds

can reduce longevity under some storage conditions, and because the long-term effects of the majority are unknown, the IBPGR Seed Storage Committee has recommended that chemical treatment of seed for long-term genetic conservation should be avoided if possible. In spite of these difficulties gene banks have a responsibility to avoid the introduction of a new seed-borne pathogen, or a new race of a seed-borne pathogen, into regions. To avoid this occurring UNFAO recommendations are that

1. All germplasm should be introduced through quarantine services.

2. Regional and national laboratories should be established for seed health testing to enable this to occur.

If the bank itself undertakes seed health examination, work could be minimised if this is undertaken the first time a sample from an accession is withdrawn for distribution, rather than delaying its acceptance into storage at receipt. However, to save seeds it has been suggested that debris removed from accessions during seed cleaning should be used for an initial investigation of seed health. As already mentioned it is best to avoid any treatment to the seeds (for example, heat or chemical treatments) before storage because these may affect rates of deterioration during long-term storage, or affect the results of subsequent monitoring tests. Where a seed health treatment is deemed essential it is suggested that, where the rules allow, it should be carried out immediately before sowing or distribution, rather than before storage. Moreover, if at all possible, the user to whom material is distributed should be made responsible for satisfying import regulations, quarantine restrictions, etc. - rather than the gene bank.

For many seed health problems, testing can be carried out on a sample of seeds drawn from the accession. This can be done when a sample is withdrawn from an accession for distribution for the first time, and will enable assumptions to be drawn concerning the health of the remaining seeds in the accession. There are, however, two drawbacks to this policy. First, such a procedure would inevitably result in some delay in responding to requests. Secondly, certain seed-borne pathogens can be actively spread from only trace infections. To test for such a pathogen requires the testing of the whole bulk - and so seed sampling procedures are inadequate. Thus in these cases each sample distributed will require testing. However, such pathogens are normally tested by post-entry quarantine and so gene banks should be able to avoid this work since the user of the distributed material will be responsible for it.

Seed health testing requires a substantial number of expertly trained, and re-trained, staff. Most gene banks are unlikely to have sufficient resources to be able to undertake seed health testing. In these cases suitable arrangements should be made for other existing institutions to assume this responsibility. The most satisfactory solution is where the gene bank and quarantine station are situated nearby each other within the same institution. Nevertheless, even if responsibility for seed health has been delegated to another laboratory, gene bank staff should continually be aware of seed health problems.

MOISTURE CONTENT DETERMINATION

Two aspects of the seed storage environment are controlled - moisture content and temperature. Both need monitoring. Measurement of temperature is relatively simple. A wide range of different types of instrument is available to give instant, remote measurement of store temperature. However, it is not so simple to measure seed moisture content.

Non-destructive methods

Equipment is available to provide instant, non-destructive, measurement of seed moisture content. One type of equipment consists of a probe which is inserted into the seed bulk and measures electrical conductivity; the recorded value needs correction according to seed temperature. However, electrical methods of determining seed moisture content are both insufficiently accurate and - because of inherent limitations in the method - are unable to measure very low seed moisture contents such as are appropriate for gene bank storage. They are nevertheless useful for checking the moisture content of incoming batches of seeds to decide whether drying is urgently needed.

Gravimetric methods

Consequently it is necessary for gene banks to use the more time-consuming, destructive, but accurate gravimetric methods of determining seed moisture content. These are destructive determinations because seeds - normally first ground - are weighed, heated in an oven and subsequently re-weighed. Moisture can be expressed as the loss in weight (i.e. less in water) as a proportion of the original weight of the seed (known as the fresh-weight basis or wet basis) or as a proportion of the final weight of the seed (dry-weight basis or dry basis).

There a problem however: the loss in weight which occurs during oven-drying is dependent upon both the temperature within the oven and the drying period. This is because - in addition to removing or failing to remove all moisture - oven drying treatments can cause oxidation, decomposition or the loss of other volatile substances (e.g. oils). The result is therefore to some extent arbitrary.

In order to make the term moisture content consistent and to minimise the above problem, agreed standard procedures are used to determine seed moisture content. Seed analysts follow the rules of the International Seed Testing Association (reproduced in part in Chapter 2). According to these rules moisture content is recorded as a percentage of the seed including the water (wet basis). Engineers follow the rules of the International Standards Organisations. They usually express moisture content as a percentage of the dry weight of the seed (dry basis). Otherwise the two sets of rules are essentially identical. A conversion scale (Chapter 2) is provided to convert between dry basis and wet basis moisture contents. In seed physiology and in gene bank applications it is normal to calculate and express moisture content values on the wet basis.

Since oil content varies between species, two methods of determining moisture content are available: (1) the high constant temperature oven method - for non-oily seeds; (2) the low constant temperature oven method - for oily seeds. Two lists of species are provided in Chapter 2 - one of species with non-oily seeds and the other of species with oily seeds. Oil content of seeds in the first category are generally less than 5% db (dry basis, otherwise the value would be confounded with moisture content!), and greater than 15% db in the second. We suggest that the high temperature method be used for species with seeds of oil contents of 5% (db) or less and the low temperature method for species where seed oil contents are greater than 5% (db). See Chapter 2 for more information.

Time taken by seeds to dry to preferred moisture contents

Moisture content determinations are expensive in terms of both seeds and manpower. Given inadequate resources and the problems of incorporating large numbers of accessions into store as quickly as

possible, how necessary is it for the moisture content of each and every accession to be determined before storage? Assuming that the gene bank is drying accessions in a drying room of known characteristics then it is possible to avoid the need for determining individual accession moisture contents in seeds of known drying characteristics by relying instead on a pre-determined fixed time within the drying room. For seeds of a given species with the same chemical composition (oil content) and the same size in a given drying environment the rate of loss of moisture is dependent upon the difference between the actual seed moisture content and the moisture content at which the seed would be in equilibrium with the drying environment. As the difference between these two values declines during drying so the rate of loss in moisture declines - and dramatically so. Consequently in a gene bank drying room operated at realistic conditions the difference between the time taken for seeds to dry to between 4 and 6% moisture content from various different levels of initial moisture will be imperceptible in practical terms. Within each species the most important factor affecting these times will be seed size. Particularly in large seeded species it may be necessary for the drying time to vary within a species depending upon seed size.

The 1982 report provides equations and nomograms which can be used to estimate approximate drying times. Nevertheless, each gene bank will need to check such times from their own practical experience. It is suggested that part of the drying room commissioning period be devoted to providing this information. When using pre-determined fixed drying times particular attention should be paid to airflow patterns throughout the drying room. It would also be advisable subsequently to sample accessions occasionally after drying to check that the expected moisture contents continue to be attained.

Having considered how to determine moisture content, or ensure it is within the preferred range of between 4 and 6%, we can now consider how seed moisture content should then be controlled during storage. There are two alternatives: either the accessions are stored in open containers and the relative humidity of the whole cold store is controlled; or the accessions are stored sealed in hermetic containers with no control of cold store humidity. In long-term stores the latter is preferable on grounds of cost, technical considerations and safety (see the 1982 report). Consequently it is necessary to consider the packaging of accessions to achieve hermetic environments.

PACKAGING

Any material which is impermeable to water vapour is theoretically suitable as a packaging medium. In practice three types of material are used: glass, metal and aluminium-plastic foil laminates. The advantages, disadvantages, potential faults, their detection and testing have been considered in the 1982 report. Here we emphasise two aspects of packaging: whether containers should be re-usable, and the importance of acting upon any observed packaging deficiency.

Re-usable or non-re-usable containers

If accessions are to be retrieved from storage frequently and sub-sampled, then re-usable containers (particularly screw-capped or similar jars or tins) are an advantage since the cost of accessing the material is essentially labour only with little or no replacement of packaging material. In contrast, if non-re-usable containers are employed then accessing material in store entails a replacement cost for the container each time. Moreover in the latter case the need to repackage may increase the period the accession remains out of the store. This may not cause a significant loss in viability but it will increase handling time. On the other hand there is potentially more chance of leaks occurring with re-usable as opposed to single-use containers. Some compromise between the two types of containers may be useful if a sequential test for

monitoring (see Chapters 12 and 15) accession viability is used. In which case it would be convenient for accessions to be stored in re-usable containers which are easy to open and reseal during the period of the sequential test when a number of samplings may be necessary over a short period, but for accessions to be sealed in single-use containers once the sequential test is terminated (if the decision is to keep the accession in store for a further monitoring interval).

Action on finding a defective container

If a container defect is observed when accessions are first placed into store then these deficiencies can and should be rectified immediately, and a close watch kept on containers from the same batch. Once a gene bank has been operating for some time it is possible that a container withdrawn from the cold store may show some defect. Obviously this container will be discarded and replaced. Further action is, however, required.

A reasonable sample of containers from the same batch which were sealed at a similar time should also be checked for deficiencies, and if necessary, replaced. In the first instance a reasonable sample might be considered to be all those containers sealed on the same date with this being extended by one day before and after as necessary until one hundred or so containers have been inspected. If no other deficiencies are found the exercise can be concluded.

If deficiencies are found, then an attempt should be made to diagnose the cause of the defect. This might be a specific operator error, a specific error in adjustment to a sealing machine, or manufacturing defects in a batch of containers. Inspections should continue until staff are confident that all containers which may have been affected by the specific error have been checked.

SYNTHESIS OF BANKING OPERATIONS

It is now possible to synthesise the preceding sections and compile examples of procedures for the incorporation of accessions into the long-term store. We shall consider two examples of the actions ideally required. In the first example a grass has been collected by an independent collector.

1. Before setting out the collector had informed the gene bank of the intended mission and asked whether the gene bank would be able to receive the material collected.
2. In reply the gene bank had confirmed their willingness, and ability, to receive the material during the period stated, and provided the collector with the following guidelines:

(i) the grasses in question show a propensity to shatter and collection should not be delayed;
(ii) the gene bank prefers the material to be returned as inflorescences;
(iii) accessions containing fewer than 4000 seeds would not be accepted for long-term storage, but ideally 12,000 seeds were required;
(iv) the inflorescences should be dried (but do not use heated air-driers) to around 10-12% moisture content and then hermetically sealed;
(v) a similar duplicate, well-documented, collection must be provided to the national gene bank;
(vi) before beginning to collect it must be confirmed that the exporting seed health authority will neither unduly delay, nor treat the material in question - the gene bank had already made a similar arrangement with the importing authority;
(vii) a copy of the passport and characterization data must accompany each collection;

(viii) the material should be despatched as soon as it is packaged at regular intervals, preferably of a week or less, and a telex containing details of each consignment should be sent to the gene bank immediately.

3. Having received a telex that another consignment was en route the gene bank was able to ensure that the courier provided the rapid service contracted.

4. When received by the gene bank the material was immediately placed in the temporary store maintained at 17°C with 40% relative humidity, whilst the accompanying documents were passed to the gene bank manager.

5. After checking that the documentation matched the packages received, the manager made arrangements for the accompanying passport and characterization data to be entered into the gene bank's files, whilst at the same time providing accession numbers and opening a management information file for each package, and informing the collector that the consignment had been received.

6. The manager then made arrangements for the package to be removed from the temporary store and for the banking procedure to begin.

7. The hermetically sealed container sent by the collector was opened in a reversed-flow laminar-flow cabinet.

8. No obvious pest or disease problems were observed within the inflorescences and the inflorescences were confirmed as being the species given on the passport data.

9. The operator assessed the inflorescences to be sufficiently dry to thresh and did this by gently pulling each inflorescence between the gloved thumb and index finger of one hand.

10. The seeds and various covering structures still attached were then repeatedly, but gently, rubbed between two gloved hands.

11. This material was then cleaned in an air-blower - adjusted so that only dust and other light material lifted off, whilst the seeds and seed-like materials remained.

12. Then, on the bench top, material of a similar density to the seeds - weed seeds, soil particles, etc. - were removed to one side using a spatula; any broken seeds were also removed at this point.

13. All discarded materials from the inflorescences - straw, dust, broken seeds, etc. - were then placed in a separate, sealed container for health testing, and labelled appropriately.

14. Using the riffle divider a sample of 100 seed-like structures was obtained, and X-rayed in real time (that is the image was observed on a video monitor).

15. Since this test indicated that about 20% of the seed-like structures were empty (that is no seeds were contained within them), the accession was returned to the cleaner/blower and the air velocity increased so that the lightest seed-like structures were removed from the seed.

16. A sample of the seed-like structures removed from the seed bulk were then cut open with a scalpel; this test confirmed that only empty seeds, not full seeds, were being removed by the cleaner.

17. Again 100 seed-like structures were sampled from the accession by using the riffle divider, and then X-rayed in real time.

18. This test indicated that more than 95% of the seed-like structures contained a seed.

19. The result of this last X-ray test was recorded on the management file, together with the total weight of the accession remaining after cleaning, the accession placed in a cotton bag and taken to the drying room maintained at 15°C with 10-15% relative humidity.

20. The empty seeds removed in the second cleaning operation were added to the bag containing all the discarded components of the inflorescences, the bag re-sealed and sent to the seed health laboratory for a preliminary investigation of the seed health hazards associated with the accession; but if this had not been possible the discarded materials would have been incinerated.

21. From the texture and brittleness of the inflorescences at threshing the operator estimated seed moisture content to be between about 12 and 14%, and from previous experience expected the seeds to dry

below 6% moisture content within a week in the drying room.

22. After a week in the drying room the accession was re-weighed and it was found that the total weight had declined to 90% of its original weight.

23. An 8 gramme sub-sample of seed was obtained using the riffle divider and this was then divided into two, weighed, dried in a mechanically-ventilated oven for 2 hours at 130° to 133°C, cooled, re-weighed, and accession moisture content calculated as 4.5%.

24. Again using the riffle divider, two large sub-samples of seed were obtained from the accession and - in a dry room - 1000 seeds counted at random from each, weighed and all these seeds then returned to the accession.

25. The accession was then re-weighed; calculations by the operator (based on total seed weight and 1000 seed weight) showed the number of seeds present to be in excess of 4000, and thus sufficient to comprise an accession.

26. The accession was then removed from the drying room, hermetically sealed in a laminated aluminium foil packet, and then placed in the long-term seed store operated at -20°C at the location specified by the gene bank manager.

27. The seed moisture content, estimate of total seed number, total accession weight, 1000 seed weight, and store location of the accession together with the date of entry into the long-term store were then recorded on the management information file, and the seed testing laboratory informed that the accession was now in store and awaiting a germination test to assess viability as soon as this was possible.

28. Provided this test result proved satisfactory the accession would then be added to the list of material available in the base collection.

By way of contrast the second example considers the routine followed when a small accession of true potato seed received from a breeder is multiplied within the gene bank because the accession is considered valuable but contains too few seeds.

1. 1000 of the 2000 seeds in the collection had been sampled, germinated and the resultant surviving seedlings transplanted into the field.

2. The growth habit of the crop and other relevant information concerning as many descriptors as possible noted down, and transferred to characterization and evaluation data forms.

3. The seed health laboratory staff were invited to inspect, and take samples from, the growing crop.

4. As no other potato crops were being grown in the vicinity no special isolation measures were taken.

5. Flowering dates were noted, and the fruits picked about six weeks later whilst still green - and certainly before the haulm began to senesce - an accession number allocated, and a management file opened.

6. The collected fruits were stored overnight in a cold room maintained at 3° to 5°C.

7. The next day the operator, wearing gloves, cut around the outside of each fruit with a scalpel and squeezed the fruit contents into a large bowl.

8. Slight pressure between gloved hands was exerted on the fruit contents within the bowl as a preliminary stage in separating the seed from the gel.

9. Small (100-200 ml) sub-samples of the gel were removed from the bowl and placed in a strainer with 1 mm square mesh.

10. A stream of water from a moderately high pressure tap was then directed at the contents of the strainer and the gel and seed separated with the assistance of gloved fingers.

11. When the majority of the gel had been removed, the seeds were thinly spread on top of blotting paper and placed in the temporary storage room maintained at 17°C with 40% relative humidity overnight.

12. The next day any remaining gelatinous covering was removed using forceps, the dried seeds brushed from the blotting paper into a cotton bag and transferred to the drying room maintained at 15°C with

10 to 15% relative humidity for 3 or 4 days.

13. Details of the harvesting, extraction, and drying procedures and dates were added to the accession management file.

14. A 1 gramme sub-sample of the seeds was obtained using the riffle divider and further divided into two, weighed, dried in a mechanically-ventilated oven for 1 hour at 130° to 133°C, cooled, re-weighed, and accession moisture content calculated as 4%.

15. Again using the riffle divider, two large sub-samples of seed were obtained from the accession and - in a dry room - 1000 seeds counted at random from each, weighed and all these seeds returned to the accession.

16. The entire accession was then re-weighed and calculations made which determined that the number of seeds present was well in excess of 4000 and thus sufficient to comprise an accession.

17. The accession was then removed from the drying room, hermetically sealed in a laminated aluminium foil packet, and then placed in the long-term seed store operated at -20°C at the location specified by the gene bank manager.

18. The seed moisture content, estimate of total seed number, total accession weight, 1000 seed weight, and store location of the accession together with the date were then recorded on the management information file, a cross-reference to the accession number and location of the parent seed material provided, and the seed testing laboratory informed that the accession was now in store and awaiting a germination test to assess viability.

19. Provided this test result proved satisfactory the accession would then be added to the list of material in the base collection.

These two examples illustrate how easy it is to incorporate good quality seed accessions into long-term stores and are intended to consolidate the information within the preceding sections.

FURTHER READING

It is outside the scope of this handbook to present detailed information on seed collecting, drying, health, regeneration/multiplication, or the management of information within gene banks.

For further information on these and other relevant topics the reader should consult the following references.

Seed collecting

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CHAPTER 2. THE DETERMINATION OF MOISTURE CONTENT AND THE NUMBER OF SEEDS IN ACCESSIONS

EQUIPMENT FOR GRAVIMETRIC MOISTURE CONTENT DETERMINATION

The following equipment is required for moisture content determinations by gravimetric methods: a forced-draught oven, drying containers, a grinder and sieves, an analytical balance, a desiccator and a thermometer.

The forced-draught oven should be ventilated by a powerful fan, electrically heated with thermostatic control, well insulated and capable of providing a uniform temperature throughout the cabinet. The heating capacity must be capable of enabling the oven to regain the operating temperature within 15 minutes of the oven door being opened at that temperature, loaded with the material to be dried and then closed. Removable perforated or wire shelves should be fitted. A thermometer, accurate to 0.5°C, should be fitted so that it is in the vicinity of the samples whilst it can be read without the oven door having to be opened.

The drying containers should be made of non-corrosive metal or glass approximately 0.5 mm thick. The bottom of each container should be flat with rounded edges. The upper perimeter should be level with snug fitting covers. The size of the container should be such that the sample to be dried can be distributed to give not more than 0.3 g/cm². Since the maximum sample size will be no more than 5 g, circular dishes of 2.5 cm diameter (internal) [$5/(\pi 2.5^2) = 0.25$ g/cm²] are sufficient.

Grinding is not necessary for small-seeded species but for gene banks dealing with larger-seeded species (a list of species where the seeds must be ground is provided later in this chapter) a grinding mill is essential. The grinder should be capable of adjustment so that the size of the ground material can be altered. Sieves of mesh sizes 0.5, 1.0 and 4.0 mm are required to enable the mill to be tested after adjustments have been made. The grinding plates should not get hot when used (otherwise moisture would be lost from the seeds before the determination). It should be constructed from non-absorbent material and protected from ambient air (particularly draughts) during grinding (that is it should be enclosed).

The analytical balance must be supported on a suitable balance bench (free from vibration), must be quick in operation (to avoid samples gaining or losing moisture during the weighing operation) and capable of weighing to 0.001 g if 5 g samples are used, or 0.0001 g if smaller samples are used (see later section).

The desiccator should be fitted internally with a thick metal plate to enable the containers to cool rapidly when removed from the oven. It should contain a suitable desiccant (e.g. indicating silica gel) which should be regularly regenerated or replaced.

METHODS FOR GRAVIMETRIC MOISTURE CONTENT DETERMINATION

The ISTA rules for procedures to determine seed moisture content are summarised below. The full rules and annexes to the rules will be found in Seed Science and Technology, 1985, Volume 13.

The essential points are as follows.

1. The sample should be exposed to the laboratory atmosphere as seldom as possible throughout the drying procedure (except in the special case of the two-stage procedure described below). Samples of seeds used to determine moisture content should not be left for long periods exposed to the laboratory atmosphere either directly or indirectly (e.g. in permeable containers).

2. Determinations are made in duplicate on two independently drawn samples - see Chapter 13 for details of appropriate sampling procedures. The size of sample required for the determination of moisture content is likely to be a problem in gene banks - see the later note on sample size - since the method is destructive. The ISTA procedures prescribe 4 to 5 g for each of the duplicate samples with two exceptions. First, if the diameter of the drying containers is greater than 8 cm then 10 g is required for each duplicate sample. Secondly, if pre-drying is required (see below) then at least 25 g of seeds must be sampled.

3. If the sample is very moist then it should be pre-dried. (This is unlikely to be necessary in gene banks, unless it is necessary to determine the moisture content of very wet seeds on receipt before they are dried.) If moisture content is more than 30% the previously weighed samples are left overnight on top of a heated oven. If the moisture content is between 17% to 30%, or 10% to 30% for soyabean, or 13% to 30% for rice, and the seeds are large, then the previously weighed samples are first placed in an oven at 130°C for 5 to 10 minutes and subsequently exposed to the laboratory atmosphere for 2 hours. To determine the loss in moisture during pre-drying, the samples - at least 25 g - are first weighed into previously weighed containers, pre-dried as above and the loss in weight (i.e. loss in moisture) determined by subtraction. This is the first stage of a two-stage moisture content determination.

4. Large seeds are then ground - unless they have a very high oil content which makes them difficult to grind - (see list of species which require grinding below). For cereals and cotton fine grinding is necessary: the ISTA prescribe that at least 50% of the ground material should pass through a sieve with 0.5 mm mesh size but not more than 10% of the ground material should remain on a sieve of 1 mm mesh size. Leguminous and tree seeds should not be ground so finely: at least 50% of the ground material should pass through a sieve of 4 mm mesh size. The settings of the grinding mill necessary to satisfy these requirements should be inscribed on or close to the grinding mill so that all staff in the gene bank are reminded of them. It is essential that seeds of the following species are ground.

Arachis hypogaea	*Gossypium* spp.	*Quercus* spp.
Avena spp.	*Hordeum vulgare*	*Ricinus communis*
Cicer arietinum	*Lathyrus* spp.	*Secale cereale*
Citrullus lanatus (*C. vulgaris*)	*Lupinus* spp.	*Sorghum* spp.
Fagopyrum esculentum	*Oryza sativa*	*Triticum* spp.
Fagus spp.	*Phaseolus* spp.	*Vicia* spp.
Glycine max	*Pisum sativum*	*Zea mays*

5. The drying containers must be dried before use. To do this they should be placed in an oven at 130°C (approximately) for an hour and then allowed to cool in the desiccator (containing a desiccant) for a further hour. The drying of containers before use is particularly important in humid regions.

6. Each dish and lid is then weighed. The sample (whether previously pre-dried and ground or not) is then placed in the container, the lid is replaced and the dish, lid and seeds are weighed. All weighings are normally to 3 decimal places (that is to 0.001 g), but see below for circumstances when greater accuracy is needed.

7. Dishes containing non-oily seeds are then placed in an oven maintained at 130° to 133°C, the lids removed and the samples dried for 4 hours (maize), 2 hours (other cereals) or one hour (all other species with non-oily seeds). This drying period commences when oven temperature has regained 130° to 133°C after the samples have been put into the oven and the oven door closed. (This period should be no longer than 15 minutes.) At the end of the appropriate drying period each lid is placed back on the dish from which it was removed and the containers allowed to cool in a desiccator for about 45 minutes. When cool the containers and (dry) contents are weighed. The ISTA provide the following list of species where seed moisture content should be determined using this method - the high constant temperature oven method.

Agrostis spp.
Alopecurus pratensis
Anethum graveolens
Anthoxanthum odoratum
Anthriscus spp.
Apium graveolens
Arrhenatherum spp.
Asparagus officinalis
Avena spp.
Beta vulgaris
Bromus spp.
Cannabis sativa
Carum carvi
Chloris gayana
Cicer arietinum
Cichorium spp.
Citrullus lanatus
Cucumus spp.
Cucurbita spp.
Cuminum cyminum
Cynodon dactylon
Cynosurus cristatus
Dactylis glomerata
Daucus carota
Deschampsia spp.
Fagopyrum esculentum
Festuca spp.
Holcus lanatus
Hordeum vulgare
Lactuca sativa
Lathyrus spp.
Lepidium sativum
Lolium spp.
Lotus spp.
Lupinus spp.
Lycopersicon esculentum
Medicago spp.
Melilotus spp.
Nicotiana tabacum
Onobrychis viciifolia
Ornithopus sativus
Oryza sativa
Panicum spp.
Papaver somniferum
Paspalum dilatatum
Pastinaca sativa
Petroselinum crispum
Phalaris spp.
Phaseolus spp.
Phleum spp.
Pisum sativum
Poa spp.
Scorzonera hispanica
Secale cereale
Sorghum spp.
Spinacia oleracea
Trifolium spp.
Trisetum flavescens
Triticum spp.
Valerianella locusta
Vicia spp.
Zea mays

Note that the above list contains a few species whose seeds have a fairly high oil content, e.g. Lactuca sativa.

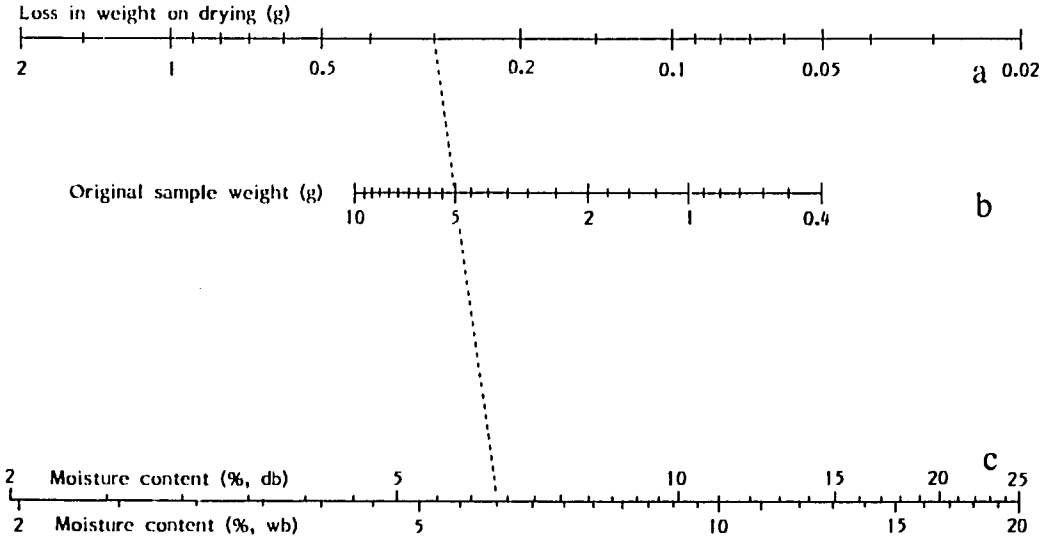
8. The low constant temperature oven method for oily seeds is carried out in exactly the same way as the above method except that the oven is maintained at 103° ± 2°C for 17 ± 1 hours. (Consequently this procedure is usually carried out overnight.) The ISTA provide the following list of species with oily seeds whose moisture content should be determined using this method - the low constant temperature oven method.

Allium spp.	Glycine max	Ricinus communis
Arachis hypogaea	Gossypium spp.	Sesamum indicum
Brassica spp.	Linum usitatissimum	Sinapis spp.
Carnelina sativa	Raphanus sativus	Solanum melongena
Capsicum spp.		

In addition the current ISTA rules prescribe that the moisture content of seeds of all tree species should be determined by the low constant temperature oven method. The 1976 ISTA rules (Seed Science and Technology, 1976, Volume 4, pages 40 to 43 and 160 to 163), however, prescribed that the moisture content of seeds of Abies, Cedrus, Fagus, Picea, Pinus and Tsuga spp. should be determined by the (toluene) distillation method. A few notes on the distillation method are provided here because gene banks might at some time wish to use this method to check the accuracy of their oven determination methods (for any species), but see the above reference for more detailed instructions on the operation of the method.

The seed sample and solvent (toluene is usually recommended) are placed in a distillation flask. The flask is then heated, gently, and the evaporated moisture and solvent condensed in a water cooled reflux condensor and collected in a trap (receiver). The trap is graduated at intervals of 0.1 ml. The solvent and water separate in the trap allowing the volume of water evolved by a known weight of seeds to be measured. Although the method, when carefully applied, is accurate, it is only rarely used to determine seed moisture content because it is extremely time-consuming.

Figure 2.1 Seed moisture content nomograph. Use a straight rule to connect appropriate points on scales a, b and c. The dotted line shows an example calculation: extrapolating the straight line connecting the values 0.3 g on scale a (loss in weight on drying) and 5 g on scale b (original sample weight) gives the moisture content on scale c which can be expressed on either the dry basis (6.4%) or on the wet basis (6.0%). Thus scale c also serves to convert between moisture content values calculated on either basis.



Calculation of seed moisture content

In seed testing work the seed moisture content is calculated as the loss in weight as a percentage of the original weight of seeds. This is known as wet basis (wb) or fresh-weight basis. The ISTA rules prescribe calculation to one decimal place. Algebraically, if M_1 is the weight of the container (with cover), M_2 the weight of container and contents before drying, and M_3 the weight of container and contents after drying then

$$\text{percentage moisture content (wb)} = 100 \times (M_2 - M_3)/(M_2 - M_1)$$

This method of expressing seed moisture content is also recommended for use in gene banks.

In some laboratories (particularly botany and biochemistry laboratories) seed moisture content is often calculated on the dry basis (db): that is the loss in weight is expressed as a percentage of the dry weight of the seeds. Algebraically, using the same notation as above,

$$\text{percentage moisture content (db)} = 100 \times (M_2 - M_3)/(M_3 - M_1)$$

The seed moisture content nomograph in Figure 2.1 can be used to calculate moisture content on either basis. In addition scale c allows easy conversion between moisture content values determined on either basis.

In a two-stage drying procedure the moisture lost in each stage can be determined as above on the wet basis. If S_1 and S_2 are the percentage loss of moisture (wb) in the first and second stages respectively, then the

$$\text{Original percentage moisture content (wb)} = (S_1 + S_2) - (S_1 \times S_2)/100$$

To determine seed moisture content on the dry basis from a two-stage drying procedure, first calculate as above on the wet basis and then convert the result to the dry basis using scale c in Figure 2.1.

According to the ISTA rules the differences between the two determinations of moisture content (remember the samples were duplicated) should be no greater than 0.2%. If it is greater the determination should be repeated.

Weight of seeds required for moisture content determinations

If moisture content is to be meaningful - that is, estimates are reliable, reproduceable and standardised (see Chapter 1) - there is currently no alternative to the adoption of the low and high constant temperature oven methods (assuming the toluene distillation method to be too time-consuming). One area where the rules can, however, be altered slightly to the benefit of gene banks concerns the amount of seed used for each determination.

For accessions with very small seeds the ISTA requirement of at least 8 to 10 g of seeds for the destructive determination of moisture content is undesirable for gene banks, if not impossible. One simple approach for very small seeds, which has been sufficiently accurate in our experience, is to reduce the

weight of seed used by a factor of ten, but at the same time to increase the accuracy of all weighings by a factor of ten; that is to use two samples of 0.4-0.5 g each and make all weighings with a 4 decimal place balance (that is to 0.0001 g).

Recent work by staff at the Wakehurst Place Gene Bank supports our view that the weight of seeds required for the gravimetric determination of accession moisture content can be reduced greatly below that prescribed by the ISTA. They have found that only when sample weights are reduced below 0.2 g is there an appreciable increase in the error of moisture content determinations.

SEED NUMBER ESTIMATION

Unless the number of seeds within an accession is known when the accession is first placed in the seed store and this number is updated subsequently whenever seeds are removed (e.g. when monitoring or distributing samples for other purposes), there is a risk that the accession will be exhausted without sufficient seeds for regeneration. The only way to determine seed number accurately is to count all the seeds within the accession.

Seeds can be counted manually using a spatula on a surface which provides a contrasting background to the seeds, but this requires considerable effort. As an alternative the use of automatic seed counters can be considered. These are accurate to within about ±5% of the true seed number when used correctly. They work best when the accession is free from debris and the seeds are of uniform size and shape. Consequently their use with genetically heterogeneous accessions in particular can result in considerably more error than ±5%.

It is suggested that for most gene banks it will be sufficiently accurate to estimate the seed number in an accession by counting and weighing a sample withdrawn from it and calculating the mean seed weight. Then

number of seeds in accession = total weight of accession/mean seed weight.

Note in passing that the weight of a given number of seeds (for example, the number of seeds to be sampled for distribution) can, of course, be determined by the following formula:

weight of seeds = mean seed weight x number of seeds required.

The mean seed weight is an estimate since it is determined from a sample of seeds drawn at random from the accession as a whole. (It is important to sample seeds from the accession at random; see Chapter 13.) The seed number derived from the above calculation is also, therefore, an estimate. Both are subject to error. The size of the error needs to be considered for, if the number of seeds in the accession is overestimated, subsequent accounting will be misleading and the accession might be depleted to a lower number of seeds than is safe before it is regenerated. A number of alternative methods for dealing with this problem are considered below.

Estimation of mean seed weight by ISTA procedures

In ISTA procedures either the whole working sample (at least 2500 seeds, that is roughly equivalent to the number of seeds within an accession) is counted in an automatic counter and then weighed; or

replicated samples are drawn, counted and weighed. In the latter case eight replicates of 100 seeds each are drawn at random from the accession. Each replicate is then weighed to an accuracy of between one ten thousandth and one thousandth of the weight of the working sample. (For example, if the working sample weighs between 100 and 999.9 g then each replicate of 100 seeds is to be weighed to 0.1 g). For gene bank purposes the total accession weight could be used in place of the working sample weight in the above calculation. An alternative suggestion is to weigh each replicate to an accuracy of between one hundredth and one thousandth. (For example, if the 100 seed replicate weighs 2.5 g weigh to 0.01 g.)

The coefficient of variation of 100 seed weight between the eight replicates must then be calculated according to the following formulae:

$$\text{Standard deviation (s)} = \sqrt{[\{n(\Sigma x^2) - (\Sigma x)^2\}/n(n-1)]}$$

$$\text{Coefficient of variation} = (s/\bar{x})\ 100$$

where x = weight of each replicate
$\bar{x}$ = mean weight of all replicates
n = number of replicates
Σ = sum of

According to the ISTA rules if the coefficient of variation does not exceed 6.0 for chaffy grass seeds, or 4.0 for other seeds, then the thousand seed weight is calculated as ten times $\bar{x}$. The mean seed weight is given by $\bar{x}/100$. If, however, these values for the coefficient of variation are exceeded then a further eight 100 seed replicates must be sampled at random, counted and weighed. The standard deviation for all 16 replicates is then calculated. If any replicate diverges from the mean of all 16 replicates by more than two standard deviations (i.e. twice the standard deviation) then these replicate values are ignored and the mean weight of 100 seeds calculated from the remaining replicate values. The thousand seed weight is then calculated as ten times this value, but to calculate mean seed weight divide the mean value by 100.

Estimation of mean seed weight by the Wakehurst Place procedure

Linington, Smith and Butler (1985) of the Wakehurst Place Gene Bank have suggested an alternative procedure. Their results with diverse species with widely varying seed weights show that the coefficient of variation for the estimation of seed weight is reduced substantially by increasing the number of seeds counted and weighed per replicate from 10 to 200, but further increase beyond 200 seeds only reduces the coefficient of variation marginally. They suggest that, rather than counting out and weighing 800 (as in the ISTA procedure above) or 1000 seeds (to determine thousand seed weight), it is only necessary in gene banks to count and weigh a single group of 200 seeds, and this would save considerable time.

Correction of the estimate of mean seed weight

Whether mean seed weight is estimated by the ISTA procedure (8 x 100 seeds), a simple thousand seed procedure (1 x 1000 seeds), or the Wakehurst Place procedure (1 x 200 seeds) it is likely that half of the estimates will underestimate mean seed weight and consequently overestimate the number of seeds present within the accession. This is a serious potential problem which gene bank staff must seek to avoid. Linington et al suggest that gene banks using their procedure should assume (arbitrarily) that they have sampled a group of seeds which is lighter than the true mean for the accession and which is in fact the

fifth percentile sample in terms of seed weight (that is it is assumed that 95% of samples drawn from the accession would be heavier than this estimate). To correct for this potential error then requires the addition of almost two (1.96) standard deviations to the original estimate.

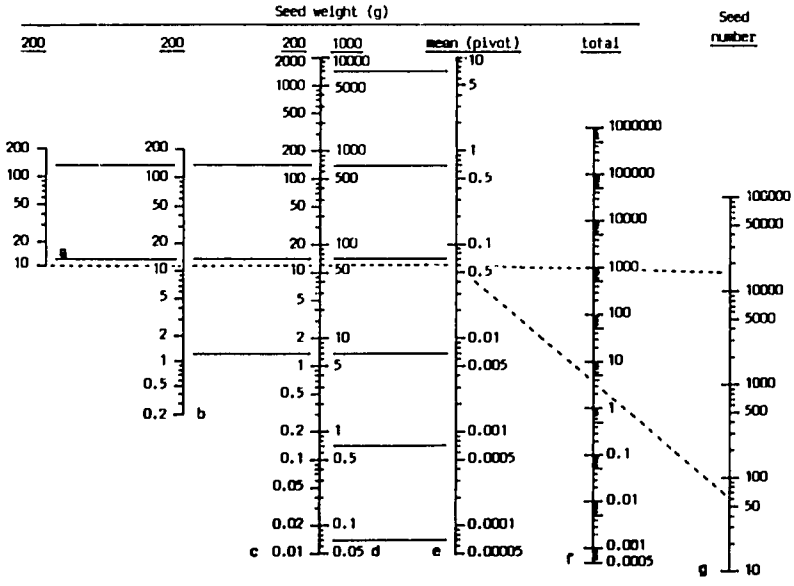
However, since only one 200 seed group has been counted and weighed no estimate of the standard deviation is available. To overcome this problem Linington et al suggest that the relationship between the standard deviation and mean seed weight determined from their study could be applied, but with the further security of using the upper 95% confidence limit of their estimates. Figure 2.2 is provided here to allow the determination of this security factor from the estimate of mean seed weight derived from counting and weighing a single group of 200 seeds: an example of how to estimate mean seed weight by this procedure is given in the caption to this figure.

Of course if the ISTA procedure is used then an estimate of the standard deviation will be available and can be applied directly: a security factor of twice this standard deviation of 100 seed weight can be added to the estimate of the mean weight of 100 seeds. This value can then be divided by 100 to obtain a safe estimate of the mean seed weight of the accession.

Monitoring accession seed number in the store

In addition to initial errors in the estimation of the number of seeds in an accession, there is also the problem that subsequent withdrawals of seeds from the accession for any purpose may also be subject to error. For example if it is intended to distribute batches of 100 seeds from an accession a few more seeds may be inadvertently included each time. These errors taken together constitute a serious potential

Figure 2.2 Seed weight/number nomograph. Scales e-g can be used to determine seed number if the mean seed weight (e) and total seed weight (f) are known. The two dotted lines show example calculations where the mean seed weight is 0.6 g. Straight lines perpendicular to the axes of scales a-e enable mean seed weight (e) to be estimated from the 200 (a-c) or 1000 seed weight (d). Conversion of 200 seed weight to mean seed weight from scale c to e provides for division by 200; conversion from scale b to e also incorporated the correction factor of 2 standard deviations (see text); conversion from scale a to e also incorporates the correction factor of the 95% confidence limit of 2 standard deviations (see text). The horizontal broken line between scales a and e provides an example of the latter correction factor: the weight of a 200-seed sample was 10 g, providing a mean seed weight estimate of 0.5 g (draw a horizontal line between scale c at this value and scale e). Inputting the value 10 g on scale a, however, corrects the mean seed weight estimate to 0.6 g (scale e), thereby reducing the estimate of accession seed number. (The relationship between scales a, b and c was determined from equations generously provided by Linington et al.).



problem to gene banks; in particular the seeds in an accession may fall below the threshold number required for regeneration before the records indicate this danger.

To avoid this it is important that the number of seeds in an accession should be re-estimated before there is danger of the accession running out. In order to consider how this should be done it is necessary first to discuss when regeneration of the accession should be carried out. Since an attempt at regeneration might fail (because of accidents or attack by pests or disease) enough seed should be left for a second attempt if the first should fail. Allowing for other errors, it is therefore suggested that the aim should be to regenerate when the number of seeds in the accession has fallen to three times the number required for regeneration. The number of seeds required for regeneration depends mainly on the number of seeds required to replace the accession (ideally, according to IBPGR, 4000 seeds for a genetically homogeneous accession, or 12,000 seeds for a genetically heterogeneous accession), divided by the seed multiplication factor (i.e. the average number of seeds expected to be produced from each plant), divided by the proportion of viable seeds (this should be the regeneration standard or higher, typically 85% viability or 0.85 if expressed as a proportion), and divided by the field factor (the proportion of viable seeds sown which are expected to emerge). It would be as well to double this number to allow for variation in the seed multiplication factor and the field factor due to variations in weather and seedbed conditions. If there is considerable genotypic variation in multiplication factor within the species then it may be wise to quadruple the calculated number of seeds. The final figure calculated should be subject to the constraint that the number of plants involved in the regeneration process should be sufficient to represent adequately the genetic variation in the accession. For most practical purposes this may be taken to be between about 50 to 100 plants (for a discussion of this problem, see Chapter 13 and the reference to Singh and Williams at the end of this chapter).

So, for example, for a genetically heterogeneous accession with considerable genetic variation in seed multiplication factor but thought to average 150, a viability of 85%, (or 0.85 as a proportion) and an expected field factor of 0.6, the number of seeds required for regeneration could be calculated as follows:

$$[12000/(150 \times 0.85 \times 0.6)] \times 4 = 627$$

This number would provide a greater number of plants (320) than is considered necessary to represent the genetic variation in the accession and, therefore, can be accepted. Regeneration should be carried out when the number of seeds in the accession falls to three times this number, i.e. 1881 seeds. For convenience this would be rounded up to 1900 seeds.

Thus it would be prudent to re-calculate the number of seeds in the accession when it is thought that the number remaining approaches this value. How should this be done? Two approaches have been suggested.

The most straightforward would be to weigh the seed container before it is filled, then re-weigh it each time a sample of seeds has been withdrawn from it (this assumes the container is re-usable, see Chapter 1). From this the weight of seeds remaining is obtained by subtraction and the number of seeds remaining can be calculated by dividing by the mean seed weight of the accession. In this way the estimate of the number of seeds remaining in the accession could be kept up-to-date and recorded in the accession file.

An alternative approach has been suggested by Linington et al. Their approach avoids weighing the accession, except initially, and depends upon determining in advance a reasonable estimate of the error in subsequent withdrawals when removing a notional seed number (for example, for distribution). If:

the original estimate of accession seed number = e;
the number of seeds required for regeneration = f;
the number of seeds intended for distribution to each customer = g;
the actual number of seeds distributed to each customer = h;

then the notional recount number = $e - [(e - f)/h] \times g$.

In other words the maximum number of samples which can be distributed is given by $(e - f)/h$. However, according to the records the number of seeds distributed will be $[(e - f)/h] \times g$ and not $(e - f)$. Thus when the records show that only $e - [(e - f)/h] \times g$ seeds remain the accession should be recounted.

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CHAPTER 3. SEED MORPHOLOGY

There are several reasons why it is necessary for anyone handling seeds in gene banks to have some knowledge of the structure of seeds and fruits. For example, many of the problems of poor seed germination arise from or are associated with different types of seed or fruit morphology (Chapters 4, 5 and 7), many of the difficulties in identifying empty seeds result from seed morphology (Chapter 8), and alternative techniques to germination tests for estimating viability require an ability to recognise various seed structures (Chapter 11). The main purpose of this chapter is to outline briefly the major features of fruit and seed morphology and how they develop from the flower, and to provide an explanation and glossary (Table 3.1) of the terms used.

SEED DEVELOPMENT

In the strict botanical sense a **seed** is a fertile and ripened **ovule** which contains an embryonic plant, usually supplied with food storage tissue, and surrounded by a protective coat **(testa)**. **Seeds** are produced by two taxa, Gymnospermae and Angiospermae.

The basic difference between the seeds of the gymnosperms and those of the angiosperms is that the **seeds** of angiosperms are the product of a double fertilization within the **ovary**, whereas the **seeds** of gymnosperms are the product of a single fertilization and there is no **ovary**. Because there is no **ovary**, the **seeds** of gymnosperms are naked; in fact the term gymnosperm literally means naked **seed**. In angiosperms, the ovules are protected by the ovary which, after fertilization develops into the fruit.

The ovule

The young ovule in gymnosperms contains a central body, called the **nucellus**, which is surrounded by between one and three envelopes, the **integuments** (one **integument** in cycads, Ginkgo, conifers and taxads, two in Ephedra and Welwitschia, and three in Gnetum). In angiosperms the **nucellus** is surrounded by either one or two **integuments**: the Sympetelae (where the **petals** are united, at least at their base) generally have one, and the Polypetalae (where the petals are separate) and monocotyledonous plants generally have two. In both gymnosperms and angiosperms the **integuments** do not completely surround the inner tissues but leave the **nucellus** exposed by a short narrow passage called the **micropyle**. Typically one of the cells of the **nucellus** becomes the **megaspore mother cell** (in gymnosperms) or **embryo sac mother cell** (in angiosperms) which then undergoes a meitotic cell division so that the normal diploid number of chromosomes of the parent plant is halved. One of the four haploid cells resulting from meiosis develops into the female **gametophyte** - the minute female haploid plant which is entirely dependent on and nurtured by the mother diploid plant. In angiosperms the female **gametophyte** is extremely reduced and consists initially of a single cell, the **embryo sac**, whose nucleus normally undergoes three consecutive mitotic divisions to form eight (but sometimes less) haploid nuclei. The eight nuclei become organised into two quartets at opposite ends of the **embryo sac**. Those at the end near the **micropyle** form the egg apparatus consisting of two **synergid nuclei**, an egg cell, and a **polar nucleus**; the four at the other end give rise to another **polar nucleus** and three **antipodal nuclei**. At least this is the case in the majority of angiosperms but there are many odd variations on this basic theme.

Pollination and fertilization

After pollination, a pollen tube grows out from the pollen grain down the **stigma** to the **ovule**, and two sperm nuclei are discharged into the **embryo sac**. In some way, not entirely understood, the **synergid nuclei** seem to play some active role in stimulating the proper discharge of the contents of the pollen tube. One sperm nucleus fuses with the egg cell to form the diploid zygote which then undergoes cell division and develops into the **embryo**. The other sperm nucleus fuses with the two **polar nuclei** thus forming the start of a cell line which develops into a triploid food-storage tissue known as the **endosperm**. At least this the normal pattern but in some cases the **endosperm** may show larger chromosome numbers. For example in Lilium species one of the **polar nuclei** is already triploid before fusion so that after combining with the other haploid nucleus and the haploid sperm, the resulting **endosperm** is pentaploid.

Meanwhile the **antipodal nuclei** undergo several divisions and form antipodal tissue which develops large nuclei containing polytene chromosomes. This antipodal tissue seems to play a role in providing RNA for controlling the development of the **endosperm** which is meanwhile undergoing such rapid nuclear division that the transcription of DNA to RNA by those nuclei may be constrained. The antipodal tissue is short-lived, however, and disappears as the **embryo** and **endosperm** develop. Eventually the nucellar tissue may also disappear but, if not, it becomes another food storage tissue in the seed known as the **perisperm**.

In gymnosperms the haploid cell destined to become the female **gametophyte** also starts its development with free nuclear division, but eventually a cellular structure develops as cell walls are laid down. The female **gametophyte** is structurally more substantial than in the angiosperms since the **gametophyte tissue** ultimately develops simple **archegonia**. Each **archegonium** contains a large cell which initially contains a single haploid nucleus. This usually divides to form an ephemeral ventral cell or nucleus, and an egg cell or nucleus.

Pollination takes place in gymnosperms when a pollen grain lands directly on the **nucellus**. The pollen tube grows towards the egg cell in an **archegonium**; eventually the contents of the tube, which includes two male gametes, are released. In some species however, pollination occurs before the **archegonia** are formed, and the arrival of the pollen stimulates their development. One peculiarity of the gymnosperms is that there is usually a long delay between pollination and fertilization, often up to one year (except in Ephedra where the delay is no more than a few hours), as compared with a few minutes or hours in the angiosperms. Fertilization occurs when one of the male gametes fuses with the egg nucleus thus forming a diploid zygote which develops into an **embryo**. Unlike the case of the angiosperms, the second male gamete appears to have no function except, possibly, in Ephedra, where it fuses with the ventral canal nucleus which may undergo a few irregular divisions.

Apomixis

Although the production of a **seed** is normally initiated by a sexual process - the fertilization of the egg cell - in some angiosperms a non-sexual process, called **apomixis** is responsible. In this case the egg cell is formed from one of the diploid cells of the **nucellus**. A common case is in Citrus where several apomictic **embryos** can develop alongside a normal sexually produced **embryo** in the same **seed**, thus leading to **polyembryony** - several **embryos** within the same seed. Apomictic seed development is also common in many of the Poa grasses including Poa annua (annual meadow grass) and Poa pratensis (Kentucky blue grass), and the common dandelion, Taraxacum officinale. Although **polyembryony** is often associated with **apomixis**, this is not always the case. **Polyembryony** can also arise by division of the zygotic **embryo**:

this type of polyembryony is quite common in the orchids, for example.

Embryo growth and development

In all seed plants, whether the embryos are produced sexually or apomictically, the embryos grow and develop to a particular stage which is characteristic of the species. Growth then suddenly stops, i.e. the developing seed becomes dormant, and in many cases the seed then dries out. At this stage the seed is usually shed from the parent plant. It is now an independent organism which usually continues in its dormant state for some time until it starts to germinate and establish a new plant. There are some cases, however, in which the embryo does not complete its growth and development until some time after the seed has been shed from the plant; this is the case, for example, in some species of Ranunculus and Fraxinus. The size and complexity of the seed when it is shed from the parent plant varies enormously from species to species. The embryo of the ripe seed may consist of a small group of 100 cells or less in the smallest seeds to quite complex structures containing several embryonic leaves and lateral roots, as is typical of the grasses, for example. Typically the embryo consists of a radicle (embryonic root), a plumule or epicotyl (embryonic shoot), and a hypocotyl which connects the radicle with the plumule. Usually there is an embryonic leaf or leaves of special form called cotyledons. In angiosperms one cotyledon is typical of monocotyledons, and two of dicotyledons (hence the names). Gymnosperms have one to several cotyledons, depending on the species.

All except the smallest seeds carry a store of food in order to provide material and energy for germination and early seedling growth and development. Once the seed has germinated and established itself it finally becomes an independent photosynthesising organism depending on its own activities for its food and energy rather than that provided by its parent. The smallest seeds do not contain food stores, either because they quickly become parasitic plants and depend on their host for a supply of organic materials (e.g. in Striga and Orobanche) or, like the epiphytic orchids, they form a symbiotic association with fungal mycorrhiza which aid nutrition during germination.

Food storage tissues

In larger seeds the food is stored in special tissues. In the gymnosperms and some of the angiosperms the perisperm, derived from the remains of the nucellus, provides the main store of nutrients. However, in the angiosperms it is generally more usual for either the endosperm (e.g. in Allium, Figure 3.1, or the grasses) or swollen cotyledons (e.g. in legumes) to be employed as food stores. But virtually any tissue may be used for this purpose, for example the food of the Brazil nut (Bertholletia excelsa) is stored in an enlarged hypocotyl. Sometimes two or three tissues may be involved, as in Beta vulgaris (Figure 3.2) where there is both a well-developed endosperm and perisperm. The three most commonly used storage tissues have quite different genetic constitutions: the perisperm is diploid maternal tissue; the endosperm is generally a triploid tissue in which two-thirds of the genetic material is contributed from the maternal parent and one-third from the paternal parent; and the cotyledons are a diploid tissue in which the genetic material is derived equally from the maternal and paternal parent.

Endospermic and non-endospermic seeds

There is therefore considerable variation in true seed anatomy, morphology and composition. A consideration of these differences is necessary since they may affect decisions on dormancy-breaking, embryo-excision, and tetrazolium staining procedures. We can identify at least nine basic forms of seed

Figures 3.1 - 3.13 Seed structure in diverse species.

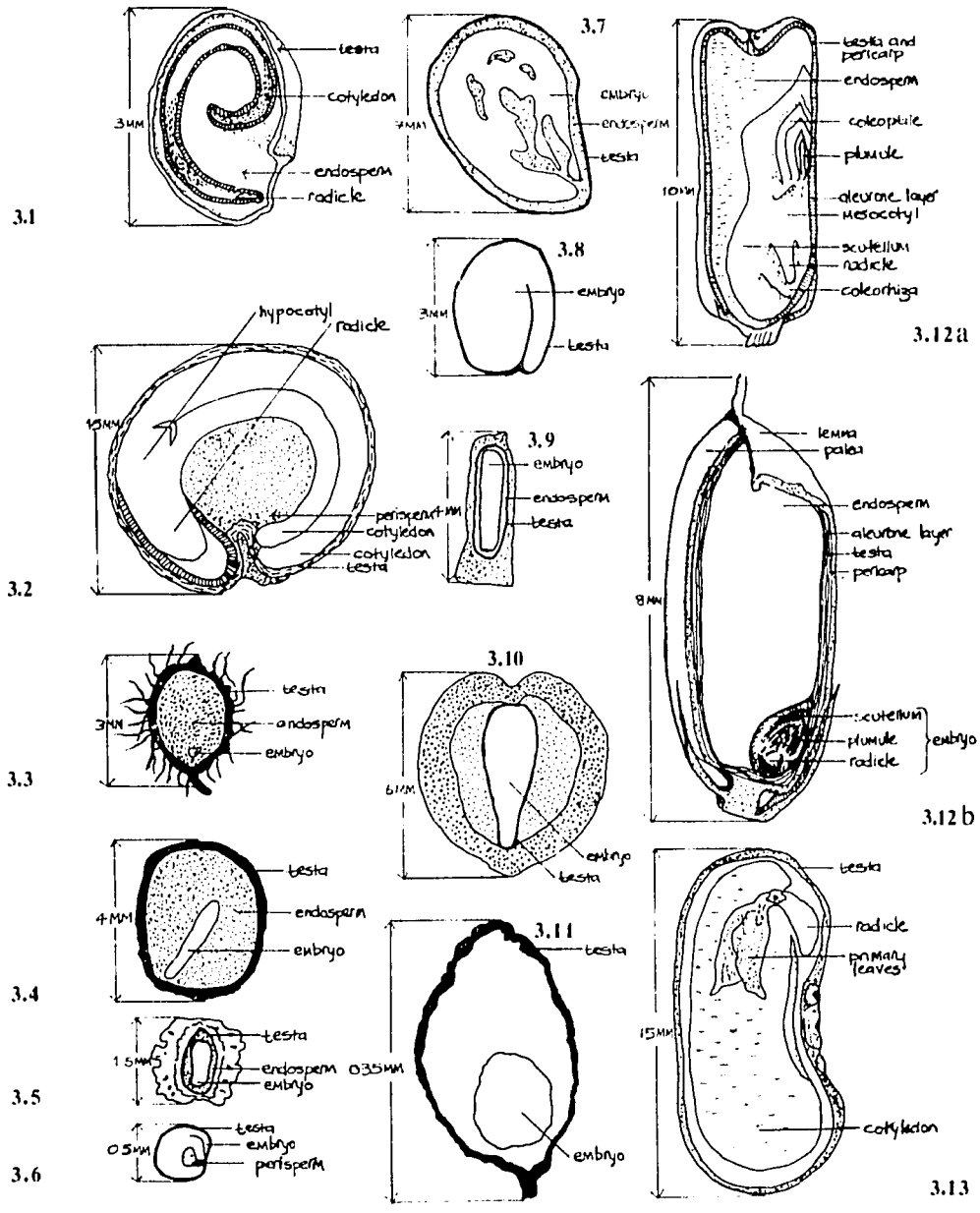


Figure 3.1 Allium spp.
Figure 3.2 Beta vulgaris
Figure 3.3 Anemone coronaria
Figure 3.4 Cyclamen persicum
Figure 3.5 Nemesia strumosa
Figure 3.6 Portulaca grandiflora

Figure 3.7 Ipomoea purpurea
Figure 3.8 Iberis amara
Figure 3.9 Verbena x hybrida
Figure 3.10 Dimorphotheca sinuata
Figure 3.11 Striga spp.

Figure 3.12a Zea mays
Figure 3.12b Oryza sativa
Figure 3.13 Phaseolus vulgaris

Figures 3.3 - 3.10 are based on the work of B.R. Atwater (see Chapter 17, Volume II).

structure. The first anatomical distinction concerns the presence or absence of an endosperm.

Endospermic seeds store the food material for the developing plant within the endosperm, which is separate from the embryo. In these seeds the endosperm is said to be dominant since the embryos are dependent upon the endosperm for the provision of nutrients for both development and germination. Some embryos of endospermic seeds may not be differentiated into visible plant organs; whilst in others the organs may be only partly developed; whilst in the Gramineae, for example, the embryos may be highly developed and contain several embryonic leaf initials.

In contrast, non-endospermic seeds either have no endosperm or only a vestigial endosperm. In these seeds the embryo is mature (since it is ready to germinate) and is said to be dominant since, where present, the food reserves are contained within the cotyledons.

Endospermic seeds can be classified into at least four categories depending upon the shape and location of the embryo, viz: basal rudimentary embryos, Figure 3.3; axillary linear embryos, Figure 3.4; axillary miniature embryos, Figure 3.5; or peripheral linear embryos, Figure 3.6. Similarly, non-endospermic seeds can be classified into five categories depending upon the characteristics of the seed coat for those seeds with cotyledons or the absence of cotyledons, viz: hard seed coats, Figure 3.7; thin seed coats with a mucilaginous layer, Figure 3.8; woody seed coats with an inner semi-permeable layer, Figure 3.9; fibrous seed coats with a separate semi-permeable membranous coat, Figure 3.10; or non-cotyledonous, Figure 3.11. The majority of these categories are derived from B.R. Atwater's work; the possible utilisation of this classification of seed morphology to select suitable dormancy-breaking procedures is discussed in Chapter 17, Volume II.

ASSOCIATION OF FRUIT STRUCTURES WITH SEEDS

In very many species it is not just the true seed which is maintained in the seed store, but intact dispersal units or fruits containing the true seeds. According to the strict botanical definition of a seed the parts of the fruit or surrounding flower parts outside the seed are clearly not a part of the seed itself. However, in normal and traditional usage the whole fruit or dispersal unit may be called the seed when it is dry, indehiscent and (usually) one-seeded. For example, the following fruits are normally called seeds: the caryopsis (the fruit of the Gramineae) (Figure 3.12); the achene (characteristic of the Ranunculaceae, Compositae and Polygonaceae) which differs from the caryopsis in that the pericarp can be removed from the mature seed; the schizocarp (characteristic of the Umbelliferae and Geraniaceae) which is composed of two or more carpels which separate at maturity to fruits resembling achenes; and the nut (characteristic of the Fagaceae) which also resembles the achene except that the pericarp is thick and hard. The carpel (Malvaceae), floret (Gramineae), endocarp (Palmae), loment segment (Leguminosae) (Figure 3.13), samara (Ulmaceae) and utricle (Chenopodiaceae) are also often classed as seeds under this wider definition. Note that in the case of the floret, for example, the dispersal unit includes several structures from the flower in addition to the fruit including, possibly, bracts, calyx, corolla, bractlets and pedicel as well as arils and awns.

EXPLORATORY DISSECTION OF SEED-LIKE STRUCTURES

Where there is some uncertainty about the morphology of the seed-like structures present within an accession a small sub-sample of the seeds can be subjected to an exploratory test. First this test will identify whether the accession consists of true seeds or not. If not the dissection can determine whether

TABLE 3.1 Glossary of Seed Development and Fruit and Seed Structure

Achene A dry, **indehiscent** one-seeded fruit.
Adventitious root A root arising from any structure other than a root (e.g. a stem-borne root).
Aggregate fruit A type of **fruit** formed from several **pistils** that were distinct in the **flower**.
Antipodal nuclei Three of the eight nuclei which result from mitotic divisions of the **megaspore**.
Apomixis **Seed** development from female **gametophyte** without fertilization.
Archegonium In gymnosperms the organ corresponding to the **pistil** of angiosperms.
Archesporial cell The cell of the **nucellus** which differentiates into cells which ultimately undergo meiosis and produce the **megaspore**.
Aril A loose, papery or pulpy appendage growing out from the **hilum** or **funiculus** of the **seed**.
Awn A bristle-like **seed** appendage often described as a **beard**.
Axillary bud A bud in the axil of a leaf.
Axillary shoot A shoot developing from an **axillary bud**.
Axis Depending on context either the main stem of the **embryo** or the main stem of the **flower**.
Beard An **awn** or bristle-like hair.
Berry A pulpy, **indehiscent** few- or many-seeded **fruit** resulting from a single **pistil**.
Bitegmic testa A **testa** derived from two layers of **integuments**.
Bract Small or scale-like leaves in, or associated with, the **flower**.
Bractlet **Bract** borne on the **pedicel**.
Callus In this context a hard prominence or protuberance at the base of certain grass **florets**.
Calyx The circle of **sepals** around the **flower** which may form part of the covering structures of the **seed**.
Capsule A dry, **dehiscent fruit** of more than one **carpel** with a dry **pericarp**.
Carpel The basic female reproductive unit: a compound **pistil** or **ovary** may contain many **carpels**, but a simple **pistil** has one **carpel**.
Caruncle A fragile appendage of the outer **integument** of the **seed** in some species.
Caryopsis A dry, **indehiscent** one-seeded **fruit** where the **pericarp** and **integuments** are tightly fused to the **seed**.
Chaff Small, thin, dry, membranous scales or **bracts** associated with the mature **inflorescence**.
Chalaza The basal part of an **ovule** where it is attached to the **funiculus** and where the **integuments** originate: in legumes this region can sometimes be distinguished on the outside of the **seed** near the **hilum**.
Cob The **rachis** of a maize **inflorescence**.
Coleoptile The membrane which protects the developing **plumule** of Gramineae during emergence through the soil.
Coleorhiza The membrane in some species which protects the **radicle** as it emerges through the **seed** covering structures.
Corolla The circle of **petals** around the **flower** which may form part of the **seed** covering structures.
Cotyledon **Seed** leaf or leaves of the **embryo** which function as either a storage organ or as an absorptive organ - also see **scutellum**.
Cremocarp The dry, **dehiscent** two-seeded **fruit** of umbellifers which comprises of two **mericarps**: synonym of **schizocarp**.
Cupule Cup-like structure at the base of some **fruits**.
Degiume Term used to describe the removal of the **glumes** from cereal **caryopses**.
Dehiscent Opens at maturity to shed the **seeds**.
Dehull Term used to describe removal of the **hull** from the **seed** or **caryopsis**: the actual covering structures removed depend upon species.
Drupe The stone fruit: a fleshy, single-seeded, **indehiscent fruit** where the **seed** is enclosed within a stony **endocarp**.
Drupelet A single **drupe** within a **fruit** comprised of aggregated **drupes**.
Ear The **inflorescence** of cereals.
Embryo The part of the **seed** normally formed by fertilization of the egg cell by the sperm cell which develops into the young plant at germination.
Embryo sac The female **gametophyte**.
Empty seed A seed-like structure which may comprise of **bracts**, **integument(s)** and/or **pericarp** but lacks an **embryo**.
Endocarp The inner layer of the **pericarp**.
Endosperm The starch- and oil-containing tissue within many **seeds** which is outside the **embryo** and provides nutrition to the developing **embryo**.
Epicarp The outer layer of the **pericarp**: synonym of **exocarp**.
Epicotyl The part of the **embryo** or seedling between the **cotyledons** and the first true leaf; it elongates during hypogeal germination.
Exocarp The outer layer of the **pericarp**.
Floret The smallest unit of a **flower**: in the grasses after fertilization it consists of a **caryopsis** enclosed within the **lemma** and **palea** and in agronomic terms is often incorrectly described as the **seed**.
Flower The organ bearing one or more **pistils**, or one or more **stamens**, or both.
Follicle A dry, **dehiscent fruit** resulting from a single **pistil** which splits along only one groove at maturity.
Fruit The mature (ripe) **ovary** together with associated structures: that is the **seed**-bearing organ.
Funiculus The stalk attaching the **ovule** to the wall of the **ovary**.
Gametophyte The part of a flower which produces either male or female sex cells.
Gametophyte tissue The nutritive (haploid) tissue occurring within conifer **seeds** which exists before fertilisation occurs.
Germ The **embryo** of a **seed**.
Glume A small **chaff**-like **bract**: commonly the **lemma** and **palea** at the base of a grass **spikelet** which may, or may not, completely enclose the **caryopsis**.
Grain A cereal **caryopsis**, either naked or enclosed between a **lemma** and a **palea**.
Hilum The scar or mark on the **testa** at the point of attachment to the **funiculus**.
Hull The tightly adhering outer covering of the **seed** or **caryopsis**; the **pericarp** in some species, and **lemma** and **palea** in others.
Hulling See **dehull**.
Husk The leaf sheath enclosing the **ear** of maize, but also used to describe the **lemma** and **palea** of other cereals.
Hypocotyl The **axis** of the **embryo** below the **cotyledons** and above the **radicle** which elongates during epigeal germination.
Indehiscent Does not split open at maturity.
Inflorescence In this context the flowering structure of the plant.
Integument The outer envelope of an **ovule**, usually consisting of an inner and an outer layer which comprise the **seed coat**, or **testa**, of the mature **ovule** - see **bitegmic** and **unitegmic testa**.
Involucre One or more whorls of small leaves or **bracts** surrounding an **inflorescence** or **flower**.
Kernel The **caryopsis** of a cereal, particularly maize or barley.
Lateral root A root arising from another root.

TABLE 3.1 (continued)

Lemma The flowering glume of grasses; lower and outer (opposite the rachilla) of the two bracts enclosing the flower/caryopsis, the upper glume being the palea.
Locule In this context the cavity, compartment, or cell of an ovary.
Lomentum A dry schizocarpic fruit in the form of a legume or siliqua with constrictions formed between the seeds as the fruit matures, forming one-seeded indehiscent loment segments.
Loment segment A single-seeded part of a lomentum.
Megagametogenesis The development of the female gametophyte from a megaspore.
Megaspore One of the four cells derived from the archesporial cell formed in the ovule as a result of meiosis.
Megasporogenesis The development of the megaspore from the archesporial cell.
Mericarp One half of a schizocarp: it has a dry, indehiscent pericarp which encloses the ovule loosely; part of a dry simple fruit.
Meristem Plant tissues composed of unspecialised cells capable of cell division.
Mesocarp Middle layer of the pericarp.
Mesocotyl In some highly specialised monocotyledons (e.g. certain Gramineae) the part of the seedling axis between the point of attachment of the scutellum and the shoot apex. It is recognised to be a compound structure, formed by the growth together of part of the cotyledon and the hypocotyl.
Micropyle The opening of the integument of the ovule through which pollen enters.
Monogerm seed In common use to describe a sugar beet fruit (cluster) which contains a single seed.
Multigerm seed In common use to describe a sugar beet cluster which is an aggregate fruit containing several seeds.
Multiple fruit A fruit formed into a single structure from a cluster of flowers on a common base.
Naked flower A flower which lacks perianth.
Nucellus The tissue within the young ovule in which the archesporial cell arises and where megasporogenesis, megagametogenesis and ovule development occur.
Nut A dry, indehiscent, single-seeded, hard and bony fruit.
Nutlet A small, dry, indehiscent, single-seeded, hard and bony fruit.
Operculum A cap or plug derived from the integuments which is deposited inside the ovule and closes the micropyle or chalaza.
Ovary The ovule-bearing part of the pistil within the flower which develops into the fruit: it may contain more than one ovule.
Ovule The part within the ovary which develops into a seed after fertilization.
Palea The upper and inner (towards the rachilla of the spikelet) of the two bracts in the flower of the grasses, the lower bract being the lemma.
Panicle A branching inflorescence.
Pedicel The stem of one flower in a cluster which in the case of grass florets can be mistaken for the seed on superficial inspection.
Pepo A type of fruit with a hard rind without internal separations.
Perianth The two floral envelopes ; the calyx (the outer floral envelope) plus the corolla (the inner floral envelope) - surrounding the flower which may form part of the seed covering structures.
Pericarp The wall of the ovary or fruit comprising endocarp, mesocarp and exocarp which has many different forms, viz. thin and dry, fleshy, or hard and dry.
Perisperm A type of storage tissue similar to the endosperm but derived maternally which is present in some species.
Petal One unit of the corolla.
Petiole The stalk of a leaf.
Pistil The female organ of the flower: it is simple if comprised of a single carpel and compound if comprised of several carpels.
Plumule The major leaf bud of the seed or seedling.
Pod A dry, dehiscent, non-fleshy fruit which, when ripe, splits open to release its seeds.
Polar nuclei Two nuclei of the female gametophyte derived from the megaspore which form the endosperm of the developing seed after fertilization.
Polyembryony The presence of more than one embryo within an ovule: it may be the result of apomixis, or division of the zygotic embryo.
Pome A type of fruit in which the receptacle forms a fleshy layer around a papery endocarp forming a multi-seeded core.
Primary leaf The first foliage leaf (or pair of leaves) of a seedling.
Primary root The main root of the seedling which develops from the radicle of the embryo.
Pyrene The nutlet in a drupe.
Rachilla A secondary rachis within a grass spikelet to which the florets are attached.
Rachis In this context the main stem of the flower.
Radicle The embryonic root of a developing seed which becomes the primary root of the seedling..
Raphe A ridge which may be visible on the seed where the funiculus joins the ovule.
Receptacle The basal structure, often enlarged or elongated, to which the parts of the flower are attached and which in some species can form part of the mature fruit.
Root hair The fine protuberance of an outermost root cell which absorbs water and mineral salts from the soil.
Samara An indehiscent, winged fruit in which the seedcoat is loose inside the pericarp.
Scale-leaf A reduced leaf, usually adpressed to the stem.
Schizocarp A dry, dehiscent, two-seeded fruit which separates at maturity into two mericarps: synonym of cremocarp.
Scutellum The highly modified cotyledon in some Gramineae which absorbs nutrient from the endosperm and thus provides nutrient to the embryo.
Secondary root A term used in seed testing to mean any root other than the primary root.
Seed A mature ovule: the essential part is the embryo contained within the integuments; but agronomically the term may be used less critically to describe planting material.
Seedcoat Normally the inner and outer integuments which protect and cover the seed which is also described as the testa; but the term is sometimes misused to include other covering structures such as bracts and pericarp.
Seminal roots The seedling root system including the primary root and a number of adventitious roots which appear more or less simultaneously in germination.
Sepal One of the separate leaves of the calyx.
Shell To separate the pericarp from the enclosed seeds (e.g. legumes) or to separate maize caryopses from the cob.
Shoot apex The main growing point of the seedling shoot axis.
Silicle The broad, short fruit of certain Cruciferae.
Silique The long, thin fruit of certain Cruciferae.
Spike A type of inflorescence in which the flowers arising along the rachis lack stalks.
Spikelet A part of an inflorescence: in grasses the unit of the flower including bracts and one or more florets.
Stamen The pollen-bearing organ within a flower.

TABLE 3.1 (continued)

Stigma The part of the **pistil** which receives pollen.
Style Elongated part of the **pistil** between the ovary and the **stigma**.
Synergid nuclei Two of the eight nuclei derived from the **megaspore** within the female **gametophyte**.
Terminal bud The shoot apex enveloped by several more or less differentiated leaves.
Testa The outer coat of a seed derived from the **integuments**, that is the **seedcoat**: it can comprise more than one layer - see **bitegmic** and **unitegmic testa**.
Thresh To separate the seeds from the stems and other plant material: the resultant material may, however, consist of more than just the seeds and/or contain seed-like structures lacking a seed.
Umbel A type of **inflorescence** where the flowers form flat or umbrella-shaped heads in which the **pedicels** and rays arise from a common point as in the Umbelliferae.
Unitegmic testa A **testa** made up of only one **integument**.
Utricle A bladdery, single-seeded, usually **indehiscent fruit** in which the seed is only loosely attached to the **pericarp**.

the seed-like structures contain either no seed (see Chapter 8) or several seeds. Secondly the dissection can enable seed morphology to be classified into one of the nine categories outlined in Figures 3.3-3.11, which may be of potential importance in selecting suitable germination test and dormancy-breaking procedures (see Chapter 17). Thirdly the information obtained can be helpful (particularly if combined with the results of an X-ray analysis - see Chapter 8) in determining the maturity of embryos, particularly the variation in embryo maturity within the accession, which also may influence the selection of germination test procedures. Fourthly this exploratory dissection will indicate the degree of infestation with insect larvae and, particularly, bruchids.

CHAPTER 4. THE GERMINATION OF NON-DORMANT SEEDS

It is essential that plants capable of growing to maturity can be produced from each seed accession maintained in a gene bank. That is the seeds must be alive (viable) and capable of germination when regenerated or when sown by users of the material. Consequently tests for germination are carried out in the laboratory on accessions in storage from time to time to check that percentage viability is above the regeneration standard. These germination tests are thus tests of percentage viability (alternative tests of viability are described in Chapter 11), but only if the major factors which may prevent the germination of viable seeds are avoided. One of the major factors which may prevent seed germination is seed dormancy. This is such an important problem that a considerable proportion of this book is devoted to it: Chapter 5 deals with the principles of seed dormancy and main features of interest to gene banks; Chapter 7 includes guidance on techniques for removing covering structures which are sometimes employed for removing dormancy; Chapter 6 provides guidance on appropriate light regimes for seeds whose dormancy is affected by light; and Volume II provides practical advice on removing dormancy.

CRITERIA OF GERMINATION

Germination involves first the activation of the quiescent, resting, embryo. In air-dry seeds (that is all seeds of orthodox species in long-term storage) metabolism is almost imperceptible. Consequently for germination to occur the seed must take up water, that is, imbibe. The uptake of water is normally sufficient to trigger germination (providing the seed is held at an adequate temperature and supplied with oxygen, and providing it is not dormant).

However, although it is reasonably clear when germination begins there are considerable differences in opinion as to at what point a seed can be said to have germinated. These differences concern the relative timing of the cessation of germination and the onset of seedling growth.

Radicle emergence

The first visible sign of embryo growth is the protrusion of some part of the embryo, usually the radicle, through the seed coat. Thus this stage of germination is often referred to as **radicle emergence.** It is a clear, objective, criterion of germination. Radicle emergence is often qualified by describing a dimension to the minimum extent of radicle protrusion; for example, by 2 mm. It is an adequate criterion for determining that a given proportion of seeds within a sample from an accession is alive, i.e. viable.

Seedling evaluation

However, a seed capable of achieving radicle emergence may not necessarily be able to produce a seedling capable of independent growth (independent that is of stored food reserves within the seed) such that a plant can can be produced. Consequently seed analysts in seed testing stations delay their decision on whether or not a seed has germinated to a point beyond the radicle emergence stage. To a seed analyst, germination in a laboratory test is the emergence and development from the seed embryo of those essential structures which, for the species being tested, indicates the ability to develop into a normal plant

under favourable conditions in soil. Two classes of seedlings are therefore created by this definition: **normal seedlings**, those which have all the essential structures (well-developed, intact, root and shoot systems with no major damage) required for continued growth and development; and **abnormal seedlings**, those which are not considered capable of continued development because of damage, deformation or decay. Percentage normal germination is therefore assessed at a later stage to radicle emergence and estimates the proportion of seeds capable of producing normal seedlings. The process of classification of seedlings into either normal or abnormal seedlings is known as **seedling evaluation**.

In contrast to radicle emergence where classification is objective, seedling evaluation is subjective requiring a certain degree of training. A further problem is that seedling development is quite sensitive to the environment of the germination test and a deficiency in the test environment can greatly increase the proportion of abnormal seedlings. All of this would appear to suggest that the radicle emergence criterion of germination is more suitable for adoption by gene banks.

This, however, may not be so. In many species a considerable proportion of abnormal seedlings can be produced at germination. For example, in a soyabean accession showing 90% radicle emergence it is possible that as many as 60% of the total number of seeds tested will produce abnormal seedlings whilst only 30% will produce normal seedlings. Moreover those few seedlings capable of continued growth and development would have poor field emergence ability if sown out for regeneration or multiplication, and poor seedling growth rates. Thus for many species it may be better to use normal germination as the criterion of germination. In addition for those species where the maximum proportion of abnormal seedlings is low the adoption of the normal germination criterion would enable seed analysts, plant breeders, and gene bank personnel to use the same - albeit subjective - criterion of germination and reduce the scope for misunderstandings. Accordingly Chapter 10 considers seedling evaluation and the differences between the structure of normal and abnormal seedlings.

THE GERMINATION TEST ENVIRONMENT

The purpose in specifying a germination test environment is to attempt to provide those conditions which will enable all viable seeds in the test to germinate. A secondary requirement for practical purposes is for germination to be achieved within a reasonable period.

There are three basic problems. First dormancy may prevent seeds from germinating: seeds may be dormant before the germination test; or seeds may become dormant during the germination test (as mentioned in the introduction to this chapter, these problems are dealt with in detail elsewhere). Secondly, certain seeds (thought to be those closest to death) can be very sensitive to test conditions and, although alive at the beginning of the test, they may die before germination can occur in certain, stressful, environments; that is they may die during the germination test. Thirdly, the range of environmental conditions over which germination can occur may be to some extent under genetic control. Now inter-specific variation can be taken into account where necessary by specifying environments for each species. But intra-specific variation is more difficult to deal with. Although there is evidence that genetically controlled intra-specific variation does exist, many differences observed between accessions may also be due to differences in maternal, or post-harvest environment. Either way it is essential that gene banks are able to provide germination test environments that are neither genotype nor accession specific. In other words the requirement is for test conditions suitable for all accessions of a given species.

Germination test substrates

Five alternative types of substrate are used in germination testing: soil, sand, compost, paper, and agar. Soil and compost have serious deficiencies from the point of view of the seed tester. Their composition is subject to considerable variation and this affects their moisture retention capacity. They may contain pathogens which can reduce the germination of seed tested. Both are bulky, messy materials to handle in laboratories and particularly within incubators. All of which severely limits the number of tests that can be run at one time. Sand too is bulky but, if prepared in the manner described in Chapter 9, can be a satisfactory germination substrate but the laboratory must be designed and equipped to handle it.

If seeds have been chemically treated in some way (e.g. by fungicides or insecticides), testing in sand, agar or on paper can result in abnormal germination (stunted growth is a particularly common symptom). In this case it may be necessary to test on compost where such effects are minimised. A standard seedling compost should be used - see Chapter 9.

Paper is the most widely used of all substrates. Seeds are either tested on top of paper or between sheets of paper. Three types of paper are used - blotting paper, filter paper, and paper towels - usually in the form of sheets or discs. However, filter paper is also used for some seeds in the form of pleated (many folded) sheets. Usually the papers are white but they are sometimes coloured (with a dye which should be non-toxic to germinating seedlings) to enable the germination of small seeds to be seen more easily. The paper should be porous (to enable water to be held), but strong (to maintain structure when wet), free of fungi and bacteria, and should not contain any toxic substances which would injure germinating seedlings. The texture should be such that roots grow on the surface of the paper, not into the paper. Chapter 9 provides details of tests which should be applied to all new batches of paper, and methods of using paper in seed germination tests.

Sufficient water must be supplied to the seeds to enable their moisture content to be raised sufficiently to allow germination to occur. Thereafter water is required for seedling growth. Chapter 9 considers water quality for seed germination tests and methods of ensuring that the germination test substrates maintain sufficient supply of moisture to the seeds.

Water sensitivity

An excess of water is problematic. It reduces oxygen availability which can cause abnormal germination or result in the failure of a seed to germinate. Reduced oxygen availability can also increase microfloral competition for oxygen. The latter phenomenon can be particularly pronounced in some accessions of barley (Hordeum vulgare L.) and results in the phenomenon of 'water sensitivity'. Water sensitivity in barley is avoided by reducing the risk of excessive microfloral competition for oxygen. This can be done by supplying only sufficient water to moisten the germination test medium without excess water being present, or removing the seed covering structures (lemma and palea), or treating the seeds with a combined bactericide and fungicide. The first method is the easiest and most reliable.

Imbibition injury

An excess of moisture at the onset of imbibition can also result in a type of damage which has been described variously as water injury, soaking injury, imbibition injury, and chilling injury. Here we will use

the term imbibition injury.

Dry seeds placed in a moist environment absorb water in three phases: an initial phase of extremely rapid water uptake; a second lag phase in which little or no water is absorbed; and a third phase of water uptake which is generally less rapid than the first phase and which is associated with embryo growth and emergence through the seed covering structures. In good quality non-dormant seeds the second phase of water uptake may be of minimal duration and the first and third phases may resemble a continuum, but it is the first phase - imbibition - that concerns us here. It is thought to be the result of the physical absorption of water by colloidal materials within the seeds. Consequently with an adequate supply of water the only limitation to the diffusion of water into the seed is provided by the seed covering structures.

For the time being we shall consider seeds of species within the Leguminosae, since in this family imbibition injury is particularly pronounced and well documented. In the Leguminosae the seed covering structure is the testa. The testa can be a very effective regulator of water uptake; indeed often it can act as a complete barrier to moisture and if the hilum (a small hole in the testa) is closed the seed will fail to imbibe. This phenomenon is known as hardseededness and is discussed in the next section. Soaking dry seeds which have had their testae removed in water results in the death of cells on the surface of the cotyledons (this is observed by tetrazolium staining techniques - see Chapter 11). It is thought that this cell death results from the rapid inrush o. water and that most damage occurs within the first few minutes of imbibition. There are three reasons for these assumptions. First, seeds with undamaged, intact, testae do not show this damage when soaked in water. Secondly, seeds without testae imbibed in media of low osmotic potential - where water uptake is very slow - generally do not show this damage. Thirdly, seeds from which the testae have been removed can be damaged (fail to germinate) if they are immersed in water for as little as two minutes.

Now although undamaged intact testae seem to be able to limit the rate of water uptake and thereby avoid death of cells on the outside of the cotyledons it is unfortunately a common occurrence for testae to be damaged (and consequently not intact). In general the problem appears to be greatest in cultivars where the testae tend to be thinner than in wild lines. The damage is often in the form of cracking either visible by eye or by microscopic investigation. These cracks may be present in the dry seeds (resulting from either the drying process or mechanical treatment of the dry seeds - see later section) before imbibition, or they may be caused during the imbibition process due to unequal expansion and folding of the testa as it takes up moisture. In addition these movements of the testa relative to the embryo can, by abrasion, also cause cell death on the outside of the cotyledons.

The cell death on the outside of the cotyledons that occurs when the testae are either damaged or removed and the seeds soaked in water can be sufficient to reduce germination. The effect is twofold: first death of these cells can result directly in abnormal germination or failure to germinate at all; secondly, solutes are leached from these cells and these solutes can stimulate and increase the activity of the seed microflora which either cause damage directly or result in increased competition for oxygen which, if oxygen is limiting, can also result in reduced germination.

Two further factors influence the expression of imbibition injury - the temperature at which imbibition occurs and the dryness of the seeds. Chilling injury was the term used to describe the observation that dry seeds of lima bean (Phaseolus lunatus) were injured (either the seeds failed to germinate or the resultant seedlings showed a reduced subsequent growth rate) if they were imbibed at low temperatures

(5°-15°C) compared to a slightly higher temperature (20°C). However, it is now recognised that the two phenomena - imbibition injury and chilling injury - are one: seeds of many grain legumes are sensitive to injury during imbibition where moisture is in excess, and this sensitivity is increased by a lowering of the temperature of imbibition.

The dryness of the seeds is also critical to the occurrence of imbibition injury. If seed moisture content is high (18% +) then imbibition injury is usually avoided. In some experimental observations there appears to be a critical moisture content - below which a constant proportion of seeds fail to germinate as a result of imbibition injury. In other observations the proportion of seeds affected increases with decrease in seed moisture content at imbibition. In either case it is clear that very dry seeds, as is the case of accessions within gene banks, are particularly susceptible to imbibition injury.

So far we have discussed imbibition injury as a phenomenon which occurs if (dry) seeds are set to imbibe in excess water - for example if seeds are immersed in water. Imbibition injury, however, is now known to occur in conditions where the supply of water is not grossly excessive. The problem can occur in standard germination tests of very dry seeds, even when the germination medium is of low osmotic potential. Further, although most experimental work has been concerned with imbibition injury in large-seeded legumes (because it is particularly pronounced in these species), it is now clear that the problem can occur in other species. For example, similar problems have been encountered with forage legumes, cotton (Gossypium spp.) and sorghum (Sorghum vulgare).

Since gene banks will be handling very dry seeds, imbibition injury is potentially a substantial problem. How can damage to seeds from imbibition injury be avoided or minimised in gene bank monitoring tests or regeneration operations?

First there should not be any excess moisture in the germination test. All the usual precautions (see section on germination substrates in Chapter 9) should be adhered to, to ensure that the germination medium is not too wet. In some cases the likelihood of imbibition injury may influence the choice of germination substrate. Agar is probably the best choice to minimise imbibition injury, but satisfactory results can be obtained with paper, which is the medium currently used by the majority of gene banks.

Secondly, after removal from storage the moisture content of the very dry seeds can be raised slowly to about 18-20% by humidification (see Chapter 11) prior to imbibition - either before a germination test or before sowing in the field or glasshouse for regeneration or multiplication. Instead of humidification an osmoticum could be used to raise seed moisture content slowly, although this has not been so extensively tested.

Finally, if the species is one which requires a low temperature for germination or for breaking dormancy, the first few hours of imbibition can be carried out at around 20°C - to avoid the greater sensitivity of the seeds to imbibition injury at low temperatures.

Hardseededness

As with imbibition injury, hardseededness can be a particularly difficult problem for accessions of species in the Leguminosae, but can also be a problem in other families; for example, Rosaceae and Malvaceae. In some senses it can be regarded as the opposite problem to imbibition injury: hard seeds possess impermeable seed coats, therefore cannot imbibe water and as a consequence cannot germinate.

Although the phenomena of hardseededness and imbibition injury are so opposed, it should be noted that both result from the properties of the seed coat and that on occasion individual seeds within a single accession may show symptoms of imbibition injury whilst others are hardseeded.

When legume seeds, for example, are dried below about 15%¶ moisture content the testae in some species, if intact and undamaged, become impermeable to moisture. Further loss in moisture occurs through a part of the hilum - this is a large oval scar near the middle of one edge of the seed coat which marks where the seed broke away from the stalk (see Chapter 3). This area of the hilum functions as a hygroscopic one-way valve, letting moisture out of the seed if ambient relative humidity is low but closing and preventing uptake of moisture if ambient relative humidity is high.

Two levels of hardseededness are recognised. Reversible hard seeds (also known as conditionally hard seeds) soften if exposed to high relative humidity for a long time by the uptake of moisture over the whole seed coat. Reversible hardseededness occurs because the relative humidity is not quite high enough to close the valve completely, moisture is absorbed, the seed begins to swell and thus the testae soften and become permeable. One trick used to ensure loss of reversible hardseededness is to raise relative humidity slowly. Irreversible hard seeds (absolute hardseededness) do not soften if exposed to high humidity and remain hardseeded throughout germination tests.

The two forms of hardseededness appear to be quite distinct. Techniques which remove reversible hardseededness may not overcome irreversible hardseededness and vice-versa. As seeds are dried they tend to become first reversibly hardseeded (around 10-12%¶ moisture content) and then irreversibly hardseeded (around 5-7%¶ moisture content). Thus irreversible hardseededness is likely to be a substantial problem for gene banks handling species which can show this characteristic. The problem is likely to be most severe in wild accessions, since these tend to have thicker seed coats than seeds of accessions of cultivated lines. Methods to remove hardseededness are described in Chapter 7.

Hard seeds are easily recognised after a short period in a germination test by appearance and feel - they are very hard and fail to swell. If one is uncertain the seeds can be dropped on a china plate. Hard seeds will 'ping' whilst dormant or dead seeds will give a 'thud'. It is probably better to remove hardseededness from those seeds identified as hard during the germination test, rather than to treat all seeds whether hard or not before the germination test. First the treatment to remove hardseededness may damage permeable seeds which do not require any treatment. Secondly this would save effort on the part of those testing the seeds. It is important that those to whom the seeds are distributed are informed that a proportion of seeds are hard and are advised on a procedure to overcome this.

Germination test temperature

The temperature of the germination test affects both the total proportion of seeds which will germinate and the time taken by those seeds to germinate. Non-dormant good quality seeds usually show full germination over quite wide temperature ranges. The usual method of investigating the effect of temperature on germination is to test sub-samples of the seed lot at a number of different constant temperatures, check each test daily and record the number of seeds germinating on each day, and plot the results for cumulative percentage germination (ordinate axis) against the germination test temperature

¶ These values are only approximate and are provided as a rough guide.

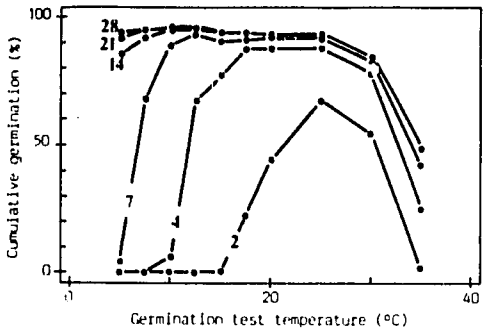


Figure 4.1 Response of germination of a barley seed lot[1] to temperature after successive periods in test (2-28 days). The cardinal temperatures for germination (minimum, optimum and maximum) are <5°C, 5°-30°C and >35°C at 28 days.

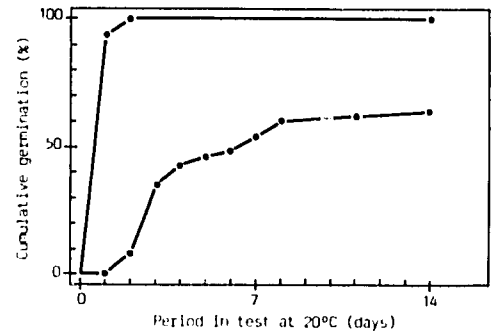


Figure 4.2 Germination progress curves of 2 barley seed lots[2]. Within each lot the slope indicates the variation in time taken to germinate between individual seeds.

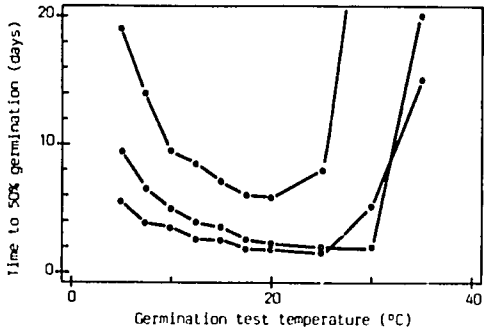


Figure 4.3 U-shaped "germination character curves" of 3 barley seed lots[1].

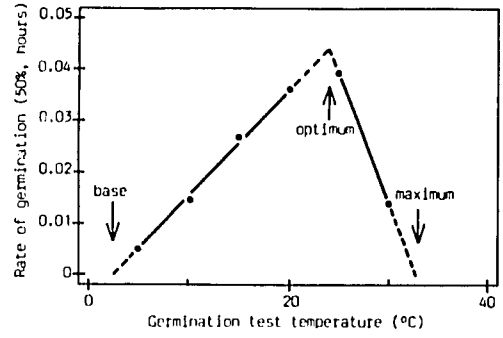


Figure 4.4 Influence of temperature on the rate of germination of a lentil seed lot[3]. The arrows indicate base, optimum and maximum temperatures for rate of germination.

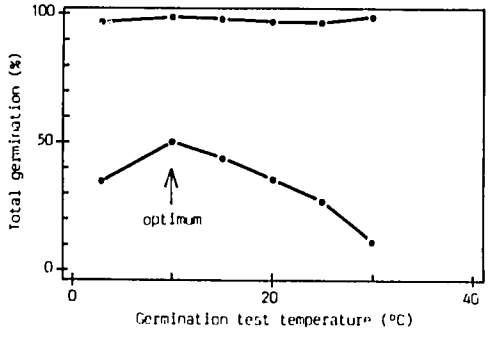


Figure 4.5 Influence of temperature on the germination of 2 barley seed lots[4]. The arrow shows the optimum temperature for maximal germination (compare with Figure 4.6).

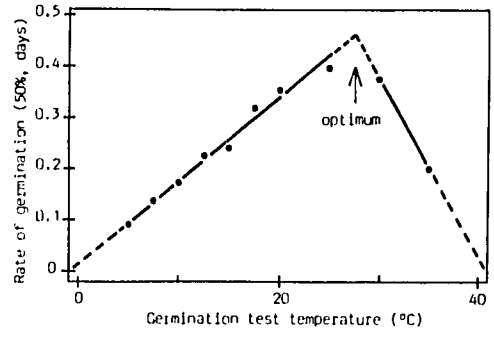


Figure 4.6 Influence of temperature on the rate of germination of a barley seed lot[1]. The arrow shows the optimum temperature for rate of germination (compare with Figure 4.5).

[1] Ellis, R.H., Hong, T.D. and Roberts, E.H. (unpublished).
[2] Ellis, R.H. (unpublished).
[3] Covell, S. and Ellis, R.H. (unpublished).
[4] Ellis, R.H. and Roberts, E.H. (1981). The quantification of ageing and survival in orthodox seeds. Seed Science and Technology, 9, 373-409.

(abscissa) for each day of the germination test. Figure 4.1 demonstrates data presented in this manner. Using this diagram it is possible to describe the temperature range over which germination occurs by a minimum, an optimum, and a maximum temperature. Such values have been described as cardinal temperatures. Note that the actual values attributed to these cardinal temperatures depend critically upon the germination test period. Also the longer the test period the wider the range of optimum temperatures (in terms of cumulative percentage germination). However the optimum temperature should really be the temperature at which both the highest value for cumulative percentage germination and the greatest rate of germination occur - if they coincide. The rate of germination is important for gene banks since this is related to the germination test period required. How can we describe the rate of germination?

First it should be pointed out that the slope of the germination progress curve (for example, as shown in Figure 4.2) does not give the rate of germination. Rather this slope is a measure of the variation of germination times between individual seeds within the lot. The true rate of germination for an individual seed is the reciprocal of the time it takes to germinate, and probably the best single measure of the rate of germination of a population of seeds is the reciprocal of the mean time taken by all the seeds in a representative sample from the population to germinate.

One method of analysing the effect of temperature on germination is to plot the mean time taken to germinate against the temperature of the germination test. In Figure 4.3 the time taken for germination to reach 50% (this is often similar to the mean germination time value and easier to work out) has been plotted against temperature. Note that at extremes of temperature the time taken to germinate increases providing characteristic U-shaped curves. When different cultivars and/or seed lots are compared we can see that although the U-shaped curves are not identical there is a common temperature range that exists over which all the cultivars and/or seed lots germinate most rapidly. In Figure 4.4 the reciprocal of the time taken to germinate is plotted against temperature: this makes it easier to identify the temperature at which the seeds germinate most rapidly.

So far we have considered non-dormant, good quality seed lots. Now we must consider non-dormant poor quality seed lots. It turns out that individual seeds close to death are much more sensitive to germination-test temperature than other seeds. Aged seeds which are close to death will often only germinate over a narrow temperature range. In a seed lot there are varying proportions of seeds close to death, with the greatest proportion of seeds in this category for populations approximately 50% viable. Thus, the effect of temperature on cumulative percentage germination can have a much more marked effect on a population which is 50% viable than on one which is 100% viable (Figure 4.5). This observation leads us to the conclusion that any attempt to determine the suitable germination temperature for all accessions of a particular species should include observation of the response to temperature of populations only 50-70% viable. It should be noted that the temperature at which poor quality seed lots show maximum germination (Figure 4.5) is not necessarily the same temperature as that at which good quality seed lots germinate most rapidly (Figure 4.6). Consequently it should not automatically be assumed that the germination test environments developed by seed analysts for the rapid germination of commercial, high-viability seed lots in seed testing stations may necessarily be ideal for potential use in gene banks.

The temperature regime also influences the expression of seed dormancy: this is considered in Chapter 5. Of course the final choice of germination test temperature(s) will require evaluation of the effect of temperature on the germination of both dormant and non-dormant seeds: subsequent chapters on individual families in Volume II of this book attempt to provide information on optimal temperatures for different species where information exists, or a simple approach where it does not.

Light

Provided the seed is not dormant (see Chapter 5) there is no essential requirement for light; non-dormant seeds will germinate in the absence of light. Light will be essential for removing dormancy in some species but, even where it is not, it aids the development of seedling morphology. Thus the provision of light for a part of the day is helpful. Since non-dormant seed may become dormant under certain lighting regimes care should be taken when providing light to assist seedling development; for more details see Chapter 6.

Additives to the germination media

In the vast majority of species there is no requirement for externally applied nutrients since the process of germination involves the mobilisation of food reserves within the seed (but certain additives may be useful in removing dormancy - see Chapter 5). There are three situations, however, in which nutrient additives to the germination medium are required.

1. There are a very few parasitic flowering plant species where the germination process does require the application of exogenous nutrients. Striga is one such genus. It is unlikely, however, that such species would be deliberately maintained in a genetic resources centre.

2. Orchid seeds are extremely small with limited food reserves and are probably best germinated on synthetic media containing organic and inorganic nutrients.

3. In certain species excised embryos are sometimes tested for germination (see Chapter 11). In a few cases nutrients may be required. If further growth is required then a nutrient supply is essential. If this technique is used as a method of propagation it is sometimes considered to be a type of tissue culture (see Withers, L.A.C., (1980). Tissue Culture Storage for Genetic Conservation, 91pp. IBPGR, Rome).

Germination test period

As can be seen from a germination progress curve (Figure 4.2) individual seeds vary in the time taken to germinate; some seeds can take considerably longer to germinate than other seeds within the same population. The germination test period should be sufficient for every viable seed to germinate, including the slowest seed - the extreme individual, with respect to germination, within the sample. As seed populations age the mean time taken to germinate increases and also the variation in germination time amongst the individual seeds increases. Consequently the time taken by the last viable seed in the population to germinate increases considerably (Figure 4.2). Again poor quality seed lots should be tested to determine suitable germination test periods (but the germination test period required by dormant seeds - see Chapter 5 - is likely to extend beyond this period).

Germination tests should be examined at regular intervals. At these intermediate examinations the germination medium may require rewetting. Also germinated seeds should be removed (i.e. seedlings evaluated, counted and removed). This is because normal seedlings in germination tests eventually die and decay in the absence of nutrients, and excessive seedling growth will dehydrate the germination medium and form a dense mat of roots and shoots which greatly hinders seedling evaluation. Unless classification as abnormal is certain, it is suggested that only normal seedlings be removed at the intermediate counts to provide other seedlings with sufficient time to develop normal seedling structures. However, decaying

seeds (caused by either bacteria or fungi) should be removed to prevent the infection of healthy seeds in the germination test.

For species which germinate rapidly inspection every 3 or 4 days (i.e. twice weekly) is recommended. However after the first two weeks, once-weekly inspection should be sufficient for the remaining seeds which are presumably slow germinators or dormant. Species whose seeds germinate less rapidly should be inspected weekly.

Inspections at the end of the standard germination test period may sometimes suggest that the test duration should be extended. For example, a few seeds may have only just begun to germinate and a further period of growth may be necessary before seedling evaluation is possible. Or seeds may remain in the test which are thought to be dormant and which may germinate given a further period in test.

This introduces another question: how does one evaluate the seeds in test which have thus far failed to germinate? The simple procedure for discerning hard seeds has already been noted. But how do we distinguish at the end, or possible end, of a germination test between dead seeds and dormant seeds? Suitable viability tests for this circumstance are provided in Chapter 11.

EQUIPMENT FOR GERMINATION TESTS

The main equipment required in the seed testing laboratory is some means of controlling the germination environment. Temperature control is the most important facet of the germination environment; of lesser importance is illumination, humidity control and ventilation. Of course germination tests could be conducted in the ambient laboratory environment. However, this would not enable all viable seeds to germinate (for reasons already discussed concerning suitable temperatures for germination) besides the additional potential pro[illegible] of contamination and drying out. Furthermore, variations in the laboratory environment on different occasions would lead to different results. Control of the germination environment can be achieved by the use of Jacobsen apparatus (Copenhagen tanks), controlled temperature cabinets (incubators), or controlled temperature rooms (walk-in germinators).

Copenhagen tanks

The Copenhagen tank consists of a tank containing water, the temperature of which is controlled by a water bath. Seeds are planted on top of (filter) paper and placed on a metal or glass strip which is suspended above the water. A paper (or cotton) wick extends from underneath the seeds to the water below. The moistness of the paper germination medium is altered by increasing or decreasing the vertical distance between the seed and the water. A high level of humidity above the seed - to prevent seeds or seedlings drying out - is maintained either by covering the entire tank with a transparent lid or by covering individual filter papers with bell jars (each with a ventilation hole in the top). The latter is preferable as individual tests can be inspected, removed or replaced without disturbing the environment of other tests.

Overhead lighting can be provided easily. Alternating-temperature regimes can be imposed on the seeds by altering the water-supply arrangements to the tank so that two water baths, controlled at different temperatures, provide water in the tank for different periods in each 24-hour cycle.

The major disadvantage of the basic design is that seed temperatures are dependent upon the water temperature, the distance between the seeds and the water, and (variable) ambient laboratory temperature. The latter can be overcome by controlling laboratory temperature. In humid countries it may prove difficult to operate Copenhagen tanks. Fungal and bacterial contamination is difficult to eradicate. Furthermore, in relation to the number of seeds which can be tested at any one time, Copenhagen tanks take up considerable space.

Incubators

Controlled temperature incubators are flexible, readily available and comparatively cheap. For most applications cooled incubators (i.e. incubators with both heating and refrigeration systems) with forced air circulation are required. The normal operating regime of such incubators is approximately -10°C to +35° or 40°C. The biggest problem with this design of equipment is the variation of temperature between different locations within the cabinet, particularly when full. Manufacturers often quote variation in temperature of this kind when tested in empty incubators. The spatial variation in temperature should be checked while germination tests are in progress inside the cabinet (since these will affect air flow). If any great disparities are noted the air circulation within the cabinet should be increased. Solid-state temperature control enables temperatures to be maintained with little ambient temperature drift and is recommended. Adequate cooled incubators can be provided by converting refrigerators. Since the latter are mass-produced, these may prove cheaper to acquire than purpose-built incubators - but any cost comparison should take into account the useable volume and the conversion time. Again it is important to minimise the spatial variation within the incubator.

An alternative to cooled incubators is to use several conventional heated incubators inside a refrigerated room, a technique often adopted in a number of warmer climates. However, for alternating temperatures it may be better to use cooled incubators designed for the purpose.

Alternating-temperature incubators (sometimes described as incubators with temperature cycling) are also readily available. It is suggested that an achievable standard for specification is that the changes from high to low and low to high temperatures should be achieved within 30 minutes (although temperatures may not stabilise until after 60 minutes or so). A very useful modification is for the high-temperature part of the cycle to be operated with the refrigeration equipment turned off - provided this temperature is more than 5°C above the laboratory temperature. Illumination as uniform as possible, with fluorescent tubes should be provided. If seeds are to be germinated in open containers then air humidity should be controlled to between 90 and 100%, but condensation should be avoided. A cheaper alternative is to control the humidity of each germination test - for example by placing the paper towels inside a loosely-wrapped polyethylene bag - thereby avoiding the need and expense of humidity control - see Chapter 9.

Several cooled incubators, each with the facility for temperature cycling and control of light, probably represent the most sensible form of germination equipment for gene banks dealing with a large number of different species. It should be remembered that cooled incubators dissipate heat: in an air-conditioned laboratory the additional heat load imposed by cooled incubators must be allowed for; and adequate ventilation must be provided in all laboratories.

Walk-in germinators

Walk-in germinators are whole rooms which are used as germinators where temperature, light and humidity are controlled. They are best suited to gene banks where large numbers of tests are all carried out at a single constant temperature, although of course more may be constructed to provide a few additional constant temperatures. However, unless they are well-designed, problems of temperature variation within these large germinators can be considerable and worse than those encountered in incubators. Similarly condensation problems can be excessive - particularly if one wall abutts a colder environment (e.g. an outside wall in a temperate location, or a cold storage room).

Illumination

As already noted in this chapter, light is necessary for normal seedling development, and it may also be required to promote the germination of dormant seeds (Chapter 5). Thus in many germination tests it is necessary to provide sufficient radiant energy of the correct quality. Specific directions on light intensity and quality are provided in Chapter 6. Warm-white fluorescent light sources are generally adequate, but it is important to check that the temperature of the test environment remains at the nominal temperature during the period of illumination.

CHAPTER 5. CHARACTERISTICS OF SEED DORMANCY AND FACTORS WHICH INFLUENCE IT

When viable seeds are set to germinate some may fail to do so because they are dormant. This problem needs considerable attention in gene banks, not only because it can seriously interfere with the results of germination tests designed to assess the viability of accessions, but also it can reduce the amount of precious seeds which emerge when sown out for regeneration, multiplication or other purposes. Consequently when seeds are sent from a gene bank if possible instructions should be sent concerning any special techniques needed to break dormancy. If dormancy is not removed when a sample is used for regeneration or multiplication there will be preferential genetical selection for less dormant types, the progeny of which may carry a tendency to germinate viviparously. Apart from concentrating the agronomically undesirable characteristic the selection will lead to a general genetic erosion of genetically heterogeneous accessions.

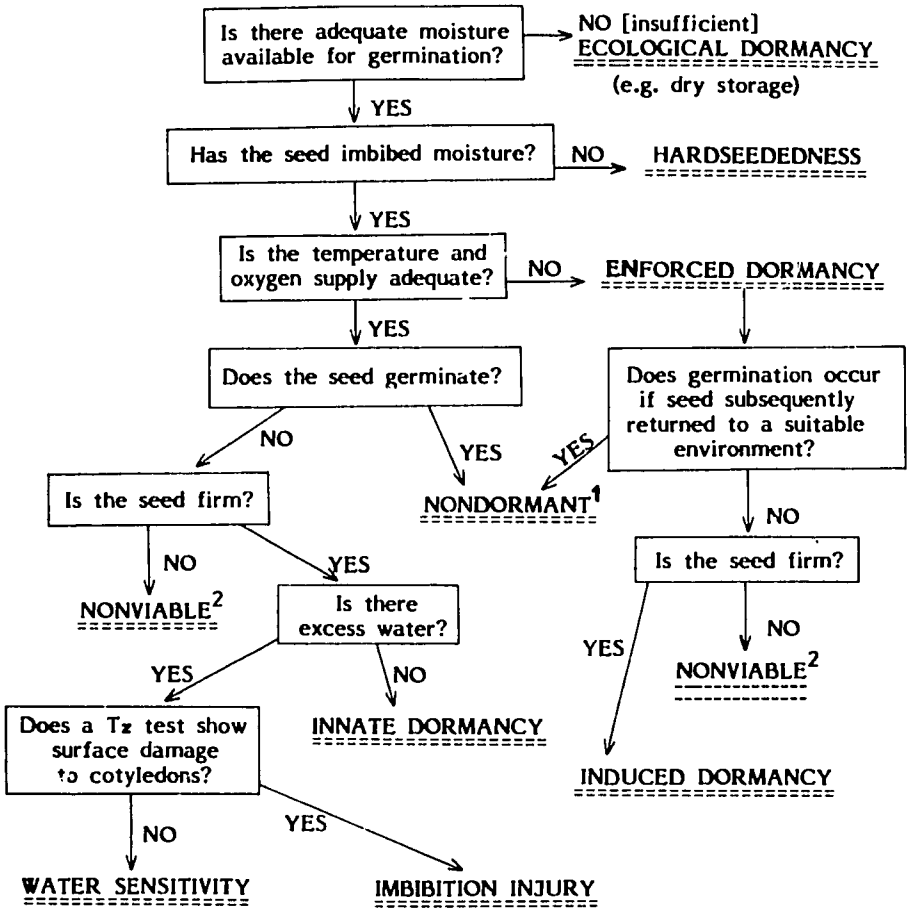
As mentioned briefly in the Preface, seed dormancy is a greater problem in gene banks than in ordinary agricultural or seed-testing practice. There are four reasons for this. (1) In order to maximise seed quality and therefore seed longevity, attempts should be made to store seeds in gene banks as soon as possible after harvest which, coincidentally, is when they are at their most dormant. (2) Again in order to maximise seed quality the 1982 Report recommends that seed drying in gene banks is carried out at a lower temperature than is common in commercial practice. Low-temperature drying will also minimise loss of dormancy. (3) The sub-zero temperatures used in long-term seed storage not only prolong viability but also prolongs the dormant state. (4) Gene banks typically include primitive land races and wild relatives of crop plants in their collections and these usually show much greater dormancy than modern cultivars.

Seed dormancy, then, is a major problem in gene bank operations and accordingly needs to be given considerable attention. This chapter discusses the principle features of seed dormancy of relevance to gene banks, and the main ways in which it may be minimised. Chapter 6 is devoted to the effects of light on the dormancy of light-sensitive seeds and suggests practical procedures for applying light in germination tests. Practical advice on removing, chipping or scarifying seed covering structures - techniques which are sometimes incorporated in dormancy-breaking procedures - is given in Chapter 7. Chapter 11 provides guidance on alternative criteria of viability other than germination when dormancy is an insuperable problem, and provides further advice on viability testing in these circumstances. Finally the entire emphasis of Volume II is on practical methods of overcoming seed dormancy: Chapter 16 introduces alternative approaches to the problem where specific advice for an accession is not available; the subsequent chapters give specific advice, organized taxanomically family by family on individual crops and, where available, general methods for species within each family.

THE DEFINITION AND ROLE OF SEED DORMANCY

Dormancy is an imprecise term which has many different meanings. In its broadest (and most imprecise) sense it can be applied to describe the period from when the seed embryo discontinues growth on the mother plant to when it begins to germinate (ecological dormancy). In this sense one could

Figure 5.1 Schematic representation of the different categories of dormancy, together with associated problems



Notes

1) If the seed takes a long time to germinate, this may be either due to a slight degree of dormancy which is 'broken' under germination test conditions, or due to low seed vigour.

2) If the seed is non-viable at the end of the germination test then either the seed was dead before the germination test began, or the seed died during the germination test presumably as a result of the germination test conditions.

consider all seed in long-term seed stores for genetic conservation to be dormant.

However, to seed technologists - and in particular those who test seeds for germination - seed dormancy has a narrower meaning which is more useful: seed dormancy is the condition in a viable seed which prevents it from germinating when supplied with those factors normally considered adequate for germination, i.e. when it is at a suitable temperature in a medium providing adequate water and access to a gaseous environment approximating to air. It is this definition which we find most satisfactory. The accompanying diagram (Figure 5.1) attempts to clarify the divisions between those phenomena which collectively comprise dormancy in its broadest sense (innate dormancy, enforced dormancy, induced dormancy, hardseededness) together with those phenomena which may mistakenly result in a seed being classified as dormant (water sensitivity, imbibition injury, non-viability). For more information on hardseededness, water sensitivity, and imbibition injury see Chapters 4 and 7; the testing of seed for viability at the end of germination tests by pressure or by cutting is considered in Chapter 11.

Dormancy is often divided into several categories. Most physiologists would now agree that there are three main types, although some confusion has arisen because different words have sometimes been used to describe the same phenomenon.

Innate dormancy

Innate dormancy (also known as primary dormancy, natural dormancy, inherent dormancy and endogenous dormancy) describes the dormancy which is present immediately the new embryo ceases to grow when it is still attached to the parent plant: such dormancy prevents the seed from germinating viviparously (i.e. on the mother plant) and also usually for some time after the ripe seed is shed or harvested. Seed accessions in gene banks are likely to exhibit considerable innate dormancy (for reasons already explained) and, thus, innate dormancy may represent a formidable problem to gene bank staff.

Innate dormancy is divided into two categories by some workers; seed coat dormancy, and embryo dormancy. If a viable seed imbibes water but fails to germinate until the seed coat is removed or the embryo excised, then dormancy is said to be imposed by the seed coat: that is it is the seed coat which is preventing germination. Note the difference between the definitions of seed coat dormancy and hardseededness: both phenomena result in the failure of viable seeds to germinate, but with seed-coat imposed dormancy the seed has imbibed moisture, whereas with hardseededness the seed is unable to imbibe. Embryo dormancy is said to occur when a viable seed from which the seed coat has been removed fails to germinate: it is also described sometimes as physiological dormancy. We believe that there are at least four reasons why gene banks need not be particularly concerned about the division of innate dormancy into two categories:

(1) The embryo fails to germinate whether it is embryo dormancy or seed coat dormancy which is imposed.
(2) Seed coat removal often enables the treatment level or concentration of another dormancy-breaking agent with which it is combined to be reduced as compared with that necessary for the treatment of the intact seed.
(3) Different seeds within an accession may exhibit seed coat dormancy and embryo dormancy.
(4) An individual seed can exhibit both seed coat dormancy and embryo dormancy to a degree: seed coat removal is unable to result in germination; but, equally, a treatment which overcomes the effect of embryo dormancy is unable to result in germination; whereas combining seed coat removal with a treatment which

overcomes the effect of embryo dormancy enables germination to occur.

Some authors refer to the last condition as double dormancy; that is both seed coat dormancy and embryo dormancy. The term double dormancy has also been used to describe the condition in which some individuals within an accession exhibit seed coat dormancy whilst some others exhibit embryo dormancy.

Enforced dormancy

Enforced dormancy (also known as environmental dormancy) describes the condition when viable seeds do not germinate because of some limitation in the environment. The term has often been applied to describe the condition of natural populations of seeds which remain dormant while buried beneath the soil but germinate immediately the soil is disturbed and the seed exposed at the soil surface. Absence of light and reduced temperature alternations beneath the soil surface appear to be the major factors enforcing dormancy in this instance. Dormancy can be enforced on seeds of this type in the laboratory by setting them to germinate in the dark at a constant temperature. This type of dormancy disappears immediately the missing factors are provided.

Induced dormancy

After a seed has lost its innate dormancy it is possible for a similar type of dormancy to be induced. Induced dormancy (also known as secondary dormancy) is usually the result of seeds being supplied with water but exposed to an environment where some other particular factor is unfavourable for germination (e.g. high temperature, low oxygen); the seeds fail to germinate in this environment, and also subsequently fail to germinate when transferred to an environment which is favourable for the germination of non-dormant seeds. Dormancy which has been induced persists for a considerable time: it is this persistence of dormancy after removal from the inhibitory environment which distinguishes induced dormancy from enforced dormancy. Induction of dormancy within seeds of accessions maintained in gene banks is possible during the storage and drying of very moist seeds, during poorly designed germination tests to monitor accession viability, and in field or glasshouse sowings under unsuitable conditions where it was intended to regenerate or multiply an accession.

Dormancy as a distributive strategy

Dormancy forms part of the distributive strategy of higher plants, providing a delay mechanism which enables dispersal from the maternal parent before germination occurs. Moreover, dormancy can also enable the seed to germinate at more opportune times or positions - since the dormant state is often removed by those conditions of the environment which are associated with the ecological niche of the particular species. The majority of seed dormancy research and literature has been concerned with investigating its ecological importance and physiological basis. No attempt is made here to discuss these issues. Instead dormancy is discussed solely in relation to the practical objective of gene banks; to identify dormant seeds and provide procedures which remove dormancy from all seeds without damage to non-dormant seeds.

DISTRIBUTION OF DORMANCY WITHIN AN ACCESSION

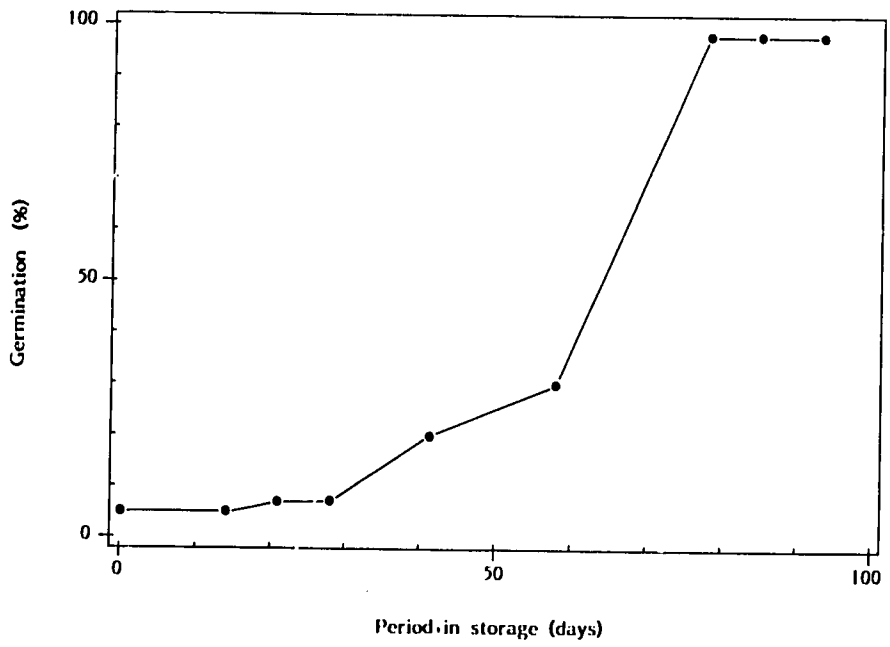
Seeds within accessions are not uniform. Rather they show individual variations in very many characters; for example, in weight and period of survival. Similarly individual seeds vary in dormancy. Thus although an accession may be described as dormant, this does not necessarily mean that each and

every seed within this accession is dormant or, indeed, equally dormant.

The last point needs further consideration since dormancy has often been considered to be a simple binary phenomenon: a seed is said to be either DORMANT (ON) or NON-DORMANT (OFF). It is true that often the expression of dormancy can be binary within a single germination test; those viable seeds which fail to germinate being defined as dormant and those which germinate being defined as non-dormant; there is no intermediate class. But suppose the germination test period were to be extended by a further seven days and a few more seeds germinated. Would these seeds be dormant or non-dormant? To which question some would answer partially dormant! (Other terms used to describe this include relative dormancy and conditional dormancy.) In other words there are circumstances in which some individual seeds would germinate under a wide range of conditions but would behave as dormant seeds under other conditions. These would be considered to be only lightly dormant. On the other hand other seeds may only germinate under relatively few environments which may involve special pre-treatments. These could be considered to be deeply dormant. Since even deeply dormant seeds will germinate under some circumstances, there is a sense in which dormancy is always 'relative' or 'conditional'.

Consequently there are two dimensions to the problem of dormancy within each accession: first there is the proportion of individual seeds within the accession which are dormant under specified conditions; and, secondly, there is the relative tenacity with which the state of dormancy is maintained by an individual seed. These two aspects are demonstrated in Figure 5.2 where germination is plotted against time in storage. Initially 95% of the population were dormant and only 5% non-dormant. Thus in the control population dormancy is not exhibited by all members of the population. Storage of the seed

Figure 5.2 Germination of rice seeds (Oryza glaberrima Steud.) withdrawn from storage at 30°C with 11.7% moisture content (wb) after various periods (●) which demonstrate the phenomenon of after-ripening. Seeds were tested for germination at 20°/30°C (16h/8h) for 14d. Note the variation in period required to remove dormancy from individual seeds in the population: i.e. there is a steady increase in the proportion of seeds able to germinate with increasing storage period. (From Ellis, R.H., Hong, T.D. and Roberts, E.H. (1983). Procedures for the safe removal of dormancy from rice seed. Seed Science and Technology, 11, 77-112.)



resulted in loss in dormancy such that after 100 days storage 100% of the seed population was non-dormant. However, note that some seeds required only a short period of storage to break dormancy, whilst others required considerably more. Thus the tenacity with which the individual seeds retained dormancy differed.

At this point it is worth noting that this difference in the tenacity with which individual seeds retain dormancy can also be reflected in the time taken by the seed to germinate in a germination test regime in which dormancy is broken. Consequently many authors will define a seed as dormant, or partially dormant despite the fact that it has germinated because there has been a delay to germination.

DIFFERENCES IN BOTH THE PROPORTION AND THE DEGREE OF SEED DORMANCY BETWEEN POPULATIONS

Dormancy, though under genetic control, is extensively modified by both the pre-harvest and the post-harvest environment to which the seeds have been exposed. Consequently the progeny of a single stock can show vastly different dormancy characteristics if the seed is multiplied in two different environments. Moreover, in our experience there is no simple relationship between populations with regard to the proportion of individual seeds which are dormant (in a given germination test environment) and the tenacity with which these individuals retain dormancy. Thus there can be no guarantee that a treatment which successfully removes dormancy in one accession will be as successful with another accession. This is why one must be very careful to avoid generalisations when discussing dormancy-breaking methods. Nevertheless, a wide range of diverse dormancy-breaking agents do have qualitatively similar effects not only for different seed lots within a species but also for seed lots of very many different species. The following section attempts to catalogue many of the treatments which often result in a loss of seed dormancy.

TREATMENTS WHICH REMOVE DORMANCY

After-ripening in dry storage

After-ripening, or post-harvest maturation, describes the loss in dormancy that gradually occurs when seeds are stored after harvest in the air-dry state. In commercial seed practice it is extremely common for dormancy to be removed in this way. As with other factors which lead to loss in dormancy, there is variation between individual seeds within a seed lot in the time taken for loss in dormancy to occur (Figure 5.2), but in addition the rate of loss can be influenced by both the temperature and the moisture content of the after-ripening environment. In general the higher either of these factors are the more rapid is the loss in dormancy, although in several investigations it has not been possible to discern any effect of seed moisture content. However, increase in either temperature or moisture content also results in greater loss in viability. Consequently it is not advisable for gene banks to use after-ripening as a dormancy-breaking technique on a complete accession, for this could result in loss in viability, genetic integrity, and a substantial reduction in potential storage life. Under some circumstances, however, after-ripening could be used on samples withdrawn for germination tests, providing the combination of conditions used is not sufficient to affect viability detectably.

Pre-drying treatments are similar to after-ripening treatments. They consist of a period of exposure to a drying regime during which seed moisture content is lost. It is often suggested that such treatments be applied after pre-soak or pre-wash treatments - see a later part of this chapter.

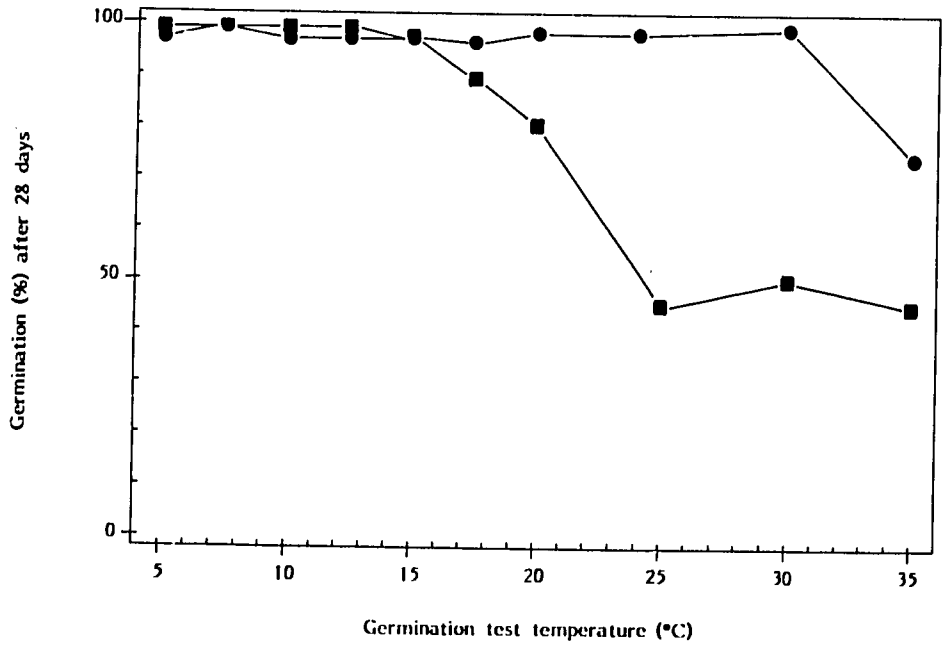
The effect of removal or puncture of structures covering the seed

In addition to preventing imbibition (hardseededness), seed covering structures can limit oxygen diffusion and act as mechanical barriers to radicle emergence - seed-coat imposed dormancy. Thus in many species removal, puncture or scratching (scarification) of seed coats may enable imbibed, dormant seeds to germinate. In a number of species such treatments can be quite effective in promoting germination. However, the procedures can be very labour-intensive - particularly in those small seeded species where dormancy can often be quite pronounced. Moreover, seeds can be damaged by these treatments. Chapter 7 gives further information on the partial or complete removal of seed coats. In a few fruit species seed coat removal can enable dormant seeds to germinate, but subsequent seedling growth may be restricted (dwarfism) unless embryo dormancy is also removed - see the information on Prunus and Pyrus in Volume II. It should also be remembered that removal of the seed coat can affect other germination-related phenomena - see the information on hardseededness and imbibition injury in Chapters 4 and 7.

The effect of gaseous atmosphere during germination tests on dormancy

As noted above, seed covering structures can limit oxygen diffusion into the embryo. Increasing oxygen partial pressure around intact seeds can increase the rate of oxygen uptake and result in loss in dormancy compared to testing the seed in an atmosphere containing 21% oxygen (i.e. in air). However, in other cases increasing oxygen partial pressure above 21% increases the proportion of dormant seeds whereas reduction below this value results in a greater loss in dormancy. Similarly pre-treatment in the complete absence of oxygen (pure nitrogen) may also result in loss in dormancy.

Figure 5.3 Influence of temperature on the germination of a barley seed lot at harvest (■) and after 10 days treatment at 40°C with 15% moisture content (●). The depression of germination of the freshly harvested seeds at 17.5°C and above is an expression of dormancy (relative or conditional dormancy). The after-ripened seeds are able to germinate fully over a wider range of temperatures than the freshly harvested seeds.



Other gases can also affect dormancy. High concentrations of carbon dioxide can result in both induction of dormancy or loss in dormancy whilst lower (3-5%) concentrations usually tend to lead to loss in dormancy. The presence of hydrogen sulphide, carbon monoxide, phosphine, ethylene and certain anaesthetics (ethanol, methanol, acetone, chloroform, ethyl ether) can also result in loss in dormancy.

The expression of dormancy in germination tests at constant temperatures and the effects of pre-chilling or stratification

Temperature during the germination test affects the expression of dormancy considerably. Although dormant seeds of some species may not germinate at any constant temperature (in the absence of specific dormancy-breaking treatments) seeds of many crop species tend to show some germination over a very narrow band of constant temperatures. Some workers refer to such differential germination at constant temperatures as relative dormancy or conditional dormancy. The conventional representation of the relationship between germination at constant temperatures and dormancy is shown in Figure 5.3; dry storage of seed results in a loss in dormancy which enables the seed to germinate over a much wider range of temperature than the dormant seed. In these circumstances it is difficult to be certain whether those seeds which germinated over the narrow band of constant temperatures before storage were not dormant to begin with or whether dormancy was broken during the brief exposure to temperatures within this range. One guide can be to examine the time taken to germinate. Often if dormancy is first broken by some other means (in this example by dry storage) the subsequent germination at these temperatures can occur sooner.

It would be fortunate if all dormant seed of all species showed germination over the same, narrow, band of temperatures. This is not the case however. Species vary quite considerably in their response to constant temperatures, as an examination of Volume II of this manual will indicate.

A period of pre-treatment of imbibed seeds at a different constant temperature to that applied in the subsequent germination test can be an extremely effective dormancy-breaking treatment. Pre-treatment at a low, but not freezing, temperature is a particularly important dormancy-breaking treatment which is widely applied in practice. For example, in the temperate cereals exposure of imbibed seeds to temperatures below 7°C for up to seven days before transfer to a higher temperature for the subsequent germination test is the normal method of breaking dormancy in seed testing stations. In other species much greater periods of exposure are used; for example, see the information provided in Volume II for seeds of the rosaceous fruits. It should also be pointed out that in many of the species where pre-chilling seeds is beneficial, the subsequent transfer to a higher temperature is not necessarily essential for germination; often the seeds will germinate if they remain at the pre-chill temperature but, of course, germination will occur more slowly than at higher temperatures. Normally seeds are pre-chilled on, or in, the same medium as used for the germination test. A few workers, however, pre-chill seeds by submerging in water. Whilst this can be beneficial to seeds that are slow to imbibe, in view of the problems that can arise when very dry seeds imbibe moisture too rapidly - see information on imbibition damage, Chapter 4 - it is recommended here that seeds should not be pre-chilled by submerging in water: if the seeds are slow to imbibe then the pre-chill treatment period can be extended. It is worth noting that the response to pre-chill treatments is not limited to species from temperate regions; the germination of dormant seeds of many species from semi-tropical and tropical regions can also be promoted by pre-chill treatments, e.g. rice and many tropical forage species.

Throughout this book we use the term pre-chill to describe an exposure of the imbibed, or imbibing, seeds to a low temperature; pre-chilling has also been described as stratification, layerage or - more rarely - after-ripening. Stratification and layerage are apt terms to describe the horticultural practice of exposing seeds in alternate layers between moist peat or sand to temperate ambient (low) winter temperatures - with the pre-treated seeds being sown out the following spring. (Throughout this report we only use the term after-ripening to describe the storage of air-dry seed under various conditions intended to reduce dormancy.)

Similarly pre-treatment of imbibed seeds at a high temperature, with subsequent transfer to a lower temperature can be a powerful dormancy-breaking treatment; though widely used, the practical importance of such treatment is less than pre-chilling. Throughout this report we use the term warm stratification to describe such treatments. In some accessions alternating warm stratification and pre-chill treatments can be particularly promotory. The information provided in Volume II for seeds of Prunus species gives examples where warm stratification, or alternating exposure to warm stratification and pre-chill treatments promote the germination of dormant seeds.

However, a further complicating factor when examining the influence of temperature on germination is the induction of dormancy. Often if seeds are exposed to temperatures outside the narrow band of temperatures at which they can germinate and then transferred to a temperature within this range they fail to germinate: that is dormancy is induced. In the majority of cases such induced dormancy is observed after exposure to temperatures above the narrow band at which they will germinate. For example, in lettuce (Lactuca sativa L.) this phenomenon provides a very real practical problem for growers: if seeds are sown when soil temperatures are at or above about 20°C seeds that have not been after-ripened will normally fail to germinate and emerge even though the soil temperature subsequently becomes cooler; in lettuce the phenomenon is described as thermodormancy. It should also be noted that it is possible for dormancy to be induced during exposure of imbibed seeds to low temperatures, but this is comparatively rare and unlikely to be a problem in gene banks.

The somewhat paradoxical effect of high temperature pre-treatments of imbibed seeds needs some explanation. Prunus provides good examples of species where dormancy can be both broken and induced by a pre-treatment at a high temperature, prior to a subsequent germination test at a lower temperature: it is possible to see both effects within one accession. An exposure of the imbibed seeds of a typical Prunus accession to 25°C, or so, for 2 weeks, with subsequent transfer to a lower temperature can increase the total proportion of seeds which germinate (that is, dormancy is broken), but the time taken by the less dormant individuals within the population to germinate can increase quite substantially (that is, dormancy is induced - the low germination test temperature itself being a dormancy-breaking treatment). In view of the final outcome of the test (a higher proportion of seeds germinating) the warm stratification treatment is, thus, beneficial. However, in less dormant accessions the most notable effect would be the substantial increase in germination time and we would, thus, not consider the warm stratification treatment to be worthwhile in this case.

The influence on seed dormancy of alternating-temperature environments in the germination test

Of course soil temperatures are anything but constant and in the surface layers there is a very distinct diurnal alternation. Alternating-temperatures can be extremely effective in breaking dormancy, and are widely used. For example, various alternating-temperature regimes are prescribed in the rules of the International Seed Testing Association; details of these regimes are provided in Volume II. However,

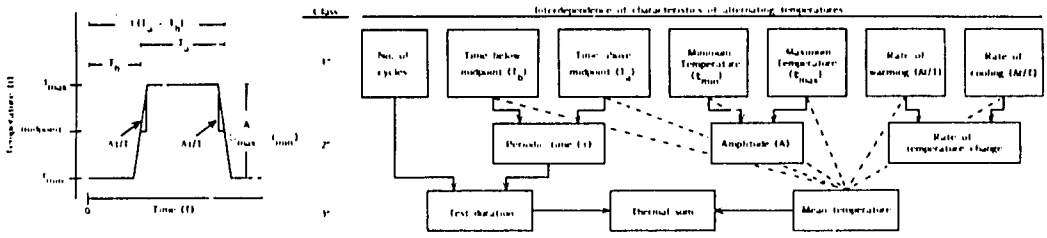
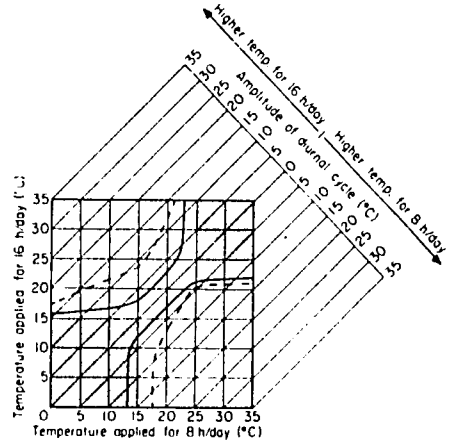


Figure 5.4 Characteristics of alternating temperature regimes and their interdependence. Constant temperature regimes can be described by the above three tertiary characteristics whereas alternating temperature regimes have many more characteristics, as shown above. The seven primary characteristics can be controlled experimentally. They are independent of each other, but affect the six secondary and tertiary characteristics. All characteristics affect dose. An accession's response to an alternating temperature regime is a function of dose and receptivity. The factors which affect an accession's receptivity to dose exist before treatment (genotype, maturation environment, after-ripening) and develop after imbibition (interacting factors - e.g. nitrate, light-ontogenetic change in receptivity). (Classification based on unpublished work of A.J. Murdoch and E.H. Roberts.)

Figure 5.5 Response of Rumex obtusifolius to alternating temperature. The solid and broken lines represent 50% and 90% germination respectively. Stippling indicates those areas where the temperature is always below 15°C or always above 25°C - where little germination occurs. (From Totterdell, S. and Roberts, E.H. (1980). Characteristics of alternating temperatures which stimulate loss of dormancy in seeds of Rumex obtusifolius L. and Rumex crispus L. Plant, Cell and Environment, 3, 3-12.



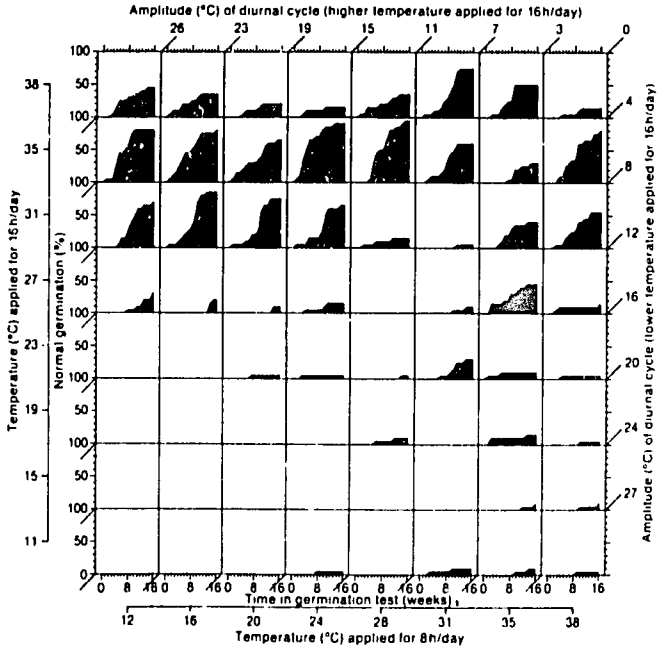


Figure 5.6 Germination progress curves of Oryza glaberrima at various constant and alternating temperatures provided by a two-dimensional temperature gradient plate. Note the differences in the population response to different regimes. (Source: as for Figure 5.2.)

it should be noted from the outset that the adoption of any alternating-temperature regime in germination tests will not ensure the germination of dormant seeds; promotory alternating-temperature regimes require accurate definition.

A major problem when considering such regimes is the number of attributes of alternating-temperatures which could conceivably stimulate seed germination. These are: the length of each cycle; the minimum and maximum temperature in each cycle; the period of exposure to the minimum and maximum temperatures; the range between the minimum and maximum temperature; the cumulative effect of the number of cycles; the efficacy of cycles with respect to time from the onset of imbibition; the rate of heating or cooling from one temperature to another; and the mean temperature. Moreover it is not possible to alter one attribute without confounding it with a change in at least one other. For clarity Figure 5.4 illustrates these various interrelated attributes of alternating temperature regimes. For practical purposes, however, we can assume diurnal alternations (i.e. a 24 hour cycle) and limit the attributes of interest to: the minimum and maximum temperatures, the period spent at each temperature, the temperature range and the mean temperature. Figure 5.5 provides a stylized example of how each attribute can be specified to provide adequate conditions to remove dormancy in Rumex. Figure 5.6 provides the results of an investigation with rice as a further example, which illustrates that we are witnessing the removal of dormancy from a population of seeds in which the responses of individuals vary. In some individuals only a small alternation is required to break dormancy, whilst in others the alternation required is considerable.

The influence of light on seed dormancy

It is well known that light can have a powerful influence on the expression of seed dormancy in many species (although the detection of its influence may be dependent on the presence of other dormancy-breaking agents - see a later part of this Chapter). Earlier reviews tended to divide light-sensitive species into two categories depending on whether germination was promoted or inhibited by light (often described as positively or negatively photoblastic species respectively). To some extent these responses could be related to the ecology of the species. Generally speaking it could be seen as an adaptive advantage for small seeds with inevitably small food reserves to remain dormant unless they are at or near the soil surface, otherwise germination could result in seedling death because food reserves are depleted before the shoot reaches the soil surface and starts to photosynthesise. On the other hand for some larger seeds of more arid climates it may be more important for seeds to avoid germination on the soil surface. In the first case one would expect to find a positive response to light and in the second a negative response.

However, the division of seeds into those which show a positive response to light and those which show a negative response to light is now known to be an over-simplification. First, light quality is important. Secondly, many seeds which germinate in response to low levels of light may be inhibited (prevented from germinating) if exposed to prolonged periods of high-intensity light. Chapter 6 provides information concerning the various responses of seeds to light and provides suggestions for optimal practical seed dormancy-breaking regimes for use in germination tests designed to assess accession viability.

Effect of soaking in water before the germination test

Pre-soaking seeds in water before the germination test sometimes has a promotory effect on seed germination which is utilised by seed testers. For example, the 1976 Rules of the International Seed Testing Association recommended that dormancy in rice (Oryza sativa) seeds be removed by soaking the seeds in water at 40°C for one to two days (but see the information on the genus Oryza in Volume II for our comments on this treatment). Throughout this report we refer to such treatments as pre-soak treatments.

A second procedure in which soaking in water alone can promote the germination of dormant seeds is more akin to rinsing than soaking. Certain species may have inhibitors to germination present within the seed or fruit coat. These are removed by rinsing the seeds in constantly changed water. Such treatments are referred to as pre-wash treatments throughout this report. Examples of species in which pre-wash treatments can increase seed germination in subsequent germination tests include Vitis spp. and Beta vulgaris. In addition to removing germination inhibitors, pre-wash treatments may soften the seed coat or surrounding fruit structures, lessening the physical resistance to germination.

Care should be taken with both pre-soak and pre-wash treatments, however. First, the water supply (whether standing or running) must contain sufficient dissolved oxygen to prevent the death of or induce dormancy in the imbibed, or imbibing, seeds. Secondly, the treatment should not be applied for so long that some seeds begin to germinate, otherwise the chitted seeds may be damaged when they are transferred to the germination test regime; moreover, very often it is recommended that pre-soak or pre-wash treatments be succeeded by pre-dry treatments (that is the seeds are dried back down again) and such treatments usually kill chitted seeds. Finally, there is the risk of imbibition injury to very dry seeds: consequently it is suggested that seed moisture content be increased by humidification before pre-soak or pre-wash treatments.

The effect of pre-applied and co-applied compounds in solution on dormancy

Very many compounds have been applied to seeds in an attempt to break dormancy. Many have been found to have at least a small effect in promoting the germination of dormant seeds. The normal method of applying such compounds to seeds is in solution in water, although other solvents such as acetone have also been used. Two different application procedures are used; pre-application or co-application. A pre-applied treatment refers to a treatment of seeds to the compound in solution in water (unless otherwise stated) prior to transfer to the germination test environment: treatment may be either by immersing the seeds in the solution, or by placing them on top of a germination-test substrate in which the solution is used instead of water. A co-applied treatment is one in which the compound - normally dissolved in water - is applied to the seed in the germination environment - that is the germination-test substrate is moistened with this solution. With both methods of application the concentration of the compound in solution can be critical: too low a concentration and no effect on germination is recorded; too high a concentration and the non-dormant seeds may be temporarily prevented from germinating or killed. Not only is it necessary to determine the most suitable concentration but, even so, optimum concentrations rarely break the dormancy of all seeds and the optimum concentrations may vary quite considerably between accessions. With the pre-applied method higher concentrations are generally necessary to promote the germination of dormant seeds compared to the co-applied method. Further factors requiring definition with the pre-applied method is the duration and temperature of the treatment.

The following chemical agents are among those most commonly applied to seeds which have been reported to promote the germination of dormant seeds in one or more species: hydrogen peroxide, ethyl alcohol, sodium hypochlorite, thiourea, mercaptoethanol, nitrates, nitrites, hydrogen ions (i.e. low pH), cyanide, azide, gibberellins, cytokinins (e.g. kinetin) and hydroxylamine. See also the list of gases in an earlier part of this chapter, and for detailed comments on the application of these and other chemicals to different species see the chapters on the various plant families in Volume II.

Interactions and substitutions between the various factors which can promote the germination of dormant seeds

It is important to note that many of the factors which are said to promote the germination of dormant seeds may only work under certain circumstances. For example, the phenomenon of thermo-dormancy in lettuce was noted under the section on the effect of temperature on dormancy, where at high temperatures (close to 30°C) no germination occurs in some accessions - indeed dormancy is induced: however, if light is present then the seeds may well germinate, despite the supra-optimal temperatures. In other words the action (or inaction!) of one factor can be modified by the presence of a further factor.

Interactions. Interactions can be either positive or negative. The meaning of the term interaction is probably best shown by an examination of some experimental results. The following results (from E.H. Roberts, 1973. Oxidative processes and the control of seed germination. In Seed Ecology ed. W. Heydecker, pp. 189-218. Butterworths, London.) provides examples of positive interactions between the effects of light, nitrate, and alternating temperature on the promotion of germination of dormant seeds of Rumex crispus. Similar interactions are very common in a wide range of species.

To determine the effect of each potential dormancy-breaking agent and the interactions between these effects requires the testing of different sub-samples of the accession under all possible combinations of these agents. In this case, where there are three potential dormancy-breaking agents, eight (i.e. 2^3) different treatment combinations are required. The treatments and results are given below.

Treatment	Germination Environment	Percentage Germination
1	25°C, dark, water	0
2	25°C, light, water	0
3	25°C, light, 10^{-2} M KNO_3	0
4	25°C, dark, 10^{-2} M KNO_3	0
5	25°/1.5°C*, dark, water	1
6	25°/1.5°C*, light, water	68
7	25°/1.5°C*, dark, 10^{-2} M KNO_3	4
8	25°/1.5°C*, light, 10^{-2} M KNO_3	100

* diurnal alternating temperature regime 25°C for 8 hours, then 1.5°C for 16 hours.

Treatment 1 is the control germination test: that is the test was conducted in the absence of all three potential dormancy-breaking agents; in this investigation, no seeds germinated under these conditions. How is germination affected by treatment with each dormancy-breaking agent on its own? At a constant temperature in the absence of nitrate light has no effect on germination (result of treatment 2 minus result of treatment 1). At a constant temperature in the dark, nitrate has no effect on germination (result of treatment 4 minus result of treatment 1). In the dark in the absence of nitrate, alternating

temperature has only a marginal effect, viz. 1% germination (result of treatment 5 minus result of treatment 1). Each of these subtractions is said to describe the action of each factor.

How is germination affected if one dormancy-breaking treatment is combined with another? There are three different treatments possible which combine two different agents: light and potassium nitrate, light and alternating temperature, and alternating temperature and potassium nitrate. Light and nitrate combined at a constant temperature has no effect on germination (treatment 3 minus treatment 1). Consequently since the action of nitrate is nil and the action of light is nil, there was no first order interaction between the action of light and nitrate since the combined result (0) is neither greater nor less than the addition of the two separate actions (0 + 0 = 0). In the presence of light and alternating temperature (treatment 6) 68% germination is recorded. There is, thus, a powerful positive first order interaction between the actions of light and alternating temperature (0 + 1 ≠ 68), the effect of this interaction being a 67% increase in germination compared to the total effects of light (treatment 2) and alternating temperature (treatment 5) when applied alone and the control germination test result (treatment 1). In the presence of alternating temperature and potassium nitrate (treatment 7) only 4% germination is recorded. The interaction is thus 3% (4 - (1 + 0)). Finally, 100% germination is recorded in the presence of all three dormancy-breaking agents (treatment 8). There is, thus, a powerful positive second order interaction between the combined action of alternating temperature, light, and nitrate (individually giving 1 + 0 + 0%) and the first order interactions (giving 0 + 67 + 3%) since (1 + 70 ≠ 100).

These results illustrate a common feature of dormancy investigations. Often investigations with a single dormancy-breaking agent alone show no effect. Only when this agent is combined with another promotory agent is any effect seen. Both light and alternating temperatures show this feature quite clearly (examine the results of treatments 1, 2 and 5 with that of treatment 6). Although nitrate on its own or in combination with any one of the other two agents had little or no effect, when combined with both other agents it had a very considerable effect (compare treatment 8 with treatment 6).

This is a conventional representation of interactions between the effects of different dormancy-breaking agents on seed germination, but it should be noted that there are more complicated definitions and explanations of interactions. Interactions can be quite powerful, for example that shown by light and alternating temperature on the germination of Rumex crispus described here. But any significant increase in germination which results from combining dormancy-breaking agents is worthwhile, even if no interaction is observed. Much use is made of the potential benefits to be obtained by combining different dormancy-breaking agents to promote the germination of dormant seeds in the suggestions made for seeds of particular crops in Volume II; for example, see the information provided for rice (Oryza spp.).

Substitution. In certain cases it is thought that two different dormancy-breaking agents break the dormancy of the same individual members of the dormant seed population; in which case one agent is said to substitute for the other. Thus when both are applied together the percentage germination is no higher than the result with either agent alone (i.e. result in treatment A = result in treatment B = result in combined treatments A and B). Gibberellic acid is often said to substitute for light in breaking the dormancy of many light-sensitive seeds, but sometimes the expression has been used uncritically.

CHAPTER 6. LIGHT, PHYTOCHROME AND SEED GERMINATION

In Chapter 5 it was noted that light can influence the germination of dormant seeds. Consequently gene bank staff need to decide what light environment should be provided to promote the maximum germination of seeds. Critical information for individual species is seldom available but this chapter attempts to provide general guidance on suitable light regimes and how to measure them.

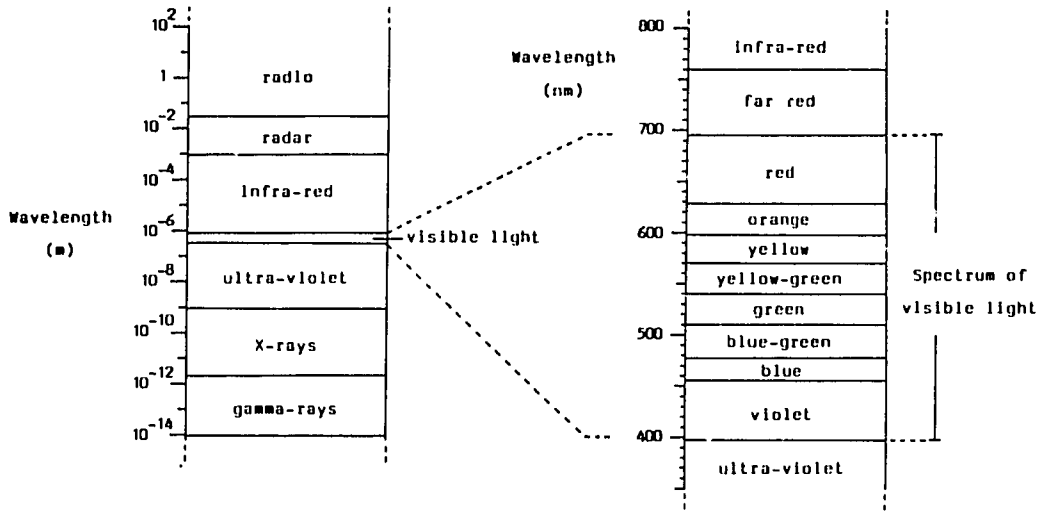
Historically it used to be thought that species could be classified into three categories regarding their seeds' response to light: those where germination was promoted by light; those where germination was inhibited by light; and those species which were unaffected by light. However, the division of seeds into those which show a positive response to light and those which show a negative response to light is now known to be an over-simplification. Many seeds which germinate in response to low levels of light may be inhibited if exposed to prolonged periods of high intensity light. The response to light within a seed lot may change during storage. Different accessions of a given species can differ in their qualitative and/or quantitative response to light. Even within a single accession there can be a thousand-fold variation between individual seeds in the minimum amount of light needed to stimulate germination. And any response to light is usually critically dependent upon the status of other aspects of the germination test environment, such as temperature. In order to understand these various responses and arrive at an optimal practical seed dormancy-breaking regime for use in germination tests designed to assess viability, it is useful to understand what is meant by the term light and to consider the main characteristics and mode of action of the photoreceptor pigment (phytochrome) involved in light reactions.

LIGHT QUALITY

Light is both a physiological and a physical term. In physiological terms light is usually described as the energy detected by the average human eye. In physical terms light appears to act in two ways. The passage of light through space is described as waves of electromagnetic radiation. The emission or absorption of light by matter is, however, described as a corpuscular (packet) phenomenon. These two aspects of the physical description of light are explained below.

Since light travels in the form of a wave it can be described by any of the three following parameters: wavelength - the distance between two consecutive wave crests; frequency - the number of wave crests passing a stationary point in one second; or wavenumber - the number of wave crests present in a one centimetre length of the wave. These parameters are inter-related. If wavelength is measured in centimetres then the wavenumber is the reciprocal of the wavelength, whilst the frequency is simply the wavenumber multiplied by the speed of light - a constant. For seed physiology light quality is most usefully described by the wavelength. Wavelengths of visible light are generally given in nanometres (10^{-9}m, symbolically nm). Ångström units (10^{-10}m, symbolically Å) were commonly used until the introduction of S.I. units. In Figure 6.1 the electromagnetic spectrum of solar radiation is described according to the wavelength of its constituents. Note that visible light is but a small part of the spectrum.

Figure 6.1 The components of solar radiation by wavelength (m, left) and the spectrum of visible light (nm, right).



Within the spectrum of visible light (Figure 6.1) the human eye identifies energy of specific wavelengths as colours. For example if the light energy received by the eye is only within the range 400-440 nanometres (nm) then this is perceived as violet light. White light is perceived when the eye receives light energy across the continuum of 400-700 nm. The sensitivity of the human eye decreases rapidly to wavelengths outside this range. In biology it is common to describe light within the visible spectrum by its perceived colour rather than its wavelength - for example, as red light rather than 650-700 nm. Conceptually the wavelength can be considered to be the quality of light energy. How then do we describe the amount of light energy?

Light absorbed or emitted by matter (as opposed to that travelling through space) behaves as a stream of discrete energy packets. The smallest energy packet is described as a quantum; if this is within the visible spectrum (or slightly outside) then it may be more specifically referred to as a photon.

The use of the term photon (or quantum) relates to the action of light energy. Einstein's law of photochemical equivalence states that in the primary photochemical reaction one quantum of light energy must be absorbed for every atom or molecule reacting. Thus the amount of light reaching a given area per unit time in a germination test - irradiance - can be quantified in terms of the number of quanta received in a given area per unit time. [Similarly the amount of light given out by an artificial light source per unit time - emittance - can be described in terms of the number of quanta radiated per unit solid angle per unit time. (The solid angle - steradian - is the surface area on a sphere which is equivalent to the square of the radius.)] However, one problem that arises when quantifying irradiance and emittance is that other quite different systems of measurement are also used. The units used and terminology of the three distinct systems will be discussed later in this chapter.

The energy contained within a quantum depends upon the wavelength of the radiation. The shorter the wavelength the more energy per quantum. Thus from Figure 6.1 each quantum at X-ray wavelengths contains considerably more energy than each quantum at infra-red wavelengths. The absorption of the large amount of energy contained within a quantum at X-ray wavelengths by a molecule can result in the complete ejection of an electron from the molecule, thus causing ionization. At far infra-red wavelengths the energy per quantum is low and only leads to an increase in the rotational or vibrational energy of the absorbing molecule (that is an increase in temperature). Photochemical reactions are those which occur when the absorption of a photon by a molecule leads to a change in the energy level of an outer electron - i.e. it moves to an orbit of higher energy. These are caused by radiation between about 290 nm (the mid-ultraviolet) and about 800 nm (the near infra-red). That is only slightly wider than the wavelength range detectable by eye (Figure 6.1). A photochemical reaction consists of first the absorption of a photon by a molecule resulting in the photochemical activation of that molecule and a subsequent reaction in which the activated molecule takes part.

The molecules which absorb photons are described as photoreceptors, or pigments. The reason why they are sometimes described as pigments is that they only absorb light within certain narrow wavelength bands; the rest is transmitted or reflected and therefore the pigment appears as a different colour from the incident light. The wavelength range over which energy is absorbed by a photoreceptor is described as the action spectrum. The main wavelengths which can influence seed germination are 600-680 nm (red light, generally promotory) and 700-760 nm (far-red, generally inhibitory). In addition wavelengths of 400-500 nm (blue light) have been found both inhibitory or stimulatory in a few cases, and prolonged exposure to other wavelengths can have an effect. The main (and possibly only) pigment involved in these germination responses is known as phytochrome.

PHYTOCHROME

Phytochrome is a protein of molecular weight 120,000 with a tetrapyrole chromaphore attached. Phytochrome occurs in two main inter-convertible forms. Pr absorbs mainly red light with a peak absorption at about 660 nm whereas Pfr absorbs more strongly in far-red light with a peak absorption at about 720-730 nm. Pfr is the active form which promotes germination whilst Pr is inactive. Under any given light conditions the situation is dynamic with continuous interconversion of the two forms with a particular equilibrium ratio of Pfr to Pr which is characteristic of the spectral quality of the light. For example, under red light the ratio of Pfr to total phytochrome [Pfr/(Pfr + Pr)] is 0.75, under far-red light it is about 0.03, and under sunlight (which contains red and far-red light in approximately equal proportions) it is about 0.60. Where seeds respond to light, short exposures to red light or daylight (white light) generally promote germination and exposure to far-red light does not. Note that exposure to far-red light can also inhibit the germination of many seeds otherwise capable of germinating in the dark. This is because their ability to germinate in the dark may have depended on a sufficient quantity of pre-existing Pfr.

The continuous interconversion between the two forms of phytochrome is also affected by other factors. A simplified diagram of the transformations undergone by phytochrome is given in Figure 6.2 and outlined below.

1. Inactive existing phytochrome is activated to Pr.
2. Pre-cursors to phytochrome are synthesized to Pr.
3. Pr is transformed to Pfr by red, or possibly blue, light.

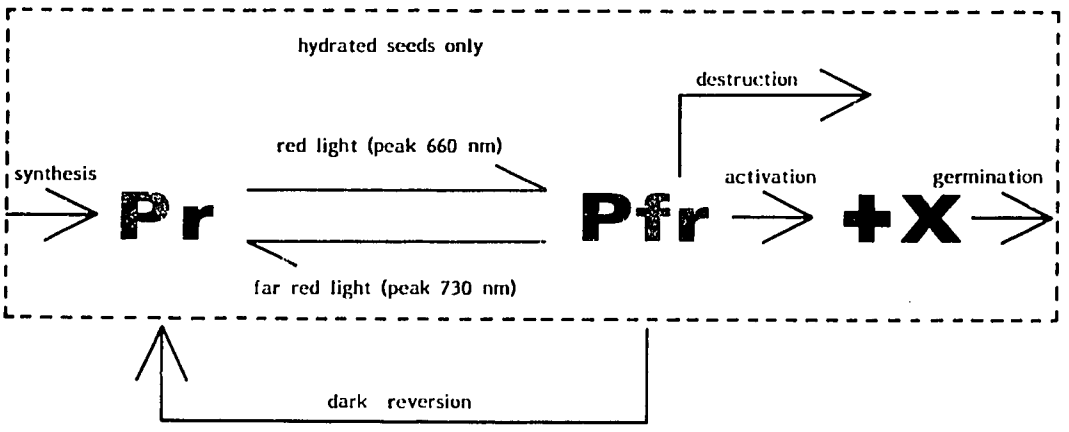
4. Pfr is transformed to Pr by far-red light.
5. In the dark Pfr may be transformed to Pr in hydrated seeds and sometimes slowly in dry seeds.
6. In dry seeds an inverse dark reaction may occur whereby Pr is transformed to Pfr.
7. Pfr may be destroyed, possibly by enzymes.
8. The reaction between Pfr and some unknown substance in the seeds causes the promotion of germination.

This simple account (above and Figure 6.2) ignores the fact that all pathways between Pr and Pfr and vice versa go through a series of intermediate forms of phytochrome whose stability varies.

In general, germination is promoted if the amount of Pfr as a proportion of the total phytochrome is high. Only small amounts of light are required to affect this equilibrium; i.e. only a small amount of red or white light is necessary to promote germination in a light-sensitive seed, and only a small amount of far-red light is necessary to inhibit germination.

There is however a further effect of light on seed germination which occurs at a subsequent stage before radicle emergence, and which can inhibit germination: this is known as the high irradiance reaction because it usually needs continuous application of light at high irradiance. It is thought to be due to the high rate of interchange (rate of cycling) between Pr and Pfr and thus can occur in any quality of light (even when the dynamic equilibrium in white or red light ensures that the majority of phytochrome is in the Pfr form). Although any quality of light can be inhibitory, blue light is often found to be more inhibitory than other wavelengths; and this has led to the suggestion that a blue-absorbing pigment may also be involved.

Figure 6.2 An outline of the transformations undergone by phytochrome.



LIGHT ENERGY

Consequently in addition to the quality of light we must, if we wish to promote seed germination, consider the amount of light energy reaching the seeds. What units are used to measure the quantity of light reaching seeds? Unfortunately the answer to this question is not simple. The problem is that three separate terminologies have been used to quantify the light environment; viz. photometric, radiometric and quantum.

Quantum units

For our purposes - where we are interested in the photochemical reaction of Pr to Pfr - quantum units are the most appropriate. As described already, the smallest unit is the single photon or packet of energy which is absorbed by one molecule. This causes a change in the energy levels of outer electrons thereby photochemically activating the molecule. In chemistry a useful concept is that of the gram-molecular weight or mole - the mass in grams of a substance numerically equal to its molecular weight. The number of molecules in a mole is a constant for all substances and is called Avogadro's number. Its value is approximately 6.02×10^{23}. Similarly, 6.02×10^{23} photons are also described as a mol(e) since this number of photons (at the correct wavelength of course) could activate a gram-molecule of the receptor, if every molecule absorbed a photon. Thus the photon density reading at a surface is quantified as mol m^{-2}. It is described as photon fluence. The photon 'flux' is described as photon fluence rate (mol m^{-2} s^{-1}). Photon fluence rate has also been described as photon flux density or quantum flux density and measured as Einsteins m^{-2} s^{-1}. To convert between the two is easy: 1 mol m^{-2} s^{-1} = 1 Einstein m^{-2} s^{-1}. The term Einstein is now used only rarely.

Radiometric units

Radiometric units are most suited to studies of power balances. The energy emitted from a light source or received by, for example, a seed is described as radiant energy if radiometric units are used, and is measured in joules (J). The other unit which may be encountered is the erg; the joule = 1×10^7 ergs. Radiant flux is the rate of flow of energy (i.e. power) from a source of radiation and is measured as joules per second (J s^{-1}) or watts (W). (1 J s^{-1} = 1 W). Energy fluence (which corresponds to photon density in quantum units) is expressed as joules per square metre (J m^{-2}) and energy fluence rate as joules per second per square metre (J s^{-1} m^{-2}) or - since one watt is one joule per second - watts per square metre (W m^{-2}). Energy fluence rate is the radiant energy received by a surface and is called irradiance or radiant flux density. All the above units are normally only specified, or measured, over limited, specified, wavelength ranges - generally the visible range (400-700 nm).

Photometric units

Photometric units were introduced by lighting engineers. These units measure light as it is perceived by human beings. The measuring system is based on the visual appearance of light given out by a standard source and relies on the judgement of a hypothetical observer (described as the Standard Photometric Observer) about the relative brightness of different light sources. Photometric units therefore not only take into account the energy fluence rate of the visible spectrum, but also the fact that the human retina is more sensitive to wavelengths in the mid-range of this (yellow-green light) than at the extremes. For these reasons photometric units are of no direct interest to plant biologists. However, because so much information is available in photometric units and because reliable and relatively cheap

instruments are available to measure light in photometric units, a considerable number of publications which describe the results of investigations into the effect of light on seed germination have used photometric units. It is for this reason that information on these units are provided here.

Luminous energy is the quantity of light, weighted for its action upon the human eye (this weighting being a major problem in making sensible use of such information), radiated or received for a period of time. The unit of measurement is the lumen-second (lm.s). Luminous energy corresponds to radiant energy in radiometric units. Luminous flux is the rate of emission of light. It can be derived from radiant flux in radiometric units but again must be weighted according to the sensitivity of the human eye. Luminous flux is measured in lumens (lm). Illuminance (or illumination) is the term most often encountered in descriptions of light environments. It is the luminous flux density incident on a surface. It is measured in lumens per square metre (lm m^{-2}). It is also described in lux (lx), where 1 lm m^{-2} = 1 lux. Unfortunately several other units are also used to quantify illuminance in photometric units. Although these are at first sight confusing it is at least simple to convert between them. The phot is one lumen per square centimetre (lm cm^{-2}). Thus 10,000 phot = 1 lux. Sometimes illuminance is measured in British rather than metric units - that is lumens per square ft (lm f^{-2}). This unit is also described as the foot-candle (fc). Illuminance in foot-candles can be converted to illuminance in lux by using the following conversion factor; 1 foot-candle = 10.76 lux. The lux has also been described as the metre-candle or candle meter.

A PROPOSED LIGHT REGIME FOR GERMINATION TESTS WHICH IS EXPECTED TO PROMOTE SEED GERMINATION

It would be convenient if a common light regime could be adopted by a gene bank for dealing with all light-sensitive species. The main difficulties on deciding on the specifications of such a regime are that there is a large variation in minimum light requirements for germination (between individual seeds within an accession, between accessions within a species, and between species). At the same time there is the possibility of inhibiting germination if the amount of light used is too large. Fortunately, however, there is usually a very considerable range (often several orders of magnitude) between the amount of light required to saturate the promotion of germination and the threshold amount required for inhibition.

Bearing these points in mind it is possible to suggest a suitable common light regime which is probably suitable for a large number of light-sensitive species. Clearly, from the preceeding discussion, a light source providing low irradiance which emits little radiation in the far-red part of the spectrum is suitable, and it would be safest to apply the light intermittently rather than continuously. For this purpose conventional domestic fluorescent 'daylight' or 'warm white' lighting is suitable - any of the several lamps with peak emission at about 580 nm will do. (These have the additional advantage that they emit relatively little heat.) Incandescent lamps are much less suitable since they emit strongly in the far-red part of the spectrum (and also emit considerable heat). Diffuse low-intensity daylight is also suitable, but less easy to control.

As a general-purpose regime it is provisionally recommended that a pulse of white fluorescent light equivalent to about 200 J m^{-2} should be applied every four hours. The irradiance should be relatively low and so, for example, this pulse could be 1 W m^{-2} for 200 second or 5 W m^{-2} for 40 seconds. If it is inconvenient to apply intermittent pulses of light, an acceptable alternative would be to apply the light for, say, 8 to 12 hours per day at about 1 W m^{-2} (86,400 J m^{-2}). In the more detailed recommendations and suggestions for specified genera in Volume II it will be noted that sometimes a suggestion is made

that the timing of the application of a light treatment from the onset of imbibition is important. Adoption of these suggestions is advised but, for the general case where no such suggestions have been made, the above light regimes can be applied throughout the germination test.

CONVERSION BETWEEN MEASUREMENTS IN QUANTUM, RADIOMETRIC OR PHOTOMETRIC UNITS

In the summary of germination test regimes provided in Volume II for specified genera it will be seen that all the possible units of light measurement have been used by different workers. Consequently it is necessary for the reader to be able to convert between the various units in order to make comparisons of the effects of different light environments.

For monochromatic light - that is light over a single narrow wavelength band (e.g. 660 ± 5 nm) - it is possible to convert between the three types of units fairly easily. This can be done by using light meters which measure in the three different units, determining the illuminance (if photometric units) irradiance (if radiometric units) or the photon fluence rate (quantum units). However, if this is done it will be found that the conversion factor differs for monochromatic light of different wavelengths. Further difficulties occur when one considers light sources which emit over a wide range of wavelengths - since, depending on the source, the relative emissions in different wavebands can differ quite markedly. For example, the spectral energy distribution of sunlight peaks at 480 nm whereas the spectral energy distribution of a warm-white fluorescent tube peaks at about 580 nm (Figure 6.3).

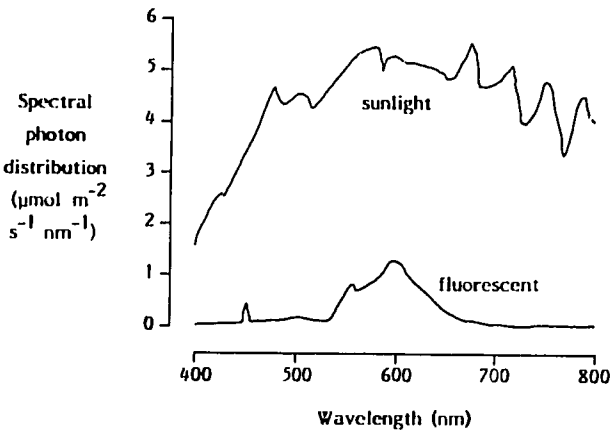
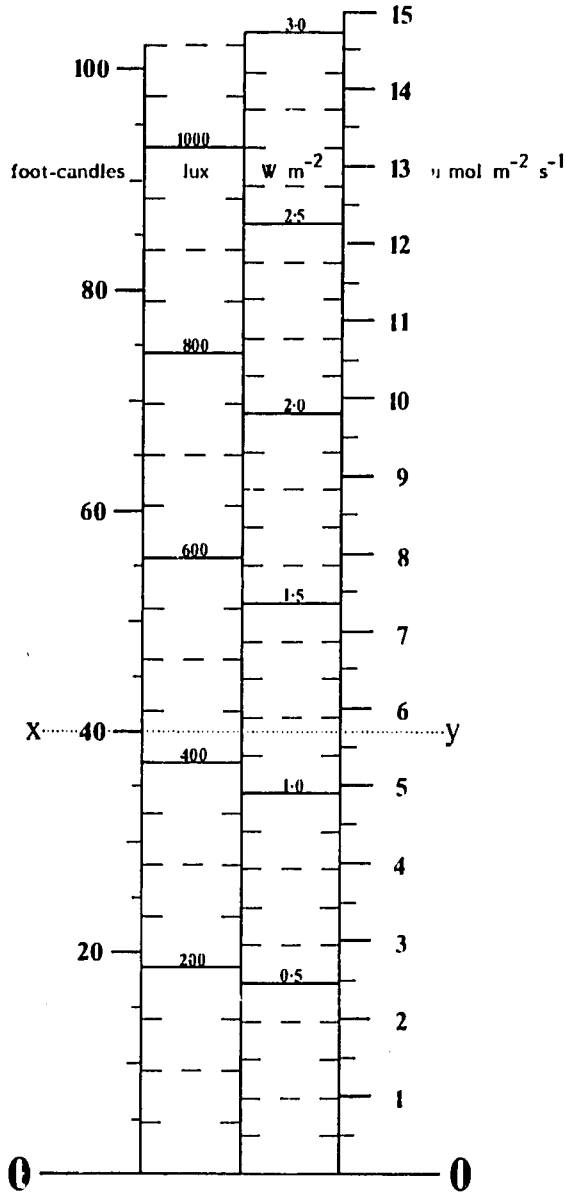


Figure 6.3 Spectral scans (400-800 nm) of sunlight (midday, summer, UK) and fluorescent light (1m from 12 'warm-white' tubes). (From data of McLaren, J.S. (1980). The expression of light measurements in relation to crop research. In Seed Production. (ed. P.D. Hebblethwaite), pp. 663-670. Butterworths, London.)

In practice we can, with one exception, limit our light sources to fluorescent tubes since in a laboratory germination test it is convenient to use an artificial light source, and the spectral energy distribution of 'white' or 'warm white' fluorescent tubes results in a Pfr/P ratio of about 0.6 which is promotory. To convert from illuminance (lux) to irradiance (W m^{-2}) for 'white' or 'warm-white' fluorescent tubes the standard conversion factor is to multiply illuminance in lux by a factor between 0.0027 and 0.0029. It is suggested that the lower value be used. Thus if illuminance from a 'warm white' fluorescent tube is 500 lux then the irradiance is (500 x 0.0027 =) 1.35 W m^{-2}. (Incidentally 500 lux or 1.35 W m^{-2} are convenient values to remember as these are typical for illuminance or irradiance within an incubator equipped with fluorescent tubes.)

Figure 6.4 Scale to convert between photometric (illuminance: foot-candles or lux), radiometric (irradiance: W m^{-2}, between 400 and 700 nm) and quantum (photon fluence rate: μ mol m^{-2} s^{-1}) units of light for 'warm-white' fluorescent light sources. To convert between non-adjacent units use a ruler parallel to the base and perpendicular to the axes. For example, the broken line between x and y demonstrates that an illuminance of 40 foot-candles or 430 lux is equivalent to an irradiance of about 1.17 W m^{-2} and a photon fluence rate of about 5.7 μ mol m^{-2} s^{-1} for 'warm-white' fluorescent light sources.



Conversion scale between illuminance (foot-candles or lux), irradiance (W m^{-2}) and photon fluence rate (μ mol m^{-2} s^{-1}) for 'warm-white' fluorescent light sources.

For many species sufficient stimulation to germinate is obtained from brief periods of exposure to diffuse laboratory daylight. (The brief periods of exposure occur in incubators without lights when incubator doors are opened and closed.) If laboratory lighting is provided by diffuse daylight then a conversion factor for illuminance to irradiance of 0.0043 (W m^{-2} lux^{-1}) is appropriate. If, however, fluorescent tubes are used to provide laboratory light then the conversion factor of 0.0027 (W m^{-2} lux^{-1}) should be used. In passing it should be noted that if a conversion factor is used to convert illuminance measured in photometric units of sunlight (diffuse or otherwise) to irradiance then the irradiance should be described as irradiance of the visible spectrum - since irradiance outside this region of wavelengths is also received from sunlight and the total irradiance of sunlight is roughly twice that within the visible spectrum.

Conversion factors from irradiance (radiometric units) to photon fluence rate (quantum units) will also be dependent upon the spectral energy distribution of the source. We have calculated an approximate conversion factor for 'warm-white' fluorescent tubes from a comparison of the spectral energy distribution and the spectral photon distribution. The method of calculation though approximate does provide a means of conversion. We estimate the conversion factor from irradiance to photon fluence rate to be 1 W m^{-2} = 0.0000049 mol m^{-2} s^{-1} for 'warm-white' fluorescent tubes. It is probably easier to think of this conversion factor as 4.9 micro mol m^{-2} s^{-1} per W m^{-2} - where 1 micro mol m^{-2} s^{-1} = 10^{-6} mol m^{-2} s^{-1}.

It is rather easy to make mistakes (for example, by an order of magnitude) when using conversion factors. Consequently a conversion scale (Figure 6.4) has been provided to enable the reader to convert easily between illuminance (lux or foot-candles), irradiance (W m^{-2}) and photon fluence rate (micro mol m^{-2} s^{-1}) where 'warm-white' fluorescent tubes are the light source. It is important to remember that this conversion scale is not valid for any other light source.

FURTHER READING

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We are most grateful to Dr. B. Frankland for his suggestions concerning the most suitable general-purpose light regimes to use.

CHAPTER 7. METHODS OF OVERCOMING THE INHIBITION OF GERMINATION CAUSED BY SEED-COVERING STRUCTURES

As mentioned in Chapter 3, in the strict botanical sense the covering structure of the seed is the testa. However in some cases the dispersal unit may consist of a dried fruit or floret which is the 'seed' in the traditional or agricultural sense, and thus the more general term seed-covering structures is used here to indicate all those structures attached to and surrounding the seed which may affect germination.

INHIBITORY EFFECTS OF SEED-COVERING STRUCTURES

There are six potential ways in which the seed-covering structures may prevent or delay the germination of viable seeds: (1) as a barrier to water uptake; (2) as a barrier to oxygen uptake; (3) as a physical barrier to radicle emergence; (4) as a source of chemical germination inhibitors or a barrier to their outward diffusion from the seed or fruit; (5) as a selective filter affecting light quality; and (6) as a source of microorganisms, especially fungi.

Barrier to water uptake

Where the seed covering structures delay or prevent imbibition and thus delay or prevent germination the problem should not be described as one of dormancy, since dormancy is usually defined as the failure of an imbibed viable seed to germinate under otherwise suitable conditions of temperature, moisture and oxygen (see Chapter 5). However the effect can be such that the delayed progress of germination is assumed (wrongly) to be the result of dormancy.

In cases where the testa is impermeable and acts as a complete barrier to moisture, thereby preventing imbibition, the seed is said to be hard (Chapter 4). This is an important topic deserving a separate section, and the morphology of hardseededness and methods of overcoming testa impermeability (that is, scarification) will be discussed at the end of this chapter. However, even when a seed is not hard, the rate of imbibition may be slow and delay germination.

First it is necessary to consider the proportion of the surface area of the seed which is in contact with the germination test substratum: this can be a particular problem where large seeds are tested on top of paper; it can also be a problem sometimes when the flower structures enclose the seed, since these may lift the seed above the germination test substratum.

Secondly several cases are known where the seed covering structures severely reduce the rate of imbibition in dry seeds. Examples of this problem are provided with the advice for germinating seeds of Citrus and Prunus accessions in Volume II.

The simplest method of overcoming reduced germination as a result of severe delays to imbibition is to increase the total germination test period. Admittedly this acknowledges rather than overcomes the delays to imbibition, but in very many cases, is the most satisfactory way in which to deal with the problem. Furthermore it automatically deals with those cases where the seed-covering structures are not

the cause of slow imbibition: tissues within the seeds such as the endosperm can also act as temporary barriers.

Where seed shape or the seed covering structures result in poor contact between the seed and the germination test substratum, contact can be improved by testing the seeds between paper or in sand. If testing on top of paper or on top of sand is necessary (e.g. in order to provide a stimulatory light regime), then the problem of poor contact can be circumvented by regularly spraying a fine mist over the seeds to enable them to absorb moisture from the atmosphere as well as from the substratum.

Several treatments are available which may lessen the effect of seed covering structures in reducing the rate of imbibition. In addition to pre-soaking, pre-washing, or surgical treatments to the seed covering structures which are considered below, other possible treatments include the use of chelating agents such as citric acid or ethylenediaminetetraacetic acid (EDTA) which are said to increase cell-wall permeability by sequestering calcium ions; however the evidence concerning chelating agents is somewhat contradictory.

Barrier to oxygen uptake

The seed covering structures may also act as a partial barrier to oxygen. The seed covering structures are often relatively impermeable to gases despite the fact that they may be permeable to water. The permeability may vary between different gases, particularly carbon dioxide and oxygen. In dry stored seeds the requirement for oxygen in metabolism is minimal, but it becomes substantial upon imbibition. The problem is increased where excess moisture surrounds the seed, or part of the seed.

Apart from a surgical treatment to remove part or all of the seed covering structures which are described later, gaseous diffusion rates can be increased by increasing the pressure of the gas in question in the atmosphere around the seeds. Generally the gas in question is oxygen; raising the partial pressure of oxygen above 21% or, more rarely, increasing atmospheric pressure is sometimes applied as a stimulatory treatment. There are a minority of cases, however, where some reduction in oxygen partial pressure (e.g. Typha latifolia and Cynodon dactylon) or increase in carbon dioxide partial pressure (e.g. Trifolium subterraneum) may be promotory. Carbon dioxide treatments to overcome hardseededness are discussed later.

Physical barrier to radicle emergence

Although the seed covering structures pose a potential mechanical barrier to the protrusion of the radicle, in general such problems are not common, and even where they occur they may be of minor importance. For example, in the absence of the seed covering structures radicle emergence may occur sooner: but the percentage germination may be no different if the germination test on intact seeds is continued for a sufficiently long period.

The germination of aged seeds may be affected by the seed covering structures because the vigour (literally, seedling strength) of the developing root and shoot may be insufficient to break through them. One example of this is provided in the barleys (Hordeum spp.) where the tips of weak coleoptiles are unable to break through the lemma and palea: instead growth occurs underneath the lemma and palea until eventually an arch of the coleoptile (and not the tip) breaks through, but the resulting seedling is abnormal. However, in general the seed covering structures (once moist) do not form an absolute barrier to radicle emergence. Nevertheless, genera in which the seed covering structures are sometimes reported

to prevent seed germination by mechanical resistance to the growing embryo include Alisma, Amaranthus, Rubus and Prunus.

Source or barrier to diffusion of inhibitors

Endogenous germination inhibitors are claimed to be a common cause of seed dormancy. When they occur these inhibitors are often located in the seed covering structures. For example, in Beta spp. the inhibitors are located in the pericarp of the fruit cluster. It has also been suggested in some species that seed-covering structures can act as a barrier to the outward diffusion of inhibitors from the seed itself. Inhibitors present in fruits and seed covering structures may inhibit germination either through their metabolic effects or through influencing the osmotic pressure so that the embryo is unable to obtain sufficient water for germination and growth. Inhibition due to chemical inhibitors may be overcome by pre-washing or surgical treatments described below.

Selective filter to light

One potential way in which the seed covering structures can influence germination is by selectively filtering light, resulting in inhibitory light quality (see Chapter 6) within the seed. Certainly there is much circumstantial evidence to suggest that this may occur in the maternal environment. Also the removal of seed covering structures tends to increase the range of light regimes over which seeds will germinate in germination tests.

Source of microorganisms

Finally it should not be forgotten that the seed covering structures can be a source of bacteria and fungi. After imbibition seeds may decay as a consequence of the infection. Moreover, any fungi present may compete for oxygen. In an environment where oxygen is limiting - because, for example, excess moisture may be present - germination may be prevented and the seed may die. In Hordeum this phenomenon is described as water sensitivity (Chapter 4).

GENERAL METHODS OF OVERCOMING SEEDCOAT EFFECTS

Alternative treatments

It should not be assumed that where the seed covering structures are hindering germination that they must be removed (either in part of in whole) for germination to occur. Alternative dormancy-breaking treatments such as pre-chilling, light, alternating temperatures, chemical treatments or simply an extension of the germination test period are often sufficient to promote full germination without removing the seed covering structures. Not only do these alternative treatments reduce the labour involved in setting up germination tests compared to that where removal of the seed covering structures is practised, but they may also avoid the potential damage to seeds that can be associated with removal of the seed covering structures. Several methods of directly overcoming the potential problems to germination posed by the seed covering structures are considered below, but the more indirect methods (such as the examples provided above) should not be forgotten.

Pre-washing or pre-soaking or seeds

Pre-washing treatments (where the seeds are continually rinsed in running water) and pre-soaking treatments (where the seeds are soaked in static water) can result in a fairly rapid uptake of moisture by the seeds and, thus, may overcome some of the delays to imbibition caused by the presence of the seed covering structures. (But rapid moisture uptake may also cause problems, see methodology below.) Pre-washing, particularly, and to a lesser extent pre-soaking treatments can also remove endogenous germination inhibitors from both the seeds and the seed covering structures.

Scarification with acid treatments

The term scarification usually describes treatments to the testas of seeds intended to overcome hardseededness and is considered later. However, the term is also sometimes used to describe acid treatments of seeds where the testa is permeable, but where the seed covering structures are considered to be preventing or delaying germination. The best example of the use of the term in this context is probably as applied to the seed dispersal units of the grasses. But in such cases the treatment is somewhat unreliable: in particular there is a tendency for it to kill some seeds in accessions; and when the same treatment (time x concentration) is applied to many accessions it may result in reduced, rather than enhanced, proportions of seeds germinating in some of the accessions. In general the use of acid-scarification treatments where hardseededness is not a problem is not recommended here; but in advice given on some genera in later chapters (Volume II) we have had to suggest acid scarification where there is little evidence currently available of superior alternatives. Nevertheless, where we have suggested the use of acid scarification, treatment periods must be severely restricted to avoid damage to the seeds, and not used as a blanket approach in testing all accessions of these genera.

Surgical treatments to the seed covering structures

The most obvious method of avoiding inhibition, or delay to germination caused by, or associated with, the presence of the seed covering structures is to remove them. Whilst total removal may be inferred by the term removal of seed covering structures, total removal is time-consuming, may be damaging to the seeds and is rarely necessary to achieve promotion of germination. Instead selective surgical treatments, which include piercing, puncturing or chipping a part of the seed covering structures, are suggested. Piercing or puncturing of seeds can be achieved with a sharp needle; in endospermic seeds it is generally done in the region of the endosperm close to the embryo; embryos may also be pierced or punctured, but whether this is damaging or promotory probably depends somewhat upon technique. The term chipping is generally used to describe the cutting, rubbing, or pulling off of a part of the seed covering structures generally at the end opposite to the embryo: the terms file or rub are used to describe a similar action.

Complete removal of the seed covering structures, or at least down to naked caryopses in the Gramineae for example, is inferred by the use of the term dehull, dehusk, deglume, or hulling. These terms are explained in Chapter 3. The complete removal of seed covering structures, particularly where these adhere tightly to the seed or fruit, can, however, be damaging to underlying tissues - particularly when the seeds are very dry - as well as time-consuming. Consequently such treatments should not be regarded as a panacea for all problems of germination in laboratory tests. Probably the most useful suggestion is to consider applying such treatments to the firm seeds which appear to be dormant during germination tests - see below.

As a general principle the removal of the seed covering structures should not be used as the first line of attack in promoting germination unless there is both ample skilled assistance able to carry this out and evidence that their removal will not damage the seeds. Instead other less labour intensive and potentially less damaging dormancy-breaking treatments such as alternating temperatures, pre-chilling, and light should be considered. In the event, however, that some removal of the seed covering structures may be unavoidable then do not do this immediately; use the first period in the germination test to enable both the non-dormant seeds and those dormant seeds which have been sufficiently promoted by the test regime to germinate, and then consider surgical treatments to the remaining firm seeds only: this should save a considerable amount of work and also avoid some of the damage which can be associated with removal of the seed covering structures.

Imbibition injury to seeds resulting from too rapid uptake of water is defined in Chapter 4. It is a particular problem for the Leguminosae and is considered in the later part of this chapter together with scarification procedures. However similar damage, particularly where the seeds are very dry, is sometimes observed in other species. The problem can be aggravated by either partial or complete removal of the seed covering structures. Consequently, where the removal of seed covering structures is practised on all seeds sub-sampled from an accession for testing germination, conditioning or humidification treatments may be necessary before placing the seeds on the germination test substrate, as discussed in detail at the end of this chapter.

TREATMENTS TO OVERCOME HARDSEEDEDNESS

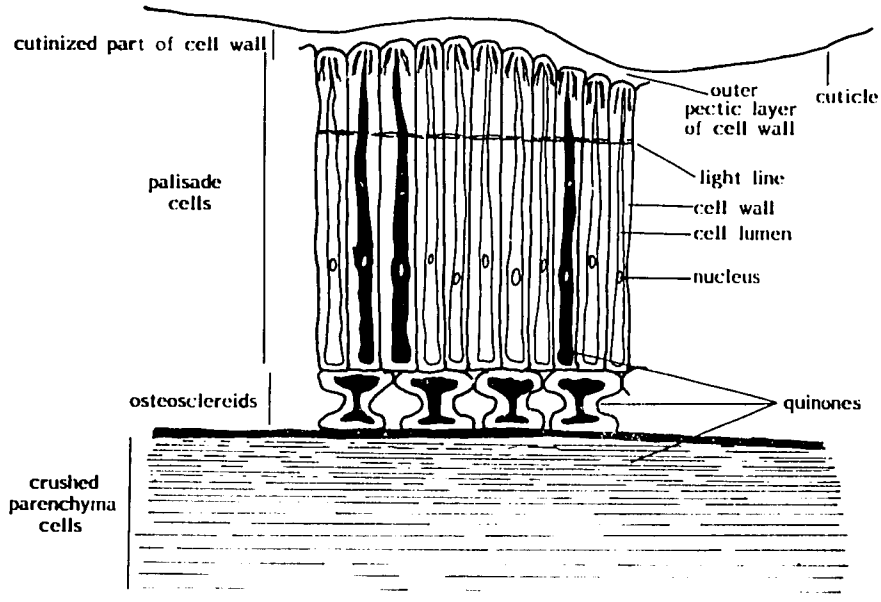
Hardseededness is particularly prevalent in the Leguminosae, but may also be observed in seeds of some species within the Malvaceae, Cannaceae, Geraniaceae, Chenopodiaceae, Convallariaceae, Convolvulaceae, Rosaceae, Solanaceae and other families. In considering the testa as a barrier to the absorption of water it was agreed at the beginning of this chapter that many seed physiologists would not consider hardseededness to be a form of dormancy, but it should be noted that the presence of hardseededness does not preclude the presence of embryo dormancy. That is, as with the influence of the seed covering structures where hardseededness is not a problem, a seed may show double dormancy.

It is worth emphasising that hardseededness is more likely to be a problem in gene banks than, for example, in commercial seed trade. First because the seeds will be considerably drier than is typical for commercial practice. Consequently absolute or irreversible hardseededness is likely to be more prevalent in gene banks than the more easily overcome reversible hardseededness which is experienced at slightly higher moisture contents. (See Chapter 4 for a discussion of reversible and irreversible hardseededness.) Secondly because the wild and weedy relatives of the cultivated crops included in gene bank collections tend to have thicker seed coats (which results in more severe hardseededness) - since with domestication there has been some (conscious or unconscious) selection for thinner seed coats.

Testa structure in papilionate legumes and hardseededness

The components of the testa which cause impermeability differ somewhat both between and within families. An example is provided here of the structure and role of the testa in papilionate legumes because they are the most important group of crop plants whose seeds exhibit hardseededness and because much is known of their structure.

Figure 7.1 Diagrammatic section through the testa of a papilionate legume to show the various constituents of the testa which contribute to the testa's impermeability to water and thus render a seed hard. (Based on a section through the testa of Pisum elatius by E. Werker: In The Germination of Seeds (A.M. Mayer and A. Poljakoff-Mayber). 211 pp. Pergamon Press, Oxford, 1982.)



The outer layers of the testae of papilionate legumes are composed of a thin waxy cuticle overlying a palisade layer of elongated macrosclerid cells (Figure 7.1). The tips (towards the outside of the testas) of these cells have thickened pectic caps and are embedded in a suberin matrix (Figure 7.1). There is not total agreement as to which part of the testa acts as the barrier to the entry of water, but the combination of the waxy cuticle and the suberin layer over the pectic caps of the macrosclerid cells is implicated, whilst the presence of dehydrated pectin and quinones towards the base of the macrosclerid cells may also contribute to impermeability.

The onset of impermeability appears to be roughly as follows. As the seeds dry moisture is at first lost through the whole surface of the seed. However, once a critical value - about 25% moisture content, say - is reached the testas begin to become somewhat less permeable: below this value the relative loss of moisture through the testas declines, whereas the relative loss through the hilum and chalaza increases. With further drying, to about 12 to 14% moisture content, the testas become impermeable and moisture is subsequently lost through the hilum and chalaza only. (These moisture contents are provided only as an approximate guide: in reality the values vary between individuals within a population, and particularly between accessions where testa characteristics - especially thickness - can vary considerably.) At this point the hardseededness of the testas tends to be reversible (see Chapter 4): if the impermeable seeds are exposed to a high humidity, the testas eventually absorb moisture over the whole area and the resultant expansion renders them permeable.

Further drying (which occurs only through the hilum) causes the testas to become absolutely hard where they are no longer able to absorb moisture from the atmosphere. This occurs at between about 5 and 10% moisture content. During this drying the hilum and chalaza act as one-way valves; allowing

moisture out, but not in. Consequently such seeds are unable to imbibe moisture via this route.

However, close to the hilum and chalaza is a region of the testa where the macrosclerid cells are both wider and considerably longer than in other regions of the testa. This area of the testa is described as the strophiole and the increased length of the macrosclerid cells in this region results in a small protuberance of the general outline of the testa. It is through this region of the testae of papilionate legumes that moisture is able to re-enter hard seeds. The longer, wider macrosclerid cells can be split apart by percussion (e.g. resulting from shaking the seeds): stresses applied to other regions of the testa cause the macrosclerid cells in the strophiolar region to split. The split region is described as the strophiolar cleft. If the relative humidity is high moisture enters the seeds via these splits and the seeds begin to imbibe. If, however, relative humidity is reduced then the splits shrink and the seeds become hard again.

Note, in passing, that there would be no point in applying percussion treatments on the complete accession before entry into the long-term store.

Other species with hard seeds do not have a strophiolar region. Instead hardseededness tends to be lost through abrasion of the testa, particularly - though not always - through the loss of the waxy cuticle.

Ecological significance of hardseededness

In dry regions the hardseededness character enables seeds to dry down and subsequently maintain a very low moisture content. Consequently survival periods of hard seeds in the soil can be considerable; reports of survival periods of 50 years or more in soil are not uncommon. High soil temperatures and alternating temperatures can result in the loss of the hardseededness character from small proportions of the seeds in the soil; in the event of rains germination can then occur for this small proportion of seeds. The hardseededness character also aids dispersal. Hard seeds ingested by animals and birds are not damaged by digestive juices and after dispersal and excretion some species are able to germinate since the waxy cuticle will have been removed from the seeds by the action of the acids and enzymes in the digestive juices.

However, this ecological strategy results in considerable problems for seed testing. In order to estimate the proportion of viable seeds in hardseeded accessions, ways of overcoming the character must be found and applied.

Hardseededness and germination progress curves

Hard seeds must be identified by gene bank staff so that the problem can be recognised in germination tests. In a germination test the hard seeds fail to swell. With large seeds this is sufficient to enable recognition, but with small seeds failure to enlarge is less obvious but hardseededness can be confirmed by dropping each seed on to a china plate; a hard seed will cause the plate to 'ping'. This enables the irreversible hard seeds to be identified. The reversible hard seeds, however, should have begun to germinate before germination tests are concluded. At the end of the germination test such seeds are normally very obvious. Whereas the non-hard seeds have produced seedlings large enough for evaluation (see Chapter 10), the reversible hard seeds have only just begun to germinate; also the texture of the testae tends to differ. When germination progress curves (see Chapter 4) are examined the delayed germination of the reversible hard seeds results in a skewed tail to the general pattern of germination.

Consequently where such skewed germination progress curves are observed it is necessary to consider hardseededness as one possible cause.

Methods of removing hardseededness

The most widely used methods of removing hardseededness rely on scarification (scratching) of testas. The scarification may be mechanical, by hand or machine, by dipping in acid, or by dipping in a solvent.

Mechanical scarification by machine usually involves momentarily forcing the testa against a grinding wheel spinning at roughly 500 to 1000 revolutions per minute. Mechanical scarification by hand can be achieved by rubbing the testa on emery paper or with a file, chipping away a small part of the testa by squeezing between forceps, cutting a part of the testa off with a sharp blade, making incisions in the testa with a sharp razor blade, or by puncturing the testa with a sharp needle. Where seeds are scarified by rubbing or filing testas by hand it is suggested that the emery paper or file be securely fixed and that each seed be rubbed across or along it once towards the operator. Chipping away a small part of the testa by squeezing between forceps is convenient for the (large-seeded) grain legumes, since each seed can be held easily.

Acid scarification is one of the more widely used treatments to remove hardseededness. Concentrated sulphuric acid is the most frequently applied acid, although other acids and lower concentrations, i.e. more dilute acids, are also used. Treatment times vary considerably, from a dip in acid (presumably a few seconds only) to several hours. In the majority of cases, however, treatment periods are between 1 and 20 minutes. Treatment in concentrated acid is obviously a severe treatment. If the concentration and/or treatment periods are too great then the seeds are damaged, that is they either germinate abnormally or fail to germinate at all. Consequently acid scarification, though convenient and easy, is not recommended here as a general treatment to remove hardseededness, since it is not possible to specify a single effective treatment combination which will not damage some seeds. However, for some genera in Volume II details of acid scarification treatments have been suggested at treatment combinations where the risk to seeds is low, but in these cases there still remains a risk that some seeds will be damaged. Moreover, in these cases the acid scarification treatment alone is often unlikely to be completely effective in removing hardseededness.

Several organic solvents have been used as scarification treatments. One example is ethyl alcohol which is thought to dissolve part of the waxy cuticle (see Figure 7.1). Treatment periods ranging from several minutes to several days have been used, but the efficacy of such treatments differs considerably between species and cannot be generally recommended: see the Leguminosae chapter in Volume II for examples of species where scarification in absolute alcohol may be beneficial.

The second most widely applied method of removing hardseededness is by impaction. Impaction (or percussion) treatments are applied by shaking the seeds (vigorously) within a container: a typical treatment would be to shake 3 times per second for 10 to 20 minutes. Standard conical flasks (with stoppers) and mechanical shakers can be used for these treatments. It should be clear from the previous section that such treatments are most effective for hard seeds which possess a strophiole. Others are less affected, but the efficacy of impaction treatments for seeds without strophioles can be increased greatly by including material such as sharp sand in the flask which will also scarify the testas. A special problem that gene banks may have in applying impaction (or combined impaction/scarification) treatments is that very dry seeds may be prone to mechanical damage. To limit this potential problem it is

suggested that the shaking should not be too severe, but the treatment period extended to 45 minutes or so.

The third most widely applied method of removing hardseededness is to subject the seeds to extremes of temperature (either in air or in water) and also, thereby, to rapid temperature changes. Temperatures of 50° or 60°C are often used, but are better avoided since such treatments can also be considered as ageing treatments. Very low temperatures are also used, generally by plunging dry seeds in liquid nitrogen (-196°C) or, less frequently, liquid air (-190°C). The effectiveness of such treatments appears to depend upon seed morphology. Again, those species whose seeds possess a strophiole are the most responsive. It appears that it is the change in temperature which is the important factor since several dips of 30 seconds or so in liquid nitrogen with 1 minute between them tend to remove hardseededness from greater proportions of seed populations than a single, longer (5 minutes or so) immersion in liquid nitrogen. However, a word of warning is necessary concerning liquid nitrogen immersion treatments to hard seeds. We have found such treatments to be very effective in enabling the seeds to imbibe, but they often also cause loss in viability. Consequently we are reluctant to advise the general use of liquid nitrogen immersion treatments.

Rapid temperature changes also occur when seeds are subjected to alternating-temperature regimes in germination tests. Consequently some irreversible hard seeds become permeable under such treatments. However the regimes best suited to increasing testa permeability are of such a wide amplitude, e.g. 10°/50°C, that they are unsuitable for germination testing. Nevertheless a long period of exposure to the narrower amplitude alternating temperature regimes which are suitable for germination tests can result in loss of hardseededness from some hard seeds.

One method of exposing seeds to an extreme of temperature which has sometimes been used to remove hardseededness is to soak the seeds in boiling water. This method is not recommended for use in gene banks, however, since there is a considerable risk of imbibition injury to the seeds which are not hard as well as some risk of loss in viability, depending upon the period of exposure, due to the high temperature.

AVOIDANCE OF IMBIBITION INJURY AFTER REMOVAL OF HARDSEEDEDNESS

The problem of imbibition injury was introduced in Chapter 4. It should be clear from that chapter that control of imbibition by the testa, although sometimes causing the problem of hardseededness, is also beneficial in that it limits the damage to dry seeds which can occur when the testa is not present to prevent excessively rapid water uptake. Whilst impaction treatments on papilionate legumes tend to mimic the mechanism of naturally softened hard seeds (where moisture is absorbed initially in a regulated manner through the strophiolar cleft) scarification and other treatments to remove hardseededness produce permeable areas in the hard seed coat almost at random. Consequently in these cases the testa is unable to regulate the water uptake over these areas and imbibition injury may occur. Consequently hard seeds which have been scarified will also require humidification (conditioning) treatments to increase seed moisture content to avoid contact of the very dry seeds with water in the liquid phase, just as seeds with cracked testas require these treatments, to avoid imbibition injury.

The aim of humidification treatments should be to increase seed moisture contents slowly to between 16 and 18% moisture content by absorption of water vapour instead of rapid imbibition of liquid water. Placing the scarified seeds on metal gauze in a sealed container above liquid water at a temperature of

20°C for 24 hours is generally adequate, but the seeds will need to be spread out to ensure that the absorption is uniform. The use of a pump to bubble the air through water and then past the seeds in a closed system is helpful, but not essential.

WHEN AND WHETHER TO TREAT SEEDS TO REMOVE HARDSEEDEDNESS

In commercial seed testing it is often the case that treatments to remove hardseededness are not applied. Instead at the end of the germination test the (irreversible) hard seeds are identified and their proportion reported. It is often assumed that the hard seeds are viable. Consequently the proportion of viable seeds may be reported as the sum of the proportions of germinated seeds and hard seeds.

There are thus three alternative ways of approaching, and solving, the problem of hardseededness. The first solution is to treat all seeds prior to the germination test. The second solution is to treat only those seeds remaining hard in the germination test after some time in test has elapsed. And the third solution is not to treat hard seeds at all, but only report their proportion.

This last solution can be discounted for the purposes of gene bank management. First it is essential that gene banks are able to promote the germination of all seeds within populations. Secondly it cannot be assumed that a seed is viable simply because it has been identified as hard.

It is suggested, however, that the decision whether to treat all seeds before the germination test or only those seeds identified as hard during the germination test will depend upon the expected proportions of hard seeds within each accession. Where the majority of seeds within an accession are known to be hard, then the treatment to remove hardseededness may as well be applied before starting the germination test (but remember to humidify the seeds). On the other hand if only a minority of seeds are hardseeded then it is a waste of effort to treat all seeds: it is more efficient to treat only those seeds identified as hard during the initial period in the germination test. Again, before returning them to the germination test remember to humidify these seeds. Where impaction treatments are applied to remove hardseededness, however, treatment of all seeds before testing requires little effort and therefore may be more efficient.

A SUGGESTED GENERAL PROCEDURE FOR REMOVING HARDSEEDEDNESS

The above sections provide many alternative approaches. Where knowledge of a species, or an accession, is limited the following general procedure is suggested for removing hardseededness.

1. Impact (shake) the seeds in a conical flask for between 20 and 45 minutes.
2. Humidify the seeds for 24 hours.
3. Carry out the germination test for 2 to 3 weeks.
4. Identify, count and remove the hard seeds.
5. Rub each hard seed on emery paper.
6. Humidify these seeds again for 24 hours.
7. Return to the germination test regime.
8. If necessary, repeat steps 3 to 7 if hard seeds still remain.

DISTRIBUTION OF HARD SEEDS FROM GENE BANKS

Where samples of seeds from accessions containing hard seeds are distributed it is important that the customer is made aware of the problem of hardseededness and provided with details of the treatments required both to remove hardseededness and to humidify the seeds. Otherwise the customer may not be able to produce seedlings from the distributed seeds.

In general it is not suggested that the seeds should be treated for hardseededness and humidified before distribution. This is because the humidified seeds will age much more rapidly than would be the case for the dry seeds and consequently some loss in viability may occur if sowing is delayed, particularly where ambient temperatures are high. However, where delivery delays are unlikely to occur and it is known that the seeds are to be sown out immediately upon arrival then prior treatment to remove hardseededness and humidification could be allowed.

CHAPTER 8. THE DETERMINATION OF THE EMPTY SEED FRACTION

It has already been noted in Chapter 1 that a seed-like structure may in fact lack a seed. In man species the seed covering structures may be firm and plump providing an impression - for example, to th farmer - that a seed is present whereas in reality it is not. This can be the case, for example, in man pasture species and many umbellifers. Seed-like structures, then, can be divided into four classes: thos containing viable non-dormant seeds which will germinate under a wide range of conditions; thos containing viable but dormant seeds which will only germinate if certain environmental stimuli have bee provided in the germination regime; those containing non-viable seeds which are incapable of germinatio in any environment; and those seed-like structures containing no seed. Where no dormancy-breakin treatment is applied in a germination test only those seed-like structures in the first category wil germinate. Where a dormancy-breaking treatment is applied in, or before, the germination test then seed like structures in the first and some, but not necessarily all, of those seed-like structures in the secon category may germinate. But in both cases the division of seed-like structures which have not germinate into the latter three categories cannot be achieved. Gene bank staff must be able to estimate thes proportions for each accession to ensure that the viability of maintained accessions is not inferior to th regeneration standard for the species. Dormancy, non-viability and empty seeds are frequently confounde by collectors and breeders: on a number of occasions we have received accessions with a high proportio of non-germinating seeds where low germination has been ascribed to dormancy, whereas subsequent test have shown the non-germinating fraction to be empty.

METHODS TO IDENTIFY EMPTY SEEDS

The determination of the empty seed fraction needs to be done fairly early in the banking of an accession since it determines the effectiveness of the cleaning operation(s): depending upon the result of the determination further cleaning may be required (Chapter 1). Whichever test procedure is used the seeds should be selected by a random sampling procedure (Chapter 13). This is because the result obtained from a test to determine the empty seed fraction is an estimate obtained from a seed sample of the accession. Since empty seeds tend to be lighter it is very easy for the empty seed fraction to vary within the seed bulk, there being a greater proportion of empty seeds at the top of the accession than at the bottom. It is thus vital to mix the accession before sampling - randomly - those seeds to be tested to determine the empty seed fraction. The accuracy of the estimate of the empty seed fraction will depend on sample size. To avoid wasting seeds it is suggested that the sample removed for the initial germination test also be used to estimate the empty seed fraction (preferably by using X-radiography): seeds identified as full can be located and subsequently tested for germination.

Irish method of determining the empty seed fraction

Where the species seeds are contained within chaffy florets, that is in many grass species, the presence of a seed can be rapidly indicated by a superficial inspection of the shape and size of the floret. The seed-like structures can be examined by feeling with the finger tip and with a spatula or by illumination from below (place the seeds on a sheet of ground glass with a light below; the full seeds are distinguished by the dark shape of the seed within the covering and flower structures). To gain expertise

of the Irish method it is suggested that gene bank staff should calibrate the method by dissecting the florets evaluated by the Irish method according to the International method (described below). Although the Irish method is comparatively rapid it lacks the precision of the International method and is not recommended.

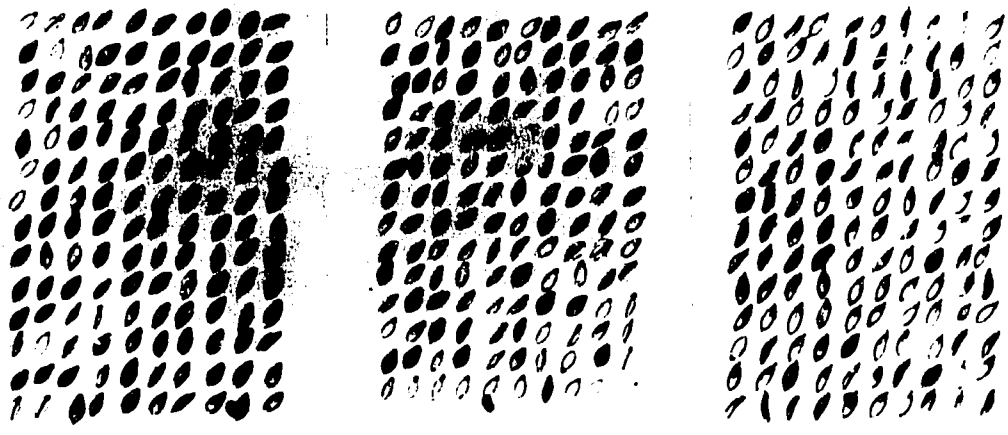
International method of determining the empty seed fraction

The International method requires the dissection of the seed-like structure to confirm the presence or absence of the seed (botanical definition) on a sample drawn from the accession. This method can be extremely time-consuming and is only likely to be useful in gene banks with a generous supply of trained assistants. Forceps and a scalpel are required for the dissection. In addition to this procedure being used to determine the empty seed fraction when an accession is banked, dissection may also be necessary before rapid viability tests are carried out on seed-like structures remaining ungerminated at the end of germination tests designed to estimate seed viability - see Chapter 11.

X-ray methods of determining the empty seed fraction

The use of X-radiography to determine the empty seed fraction within accessions maintained in gene banks is recommended. The initial high cost of the equipment is justifiable since the method is accurate and easy and quick to perform. An X-radiograph is an image of the seed-like structures obtained by those X-rays which pass through these structures: the darkest parts within the image correspond to those parts of the subject which are easily penetrated by X-rays; on a negative the lighter parts correspond to those which are the most difficult for the X-rays to penetrate. The background and seed-covering structures are easily penetrated and are thus dark; the seeds within the seed-like structures, if present, are more

Figure 8.1 X-ray photographs (i.e. positives, see text) of three seedlots of Brachiaria humidicola showing full (black) and empty (opaque) seeds. (From Goedert, C.O. (1984). Seed dormancy of tropical forage grasses and implications for the conservation of genetic resources. Ph.D. Thesis, University of Reading.)

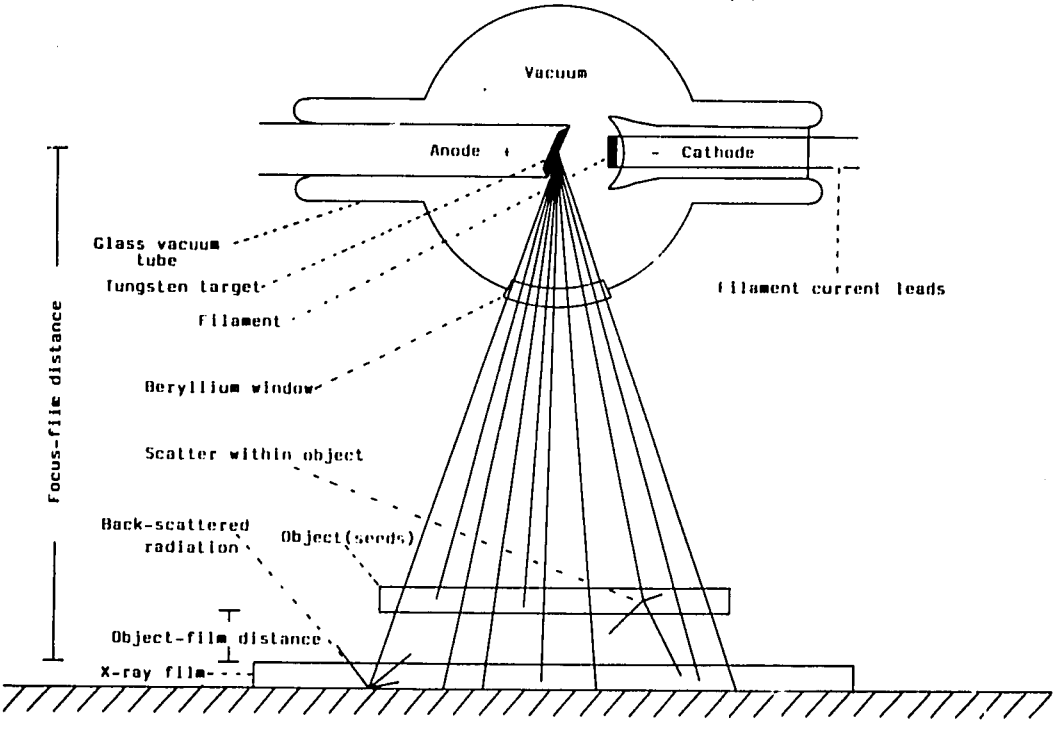


difficult for X-rays to penetrate and thus result in light images. Hence the presence of seeds within seed-like structures can be determined from an X-radiograph - provided the quality is adequate. The radiograph image can be obtained on X-ray film (negative) or on photographic paper (positive) if a permanent record is required (see Figure 8.1), or projected onto a fluorescent screen or transmitted to a video screen for immediate analysis. Whilst the Irish and International methods of determining the empty seed fraction are comparatively simple and can be self-taught, X-ray methods require some instruction before they can be used accurately and safely. It is recommended that, if possible, purchasers of X-ray equipment receive instructions from the supplier in the gene bank when the machine is installed using accessions from species maintained in the bank. Ensure that the supplier adjusts the operating parameters of the equipment to obtain sharp radiographs with sufficient contrast to indicate the presence of seeds within seed-like structures. To assist gene bank staff in the purchase of X-ray equipment and its initial operation a discussion of the factors affecting the quality of radiographs follows.

The characteristics of radiograph quality are: contrast - the degree of difference between adjacent parts of the image, in our case the seed and seed covering structures; density - the darkness of the radiograph; and definition - the sharpness of details of the image, that is the resolution of fine detail. To identify the presence of seeds within seed covering structures we are primarily concerned with contrast and definition. Before discussing the operating parameter adjustments which may be required to produce sharp contrasting images it is necessary to describe, in simple terms, the X-ray equipment. Figure 8.2 provides a simplified outline of the main features of X-ray equipment.

The X-ray tube, which generates the X-rays, consists of a filament (cathode) and a tungsten target (anode) in a vacuum contained within a glass envelope. If the cathode is heated (the first operating parameter) then the electrons in this filament are activated to high energy states. As a result of a high

Figure 8.2 Outline of main features of X-ray equipment.



potential difference in voltage between the cathode and the anode (the second operating parameter) the excited electrons travel from the cathode across the vacuum towards the anode at a high velocity. The part of the anode which these electrons strike is called the target and is composed of tungsten: the actual area of the tungsten target which the electrons hit is described as the focal spot. It is the collision of the electrons on the tungsten target of the anode at the focal spot which results in the production of X-rays. These X-rays are emitted through a metallic window (normally a beryllium window) from the X-ray tube into the X-ray cabinet as a cone of radiation. The apex of this cone of radiation is is at the focal spot of the tungsten target. Consequently the closer the object under investigation and the X-ray film to the tube the greater the energy received per unit area of film. The distance between tube and film is the third operating parameter and is called the focus-film distance; the fourth operating parameter is the distance between object and film. As in light photography the density of the image on the film is affected by the period of exposure to radiation (the fifth operating parameter). Finally, the speed of the X-ray film is the sixth operating parameter. The effects of adjustments to these six operating parameters are discussed below.

1. A current is applied to heat the cathode filament and is usually measured in milliAmps (mA). Increasing the heating current increases the number of electrons emitted and thus - see below - increases the number of X-rays generated. The number of X-rays generated affects the density but not the contrast of the image.

2. The potential difference between the cathode and anode is measured in kilovolts (kV). The greater the potential difference across the tube the greater the speed of electrons between the cathode and the anode and thus the greater the impact velocity of electrons on the tungsten target. This velocity affects the wavelength of the emitted X-rays. At higher voltages shorter-wavelength X-rays are emitted. The shorter-wavelength X-rays are more penetrating and are described as hard X-rays. Conversely at lower voltages longer-wavelength X-rays are emitted which have less energy, are thus less penetrating and are described as soft X-rays. The type of X-rays generated affects the contrast of the image: soft X-rays generated by lower voltages improve contrast.

3. Since the X-rays are emitted as a cone of radiation increasing the focus-film distance decreases the intensity and density of the radiation. In addition, of course, it also increases the total area of object radiographed. The definition of the image is improved by increasing the focus-film distance and also enables larger film to be used. To avoid reduction in density when increasing the focus-film distance the heating current and/or the exposure period should be increased.

4. For most purposes the seeds can be placed directly on the surface of the film and the distance between the object and the film is then very small. The closer the contact between the seed and the film the better the definition of the image: a blurred image may be caused by incomplete contact of the seeds with the film.

5. The exposure time of the seeds to the X-rays is measured in seconds. The greater the exposure time the greater the density of the image. Both the cathode filament heating current and the exposure time affect density. Consequently their product, milliAmp-seconds (mAs), must be kept constant to obtain the same image density if either operating parameter is altered.

6. The faster the film the shorter the exposure period and X-ray dosage required, but the poorer the contrast and definition of the image. Where seeds are to be tested for germination after X-radiography

it may be necessary to minimise X-ray dosage by using a fast film. If the seeds are to be discarded after X-radiography then a low speed film can be used for very high definition of the image, but a medium speed film is acceptable for most purposes.

Once the equipment is in day-to-day use the only changes in the operating parameters which will normally be required will be when different speed films or different seeds are used. As an example with medium speed film the average exposure conditions required for Pinus sylvestris seeds are 15 kV, 15 mAs with a focus-film distance of 30 cm. At the Wakehurst Place Gene Bank normal exposure conditions for most grass species are 11 kV, 3mA applied for 180s with a focus-film distance of 40 cm.

It is envisaged that gene banks will use X-radiography in two ways: either as a procedure to determine the empty seed fraction alone - for example, to determine whether further seed cleaning is required - in which case the X-ray dosage is unimportant since the radiographed sample will be discarded, or as an initial procedure of the germination test to identify and locate the full seeds to determine the proportion of these which germinate in a subsequent germination test. In the latter case the X-ray dosage should be minimised to avoid damaging full seeds. In the first case the status (full or empty) of the individual seed is unimportant; we are concerned only with providing an estimate of the proportion of empty seeds in the accession. In the second case, however, it is useful to identify each individual seed so that at the end of the germination test the full but non-germinated seeds can be subjected to further tests (see Chapter 11). It is suggested that a matrix of cells be prepared from card or plastic such that, for small-seeded species, 100 seeds (or whatever number is required) can be radiographed at once and each individual located. Ideally this frame should double as a counting board for the germination test (see Chapter 9) so that the radiograph image corresponds to a plan of each germination test. Remember to mark one corner of this frame and the germination test so that the radiograph and germination test tally. When constructing the frame check that the card or plastic can be penetrated by soft X-rays and that the image is not blurred or lacking in contrast. There are two potential problems: first the material used to construct the frame will absorb and reflect X-rays, the former reducing contrast and the latter causing blurring; secondly the object to film distance is increased which may cause loss of definition. If such frames are unavailable or are unsatisfactory then use double-sided adhesive tape to fix a line of seeds to the envelope containing the X-ray film - again after checking that the adhesive tape is penetrable by soft X-rays.

Although much of the above refers to the use of X-ray film, which must be developed, the radiographic image can also be projected onto a fluorescent screen. For small-seeded species some magnification of the image is necessary to avoid operator fatigue and to improve the accuracy of estimating the empty seed fraction. Developed X-ray films can be enlarged onto a screen or examined with a lens in a high-intensity illuminator. A wide variety of such equipment is available from medical radiography supply companies.

Thus far the practice of direct radiography has been described. For certain research purposes gene banks may wish to use contrast radiography in which either aqueous contrast agents or vapourous contrast agents are applied to the seeds before radiography. These techniques can be used to obtain greater contrast between the various tissues within full seeds, but are unnecessary for the detection of empty seeds in accessions and are consequently not discussed here. In addition to detecting the presence and location of empty seeds direct radiography can also locate the presence (and frequency) of insect infested seeds and mechanically damaged seeds, as well as immature seeds with undeveloped embryos.

When using X-ray machines for detecting the empty seed fraction follow all the safety precautions advised by the manufacturer and, if available, by government safety regulations. Ensure that the X-ray machine is regularly checked by the manufacturer's servicing agent and that each operator is adequately trained in the safe operation of the machine and wears a film badge (or similar) at all times to detect involuntary exposure to radiation.

It is recommended that advice be obtained from other operators of X-ray machines before purchasing one. Ideally this would be from a seed testing station or gene bank dealing with those species of concern. If no such direct independent advice is available then it is suggested that entomologists are a good source of relevant practical information since they too use soft X-rays in direct radiography. Indeed where the purchase of an X-ray machine cannot be contemplated, it is suggested that neighbouring entomologists be approached to see whether they have a machine that could be used on a part-time basis or at least to assist in the training of staff, e.g. by calibrating the Irish method against the X-ray method.

Uniform blower method to determine the empty seed fraction

The uniform blower method is used in seed testing stations for determining purity in several small seeded grass species. The seedblowers used are standardised and calibrated. The samples are blown for 3 minutes with the airflow set to a given calibration point. In this procedure the lighter seeds are blown off and lost, but the heavier seeds remain. Consequently this procedure is not useful in determining the empty seed fraction in gene banks. Air blowing is, however, a useful seed cleaning procedure (Chapter 1).

THE EMPTY SEED FRACTION

It is recommended that the empty seed fraction be reduced by cleaning when accessions are received so that 95% or more of the seed-like structures within each accession are full (i.e. true) seeds. The size of the empty seed fraction should be recorded and those receiving seeds distributed from the accession should be informed of the estimated empty seed fraction. Where insect damage is detected by X-radiography the proportion of infested seeds should also be communicated. It is also recommended that the viability or germination of accessions be reported as percentage of full seeds. To make these estimates as accurate as possible it is also recommended that empty seeds be identified, counted and removed from each germination test intended to estimate accession viability, rather than using the initial estimate of the empty seed fraction derived when the accession was banked to "correct" the germination test result. This is because the actual empty seed fraction in any one sample derived from the accession may not be equal to the initial estimate derived from a previous sample.

CHAPTER 9. THE CONDUCT OF SEED GERMINATION TESTS

This chapter provides advice on various practical aspects of the conduct of seed germination tests. Much of it is based on that provided by the International Seed Testing Association in annexes to the International Rules for Seed Testing but it is supplemented with much more detailed information relevant to gene banks.

GERMINATION TEST SUBSTRATES AND PROCEDURES

Paper quality

Paper is the most widely used substrate for laboratory tests of seed germination. The essential requirements of paper for this purpose is that it must not contain any substance toxic to developing seedlings, is able to hold, and supply to the seeds, sufficient moisture to enable the seeds to imbibe and germinate, and is strong enough neither to break up when handled nor be penetrated by the roots of developing seedlings. The ability of a paper substrate to meet these requirements can (and should) be tested within the gene bank. As a rule all new batches of paper substrate should be tested at receipt, since although the specification provided by manufacturers may be the same as previous batches, the batches may in fact differ in their suitability as a medium for seed germination tests.

The fibre content of the paper should be 100% bleached wood, cotton, or other purified vegetable cellulose and the paper structure should be open and porous in order to hold sufficient moisture for the germination test. One potential problem is that the paper may have been treated with various chemicals during its manufacture, for example to bleach the paper. Such substances may be toxic to developing seedlings and consequently it is necessary to test the paper as follows to ensure that it is free from toxic substances. Cut the paper to size (if necessary) and place in petri dishes or polyethylene boxes with two thicknesses of blotters or four thicknesses of towels or filter papers per dish or box. Moisten the papers with sufficient water to saturate them, but do not provide excess water which would need to be poured off since this might remove some toxic substances. Then test seeds of one of the following species for germination on the moistened paper under test: timothy (Phleum pratense), redtop (Agrostis gigantea), chewings fescue (Festuca rubra var commutata), Bermuda grass (Cynodon dactylon), weeping lovegrass (Eragrostis curvula), celery or celeriac (Apium graveolens), garden cress (Lepidium sativum), chicory (Cichorium intybus), or dandelion (Taraxacum officinale). These species are suggested because their root development is particularly sensitive to the presence of toxic substances. Root development should be evaluated between about 3 and 10 days in test because symptoms of slight root inhibition are more pronounced initially; later secondary root growth may mask these symptoms. The classic symptoms of toxic substances are shortened and discoloured root tips. Additionally the root hairs may be bunched and the roots raised above the paper. To ensure that these symptoms can be detected it is necessary to carry out a control test at the same time with paper that is considered reliable. During this test one can also check that the texture of the paper enables the roots to grow on the surface and not into the papers. Another potential problem which can be checked during this test is the cleanliness of the paper. If fungi or bacteria develop from the paper (not from the seeds themselves) when moistened and incubated then the paper should be rejected for use. (In certain tropical climates it has been suggested that paper may

require sterilization before use, presumably because of contamination during storage.) When moistened the layers of paper must be at least 2 mm thick and strong enough not to fall apart when handled: when moist hold from one corner in the air to test this. To test the ability of the paper to absorb moisture cut the dry paper into strips about 10 mm wide and hold vertically with about 20 mm of the paper immersed in water. After 2 minutes measure the distance up the paper that moisture has risen - this is described as capillary rise. A minimum standard is a 30 mm rise within 2 minutes.

Although most paper used for germination tests is white, some thought should be given to colour. One problem is that with very small seeds radicle growth can be difficult to detect on white paper since there is little contrast between the seedlings and background. This can make observations tiring and consequently increase errors. On coloured paper however, developing seedlings are more easily discerned. Red, blue and grey papers are available and highlight developing seedlings well. Whichever colour paper is used, however, two tests are necessary. The first is that for the presence of toxic substances and other paper qualities described above - since the dye may be toxic. The second concerns the effect of the light environment on the germination of dormant seeds (see Chapter 6). That is it will be necessary to check (with a light-sensitive seed accession) that the use of the coloured paper does not produce a less stimulatory, or an inhibitory, light environment for germination.

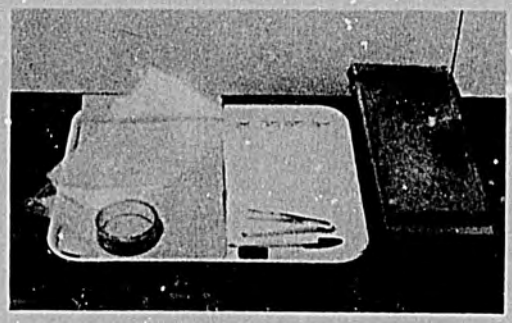
The pH of all paper substrates should be within the range 6.0 to 7.5. Four types of paper are normally used: paper towels or blotters in rolled towel tests; filter papers either in petri dishes or on Copenhagen tanks; or pleated papers in pleated paper tests. These germination test procedures using different types of paper are described below.

Rolled towel test

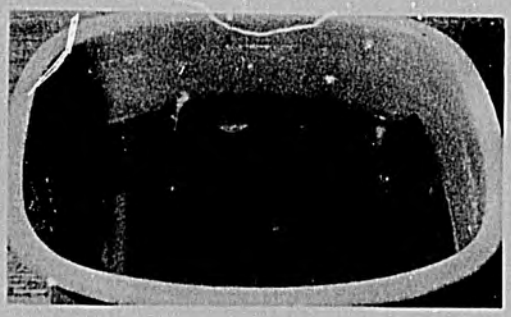
In a rolled paper towel test the seeds are germinated between paper. Medium- and large-seeded species such as the cereals, maize and the grain legumes are normally tested for germination by this procedure. The towels used are slightly larger than an A4 sheet of paper, roughly 300-360 x 230-250 mm. Whilst the blotters may be of a similar quality to filter papers, paper towels are those used in wash-rooms, etc. and have different characteristics. The towels can be laid flat on top of each other and then soaked in water. About 40 or 50 towels at a time can be removed from the water, drained for a moment, and then gently wrung between the hands until no more water is squeezed out. They can then be laid out flat close to where the germination tests are to be prepared. The towels should be strong enough to maintain their structure during the wringing operation. The towel moisture content should have been reduced sufficiently so that, when a towel is gently pressed with a finger, a film of water is not formed around the finger.

In the rolled towel test 3 towels are laid on top of one another. The seeds - normally 25 - are then placed on top of the uppermost sheet in a fairly regular pattern so that they are approximately equidistant - see the information later in this chapter on counting boards for spacing - with the exception that seeds are not placed within about 30 mm of 3 edges of the paper, and not within about 50 mm of one of the longer edges of the towel. A fourth towel is then placed on top of the seeds and the basal 20-30 mm of the longer edge of all 4 towels turned up to form a lip to prevent the seeds falling out. The towels can then be gently and loosely rolled to form a tube about 50-60 mm in diameter. The 4 towels should be strong enough when rolled to stand upright without collapsing. It is vital that the towels are rolled loosely: tight rolling will prevent air circulating between them and results in either no germination or abnormal germination. The friction between seeds and towels is sufficient to prevent the

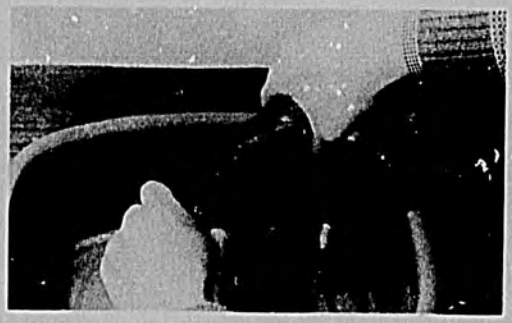
Figure 9.1 The preparation of a seed germination test between moist rolled paper towels.



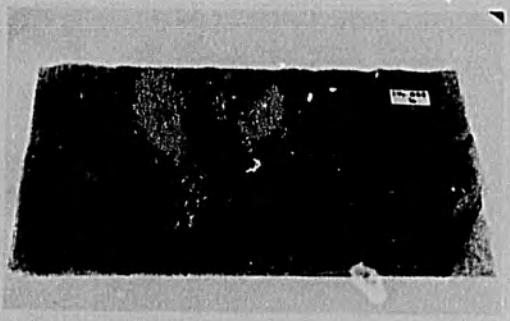
(i) Requirements (clockwise) from top left): paper towels, polyethylene bags, labels to identify each replicate, counting board (optional), form to record test details and results, forceps, spatula, pen, marker pen, adhesive tape.



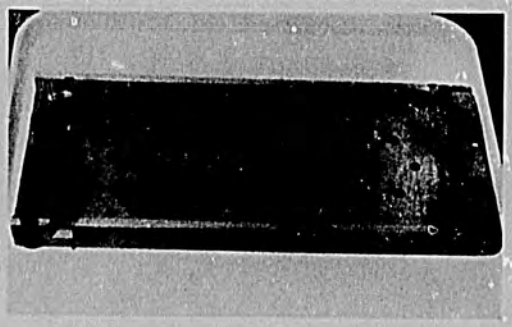
(ii) The flat towels (4 per replicate, prepared in bulk) are soaked in water (a few minutes is sufficient).



(iii) The towels are then wrung almost dry by hand.

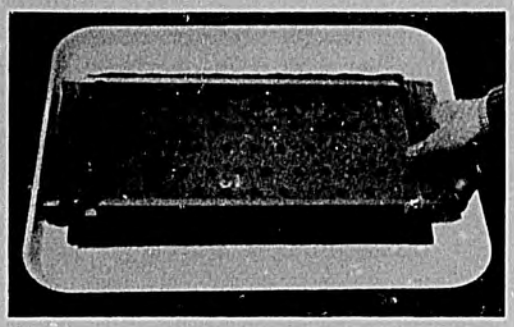


(iv) For each replicate lay 3 towels down as the base and place a label to identify the test on top.



(v) Count the seeds using a counting board (as shown) or using a spatula.

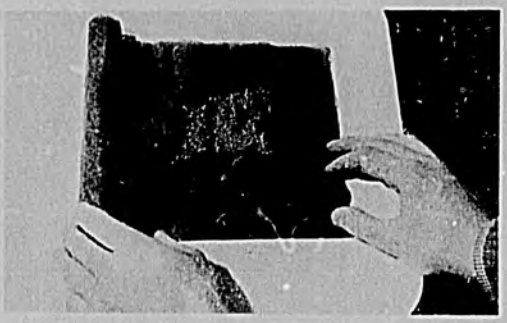
Figure 9.1 (continued)



(vi) Place the seeds on top of the towels using the counting board (as shown) or using forceps.

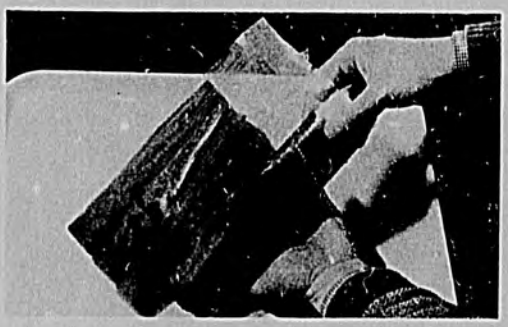
(vii) Whichever method is used the seeds should be arranged in such a way that the distance between seeds is maximised. Leave a 40-50 mm gap between the edge of the paper and the seeds.

(viii) Place a moist towel on top of the seeds and turn up the bottom 25 mm of all four paper towels.



(ix) Gently roll the towel. It is helpful if the label identifying the test replicate is to the outside of the roll.

(x) There should be plenty of space between the layers to enable air to move freely within the rolled towels.



(xi) Where a high humidity is not maintained during the test (i.e. where only temperature is controlled) the rolled towels should be placed in polyethylene bags, labelled, the top folded loosely (but not sealed) and taped, and then placed upright in the incubator.

seeds falling to the bottom of the rolled towels when these are stood upright. Keep practising until the tightness of the roll is just sufficient to prevent this happening. The commonest error is to roll the towels too tightly. It is a good idea when learning to use this technique to deliberately roll a germination test too tightly in order to observe the effect that this has on seed germination. Sometimes metal, glass or plastic frames are placed between the sheet on which the seeds are placed and the top sheet to ensure ventilation, but this is not necessary in normal practice. Once the towels have been rolled, the tests can be placed in a germinator. Where the germinator maintains a high relative humidity the rolled towels can be held with a rubber band (not too tight) and stood upright in cages (constructed from metal mesh) within the germinator. If the germination test is to be conducted in incubators where relative humidity is not controlled then the rolled towels can be placed in polyethylene bags and the top loosely folded over: spray water over the towels every 2 or 3 days in this case. To identify each germination test requires an internal label between the paper towels, and an external label, either held within the rubber band or written on the polyethylene bag.

Blotters and towels can also be used flat in polyethylene boxes. Generally in the case of blotters a single bottom layer with one layer on top is sufficient, but otherwise use 3 towels on the bottom and one on top as with the rolled towel test. The box can be labelled with the germination test identification.

One problem with both rolled towel tests and flat tests in polyethylene boxes is that they are quite bulky and great care must be taken to ensure that the airflow within the germination cabinet is not impaired, otherwise the temperature within different parts of the cabinet may vary substantially. Figure 9.1 shows the stages involved in the preparation of germination tests using blotters or paper towels.

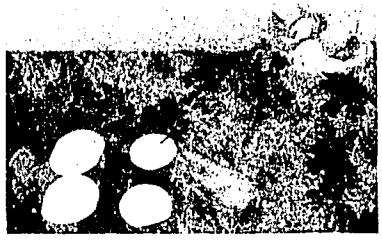
Filter papers

Filter papers are either used for germination tests in petri dishes or on Copenhagen tanks. Normally 9 cm diameter round filter papers are used although of course larger and smaller round or square filter papers and petri dishes can be obtained. Because of the smaller size of working area the small-seeded species are usually tested for germination using these procedures, e.g. the small-seeded vegetables. Filter papers can be moistened in one of two ways. They can be soaked in water for 10 minutes or so, and then hung to drip dry, or they can be placed in the petri dish and the required volume of water added using an automatic syringe. The former can be more time-consuming and requires a clean atmosphere in which to remove the excess moisture by gravity, but in the latter case the problem arises that the filter papers are never exactly equal in thickness and thus differ in their moisture holding capacity. As with paper towels and blotters, the filter papers should not be so wet that when pressed a film of water forms around the finger.

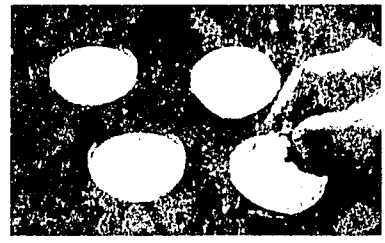
Use the smaller of the two halves of the petri dish as the base, place the filter paper in and firm down in the dish when moist using an upside-down 9 cm funnel. Normally 2 filter papers are required for each dish. If the larger half of the petri dish were to be used as the base then two problems would arise: first the filter paper would dry out more quickly since there would be a gap between the smaller lid and the base, second the moisture in the paper would form a seal with the lid preventing fresh air reaching the seeds. In fact some petri dish designs now include a ventilation gap in the (larger half) lid. After distributing the seeds on top of the filter paper the germination test identification can be written on the petri dish lid. Where humidity is not controlled the petri dishes should be loosely wrapped in a polyethylene bag to prevent the filter papers drying out before the first check. Figure 9.2 shows the stages involved in the preparation of germination tests using filter papers in petri dishes.

Figure 9.2 The preparation of a seed germination test on top of filter papers.

(i) Requirements (clockwise from top left): petri-dishes, (automatic) pipette, filter papers, form to record test details and results (a pen is also needed), wax crayon, forceps, spatula, polyethylene bags, funnel (optional), vacuum counter head (optional).

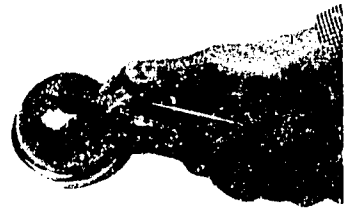


(ii) The filter papers (2 per dish in this case) are placed in the petri-dishes and moistened with the required amount of water.

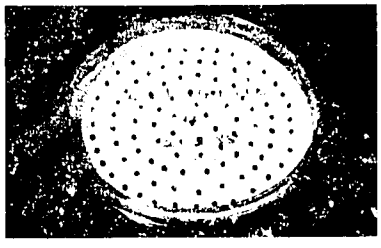


(iii) The filter papers can be made to lay flat in the dish by pushing them down with a funnel.

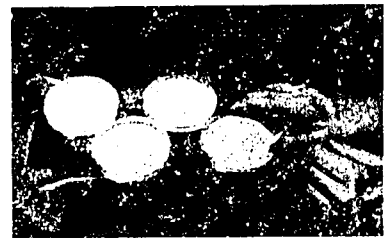
(iv) The seeds can be counted using a vacuum counter (as shown) or a spatula.



(v) The seeds are then arranged on top of the dish using the vacuum counter (as shown) or using forceps, seed by seed.



(vi) Whichever method is used the seeds should be spaced in such a way that the distance between seeds is maximised. The lid of the dish should be identified with details of the test.



(vii) Where a high humidity is not maintained during the test (i.e. where only temperature is controlled) the petri-dishes should be placed in polyethylene bags which should be loosely folded at the open end, but not sealed.

On Copenhagen tanks the filter papers can be moistened by soaking and then draining by gravity. Ensure that the germination plate is clean before placing the filter paper on top, with the wick from the water bath below pressed between the filter paper and germination plate. After the seeds have been uniformly distributed on the filter paper - see the information later in this chapter on vacuum counters for spacing - cover with a clean well-fitting bell-jar and ensure that the ventilation hole is not blocked. Remember to identify the germination test.

Pleated papers

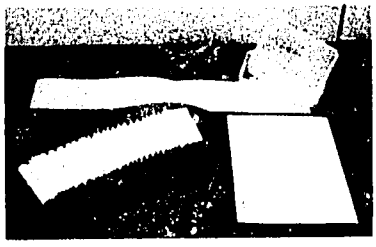
The one remaining type of paper substrate which might be used is pleated paper. This is simply a long piece of paper which has been repeatedly folded in a concertina fashion to provide many folds, or pleats, into which seeds can be placed. These papers are particularly useful in cases where the position of an individual seed needs to be identified from one intermediate count of germination to another and where it may be necessary to keep seeds isolated. In commercial seed testing they are normally used for sugar beet and for pelleted seeds. A typical pleated paper is 2000 mm long and 110 mm wide but already folded to provide 50 pleats - each 18 mm high within a reduced length of 165 mm into which seeds can be placed - see Figure 9.3. Each pleated paper is used in conjunction with a wrapping strip 580 x 110 mm and a polyethylene box 180 mm long x 120 mm wide x 50 mm high with lid.

The wrapping strip should be placed lengthwise in the box, pushed to the bottom with about 160 mm protruding on either side. Spray the wrapping strip with water so that it is moist, but when pressed a film of water does not form around the finger. Next soak the pleated paper in water, pulling apart slightly and gently to ensure moisture reaches inside every pleat. Remove from the water and squeeze the pleated paper between the thumb and fingers of both hands until all free water has been removed. Then place in the polythene box on top of the moist wrapping strip. There is a correct way up: ensure that both ends of the pleated paper face upwards, otherwise there will only be 49 pleats in which to place seeds not 50! Place two seeds (normally) in each pleat, fold the wrapping strips over and replace the lid. Information on individual seeds can be recorded at the top of the pleat above the seed in waterproof ink if this is required. The germination test identification can be written on the lid of the polyethylene box.

Agar

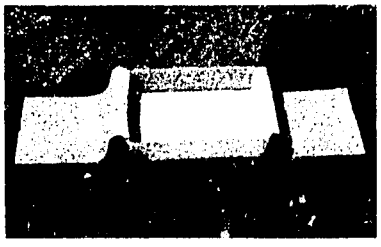
Agar is an alternative germination test substrate to paper which can be used for tests in petri dishes or in small pots with close-fitting lids. Agar is a polysaccharide complex which though insoluble in cold water dissolves slowly in hot water to a viscid solution. New Zealand agar or Japan agar is suitable for germination tests. Make a 1% solution by dissolving in water heated on a hot plate. When dissolved allow to cool partly and then pour into petri dishes or any other suitable germination test container. Dispensers are available which automate the preparation of agar dishes. A 1% agar solution forms a stiff jelly on cooling, on to which the seeds can then be placed for the germination test. The advantage of agar over paper as a germination test substrate is that the control of moisture availability is better. For example, in a germination test at 20°C the agar should stay moist for about a month, whilst in a germination test at 30°C the agar should stay moist for 2 weeks or so. Also, as noted in Chapter 4, the initial seed imbibition environment may be more favourable than paper substrates and potential problems from imbibition injury can be reduced. Remember to mark the container with the germination test identification.

Figure 9.3 The preparation of a seed germination test between pleated paper.

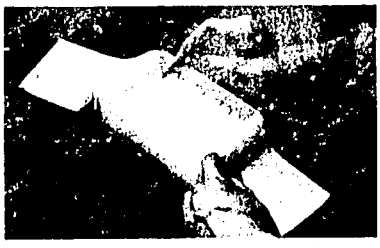


(i) Requirements (clockwise from top left): wrapping strip, polyethylene box with lid, form for recording test details and results, pen, wax crayon, forceps, spatula, pleated paper.

(ii) The pleated papers are moistened by soaking in water and then squeezing almost dry by a concertina-like action by the thumbs and forefingers.

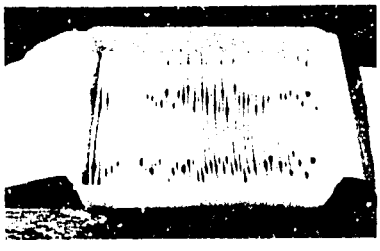


(iii) The wrapping strip is placed across the base of the box and moistened with a spray of water.

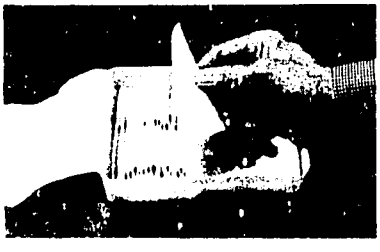


(iv) The pleated paper is placed on top of the wrapping strip and the upright, free-edges positioned to adhere to the wrapping-strip "wall".

(v) The seeds (sugar beet clusters in this case) are then positioned, two to each pleat. A slight zig-zag between each pleat can be helpful with large seeds/ clusters.



(vi) When full the pleated paper will contain 100 seeds. The pleats should not stick together at the top otherwise the pleat may become sealed.



(vii) Each end of the wrapping strip is then laid along the top of the pleated paper in turn.

(viii) Finally the lid, labelled with test details, is placed on the box and the box placed in the correct temperature regime.

If a dormancy-breaking agent is to be co-applied in a germination test on agar then the agent should be added to the 1% agar solution as it cools but before it becomes a stiff jelly.

Sand

Sand is a bulky and messy substrate for laboratory germination tests and it is suggested that it only be used for routine germination tests where there are difficulties in obtaining reliable supplies of suitable paper. The washed sand must consist of uniform particles and have a pH of between 6.0 and 7.5. Using sieves grade the sand and only use those particles which will pass through a sieve with 0.8 mm diameter holes but which are retained by a sieve with 0.05 mm diameter holes. Before use the sand must be sterilized. First pick out from the sand all obvious traces of organic matter. (For example, if the sand is re-used remove all seeds and seedlings from the previous test.) Heat the sand in steel pans, with no more than a 150 mm depth of sand, for 2 hours at 150°C. During this heating the sand must be turned regularly in order to dry thoroughly and prevent overheating. Finally, remove any plant remains (now charred and thus more visible) by sieving. As with paper substrates the sterilized sand may contain substances toxic to developing seedlings and it is necessary to test one of the sensitive species already listed in this chapter to check that the batch of sand is a suitable germination test substrate.

The amount of water added to the sand for the germination test is a critical factor in the reliable use of this substrate. The exact amount of water which should be added to the sand depends upon the size of the seeds under test and the characteristics of the sand. The first thing to do is to calculate the water-holding capacity of the batch of sand: this is the weight of water the sand will hold without run off, expressed as a proportion of the dry weight of the sand. This weight can be determined by adding water to the sand until run off occurs, then allow the sand to drain until no more run off is observed, weigh, dry in the oven at 150°C until constant weight is reached and weigh again. The water-holding capacity is then the loss in weight expressed as a proportion of the final weight. For cereals, except maize, and vegetables the appropriate sand moisture content for germination tests is 50% of water-holding capacity; for large-seeded legumes and maize the appropriate figure is 60%, but the sand should only be made up to 50% before the seeds are placed in the sand, with the final 10% being sprinkled on subsequently. Calculate the volume of water required to provide these moisture contents and always measure accurately.

Aluminium dishes of 150 mm diameter at the top, 125 mm diameter base and 40 mm deep can be used for the germination tests. The seeds should be sown on top of the sand and then covered with 10-20 mm of loose sand, although in a few species (smaller-seeded) the seeds are only pressed into the surface of the sand and not covered. The dishes should be covered with a flat plate, and placed in the germinator. Use a planting marker to label the germination test.

Although the sand can generally be re-used (after cleaning and sterilizing) vegetable seeds should always be tested in new sand, and sand should be discarded after being used to test heavily dressed seeds.

Compost

The use of compost for laboratory germination tests should only be considered when abnormal germination is observed from seed accessions which have been chemically treated - for example, with fungicides or insecticides. (See the final section of this chapter for a warning of health hazards from handling treated seeds.)

A proprietary seedling compost should be obtained consisting of a graded peat, adjusted for pH with sand and nutrients added. The moisture content of the compost will be extremely variable. For germination tests add sufficient water until a handful of the mixed compost just produces drops of water when gently squeezed and check that the ball of compost still readily breaks up when pressed with the thumb. Use standard seed trays for the germination test and sow the seeds sufficiently below the compost surface. Use a planting marker to identify the germination test. Do not add additional water to the compost after sowing.

SEED COUNTERS

When arranging the seeds on, or in, whichever germination substrate is chosen for the germination test it is important to space the seeds out so that they are as far apart as possible from one another within the limitations of the germination test container. The reasons for this are three-fold: first to reduce the chance of contamination of viable seeds from microorganism growth from adjacent decaying seeds; second to avoid leachate from one seed inhibiting the germination of another; and finally to enable the developing seedlings to be evaluated individually without the complication of disentangling - and possibly damaging - adjacent seedlings. In addition to the labour involved in distributing seeds equidistant on the germination medium there is also the task of counting the seeds for each replicate of the germination test. Seed counters can reduce the labour involved in both these stages of preparing germination tests. Three types of seed counter are discussed below: electronic seed counters, vacuum counters, and counting boards.

Automatic counters

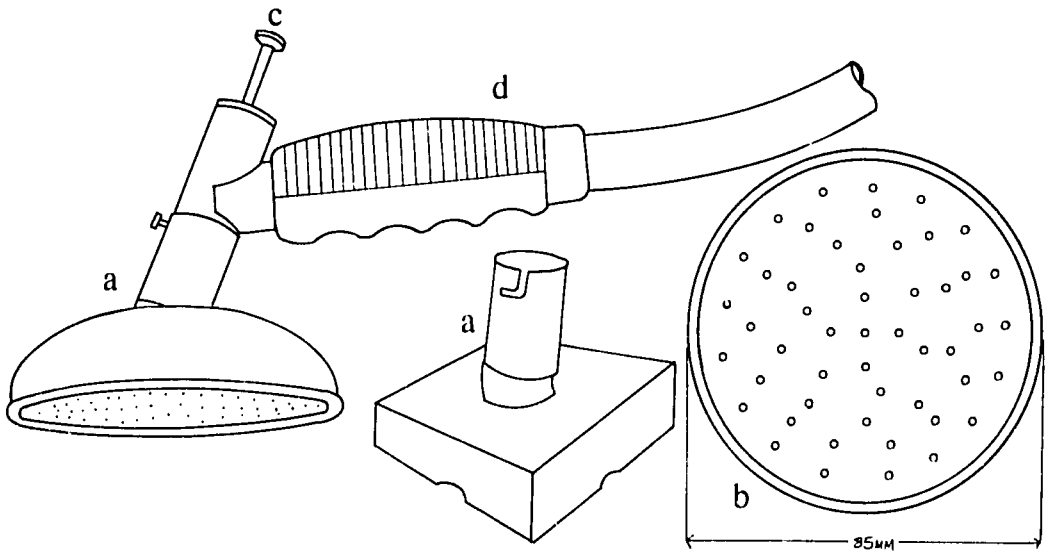
Various types of electronic seed counters are available. In essence the heart of the design is an electronic 'eye' which detects individual seeds dropping past. Mechanical means - normally by vibration - are employed to arrange the seeds in a single-file flow past the electronic eye. It is suggested that this type of seed counter is of little use in the preparation of germination tests. First because the equipment only counts the seeds and does not place them on the germination test medium. Second the mechanical means employed to arrange the seeds in a single-file results in the selection of seeds by weight - that is a biased sample is obtained. Finally the electronic 'eye' will tend to count dust or debris as a seed, and occasionally miss a seed because two pass by at once. In our experience the accuracy of such machines is about ±5% and is inadequate for the preparation of seed germination tests.

Vacuum counters

Vacuum counters are widely-used in seed laboratories to count and place seeds on top of filter papers either for tests in petri dishes or on Copenhagen tanks. Figure 9.4 illustrates the general features of a vacuum counter head. The most common arrangement is of 100 holes within a round 9 cm face, but fewer holes and different shape (square) and size faces are also used. The distance between holes should be roughly 3 to 5 times the seed diameter.

The use of vacuum counters will require a vacuum system including pipes to the benches where germination tests are prepared. It is preferable for the vacuum pump to be some distance from the operator and insulated to avoid operator fatigue from the high level of noise. To be effective the pump must be quite powerful: the system needs to be able to support between 500 and 700 mm of mercury at the laboratory benches where the counting heads are to be used and displace 0.6-0.75 m^3 of air per

Figure 9.4 General features of a vacuum counter: detachable counting heads (a); plan view of a counting head (b); plunger (c) and handle (d). (Figure 9.2 provides illustrations of a vacuum counter in use.)



minute. The pipe run from the pump to the bench should be as short as possible (subject to the consideration of working noise levels above), with no constriction in the hose. The line and connections should be less than 10 mm wide, and there should be as few right angle joints as possible to reduce loss of vacuum down the line. Where more than one vacuum counting head is to be used at a time a large vacuum tank will be required to prevent the use of one head reducing the vacuum at another head: that is the system must be capable of supporting both heads at once.

Vacuum counters are most useful for species which have regularly shaped, smooth seeds: Brassica species are particularly easy to handle with vacuum counters; other crops which can be counted with this equipment include the cereals, clovers and some small-seeded vegetable species. Great care is required when using vacuum counters to avoid biased selection of seeds. The following details of equipment and operation are provided with a view to avoiding this.

The heads should be marginally smaller than the germination test substrate the seeds are to be placed on, and the face requires an edge to prevent seeds rolling off. The seeds should be poured evenly on to the counting head (face up) with the vacuum off. The vacuum should not be applied whilst the seeds are being poured over the face because this - and the alternative of plunging the head face down into the seed bulk with the vacuum on - will result in the selection of lighter seeds from the accession. The vacuum is then applied - normally by releasing the valve at the rear of the counting face, see Figure 9.4 - surplus seeds (those not held to a hole) are removed by gently tipping them back into the seed bulk and the face checked to ensure that each and every hole is filled with a single seed. The head is then turned over on to the germination substrate (prepared previously) and the vacuum released. The seeds should fall into place on the germination test substrate as a mirror image pattern of the arrange-

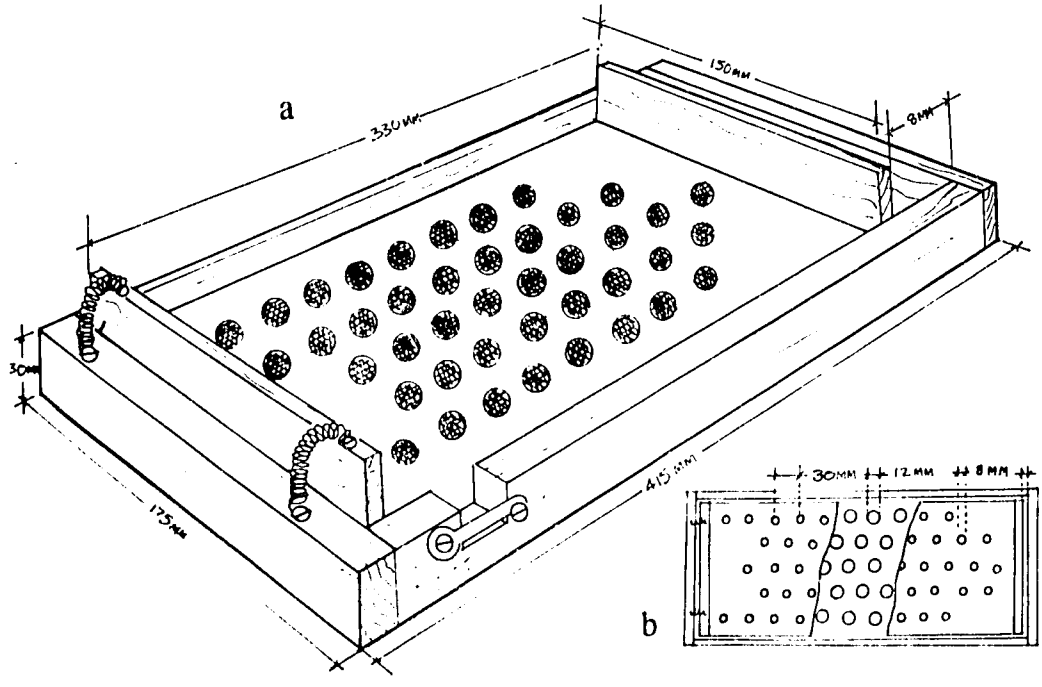
ment of holes on the counting head. Check that no seed is left attached to the counting head; seed covering structures can get stuck in the holes and may not fall off when the vacuum is released. It is important to check the plate and valve for wear regularly because small vacuum losses around the valve and at connections can make the counters unreliable and difficult to use. The final hose to the head should be very flexible so that the use of the counting head is as easy as possible. It may prove difficult to obtain counting heads. Although many potential suppliers are available they tend to supply from stock and are reluctant to manufacture more unless a large order is received. It is suggested that a seed testing station be contacted for assistance in obtaining counting heads if supply difficulties are encountered.

Counting boards

Counting boards are more useful for larger-seeded species such as maize and the grain legumes. As with vacuum counters difficulties in obtaining them may arise, but they can be easily constructed and so this represents less of a problem. Figure 9.5 shows the essential features of counting boards together with an example of suitable dimensions: this information is sufficient to enable a board to be constructed easily and cheaply. Counting boards are marginally smaller than the area of germination test substrate with which they are intended to be used and are generally used in conjunction with rolled towel tests. They are also convenient for tests in sand or compost.

There are two identical layers within the counting board. The two layers have 50, 100 or however many holes are required per replicate, arranged in a regular equidistant pattern. The distance between holes should be at least 3 to 5 times the seed diameter; remember that seeds can roughly double in size after imbibition (in the case of legumes for example) and this must be taken into account when designing

Figure 9.5 General features of a counting board with example dimensions (a) and a plan view of a board showing arrangement and dimensions of holes through which the seeds drop (b). (Figure 9.1 provides illustrations of a counting board in use.)



the board. Each hole should be marginally larger than the largest seed to be tested. The counting board is constructed so that in use the two layers are initially staggered so that each hole on the top board forms a ledge. The seeds are scattered over the board, preferably in a manner similar to that explained in Chapter 13 for the random cups sampling method. Seeds which are not caught in a ledge are removed by gently tipping the board slightly and opening the trap on the side of the board (Figure 9.5), each ledge checked to ensure that one seed, and one seed only, is present. The board is then placed above the germination test substrate. The top layer is now pulled to one side (normally against the action of a spring, Figure 9.5); the holes in the two layers line up and the seeds drop through on to the germination test substrate below.

Seed shape

Both vacuum counters and counting boards work best when used for accessions with spherical smooth seeds of uniform size. The more angular the seeds and the more variable the seed size within an accession the less satisfactory are vacuum counters and counting boards. Thus gene banks are likely to find this equipment presents more problems in operation than is the case in seed testing stations, for example. Nevertheless the use of this equipment is recommended for non-angular smooth seeded species. It may also prove advisable to use equipment similar to the top layer of a counting board for seed germination tests of chaffy species after X-radiography to detect the empty seed fraction - see Chapter 8.

THE PROVISION OF WATER IN SEED GERMINATION TESTS

The water used to moisten the germination test media must be clean: it should be free from organic and inorganic impurities and have a pH of between 6.0 and 7.5. Tap water can be satisfactory in some locations, but problems may arise from variations in its quality over time. Consequently the cleanliness and pH of tap water should be checked frequently. Distilled water is normally preferable to tap water, but it may require aeration to increase dissolved oxygen levels. Moreover problems of low pH can arise due to high concentrations of dissolved carbon dioxide forming carbonic acid. De-ionised water may be preferable to either tap or distilled water, but the pH must be checked regularly.

Whatever the source of water for germination tests it is necessary to verify its suitability for seed germination tests by testing a sensitive species in the manner previously described to test for paper toxicity. One final point relating to the provision of water to seeds in germination tests concerns the avoidance of imbibition injury - see Chapter 4. In a number of species it will be necessary to humidify the very dry seeds before setting them to germinate on the moist substrate. Details of humidification procedures are provided in Chapter 7.

FUNGAL GROWTH DURING SEED GERMINATION TESTS

In some germination tests problems may arise due to fungal growth from diseased or decayed seeds covering and overwhelming other seeds which may then be prevented from germinating normally. There is no easy way of avoiding this problem completely but it can be minimised by the following laboratory practices.

(1) Proper spacing of seeds (see earlier sections): if severe problems are experienced with a particular accession then increase the distance between seeds and use a greater number of replicates. If problems remain then use pleated papers for the germination test.

(2) Optimum environment for germination: the more rapidly the seeds germinate the shorter the time available for the fungi to take hold. Thus make sure the temperature regime is suitable, and the test environment is well aerated and that there is no excess moisture.
(3) Cleanliness of germination test media and containers: make sure that these are not a source of fungal infection.
(4) Imbibition environment: where seeds are damaged by imbibition injury (see Chapter 4) cell contents leak out and cover the seed surface, providing a source of nutrients for fungi. Avoid by prior humidification of the seeds.
(5) Prompt removal of decaying seeds: seeds which begin to decay in germination tests should be removed immediately they are identified to prevent the spread of fungi to neighbouring seeds.
(6) Seed covering structures: where seed covering structures are the source of infection it may be worthwhile to remove these from the seeds before the tests.
(7) Sprouted seeds: seeds which began to germinate before harvest and were subsequently dried can be a source of particularly severe infection during germination tests. Ideally where they can be identified in the accession at receipt they should be removed at that time. If present within the sample to be tested for germination they should be identified and removed before the test or as soon after the seeds have imbibed as possible. In this case a record of the discarded seeds must be made.

A range of different types of treatments have been applied to seeds with a view to avoiding seed-borne infections. If the infection is present only on the surface of seeds then the simplest treatment is to wash them in water. Where the infection is present within the seeds then three types of treatment are sometimes employed in an attempt to kill the pathogen, but not the seed. These are moist heat, dry heat and chemical treatments. In moist heat treatments the seeds are exposed to a high temperature, say 50°C, at a high relative humidity or, very rarely, in water for roughly 15 to 90 minutes. In dry heat treatments the seeds are first dried to a low moisture content and then exposed to very high temperatures, say 80° to 100°C, for several hours. In both these cases the treatment is something of a gamble: will the pathogen be killed but not the seed? For this reason such treatments will not be contemplated in gene banks, but staff should be aware that accessions received from some sources may have been subjected to such severe treatments.

In some laboratories seed sterilisation treatments are applied to seeds before germination tests as a routine procedure. This is not advised here since such treatments can be injurious and the presence of heavy metal salts (if used) can result in seedling abnormalities. Nevertheless, since for certain problematic accessions gene banks may wish to investigate whether there would be an advantage from seed sterilization for a particular accession, the following summary of chemical treatments which have been used is provided. Where a range of treatment periods is suggested the shorter time should be used for small seeded species and the longer time for large-seeded species: 4-10% calcium hypochlorite, 4-45 minutes (an alkaline solution, pH 12, reduces the risk of injury to seeds); 0.1-4% mercuric chloride, 0.5-15 minutes; 0.1% silver nitrate, 15 minutes; 3% hydrogen peroxide, 20 minutes-16 hours; 0.2-1% mercury chlorophenol, 1-2 hours; 0.5-5% sodium hypochlorite, 2-10 minutes. In all these cases it is necessary to rinse the seeds several times with sterile water before testing for germination. Other pre-treatments which have been useful include 50 ppm amphotericin B, a fungicide, or combining an antibacterial with an antifungal agent - 500 ppm nystatin plus 500 ppm streptomycin.

Until recently the rules of the International Seed Testing Association have not allowed seed disinfection treatments prior to germination tests. In the case of Beta vulgaris clusters, however, the ISTA rules have now been altered to allow treatment of the clusters with a fungicide. The purpose of

this treatment is to reduce secondary infection of otherwise healthy clusters from infected clusters. The treatment with thiram (tetramethylthioperoxydicarbonic diamide or TMTD) is applied to dry clusters (unless they have already been so treated) at the rate of 3 grammes of thiram per kilogramme of dry Beta clusters.

HANDLING CHEMICALLY TREATED SEED ACCESSIONS

It is not recommended that treatments with mercuric compounds be used since mercury is a health hazard. The use of mercury-containing seed treatments should by now have been phased out. This introduces a further topic of great concern to the seed testing laboratory (and also other areas) of the gene bank: the risk posed to staff health from handling chemically treated seeds. It is not recommended that gene banks apply fungicides to seeds prior to storage in an effort to increase storage longevity since these cause difficulties in the subsequent evaluation of seedlings in germination tests and can be counter-productive, reducing subsequent seed storage life. However gene bank staff may have to handle seed accessions which were treated with fungicides and insecticides prior to receipt, for example by seed health authorities. Acute poisoning of staff as a result of handling seeds treated with fungicides and insecticides is unlikely to occur since the doses applied to seeds should be very low, but a potential toxic hazard exists from repeated exposure to the laboratory environment in which treated seeds are handled since a number of the active ingredients of such treatments are suspected of being carcinogens.

The major ways in which such chemicals will accumulate in the bodies of staff working in areas where treated seeds are handled are by: absorption through the skin from direct contact with treated seeds or containers; inhalation of dust from air contaminated by the treatment; inhalation of vapours from the seeds; absorption through the mucous membranes of the eyes, nose and mouth; and the less likely risk of direct ingestion. The major ways in which the hazards can be reduced are as follows. Make sure treated seeds are always kept in sealed containers (and certainly not in a cotton bag if the accession is received in one). Open containers and handle the treated seeds in an isolated area of the laboratory which is well ventilated using an externally exhausted negative pressure system which does not recirculate the air. Some care will be needed with the exhausted air: it must not be directed towards other workers and may require a filter. Within the ventilated area the greatest air velocity is required at the point where containers are opened and the treated seeds handled. The operator should wear protective gloves and a laboratory coat which should be regularly cleaned separately from other clothing. The floor and bench should be frequently cleaned with a damp mop to reduce the risk of toxic dust spreading. After handling treated seeds the operator should wash hands and forearms thoroughly. No food or drink should be consumed in the area. Smoking must also be banned. Pregnant women should not be allowed to handle treated seeds.

The greatest hazard to health documented so far in the handling of treated seeds has occurred with the use of mercury containing treatments. Whilst their use has been largely phased out, some countries may still be using such treatments and accessions treated with mercuric compounds may be received by gene banks. Perhaps the most important advice is that information on whether seeds have been treated and if so the chemical(s) and treatment concentration(s) be consulted before the accession is opened at receipt. Where no information is available it should be assumed that the seeds have been treated until proven otherwise.

CHAPTER 10. THE APPRAISAL OF GERMINATION TESTS

Although the main purpose of germination tests in gene banks is to estimate the proportion of seeds within accessions that are viable, considerably more information than this should be recorded during germination tests. It is particularly important that full details of the way in which the germination test is conducted and the full results of the germination test are recorded. Only if this is done is it possible to gauge the accuracy of the estimate of viability obtained and to compare subsequently the results obtained in different germination tests. Examples of results likely to be compared in gene banks include the results of testing in different germination test environments to decide the most suitable regime for breaking seed dormancy, or the results from a given accession in a standard germination test environment at various times during storage in order to detect whether loss in viability has occurred. This chapter discusses how germination tests should be appraised and evaluated once underway, and provides advice on how to determine when a germination test should be terminated and what to record then.

RECORDING THE GERMINATION TEST

Table 10.1 provides an example of the information likely to be recorded during a germination test. The printed information in Table 10.1 comprises a typical germination test form together with the germination test and dormancy-breaking procedures to be followed. The information entered by pen comprises the various components of the results of the germination test together with the number of seeds tested for each replicate.

When starting the germination test the seeds to be tested are counted and divided between the replicates; the number per replicate is recorded on the germination test form and each replicate is labelled accordingly. These points may seem obvious, but in some laboratories the number of seeds is not recorded before the germination test. Instead the number of seeds tested is derived at the end of the test by adding together the number of seedlings removed during the test and the number of seeds remaining ungerminated at the end of the test. This practice is not advised in gene banks: counting the seeds before the test is to be preferred for the following reasons. First the above calculation can be used as a check of the accuracy of recording the germination test. Secondly seed covering structures often become detached from the seedling during growth and development and may remain in the test when the seedling has been removed; these may be difficult to distinguish from ungerminated seeds. Finally the proportion of seeds germinating can be determined whilst the test is in progress.

THE PROGRESS OF GERMINATION

Although many tests carried out in gene banks will be shorter than this, Table 10.1 shows that this particular germination test was evaluated on 15 occasions over a 98-day test period. The results give some idea of the trend of germination in time. It is important that germination tests be evaluated regularly whilst in progress. First because the seedlings must not be allowed to overwhelm the test by growing too large before they are removed, and secondly because the progress of germination provides additional information on various attributes of both the seed accession and the germination test environment. That is since dormancy can affect the rate of germination (see Chapter 5) monitoring the

Table 10.1 Example of the information recorded during a seed germination test of a rice seed accession.

Location	Genus	Species	Sub-species	Test date	Accession
12.24.3	Oryza	glaberrima	-	16/02/82	OR7G

1st treatment	HNO_3, pre-applied, 24 hours, 0.1 Normal	1602
2nd treatment	H_2O_2, pre-applied, 24 hours, 0.25 Molar	1702
3rd treatment	Mercaptoethanol, co-applied, 0.01 Molar	1802
4th treatment	Nil	- - - -
Substratum	Top of filter papers (2)	1802
Temperature regime	Alternating, 34°/11°C, 16 hours/8 hours	
Light regime	No special treatment, diffuse light	

Replicate	1	2	3	4	5	6	7	8	9	10	11	12	Total
No. of seeds	33	33	33	33	33	33	55	33	33	33	33	33	398
Date/Days													
22 02 / 4	1	2	-	5	1	1	-	2	2	1	1	4	20
25 02 / 7	2	6	6	7	6	2	7	6	8	6	6	6	88
01 03 / 11	3	3	4	2	3	3	6	4	1	5	4	5	13
04 03 / 14	2	1	5	1	4	3	1	2	3	1	4	1	15
11 03 / 21	7	7	3	6	2	4	3	5	9	9	7	4	22
15 03 / 28	5	2	7	4	5	5	6	5	4	5	6	1	26
25 03 / 35	5	7	1	4	3	5	6	4	1	3	3	5	32
01 04 / 42	2	1	1	1	1	2	1	-	2	-	1	-	33
04 04 / 49	1	-	-	-	1	2	-	1	1	-	-	1	34
15 04 / 56	1	-	1	-	1	-	-	1	-	-	-	-	35
22 04 / 63	1	-	-	1	-	-	-	-	-	-	-	-	35
24 04 / 65	-	-	-	-	-	-	-	-	-	-	-	-	35
01 05 / 74	-	1	-	-	-	-	-	-	-	-	-	-	35
13 05 / 76	-	1	-	-	-	-	-	-	-	-	-	-	35
13 05 / 84	-	-	-	-	2	1	-	-	-	-	-	-	35
27 05 / 98	-	1	-	1	-	-	1	1	-	-	-	-	36
Abnormal	2	-	-	-	1	-	-	-	-		-	1	4
Fresh	1	2	2	1	3	2	2	2	2	3	1	3	24
Mouldy	-	-	3	-	-	3	2	-	-	-	-	2	10
Hard	-	-	-	-	-	-	-	-	-	-	-	-	-
													398

90.5 % germination

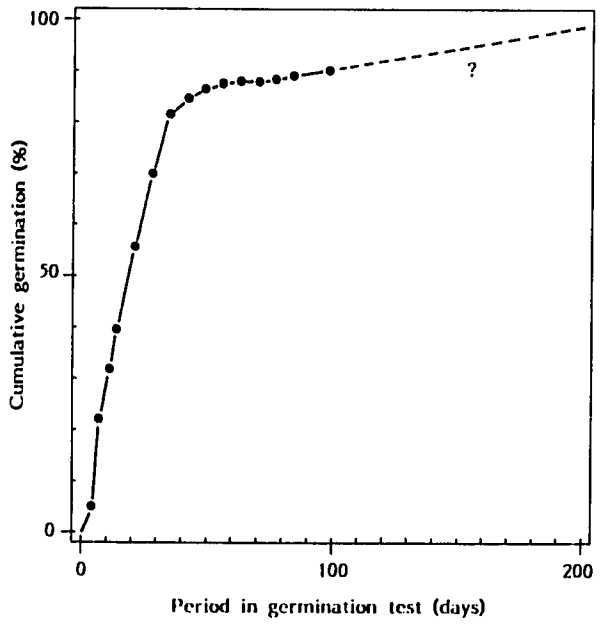
progress of germination can indicate whether seed dormancy may be affecting this and a comparison of the progress of germination of one accession in different germination test regimes may help to indicate which is the more successful dormancy-breaking regime. Similarly monitoring the progress of germination can also help in identifying the accessions where aged seeds are present and may be helpful in detecting whether ageing (see Chapter 4) has occurred (where the progress of germination has become less rapid). Similarly reversible hardseededness (see Chapters 4 and 7) can be detected by monitoring the progress of germination.

When to conclude a germination test

This additional information generated by monitoring the progress of germination is useful, if not essential, in deciding when to terminate a germination test. Perhaps the best way of explaining why this is so is to use the data shown in Table 10.1 as an example, and to discuss why the decision was taken to terminate this germination test after 98 days.

Figure 10.1 is the germination progress curve calculated from the results shown in the cumulative total column to the right of Table 10.1. It can be seen from Figure 10.1 that the majority of seeds germinated within the first 50 days or so in test and that the germination progress curve over this period shows the sort of curve expected for a uniform seed population. One criterion often used to decide when a germination test should be terminated is to conclude the test when no more seeds have germinated over a period of 7 days. According to this criterion the germination test would have been concluded at 70 days, since between 63 and 70 days in test no seeds germinated (Table 10.1). In this instance the test was not terminated at 70 days because 32 firm seeds remained in the test and we were uncertain whether these seeds would germinate eventually. From Figure 10.1 it is clear that another pattern of more

Figure 10.1 Germination progress curve of a rice seed accession. The original data is shown in Table 10.1. The broken line represents an extrapolation of the germination progress curve and is discussed in the text.



sporadic germination became evident after 70 days in test. The test was then concluded after 98 days because this later, sporadic pattern of germination provided evidence that the firm seeds would germinate, but that these remaining firm, ungerminated seeds would probably require a further 80 to 90 days in test before - we assumed from an extrapolation of the germination progress curve, see Figure 10.1 - they would have all germinated in this environment. It was therefore considered valid to include the number of firm ungerminated seeds at the end of the test with the number of seeds which had germinated normally (see below) to calculate the percentage of viable seeds.

SEEDLING EVALUATION

The seedlings removed during the progress of the germination test were those classified as normal seedlings (see also Chapter 4) by seedling evaluation. The purpose of seedling evaluation is to distinguish between those seedlings which are thought to be capable of developing into plants given favourable conditions (normal seedlings) and those seedlings which are thought to be incapable of further development (abnormal seedlings). In essence normal seedlings possess adequate root and shoot structures, whilst abnormal seedlings suffer deficiency, decay or weakness in either (or both) their root and shoot systems.

Clearly it is important that seeds distributed from gene banks are capable of producing plants. Consequently it is necessary for gene bank staff to be able to evaluate seedlings when testing accessions. The International Seed Testing Association has produced a Handbook for Seedling Evaluation, 129 pp., ISTA, Zurich, 1979. Copies of this detailed publication can be obtained from the ISTA Secretariat, P.O. Box 412, 8046 Zurich, Switzerland. The Handbook is divided into two parts. The first describes seed and seedling morphology and seedling development during the germination test. The second part deals with practical seedling evaluation and includes many photographs and drawings of normal and abnormal seedlings of many genera. This Handbook is recommended to those carrying out germination tests in gene banks, and no attempt is made here to duplicate it. Instead we describe here only the basic forms of germination, elementary seedling morphology, a basic explanation of types of abnormalities and some precautionary notes on the influence of the germination test environment on the production of abnormal seedlings.

Seed germination and seedling development and structure

In order to be able to evaluate and classify seedlings into the two categories (normal and abnormal) it is necessary to be able to recognise the essential structures of a seedling. Although in essence these are simply a functional root and shoot system, differences of detail occur between genera depending upon the form of germination and whether the genus is monocotyledonous (the embryo normally has one cotyledon) or dicotyledonous (the embryo normally has two cotyledons). As a background to this section it may be helpful to refer first to Chapter 3 which deals with seed morphology.

The usual first visible sign of germination is the piercing of the seed coat by the primary root. This subsequently elongates rapidly with secondary roots appearing later. Differences occur, however, in the development of the shoot system. In genera with epigeal germination the cotyledons are carried above the soil, whereas in genera with hypogeal germination the cotyledons remain within the seed coat (and thus below the soil surface.

The sequence of epigeal germination is as follows. After the primary root has emerged through the seed coat the hypocotyl elongates and forms an arch. Eventually this arching pulls the cotyledons and young shoot above the soil surface leaving the remainder of the seed below the surface (although parts of

the seed coat may initially remain adhering to the cotyledons). The cotyledons turn green, expand and form the first photosynthetic organs of the seedling. The epicotyl and terminal bud develop subsequently. Allium is an example o. a monocotyledon with epigeal germination (Figure 10.2). Phaseolus is an example of a dicotyledon with epigeal germination (Figure 10.3).

The sequence of hypogeal germination is as follows. In contrast to epigeal germination after the primary root has emerged - in some genera (e.g. Triticum) this may be difficult to distinguish from secondary roots - there is little elongation of the hypocotyl and the cotyledons remain within the seed coat. In dicotyledons and some monocotyledons (e.g. Asparagus) the epicotyl elongates, usually forming an arch, and pulls the young first shoot above the soil surface. In other monocotyledons (e.g. Gramineae) there is no obvious epicotyl elongation. In hypogeal germination the primary, or first, leaves which develop from the plumule are the first photosynthetic organs. Zea is an example of a monocotyledon with hypogeal germination (Figure 10.4). Pisum is an example of a dicotyledon with hypogeal germination Figure 10.5). Figures 10.2 to 10.5 illustrate the essential structures of seedlings of monocotyledons and dicotyledons with epigeal and hypogeal germination.

Characteristics of abnormal seedlings

Essentially abnormal seedlings are either damaged, defective or decayed. However, not all seedling deficiencies are critical. In Zea, for example, the primary root may fail to develop. Despite such substantial damage these seedlings can establish themselves as plants under favourable soil conditions provided sufficient (two or more) seminal roots develop. Consequently maize seedlings which have no functional primary root but which have two or more functional seminal roots are classified as normal. The list of seedling defects in Table 10.3 should help to clarify the distinction between normal and abnormal seedlings when evaluating seedlings.

POSSIBLE CAUSES OF ABNORMAL GERMINATION OTHER THAN POOR SEED QUALITY

It should be recognised that faults in the germination test procedure (extrinsic factors) may lead to the production of abnormal seedlings from seeds which under more suitable procedures would have produced normal seedlings. Six major extrinsic factors which can have this effect are listed below.

(1) Substrate toxicity. Comparison of seedling morphology in germination tests is a useful guide when determining the most suitable environment for germination tests. One example of this use of seedling evaluation is the test for toxic effects of paper used for germination tests. Seeds of Timothy (Phleum pratense), or another sensitive species, are set to germinate on the paper in question and the seedlings evaluated - specifically the roots which are particularly sensitive to paper toxicity. This test is described in the section on substrates, Chapter 9. Thus the first point to recognise is that the substrate used for the germination test may result in the production of abnormal seedlings.

(2) The duration of the germination test. Seedlings where evaluation is doubtful should be given further time for development. Only well developed normal seedlings should be removed from unconcluded tests (for example, as was the case in Table 10.1), except in the cases of badly decayed seedlings or mouldy seeds which must be removed to minimise cross-infection. But, if the test duration is particularly long, abnormal seedlings should also be removed since their continued growth and development would interfere with the germination and development of seeds which germinate later.

TABLE 10.2 Glossary of Seedling Structures (Key to Figures 10.2 - 10.5)

1.	Adventitious root	13.	Lateral root	25.	Primary leaf
2.	Axillary bud	14.	Lemma	26.	Primary root
3.	Axillary shoot	15.	Meristem	27.	Radicle
4.	Caryopsis	16.	Mesocotyl	28.	Root hairs
5.	Coleoptile	17.	Nucellus	29.	Scale-leaf
6.	Coleorhiza	18.	Ovary	30.	Scutellum
7.	Cotyledon	19.	Ovule	31.	Secondary root
8.	Embryo	20.	Palea	32.	Seminal roots
9.	Endosperm	21.	Pericarp	33.	Shoot apex
10.	Epicotyl	22.	Perisperm	34.	Terminal bud
11.	Gametophyte tissue	23.	Petiole	35.	Testa
12.	Hypocotyl	24.	Plumule		

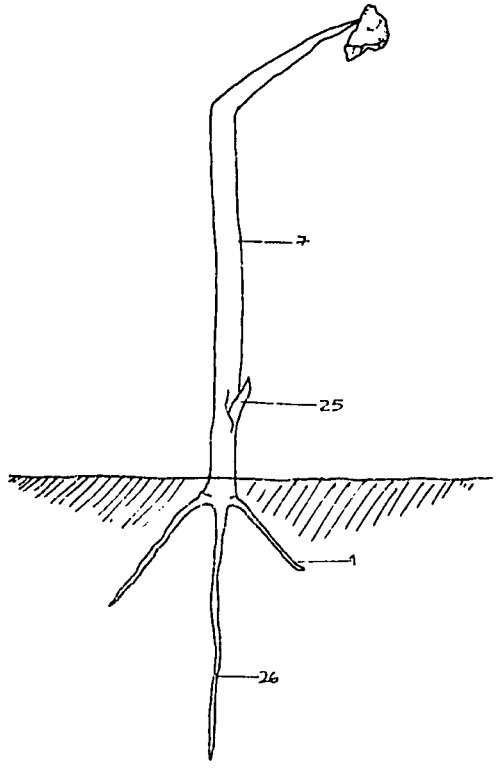


Figure 10.2 Normal seedling structure in onion (Allium cepa L.), a monocotyledon with epigeal germination. See Table 10.2 for the key to the seedling structures.

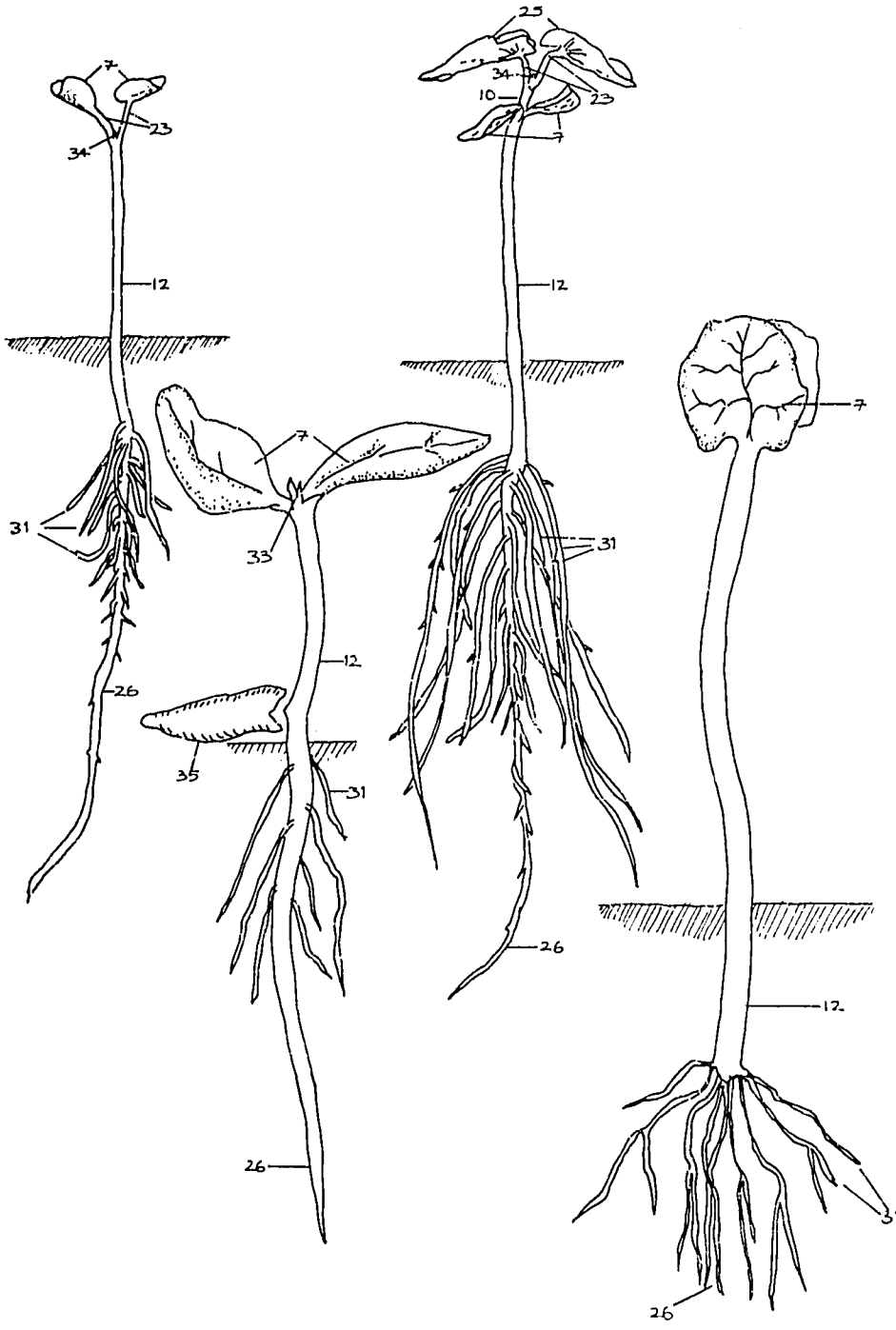


Figure 10.3 Normal seedling structure in (from left to right) sunflower (Helianthus annuus L.), cucumber (Cucumis sativus L.), kidney bean (Phaseolus vulgaris L.) and castor bean (Ricinus communis L.), dicotyledons with epigeal germination.
See Table 10.2 for the key to the seedling structures.

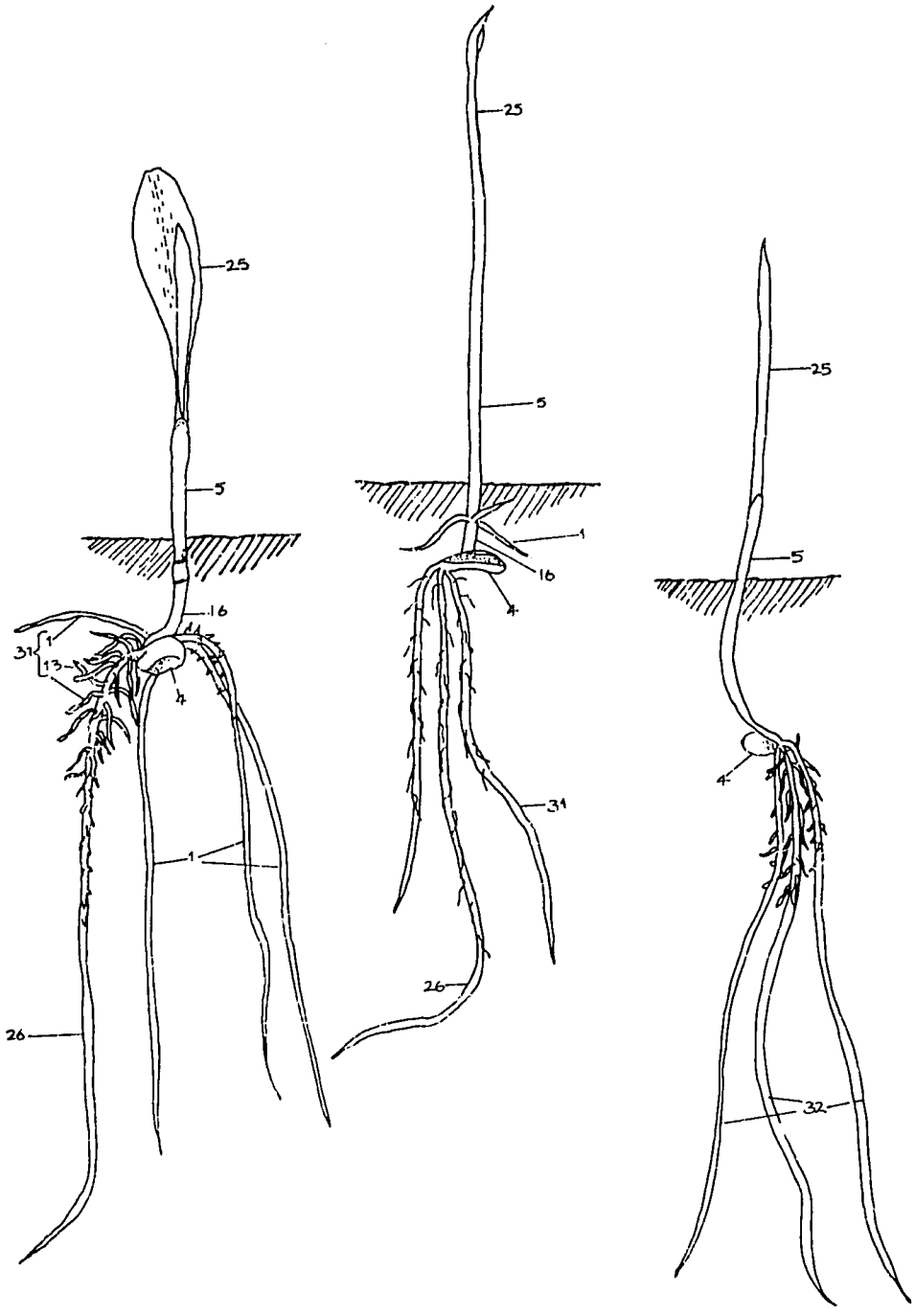


Figure 10.4 Normal seedling structure in (from left to right) maize (Zea mays L.), ryegrass (Lolium perenne L.) and wheat (Triticum aestivum L.), monocotyledons with hypogeal germination. See Table 10.2 for the key to the seedling structures.

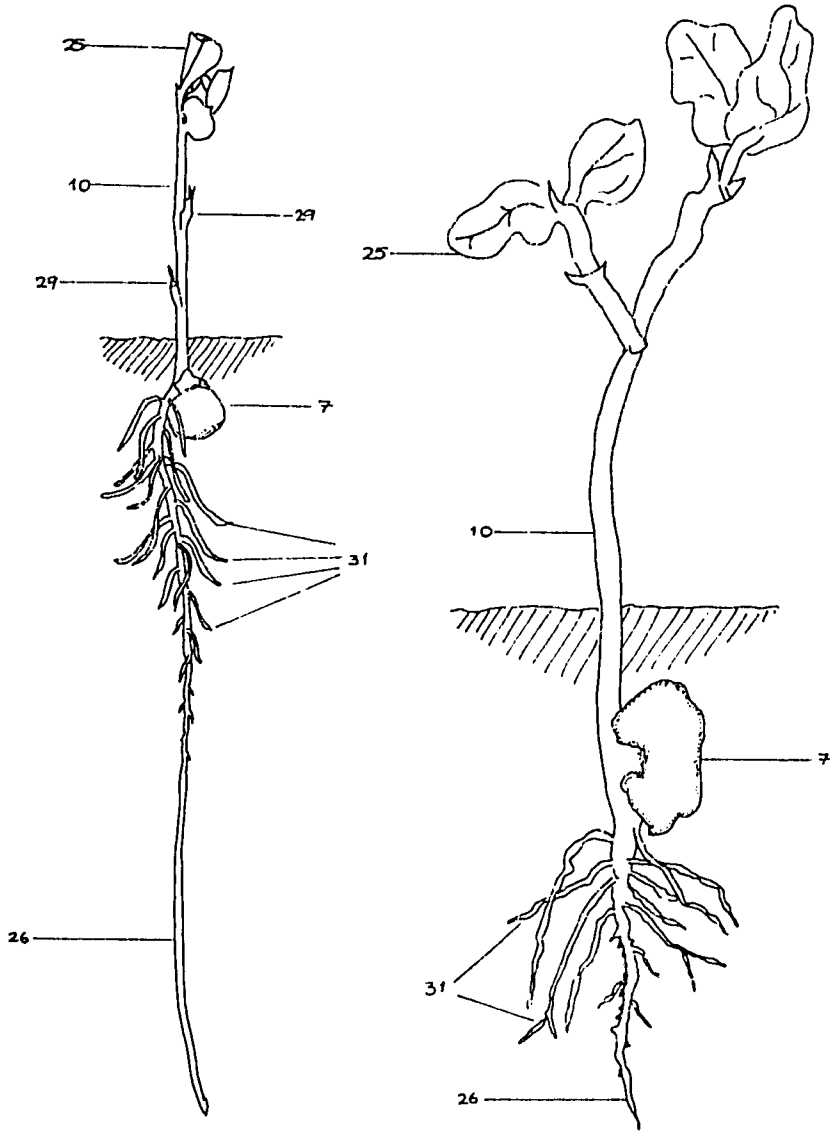


Figure 10.5 Normal seedling structure in pea (Pisum sativum L., left) and broad bean (Vicia faba L., right), dicotyledons with hypogeal germination. See Table 10.2 for the key to the seedling structures.

TABLE 10.3 List of Seedling Defects which result in Classification as Abnormal

I The primary root:

1. stunted
2. stubby
3. retarded
4. missing
5. broken
6. split from the tip
7. constricted
8. spindly
9. trapped in the seed coat
10. with negative geotropism
11. glassy
12. decayed due to primary infection (1)

the seminal roots (2):

13. less than two seminal roots

II The hypocotyl, the epicotyl, the mesocotyl:

1. short and thick (3)
2. deeply cracked or broken
3. split right through
4. missing
5. constricted
6. tightly twisted
7. bent over
8. forming a loop or spiral
9. spindly
10. glassy
11. decayed due to primary infection (1)

III. The cotyledons: apply the 50% rule (4,5)

1. swollen and curled
2. deformed
3. broken or otherwise damaged
4. separate or missing
5. discoloured
6. necrotic
7. glassy
8. decayed due to primary infection (1)

Special defects of cotyledons of dicotyledonous species with epigeal germination (e.g. Beta, Brassica, Daucus, Dianthus, Helianthus, Lactuca, Trifolium, Zinnia)

9. short and thick
10. constricted
11. bent over
12. forming a loop or spiral
13. without a definite "knee"
14. spindly

IV The primary leaves: apply the 50% rule (4)

1. deformed
2. damaged
3. missing
4. discoloured
5. necrotic
6. decayed due to primary infection (1)
7. normal shape but less than 1/4 of the average leaf size of the normal seedlings in the test

V The terminal bud (and surrounding tissues) (6):

1. deformed
2. damaged
3. missing
4. decayed due to primary infection (1)

VI The coleoptile and first leaf (leaves)

the coleoptile:

1. deformed
2. damaged
3. missing
4. with the tip damaged or missing
5. strongly bent over
6. forming a loop or spiral
7. tightly twisted
8. split for more than 1/3 of length from the tip (7)
9. split at the base
10. spindly
11. decayed due to primary infection (1)

the leaf (leaves):

12. extending less than half-way up the coleoptile
13. missing
14. shredded or otherwise deformed (7)

VII The seedling:

1. deformed
2. fractured
3. cotyledons emerging before the root
4. two fused together
5. persisting endosperm collar
6. yellow or white
7. spindly
8. glassy
9. decayed due to primary infection (1)

TABLE 10.3 (continued)

Notes

(1) In other words if the source of infection is the particular seedling being evaluated then the seedling is classified as abnormal. If, however, the source of infection is secondary - that is a neighbouring seed in the germination test - then, provided decay is the only form of abnormality, the seedling is classified as normal. The reason for the distinction between primary and secondary infection is that in the soil seeds would not be so closely spaced and cross-infection would not occur. Hence the decay observed is an artefact of the germination test environment.

(2) Two or more seminal roots can replace the requirement for the primary root (e.g. Zea). However, secondary roots or seminal roots showing one or more of defects 1 to 12 are abnormal and cannot replace an abnormal primary root in cases where the presence of several secondary roots (e.g. Cucumis) or at least two seminal roots (e.g. Triticum) determine the value of a seedling.

(3) However, in genera where the shoot system consists of a swollen, tuberous hypocotyl (e.g. Cyclamen) the opposite applies. The hypocotyl should be tuberous (that is short and thick).

(4) The 50% rule refers to the area of cotyledons or the primary leaves which are alive. Seedlings are considered normal if 50% or more of the total cotyledon tissue is functional, but abnormal if more than 50% is missing, necrotic (dead), discoloured or decayed. The rule is also applied when defective primary leaves are to be evaluated, but it is not applied if the primary leaves show their normal shape though smaller.

(5) Irrespective of the 50% rule the cotyledons must not be damaged or decayed at the point of attachment to the seedling axis or adjacent to the shoot apex. If they are then the seedling is classified as abnormal.

(6) In genera with a terminal bud (e.g. Phaseolus, Pisum) the seedling is abnormal if the terminal bud is defective or missing, even if axillary buds or shoots have developed.

(7) In Zea and Oryza splits in first leaves are common and seedlings exhibiting a split first leaf should not be classified as abnormal. If, however, the second leaf is split then the seedling is classified as abnormal.

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(3) The number of seeds tested for germination in a given area. Certain (poorer quality) seed lots may show a much greater proportion of abnormal seedlings if tested at too high a density (e.g. too many seeds on a 9 cm diameter filter paper in a petri-dish).

(4) Oxygen deficiency. Oxygen deficiency, for example, may be caused by a towel being rolled too tightly in a rolled towel test (Chapter 9), and this can result in a substantial number of abnormal seedlings.

(5) Inappropriate temperature. The temperature may be adequate for seed germination but too extreme for normal seedling growth and development.

(6) Water deficit or surplus. Either excess or inadequate moisture supply (particularly the drying out of the substrate during a germination test) can result in abnormal germination.

Commonly if one of the above factors is the cause of abnormal germination then a very high proportion of seedlings in the test will be abnormal. Indeed the cause of the problem may well be very evident. Precautions should be taken to ensure that subsequent tests (on all accessions) do not suffer from the same deficiency. The test on the particular accession in which the problem was discovered may need to be repeated if a number of the replicates of the germination test are affected. If, however, only one replicate is affected and this is confirmed by the use of the tolerance tables (Chapter 14) then the estimate of accession germination can be calculated from the results of the remaining replicates where there was no deficiency of the test environment. The above refers to a replicated fixed sample-size germination test. In the case of a sequential germination test (see Chapter 15) no such comparison will be possible and the result of this particular group of seeds should be ignored and a further group of seeds sampled from the accession in store and tested for germination.

Although seeds for long-term storage should not be treated with chemicals, some seeds received by the gene banks may have been dressed with fungicides or insecticides which result in stubby, stunted or retarded roots when the seeds are tested on paper. Similar phenomena can also sometimes result from the exudation of natural substances from the seed. Soil, however, tends to absorb these phytotoxic substances. Consequently if these abnormalities are observed a further sample of seeds from the accession should be retested in soil. For more details on handling and testing treated seeds see Chapter 9.

OBSERVATIONS ON UNGERMINATED SEEDS

In addition to evaluating the germinated seeds into the normal and abnormal categories by seedling evaluation at the conclusion of the germination test, some evaluation of the non-germinated seeds is also required. The example provided of a germination test form (Table 10.1) shows three categories of non-germinated seeds at the conclusion of the test: fresh (firm); mouldy; and hard.

The classification between imbibed, ungerminated seeds into the fresh and mouldy categories is dealt with in Chapter 11. The firm seeds identified at the end of the germination test may be subjected to further tests; e.g. a continuation of the germination test, but in another environment or after a further dormancy-breaking treatment, or a rapid viability test such as a tetrazolium staining test - see Chapter 11.

Hard seeds are those which have failed to imbibe during the germination test (Chapter 4). Methods are available which can be applied in order to enable such seeds to imbibe (Chapter 7), and it is suggested here that these seeds be so treated and then returned to the germination test environment. Rather than wait until the end of the germination test to identify and scarify the hard seeds, it is suggested that this might be done somewhat earlier (see Chapter 7).

The only other category of ungerminated seeds which may be observed at the end of a germination test but which is not shown in Table 10.1 is that of empty seeds. Methods of determining whether a seed is empty or not are provided in Chapter 8. Empty seeds can either be identified before the germination test is begun or after the germination test is concluded. Where the empty seeds are identified before the germination test they can be removed from the sample of seed-like structures and excluded from the germination test; consequently there is no need to check for empty seeds at the end of the germination test. (Hence in Table 10.1 no row was provided for this category of ungerminated seeds at the end of the test.) Whether the proportion of empty seeds is determined before the germination test is begun or at the conclusion of the germination test, it is suggested here that germination and viability (which may include both the germinable seed and fresh seed categories) are reported as percentages of the full seeds.

CHAPTER 11. TESTS FOR VIABILITY WHEN THE GERMINATION RESULTS WOULD BE MISLEADING

A germination test is usually the best method of estimating seed viability. However a number of difficulties can arise in deciding why a particular seed has not germinated when a test is concluded, viz: dormant seeds (Chapter 5); hard seeds (Chapter 4); empty seeds (Chapter 8); and slow-germinating seeds (Chapter 10). The purpose of this chapter is to discuss the procedures which can be employed to determine seed viability when dormancy is suspected of confounding the results of germination tests intended to estimate viability.

Occasions may well arise when the results of germination tests could be misleading because the dormancy-breaking treatments applied were ineffective or only partially effective. In such cases it is necessary to carry out a viability test on the seeds which remain ungerminated at the end of the test. Alternatively a fresh sample of seeds may be sampled from the accession in store and subjected to a test of viability other than germination. The purpose of either test will be to estimate the percentage of viable seeds in the accession (in order to determine whether the accession is acceptable as it is for continued storage or needs regenerating) and also to decide whether it is worthwhile to apply further dormancy-breaking treatments to the seeds in future germination tests - since the difference in results between the viability and germination tests will indicate the proportion of seeds in the accession which remain dormant despite the use of specified dormancy-breaking treatments.

It should be pointed out that one viability test which the ungerminated seeds may be subjected to is simply a continuation of the germination test - possibly in the same environment, or after the application of a further stimulus intended to break dormancy, or in another environment. For example, in Table 10.1 and Figure 10.1 (Chapter 10) it was shown that considerable extension of the germination test period might enable the most dormant seeds in some accessions to germinate eventually. This chapter, however, is concerned with procedures which provide estimates of viability by other methods.

It is to be hoped that such alternative viability tests will not need to be used frequently since they are considerably more labour-intensive than germination tests and require considerably more expertise. (This is one reason why considerable emphasis is placed in this report on testing seeds in environments where full germination is likely to be promoted.) Information is provided in this chapter on four different viability tests which may be useful in gene banks as an adjunct to germination tests. The first two rely on the different physical properties of viable and non-viable embryos after imbibition and incubation, while the other two utilise stains to distinguish between live and dead areas of embryonic tissues within the seeds. Of the four, staining tests based on the use of tetrazolium salt are the most widely applied in seed testing. Further comments are provided on a few additional rapid viability tests, but these are brief since they are of limited value in gene banks.

THE EXAMINATION OF UNGERMINATED SEEDS AT THE END OF THE GERMINATION TEST

This is the simplest viability test which should be applied at the end of every germination test. Seeds which fail to germinate are divided into four categories: those which failed to imbibe at all;

those which imbibed and are firm and free from primary fungal infection; those which are mouldy and which may, when gently squeezed, exude liquid; and those which are empty. The first category are the hard seeds (see Chapters 4 and 7).

Seeds which fall into the second category are referred to as fresh seeds. It is generally assumed that these seeds are viable but dormant. This assumption, however, may well be wrong in some cases since the seed may be empty although the seed covering structures may be firm to the touch. In the tropical pasture species, for example, it is comparatively common for empty seeds to be classified as firm seeds and mistaken for dormant seeds at the end of germination tests. One way to avoid this particular mistake is to elaborate the test of firmness at the conclusion of germination tests by dissecting the seeds (or seed-like structures) which remain ungerminated. This is often referred to as a cutting test. Even so, the combined procedure including the cutting test will only determine accurately the proportions of empty, mouldy, insect damaged or severely discoloured seeds. The proportion of viable but dormant, or dead seeds remains difficult to detect.

The third category of seeds are mouldy. It is generally assumed that these seeds are dead. This is certainly the case if liquid is released when the seed is squeezed since this is the result of the catabolism of the embryo (and endosperm if present) by hydrolytic enzymes. However, it is by no means certain that all or any of these seeds were dead at the beginning of the germination test. Moreover, there are a number of examples where the assumption that mouldy seeds are dead was made erroneously, and the error has since been repeated consistently. Again, this limits one's confidence in conclusions based on this criterion.

The fourth and final category used to classify the seeds remaining ungerminated at the conclusion of germination tests is that of empty seeds. Whilst those seeds identified by inspection as being empty are likely to be so, in some species without a dissection test (see above) it is possible that other empty seeds may not be identified. Consequently although it is reasonable to assume that if fresh seeds are present at the end of the germination the proportion of viable seeds is likely to be greater than the proportion of seeds which germinated during the test, it is not necessarily the case that the proportion of viable seeds is the sum of the proportions of hard seeds, firm seeds and germinated seeds.

Despite these criticisms the examination of ungerminated seeds is an essential adjunct to germination tests. It is especially helpful in initially detecting the presence of severe dormancy in high quality accessions where the proportion of empty seeds is low. The procedure can be made more useful by dissecting some of the remaining seed-like structures and subjecting these to a tetrazolium test (see later section). Where empty seeds are common it is strongly advised that X-ray examination is used as part of the routine procedure (see Chapter 8).

THE EXCISED EMBRYO TEST

The excision and subsequent incubation of embryos from the seed and/or fruit covering structures is another method of estimating the viability of seeds. Whilst it is not as rapid as the staining techniques described subsequently, for slow germinating species - such as certain tree species - it is considerably more rapid than germination tests. Typically an estimate of viability can be obtained within 7 to 14 days, whereas germination tests on many tree seeds, for example, may require as many as 2 to 6 months - especially if pre-chilling is required. Essentially excised embryo tests overcome the criticism that germination tests are tests of the condition of the seed covering structures (since these may prevent

germination) rather than the embryo. Considerable skill is required to carry out excised embryo tests, not so much in evaluation but in the preparation of the seeds and the excision of the embryos.

The excised embryo test has been applied to a diverse range of species in which seed size and morphology vary considerably: for example, seeds of flowers, vegetables, shrubs, vines, fruit trees, coniferous and broad-leaved trees. A summary of excised embryo test procedures is provided below, but for more detailed descriptions the reader should consult Heit (1955) and the references provided therein.

Moistening the seeds

A fresh sample of seeds is required for this test, i.e. ungerminated seeds recovered at the end of a germination test should not be used. Before embryos can be excised the seeds must be moistened so that they can be excised from the covering structures easily and without damage. The following alternative procedures have been used.

(a) Soak the seeds in slowly running water.

(b) Soak the seeds either in standing water below 15°C or at room temperature with two changes of water per day.

(c) Imbibe the seeds under germination test conditions: that is on paper, between paper, in or on sand, or on or in peat or compost.

Seed moistening periods vary from one to seven days depending upon the species and the method applied. Where the seed covering structures are thick, tough, hard or semi-hard, they may need to be cracked, punctured, slit or (possibly) removed before or during the moistening treatment. This can be done with scalpels or knives. Hard seeds, however, may need to be scarified or impacted (see Chapter 7). In the case of the stone fruits the endocarp will need to be removed before soaking. This can be done by inserting a strong, sharp blade in the suture of the endocarp and levering it apart.

Note, however, that for very dry seeds soaking treatments similar to those described above are likely to result in imbibition damage (see Chapter 4) - particularly if the seed covering structures are disrupted in some way before the soaking treatment. Consequently it is essential that very dry seeds are subjected to the humidification treatments described in Chapter 7 before soaking the seeds.

Excising the embryo

Embryos are excised using sharp instruments such as scalpels and razor blades. It is important to avoid touching embryos as far as possible, but the application of pressure by the thumb and finger nails against the seed covering structures is helpful during excision. It is vital to avoid infecting embryos during excision and so fairly sterile conditions should be used. A laminar flow cabinet would be ideal, but otherwise a clean, draught-proof room can be used and excision carried out below a fixed sheet of glass. The instruments and the working surface should be sterilized with a 50% ethanol solution in water and instruments should be cleaned and sterilized before every excision. If, despite the above, mould infections develop, seeds or fruits can be sterilized either by shaking for three minutes in a 1% solution of mercuric chloride or by shaking for 15 minutes in a 1% sodium hypochlorite solution. After either treatment the seeds should be washed well before excision.

Incubating and evaluating embryos

The excised embryos are incubated on top of filter paper in petri dishes under normal conditions of light and moisture at 20°C for up to 14 days. The principle of the test on which evaluation is based is that viable embryos will remain healthy and may also begin to grow and turn green during the test whilst non-viable embryos will decay during the incubation period. (Note the similarity between the above and the physical test of viability which is carried out at the end of germination tests by pressure or cutting described earlier.)

Thus at the end of the incubation period the following categories of embryos are considered viable:
(a) Embryos which have begun to germinate
(b) Embryos with one or more cotyledons growing or greening
(c) Embryos which remain firm (apply pressure with a spatula to test for this) and which are slightly enlarged and either white or yellow (according to species).

The following categories are considered non-viable:
(a) Embryos which develop mould, deteriorate and decay
(b) Degenerated embryos
(c) Discoloured embryos
(d) Embryoless seeds or seeds with deformed embryos detected at excision (remember to record these when the test is set up).

Some embryos may be damaged by excision. This is likely to be a considerable problem until expertise has developed. The embryos damaged during excision can be distinguished from non-viable embryos by the localised discolouration (e.g. straight lines of necrosis) of tissue after 24 hours incubation. If these embryos show no other signs of damage (as listed above for non-viable embryos they should be classified as viable. However, if a substantial proportion of embryos shows excision damage then the method of seed preparation and embryo excision should be improved before retesting the accession.

Because some embryos turn green and may also begin to germinate during the test, their classification is much easier than in staining tests. Distinguishing the remaining excised embryos as viable and non-viable is more difficult, but these remaining viable embryos provide an estimate of the proportion of seeds showing embryo dormancy (as opposed to that imposed by the seed covering structures or, where present, the endosperm) and their recognition may help to explain earlier inadequate germination test results.

VITAL STAINS TO ESTIMATE VIABILITY

A word of warning concerning viability testing using staining techniques is necessary by way of introduction. Staining techniques rely on considerable practical expertise. This can only be gained from repeated use of the tests, preferably under expert guidance. Moreover, for many species no established or well described techniques are available.

The evaluation of topographical patterns of live and dead areas within the embryo (including the shade of red colouration observed in tetrazolium stains) is difficult and subjective. Consequently a good deal of training and practice are required before competence can be achieved. It would be naive to suggest that all gene banks could apply staining tests easily as routine procedures - not least because they are

extremely time consuming (even though the results are obtained more rapidly than in a germination test). Nevertheless staining tests should be regarded as an essential back-up procedure to germination tests in gene banks. It is probably worthwhile for gene bank staff to receive specialised training in tetrazolium staining techniques and evaluation. Training with expert guidance is provided by occasional courses run by the International Seed Testing Association.

This chapter concentrates on the provision of practical information for the application of these tests in gene banks but does not attempt to provide the sort of detailed information (such as tetrazolium staining patterns species by species) which is described elsewhere. The references provided at the end of this chapter should be consulted for examples of staining patterns. Only a very basic outline of staining procedures and possible problems is provided here.

THE TOPOGRAPHICAL TETRAZOLIUM TEST FOR VIABILITY

The salt 2,3,5-triphenyltetrazolium chloride (tetrazolium) is colourless and water-soluble. As such it is able to penetrate imbibed seed embryos. Tetrazolium is an oxidation-reduction indicator which is reduced to a red, insoluble formazan by dehydrogenases which are active within viable tissues, but not dead tissues. Since formazan is insoluble it becomes trapped within these viable cells and since it is red tissues containing viable cells can be identified. Loss of activity of the dehydrogenases tends to parallel loss in seed viability. Consequently the tetrazolium test can be used to estimate seed viability.

The tetrazolium test can either be applied to a fresh sample of seeds or to the firm seeds which have failed to germinate at the end of a germination test. However, in the latter case it will not be known whether the dead seeds identified by the tetrazolium test were dead before the germination test began or whether they died during the course of the germination test. Tetrazolium tests on both a fresh sample of seeds and those remaining ungerminated at the end of a germination test allow an estimate to be made of the proportion of seeds which died during a given germination test procedure. This information can be useful in investigating which of several environments is the most suitable for germination tests.

In expert hands the tetrazolium test on a fresh sample of seeds can have several advantages over germination tests. Obviously the problem of dormancy is avoided, and the result is obtained comparatively rapidly. In addition, however, some workers are able to evaluate the staining patterns and stain intensity to indicate forms of damage to seeds other than loss in viability resulting from ageing: for example, mechanical damage, rapid drying damage, and imbibition injury and freezing injury. See Moore (1973) for a discussion of these applications.

Stability of tetrazolium salt and solutions

The reaction rate for the reduction of 2,3,5-triphenyltetrazolium chloride to formazan in the presence of dehydrogenases is affected by pH, temperature, atmospheric pressure and the concentration of the tetrazolium salt in solution. Tetrazolium salt is a white, or light yellow, powder with a finite shelf life. The powder should be stored in a deep freeze at -18°C or less. In solution ultra-violet light stimulates a slow reduction of the salt. This results in the colourless solution turning red. Such tainted solutions must be discarded. To avoid reduction by light, tetrazolium solutions should be stored in a cool (3°-5°C) dark place. A further precaution is to cover the vessel in which the solution is stored with aluminium foil. Under these conditions tetrazolium solutions are very stable. However, the bulk may be contaminated

when samples of the solution are withdrawn. Because of this sufficient solution is usually made up for about two weeks' work.

Concentration of tetrazolium solutions

Different concentrations of tetrazolium solutions are used, normally 0.1, 0.5 or 1.0 per cent depending on species. It is normal practice to make up a single concentration stock solution (1%) and to dilute this if necessary. Solution concentrations are expressed on the weight-volume basis. Thus a 1% tetrazolium solution is one gramme of tetrazolium salt dissolved in distilled water made up to 100 ml. To achieve the best results the pH value of the tetrazolium solution should be between pH 6 and pH 8. The higher concentrations of tetrazolium in solution can be slightly more acidic than this. To avoid this buffered tetrazolium solutions are prepared. One litre of a buffered 1% tetrazolium solution can be prepared in the following way.

1. Dissolve 3.631 g KH_2PO_4 in 400 ml of distilled water.
2. Dissolve 7.126 g $Na_2HPO_4.2H_2O$ in 600 ml of distilled water.
3. Mix the two solutions.
4. Dissolve 10 g of 2,3,5-triphenyl tetrazolium chloride in the 1000 ml of the buffer solution.

To dilute a 1% tetrazolium buffered stock solution in order to produce a 0.5% tetrazolium solution mix one part of the stock solution with one part of distilled water. To dilute a 1% tetrazolium buffered stock solution to produce a 0.1% tetrazolium solution mix one part of the stock solution with nine parts of distilled water.

OUTLINE OF TETRAZOLIUM TEST PROCEDURES

There are five stages to the tetrazolium test: pre-conditioning of the seeds; preparation of the seeds; staining the seeds; washing; and the evaluation of the staining pattern within the seeds. Table 11.1 summarises, according to family, recommended pre-conditioning and staining treatments, together with an indication as to whether any treatment to the seeds is required prior to pre-conditioning, staining and evaluation. The sources of this information are provided in the references at the end of this chapter. This table is provided to assist those with experience of tetrazolium staining procedures in applying the test to other species. An outline of each stage of the tetrazolium staining procedure is provided below.

Pre-conditioning of the seeds

The dry seeds must first be moistened in order that they can be easily bisected, or punctured etc. and to allow the tetrazolium solution to diffuse throughout the embryo and provide a clean, clear stain. In seed testing work in general this has been achieved by first soaking the seeds in water or by placing the seeds in a moist medium (similar to the germination test medium). To moisten seeds quickly, pre-conditioning is often carried out at 30°C. Longer periods at lower temperatures or shorter periods at higher temperatures, however, may be equally satisfactory. Pre-conditioning, particularly where very dry seeds are soaked, may result in imbibition injury. Also in some grain legumes very dry seeds may crack when soaked. Consequently it is suggested here that a humidification treatment (Chapter 7) may be required prior to pre-conditioning.

In some species an additional treatment may be required before pre-conditioning - for example, scarification or the puncture or removal of the seed covering structures - to facilitate imbibition. The

requirement for such treatments is indicated in column 2 of Table 11.1. Note that no pre-conditioning treatment is necessary where the seeds to be tested are the ungerminated, imbibed seeds remaining at the end of a germination test. During pre-conditioning various leachates (see the later section on rapid viability tests) may form a 'mucus' around the seeds. This can be removed by rubbing the seeds between two sheets of filter paper before preparing them for staining.

Preparation for staining

Before staining it is usually necessary to prepare the seeds in some way to allow rapid staining and evaluation of the embryo. Magnification will aid the preparation of small seeds. There are a few species, such as clovers and vetches, where pre-conditioning and preparation for staining are not normally recommended. In these cases we nevertheless recommend humidification treatments to avoid imbibition injury when the seeds are soaked in the tetrazolium solution, and we also recommend scarification if the seeds are hard. The requirement for preparation before staining is indicated in column 4 of Table 11.1.

For detailed information on the preparation required for each species consult the appropriate reference but the more common treatments, depending mainly on seed morphology (see Chapter 3) are as follows.

(1) Puncture. Small endospermic seeds (which cannot easily be bisected) are punctured in the endosperm above the embryo with a sharp needle, e.g. small-seeded grasses. In other species the seed coat can be pierced with a needle between the radicle and cotyledon, e.g. Beta spp.
(2) Bisect. Larger endospermic seeds and non-endospermic seeds with a linear embryo are bisected longitudinally and medially through the embryo with a razor blade in a sawing motion, e.g. large-seeded grasses and cereals, members of the Compositae. One half of the seed is discarded and the other placed in the staining solution.
(3) Cut at the distal end. Small endospermic seeds can be cut transversally with a razor blade at the opposite end of the seed to the embryo, e.g. small-seeded grasses.
(4) Seed coat removal. In non-endospermic seeds it may be necessary to remove the seed coat after pre-conditioning, e.g. legume seeds.
(5) Excise embryo. Where the embryo is sufficiently large it can be excised, e.g. oil palm.

Staining

The seeds are then soaked for a set period at a set temperature in a given concentration of tetrazolium solution in the dark (because of the effect of ultra violet light on the solution's colour). Suitable concentrations, temperatures and periods for staining are summarised in Table 11.1 (column 5). Alternative treatment combinations may, however, also be applied: longer periods at lower temperatures or shorter periods at higher temperatures may be found to be equally satisfactory or better.

Washing

After the staining treatment the seeds are washed several times in distilled water. This is to remove any excess tetrazolium solution. They can either be evaluated immediately, or stored immersed in distilled water at 3°-5°C until required for evaluation. For some species it has been suggested that the seeds be immersed in a clearing solution of lactophenol for one to two hours after staining and washing before evaluation. The purpose of this treatment is to make the tissues transparent, thereby aiding the

TABLE 11.1 Recommended tetrazolium test procedures by family

Key to the columns

Column 1: species
Column 2: need for treatment to seed prior to pre-conditioning
Column 3: pre-conditioning treatment
Column 4: need for treatment to seed prior to staining
Column 5: staining treatment (concentration, time, temperature)
Column 6: need for treatment to seed prior to evaluation of staining pattern

1	2	3	4	5	6	7
		ACERACEAE				
Acer campestre L.	-	imbibe, sand, 3°-5°C, 10-14d	+	1%, 24h, 30°C	+	ISTA
Acer ginnala Maxim.	+	soak, 18h	+	1%, 24-48h, 30°C	+	ISTA
Acer palmatum Thunb. ex Murr.	-	imbibe, sand, 3°-5°C, 10-14d	+	1%, 24h, 30°C	+	ISTA
Acer platanoides L.	+	soak, 18h	+	1%, 15-20h, 30°C	+	ISTA
Acer pseudoplatanus L.	+	soak, 18h	+	1%, 15-20h, 30°C	+	ISTA
		AIZOACEAE				
Tetragonia tetragonioides (Pall.) Kuntze	-	imbibe or soak, 18h	+	1%, 24h, 30°C	+	ISTA
		BERBERIDACEAE				
Berberis spp.	-	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
Mahonia spp.	-	soak, 18h	+	1%, 20-48h, 30°C	+	ISTA
		CAPRIFOLIACEAE				
Lonicera spp.	-	soak, 18h	+	1%, 24-48h, 30°C	+	ISTA
Sambucus spp.	+	soak, 18h	+	1%, 20-24h, 30°C	+	ISTA
Viburnum spp.	+	soak, 18h	+	1%, 24-48h, 30°C	+	ISTA
		CELASTRACEAE				
Euonymus spp.	-	soak, 18h	+	1%, 24h, 30°C	+	ISTA
		CHENOPODIACEAE				
Beta vulgaris L.	-	imbibe or soak, 16-18h	+	1%, 24-48h, 30°C	+	ISTA
Spinacia oleracea L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		COMPOSITAE				
Arctium lappa L.		soak, 24-48h, 20°C	+	1%, 6-8h, 30°C		Novotna
Calendula officinalis L.		soak, 20h, 20°C	+	1%, 4h, 30°C		Novotna
Carthamus oxyacantha Bieb.	-	soak, 20h	+	1%, 24h, 30°C	-	B&K
Carthamus tinctorius L.	-	imbibe or soak, 18h	+	0.5%, 6h, 30°C	+	ISTA
	-	imbibe or soak, 18h	+	1%, 24h, 30°C	+	ISTA
Cichorium spp.	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	+	ISTA
Cnicus benedictus L.		soak, 24h, 20°C	+	1%, 7h, 30°C		Novotna
Cynara scolymus L.	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	+	ISTA
Helianthus annuus L.	-	imbibe or soak, 18h	+	0.5-1%, 3-6h, 30°C	+	ISTA
	-	imbibe or soak, 18h	+	1%, 6-24h, 30°C	+	ISTA
Inula helenium L.		soak, 16h, 20°C	+	1%, 3h, 30°C		Novotna
Lactuca sativa L.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Matricaria chamomilla L.		soak, 16h, 20°C	+	0.5%, 3h, 30°C		Novotna
Scorzonera hispanica L.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Silybum marianum (L.) Gaertn.		soak, 20h, 20°C	+	1%, 6h, 30°C		Novotna
Tragopogon porrifolius L.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		CORNACEAE				
Cornus spp.	-	soak, 40h, or all	+	1%, 48h, 30°C	+	ISTA
		CORYLACEAE				
Carpinus spp.	-	soak, 18h	+	1%, 20-24h, 30°C	+	ISTA
Corylus spp.	+	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA

1	2	3	4	5	6	7
		CRUCIFERAE				
Brassica spp.	-	imbibe or soak, 16-18h	+	0.5-1%, 3-6h, 30°C	+	ISTA
	-	imbibe or soak, 16-18h	+	0.4-1%, 6-18h, 30°C	+	ISTA
	-	imbibe, 16h, 25°C	+	1%, 1-2h, 40°C	-	DSRL
Lepidium sativum L.	+	imbibe or soak, 18h	+	1%, 3-6h, 30°C	+	ISTA
Raphanus sativus L.	-	imbibe or soak, 18h	+	0.5-1%, 2-3h, 30°C	+	ISTA
	-	imbibe or soak, 18h	+	0.5-1%, 3-6h, 30°C	+	ISTA
	-	imbibe, 16h, 25°C	+	1%, 1-2h, 40°C	-	DSRL
Sinapis alba L.	+	imbibe or soak, 18h	+	0.5-1%, 2-3h, 30°C	+	ISTA
		CUCURBITACEAE				
Citrullus lanatus (Thunb.) Mansfield	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	+	ISTA
(Citrullus vulgaris Schrad.)	-	soak, 1h, 30°-35°C	+	1%, 1-2h, 40°C	-	DSRL
	-	soak, 1h, 30°-35°C	+	1%, 2-5h, 25°C	-	DSRL
Cucumis spp.	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	+	ISTA
Cucurbita spp.	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	+	ISTA
		CUPRESSACEAE				
Chamaecyparis thyoides (L.) BSP	-	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
Juniperus spp.	+	soak, 18h	+	1%, 24h, 30°C	+	ISTA
Libocedrus spp.	+	soak, 18h	+	1%, 24-48h, 30°C	+	ISTA
		ELAEAGNACEAE				
Elaeagnus spp.	-	soak, 18h	+	1%, 24, 48h, 30°C	+	ISTA
		EUPHORBIACEAE				
Hevea brasiliensis Muell. Arg.	+	soak, 16h	+	1%, 2h, 40°C	-	HCH
Phyllanthus emblica L.	-	nil	+	1%, 2-2.5h	-	S&R
		FAGACEAE				
Fagus spp.	+	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
		GRAMINEAE				
Agropyron smithii Rydb.	-	nil	+	0.05%, 48h	-	Bass
Agropyron spp.	-	imbibe, 6-18h	+	0.5%, 4-6h, 30°C	+	ISTA
	-	imbibe, 6-18h	+	1%, 20-24h, 30°C	+	ISTA
Agrostis spp.	-	imbibe, 6-18h	+	0.5-1%, 18-24h, 30°C	+	ISTA
Alopecurus spp.	-	imbibe, 6-18h	+	1%, 20-24h, 30°C	+	ISTA
Andropogon gayanus Kunth	-	imbibe	+	0.5%, 2h, 40°C	-	Elra
Anthoxanthum odoratum L.	-	imbibe, 18h	+	1%, 20-24h, 30°C	-	ISTA
Arrhenatherum elatius (L.) Beauv.	-	imbibe, 6-18h	+	0.5%, 4-6h, 30°C	+	ISTA
	-	imbibe, 6-18h	+	1%, 20-24h, 30°C	+	ISTA
Avena sativa L.	-	imbibe or soak, 6-18h	+	0.1-0.5%, 2h, 30°C	+	ISTA
	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	+	ISTA
Avena spp.	+	imbibe, 16h, 30°C	+	0.5%, 0.5-1h, 40°C	-	DSRL
Axonopus affinis Chase	-	imbibe, 16h, 30°C	+	0.5-1%, 8-10h, 25°C	-	DSRL
Axonopus spp.	-	imbibe or soak, 6-18h	+	1%, 24h, 30°C	+	ISTA
Beckmannia eruciformis (L.) Host	-	imbibe or soak, 6-18h	+	1%, 24h, 30°C	+	ISTA
Brachiaria decumbens Stapf	-	imbibe, 16h, 30°C	+	1%, 4h, 35°C	-	Beavis
Brachiaria spp.	-	imbibe or soak, 6-18h	+	1%, 24h, 30°C	+	ISTA
Bromus catharticus Vahl.	-	imbibe, 16h, 25°C	+	0.5%, 3-6h, 25°C	-	DSRL
Bromus spp.	-	imbibe or soak, 6-18h	+	0.5%, 4-6h, 30°C	-	ISTA
	-	imbibe or soak, 6-18h	+	1%, 20-24h, 30°C	+	ISTA
Cenchrus ciliaris L.	-	imbibe or soak, 6-18h	+	0.5%, 4-6h, 30°C	-	ISTA
	-	imbibe or soak, 6-18h	+	1%, 24h, 30°C	+	ISTA
Chloris gayana Kunth	-	imbibe or soak, 18h	+	0.5%, 6h, 30°C	-	ISTA
	-	imbibe or soak, 18h	+	1%, 24h, 30°C	+	ISTA
Cynodon dactylon (L.) Pers.	-	imbibe or soak, 6-18h	+	1%, 16-24h, 30°C	+	ISTA
	-	imbibe, 16h, 30°-35°C	+	0.5-1%, 12-4h, 25°C	-	DSRL
Cynosurus cristatus L.	-	imbibe or soak, 6-18h	+	0.5%, 6-24h, 30°C	-	ISTA
	-	imbibe or soak, 6-18h	+	1%, 16-24h, 30°C	+	ISTA
Dactylis glomerata L.	-	imbibe or soak, 6-18h	+	1%, 16-24h, 30°C	+	ISTA
Deschampsia spp.	-	imbibe or soak, 6-18h	+	1%, 16-24h, 30°C	+	ISTA
Echinochloa crus-galli (L.) P. Beauv.	-	imbibe or soak, 6-18h	+	0.5%, 6-24h, 30°C	-	ISTA
	-	imbibe or soak, 6-18h	+	1%, 18-24h, 30°C	+	ISTA
Eleusine coracana (L.) Gaertn.	-	soak, 18h, 5°C	+	0.5%, 3h, 30°C	-	A&K
Eragrostis curvula (Schrad.) Nees	-	imbibe or soak, 6-18h	+	1%, 18-24h, 30°C	+	ISTA
Festuca arundinacea Schreber	-	imbibe, 16h, 25°C	+	0.5%, 3-6h, 25°C	-	DSRL

1	2	3	4	5	6	7
Festuca spp.	-	imbibe or soak, 6-18h	+	0.5%, 4-6h, 30°C	+	ISTA
Holcus lanatus L.	-	imbibe or soak, 6-18h	+	0.5%, 4-6h, 30°C	-	ISTA
	-	imbibe or soak, 6-18h	+	1%, 18-24h, 30°C	+	ISTA
Hordeum vulgare L.	-	imbibe or soak, 6-18h	+	0.5%, 3h, 30°C	-	ISTA
	-	imbibe or soak, 6-18h	+	1%, 6h, 30°C	-	ISTA
Hordeum spp.	-	imbibe, 16h, 30°C	+	0.5%, 0.5-1h, 40°C	-	OSRL
Lolium spp.	-	imbibe or soak, 6-18h	+	0.5%, 3-6h, 30°C	+	ISTA
	-	imbibe, 16h, 25°C	+	0.5%, 3-6h, 25°C	-	OSRL
Melinis minutiflora P. Beauv.	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	+	ISTA
Oryza glaberrima Steud.	-	soak, 16h, 30°C	+	0.5%, 2h, 40°C	-	A
	+	imbibe, 16h, 25°-30°C	+	0.5%, 3-6h, 25°C	-	OSRL
Oryza sativa L.	-	imbibe or soak, 18h	+	0.5%, 3h, 30°C	-	ISTA
	+	imbibe, 16h, 25°-30°C	+	0.5%, 3-6h, 25°C	-	OSRL
	+	imbibe, 16h, 30°C	-	0.5%, 3-6h, 25°C	-	OSRL
	-	soak, 16h, 30°C	+	0.5%, 2h, 40°C	-	A
Oryzopsis hymenoides Ricker	-	soak, 24h	-	1%, 4-8h, 35°C	+	M&K
Oryzopsis miliacea Asch. & Schw.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Panicum maximum Jacq.	+	soak, 16h	+	0.5%, 4h, 40°C	-	HHEA
Panicum ramosum L.	-	imbibe, 16h, 30°C	+	1%, 4-6h, 30°C	-	Andersen
Panicum spp.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Paspalum dilatatum Poir.	-	imbibe, 16h, 30°C	+	0.5-1%, 8-10h, 25°C	-	OSRL
Paspalum notatum Flugge	-	imbibe, 16h, 30°C	+	0.5-1%, 6-8h, 25°C	-	OSRL
	-	imbibe, 16h, 30°C	+	0.5-1%, 8-12+h, 25°C	-	OSRL
Paspalum spp.	-	imbibe or soak, 6-16h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Pennisetum typhoides Stapf & Hubbard	-	imbibe or soak, 6-16h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Phalaris spp.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Phleum pratense L.	-	imbibe, 16h, 30°-35°C	+	0.5-1%, 2h, 12+h	+	OSRL
Phleum spp.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Poa pratensis L.	-	imbibe, 16h, 30°-35°C	+	0.5-1%, 12+h, 25°C	+	OSRL
Poa spp.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Secale cereale L.	-	imbibe or soak, 6-18h	+	0.5%, 2-3h, 30°C	-	ISTA
	-	imbibe, 16h, 30°C	+	0.5%, 0.5-1h, 40°C	-	OSRL
Setaria spp.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Sorghum almum Parodi	-	soak, 4-6h, 30°C	+	0.5%, 1-2h, 40°C	-	OSRL
Sorghum bicolor (L.) Moench	-	imbibe, 16h, 30°C	+	0.5%, 0.5-1h, 40°C	-	OSRL
Sorghum halepense (L.) Pers.	-	imbibe, 16h, 30°C	+	0.5%, 6-8h, 20°-25°C	-	OSRL
Sorghum sudanense (Piper) Stapf	-	soak, 4-6h, 30°C	+	0.5%, 1-2h, 40°C	-	OSRL
Sorghum spp.	-	imbibe or soak, 18h	+	0.5-1%, 3-24h, 30°C	+	ISTA
Stipa viridula Trin.	-	imbibe, 15h	+	0.1%, 2h, 35°C	-	F&L
Trisetum flavescens (L.) P. Beauv.	-	imbibe or soak, 6-18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Triticum spp.	-	imbibe or soak, 6-18h	+	0.5%, 2-4h, 30°C	-	ISTA
	-	imbibe or soak, 6-18h	+	1%, 6-24h, 30°C	-	ISTA
	-	imbibe, 16h, 30°C	+	0.5%, 1-2h, 20°-25°C	-	OSRL
Zea mays L.	-	imbibe or soak, 18h	+	0.5-1%, 2-6h, 30°C	-	ISTA
	-	imbibe, 16h, 30°C	+	0.5%, 30 min, 40°C	-	OSRL
Zizania aquatica L.	+	nil	+	0.25%, 12h	-	Simpson
Zoysia japonica Steud.	-	imbibe or soak, 6-18h	+	0.5-1%, 18-24h, 30°C	+	ISTA
		LABIATAE				
Galeopsis ochroleuca		soak, 16h, 20°C	+	1%, 6-8h, 30°C		Novotna
Hyssopus officinalis L.		soak, 16h, 20°C	+	1%, 4h, 30°C		Novotna
Lavandula angustifolia Mill.		soak, 12h, 20°C	+	0.5%, 4h, 30°C		Novotna
Leonurus cardiaca		soak, 16h, 20°C	+	0.5%, 6h, 30°C		Novotna
Marrubium vulgare L.		soak, 16h, 20°C	+	1%, 3h, 30°C		Novotna
Melissa officinalis L.		soak, 16h, 20°C	+	0.5%, 6h, 30°C		Novotna
Ocimum basilicum L.	+	imbibe or soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
		soak, 20h, 20°C	+	0.5%, 5h, 30°C		Novotna
Salvia officinalis L.		soak, 20h, 20°C	+	1%, 5h, 30°C		Novotna
Satureja hortensis L.		soak, 20h, 20°C	+	0.5%, 4h, 30°C		Novotna
Thymus serpyllum L.		soak, 16h, 20°C	+	0.5%, 4-7h, 30°C		Novotna
Thymus vulgaris L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		LEGUMINOSAE				
Arachis hypogaea L.	+	imbibe or soak, 18h	+	0.5-1%, 20-24h, 30°C	+	ISTA
	+	imbibe, 18h, then soak, 2-3h	-	1%, 24h, 40°C	+	ISTA
Cicer arietinum L.	+	imbibe or soak, 18h	+	1%, 6-24h, 30°C	+	ISTA
Galega officinalis L.		soak, 20h, 20°C	+	1%, 3-4h, 30°C		Novotna

1	2	3	4	5	6	7
Glycine max (L.) Merr.	-	imbibe, 18h, then soak, 2-3h	+	0.5-1%, 6-24h, 30°C	+	ISTA
	-	imbibe, 16h, 25°C	+	1%, 2-4h, 40°C	-	DSRL
	-	imbibe, 16h, 25°C	+	1%, 5-7h, 25°C	-	DSRL
Lathyrus hirsutus L.	+	imbibe, 16h, then soak, 2-3h	+	1%, 6-24h, 30°C	+	ISTA
	-	soak, 3-4h, 25°C	-	1%, 2-3h, 40°C	-	DSRL
	-	soak, 3-4h, 25°C	-	1%, 5-7h, 25°C	-	DSRL
Lens culinaris Medic.	+	imbibe, 18h, then soak, 2-3h	+	1%, 6-24h, 30°C	+	ISTA
Lespedeza spp.	+	imbibe or soak, 18h	+	0.5-1%, 3-6h, 30°C	+	ISTA
	+	soak, 2h, 25°C	-	1%, 3-5h, 40°C	-	DSRL
	+	soak, 2h, 25°C	-	1%, 8h, 25°C	-	DSRL
Lotus spp.	+	soak, 22h	±	0.5-1%, 6-24h, 30°C	+	ISTA
Lupinus spp.	+	soak, 22h	±	0.5-1%, 6-24h, 30°C	+	ISTA
Medicago sativa L.	-	soak, 2h, 25°C	-	1%, 3-4h, 40°C	+	DSRL
	-	soak, 2h, 25°C	-	1%, 7-8h, 25°C	+	DSRL
Medicago spp.	+	soak, 22h	±	0.5-1%, 6-24h, 30°C	+	ISTA
Melilotus spp.	+	soak, 22h	±	0.5-1%, 6-24h, 30°C	+	ISTA
	-	soak, 2h, 25°C	-	1%, 4-5h, 40°C	+	DSRL
	-	soak, 2h, 25°C	-	1%, 8-10h, 25°C	+	DSRL
Mucuna deeringiana (Bort) Merr.	+	soak, 22h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Onobrychis viciaefolia Scop.	+	soak, 22h	±	0.5-1%, 4-24h, 30°C	+	ISTA
Ononis arvensis		soak, 16h, 20°C	+	1%, 3h, 30°C		Novotna
Ornithopus sativus Brot.	+	soak, 22h	±	0.5-1%, 4-24h, 30°C	+	ISTA
Phaseolus spp.	-	imbibe, 18-24h, then soak, 2-3h	±	0.5-1%, 6-24h, 30°C	+	ISTA
	-	imbibe, 16h, 25°C	-	1%, 2-3h, 40°C	+	DSRL
	-	imbibe, 16h, 25°C	-	1%, 6-8h, 25°C	+	DSRL
Pisum sativum L.	-	imbibe, 18-24h, then soak, 2-3h	±	0.5-1%, 6-24h, 30°C	+	ISTA
Pisum spp.	-	soak, 3-6h, 25°C	-	1%, 2-3h, 40°C	+	DSRL
	-	soak, 3-6h, 25°C	-	1%, 6-8h, 25°C	+	DSRL
Pueraria lobata (Willd.) Ohwi	+	soak, 22h	±	0.5-1%, 6-24h, 30°C	+	ISTA
Trifolium incarnatum L.	-	soak, 2h, 25°C	-	0.5-1%, 2-4h, 40°C	+	DSRL
	-	soak, 2h, 25°C	-	0.5-1%, 4-6h, 25°C	+	DSRL
Trifolium pratense L.	-	soak, 2h, 25°C	-	1%, 4-6h, 40°C	+	DSRL
	-	soak, 2h, 25°C	-	1%, 6-10h, 25°C	+	DSRL
Trifolium repens L.	-	soak, 2-3h, 25°C	-	1%, 4-6h, 40°C	+	DSRL
	-	soak, 2-3h, 25°C	-	1%, 6-10h, 25°C	+	DSRL
Trifolium spp.	+	soak, 22h	±	0.5-1%, 4-24h, 30°C	+	ISTA
Trigonella foenum-graecum L.		soak, 16h, 20°C	+	1%, 4-5h, 30°C		Novotna
Vicia faba L.	+	soak, 22h	±	0.5-1%, 16-24h, 30°C	+	ISTA
Vicia spp.	+	soak, 22h	±	0.5-1%, 16-24h, 30°C	+	ISTA
	-	soak, 3-4h, 25°C	-	1%, 2-3h, 40°C	+	DSRL
	-	soak, 3-4h, 25°C	-	1%, 5-7h, 25°C	+	DSRL
Vigna spp.	+	soak, 22h	±	0.5-1%, 16-24h, 30°C	+	ISTA
	-	soak, 16h, 25°C	-	1%, 2-3h, 40°C	+	DSRL
	-	soak, 16h, 25°C	-	1%, 4-6h, 25°C	+	DSRL
		LILIACEAE				
Allium spp.	-	imbibe or soak, 18h	+	1%, 24-28h, 30°C	+	ISTA
Asparagus officinalis L.	-	imbibe or soak, 18h	+	0.5-1%, 20-24h, 30°C	+	ISTA
		LINACEAE				
Linum usitatissimum L.	+	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		MAGNOLIACEAE				
Liriodendron spp.	+	soak, 18h	+	1%, 24-48h, 30°C	+	ISTA
		MALVACEAE				
Althea rosea Cav.		soak, 24h, 20°C	+	1%, 3h, 30°C		Novotna
Gossypium spp.	+	imbibe or soak, 18h	+	1%, 6-24h, 30°C	+	ISTA
	+	imbibe, 16h, 30°C	+	0.5-1%, 1-2h, 40°C	-	DSRL
	+	imbibe, 16h, 30°C	+	0.5-1%, 2-4h, 25°C	-	DSRL
Hibiscus cannabinus L.	-	nil	-	1%, 4-6h, 40°C	+	DSRL
Malva sylvestris L.		soak, 12h, 20°C	+	0.5%, 4h, 30°C		Novotna
		MORACEAE				
Cannabis sativa L.	-	imbibe or soak, 18h	+	0.5%, 4-6h, 30°C	+	ISTA
	-	imbibe or soak, 18h	+	0.5-1%, 20-24h, 30°C	+	ISTA

1	2	3	4	5	6	7
		MYRTACEAE				
Psidium guajava L.	-	nil	+	1%, 12-14h	-	S&P
		OLEACEAE				
Fraxinus spp.	+	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Ligustrum spp.	-	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
		PALMACEAE				
Elaeis guineensis Jacq.	+	soak, 16h	+	1%, 14h, 28°C	-	Mok
Oreodoxa regia HBK	+	nil	+	1%, 24h, 30°C	-	Soetisna
		PAPAVERACEAE				
Glaucium flavum Crantz		soak, 16h, 20°C	+	0.5%, 6-8h, 30°C		Novotna
Papaver somniferum L.	-	imbibe or soak, 18h	+	0.5-1%, 24-48h, 30°C	+	ISTA
		PEDALIACEAE				
Sesamum indicum L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		PINACEAE				
Abies spp.	-	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
Pinus cembra L.	-	soak, 24h	+	1%, 24-36%, 30°C	+	ISTA
Pinus coulteri D. Don	-	soak, 24h	+	1%, 24-36h, 30°C	+	ISTA
Pinus koraiensis Sieb. & Zucc.	-	soak, 24h	+	1%, 24-36%, 30°C	+	ISTA
Pinus spp.	-	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
Pseudotsuga spp.	-	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
		PLANTAGINACEAE				
Plantago lanceolata L.		soak, 24-30h, 20°C	+	0.5%, 3h, 30°C		Novotna
		POLYGONACEAE				
Fagopyrum esculentum Moench	-	imbibe or soak, 18h	+	1%, 6-24h, 30°C	+	ISTA
Rheum palmatum L.		soak, 20h, 20°C	+	1%, 5h, 30°C		Novotna
Rheum rhaponticum L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Rumex acetosa L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		RHAMNACEAE				
Rhamnus spp.	+	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
Zizyphus mauritiana Lam.	-	nil	+	1%, 1.5h	-	S&R
		ROSACEAE				
Agrimonia eupatoria		soak, 24h, 20°C		1%, 6h, 30°C		Novotna
Amelanchier spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Cotoneaster spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Crataegus spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Malus spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Prunus spp.	+	soak, 18h	+	1%, 4-8h, 30°C	-	ISTA
	+	soak, 18h	+	0.5%, 10-12h, 30°C	-	ISTA
Pyrus spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Rosa spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Rubus spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
Sorbus spp.	-	soak, 18h	+	1%, 16-24h, 30°C	+	ISTA
		RUBIACEAE				
Coffea arabica L.	-	soak, 4d	+	1%, 4h, 35°C	-	G&V
Coffea canephora Pierre ex Froehner	-	soak, 4d	+	1%, 4h, 35°C	-	G&V
		RUTACEAE				
Citrus natsudaidai Hayata	-	nil	+	0.1%, 3h, 40°C	-	H&N
Ruta graveolens L.		soak, 16-20h, 20°C	+	1%, 6h, 30°C		Novotna
		SCROPHULARIACEAE				
Digitalis lanata Ehrh.		soak, 16h, 20°C	+	0.5-2%, 5h, 30°C		Novotna
Verbascum thapsiforme Schrad.		soak, 16h, 20°C	+	0.5%, 4-5h, 30°C		Novotna
		SOLANACEAE				
Atropa belladonna L.		soak, 16h, 20°C	+	1%, 10h, 30°C		Novotna
Capsicum spp.	-	imbibe or soak, 18h	+	0.5-1%, 24h, 30°C	+	ISTA
Lycopersicon lycopersicum Karst.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA

1	2	3	4	5	6	7
Nicotiana tabacum L.	-	imbibe, 18-24h	+	0.5-1%, 18-24h, 30°C	+	ISTA
Physalis ixocarpa L.	-	imbibe or soak, 18h	+	0.5-1%, 18-24h, 30°C	+	ISTA
Solanum melongena L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		TAXACEAE				
Taxus spp.	-	soak, 18h	+	1%, 24-48h, 30°C	+	ISTA
		TAXODIACEAE				
Taxodium distichum (L.) Rich.	-	soak, 18h	+	1%, 24-48h, 30°C	+	ISTA
		TILIACEAE				
Tilia spp.	+	soak, 18h	+	1%, 18-24h, 30°C	+	ISTA
		UMBELLIFERAE				
Anethum graveolens L.	-	imbibe, 18h	+	1%, 20-24h, 30°C	+	ISTA
Apium graveolens L.	-	imbibe, 18h	+	1%, 6-24h, 30°C	+	ISTA
Archangelica officinalis Hoffm.		soak, 20h, 20°C	+	1%, 4h, 30°C		Novotna
Carum carvi L.	-	imbibe or soak, 18h	+	1%, 24h, 30°C	+	ISTA
Cuminum cymirum L.	-	imbibe or soak, 18h	+	1%, 6-24h, 30°C	+	ISTA
Daucus carota L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Levisticum officinalis Koch		soak, 16h, 20°C	+	0.5-1%, 4h, 30°C		Novotna
Pastinaca sativa L.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
Petroselinum crispum Nym.	-	imbibe or soak, 18h	+	0.5-1%, 6-24h, 30°C	+	ISTA
		VALERIANACEAE				
Valeriana officinalis L.		soak, 16h, 20°C	+	1%, 3h, 30°C		Novotna
Valerianella locusta (L.) Laterr.	-	imbibe or soak, 18h	+	0.5-1%, 18-24h, 30°C	+	ISTA
		VITACEAE				
Vitis spp.	-	soak, 16h, 30°C	+	0.5%, 2-3h, 40°C	-	A

subsequent evaluation of the seeds. One litre of lactophenol solution can be prepared from 200 ml phenol, 200 ml lactic acid, 400 ml glycerin, and 200 ml water.

Evaluation

To evaluate the seeds it is necessary for the embryo and other essential structures (those necessary for the development of normal seedlings - see Chapter 10) to be exposed. In many cases preparation of the seeds before staining will have already exposed the embryo and other essential structures, but in other cases it may be necessary to prepare the stained seeds for evaluation. Column 6 of Table 11.1 indicates whether any preparation for evaluation is required. The types of preparation required at this stage are generally similar to those given previously for preparation for staining. Additional shaving off of small slices of tissue may be helpful.

After staining, washing and any further preparation, the seeds, or dissected seeds, are then evaluated for stain intensity and stain location. Often a low-power binocular microscope or magnifying lens is required. The seeds fall into three groups:

(1) completely stained and thus viable seeds;
(2) completely unstained and presumably non-viable seeds;
(3) partially stained seeds, the classification of which as either viable or non-viable is considerably more difficult. The references at the end of this chapter provide examples of the staining patterns observed in normal practice and should be consulted for further advice.

It should be noted that stain intensity (colour) is also important. Viable tissues should stain a bright red (sometimes described as carmine red). Both pink and very dark red stains are indicative of dead tissue. This is particularly important for seeds of dicotyledonous species where differential staining may be observed on the surfaces of the cotyledons. Note also, however, that viable seed embryos will be stained a very dark red colour if the staining period is too long.

CALIBRATION OF THE TETRAZOLIUM TEST

The tetrazolium test should not be regarded as an absolute test of seed viability. Before the results obtained can be used with confidence the test must first be calibrated. This requires a comparison, for each species, of the staining patterns observed in tetrazolium tests with the results of germination tests on the same accessions where the results of the latter have not been confounded with either dormancy or poor seed quality: that is where all viable seeds are know to have germinated. It is preferable to do this on several seed lots of differing percentage viability. Note also that, where imbibition injury, hardseededness or delayed imbibition has lead to germination tests underestimating accession viability, tetrazolium tests will suffer from the same deficiency: although the two test results will agree, viability will be underestimated. Thus a good deal of preparatory work is required before the tetrazolium test can be applied with confidence. Similar considerations apply to the indigocarmine tes described below.

THE INDIGOCARMINE TEST FOR VIABILITY

Indigocarmine (disodium 5,5'-indigtin disulphonate; soluble indigo blue) is widely used in Poland, Roumania, USSR and Yugoslavia for determining the viability of tree and shrub seeds. It is reported to be as accurate as tetrazolium staining procedures' and has the advantage of being cheaper, and safer (lower human toxicity) than tetrazolium.

Indigocarmine is a dark blue powder with a copper lustre. In solution it is blue or bluish-purple colour. Like tetrazolium it is sensitive to light and therefore should be kept in the dark. Unlike tetrazolium, however, indigocarmine solutions stain the dead areas of embryos. Stained areas are blue, whereas the live areas of embryos remain unstained. The following summary of the indigocarmine method of testing seeds is largely based upon information provided by Rostovtsev and Lyubich.

Preparation of indigocarmine solution

One gramme of indigocarmine will dissolve in 100 ml of water at 25°C. Solutions of 0.05% (weight by volume; i.e. 1 g in 2 litres of water) indigocarmine are used for seed testing. However, not all of the indigocarmine powder will be soluble; a proportion will be insoluble. Consequently for any batch of powder it is necessary to determine the proportion of insoluble material. This is done as follows. One gramme of indigocarmine is dissolved in two litres of boiling water for 30 minutes. After cooling the solution is filtered and the weight of undissolved indigocarmine (after drying at 100°-105°C to constant weight) is determined (a). The proportion of dissolved indigocarmine is thus (1-a)/1. Thus to prepare a 0.05% solution 1/(1-a) g of powder is boiled for 30 minutes in two litres of water. This solution is subsequently cooled and then filtered. Cooled boiled water (indigocarmine is likely to oxidise, hence boiling is required to remove dissolved oxygen) is then added to make the solution up to two litres. The prepared solution should be stored in the dark for not more than 15 days.

Preparation of seeds

In general seeds are soaked in water at 18°-20°C for 1 to 2 days with daily changes of water. Specific treatments have been provided for the following genera.

Tilia Crack the fruits, puncture the seeds and immerse in water for 24 to 48 hours.
Cornus Soak for up to 3 days if necessary and then crack into half. The halves with intact embryos are then immersed in water for two hours.
Corylus Crack the nuts and soak for 1 to 2 days. Split into two. The cotyledon to which the radicle is attached is extracted and the seed coat removed.
Prunus Crack the stones (see the method described for the excised embryo test) and soak for two days.
Fraxinus Soak for 2 to 3 days.

As with the excised embryo and tetrazolium tests, the soaking treatments may result in imbibition injury. Consequently it is necessary to consider the use of humidification treatments (Chapter 11).

Staining of embryos

The moist embryos are extracted and those undamaged by extraction are immersed in the dark in the 0.05% indigocarmine solution for two hours.

Evaluation of embryos

Embryos of the above species are considered viable if

(a) the embryo is complete unstained
or (b) there is only a faint stain at the radicle tip
or (c) there are only a few spots of stain on the cotyledons opposite the radicle
or (d) the embryo shows only pale surface staining.

RAPID VIABILITY TESTS

The excised embryo, tetrazolium, and indigocarmine tests are used in the commercial seed trade as rapid tests of viability, where the delay associated with germination testing is unacceptable. In contrast gene banks are only likely to apply these tests where dormancy is suspected of confounding the results of germination tests. Several other types of rapid viability tests are used in the seed trade. In general we believe their application in routine gene bank work will be limited. Consequently we have not provided full details of the procedures to be followed. Nevertheless a brief description of several tests is provided here for background information since it is conceivable that they might have some research application in gene banks.

Seed leachates

Two types of seed leachate conductivity tests are used. In these tests seeds are soaked in a given volume of water at a set temperature and for a set period. This seed steep water is then decanted and the concentration of leachates in solution determined. In the electrical conductivity test the concentration of ions is determined with electrodes and a conductivity meter. In the urine test paper method the concentration of sugars is determined by the change in colour of a urine test paper dipped in the solution. In both tests the greater the concentration of ions or sugars in the leachate the poorer quality the seed lot. However, it should be noted that it is difficult to interpret the results solely in terms of viability, since the results are also influenced greatly by the condition of the seed covering structures. (See Chapters 4 and 7 on imbibition injury to understand why this is so.)

Glutamic acid decarboxylase activity

The glutamic acid decarboxylase activity (GADA) test is made on unimbibed, homogenised seeds. In this test glutamic acid is added to the homogenate and the resultant evolution of carbon dioxide is measured; the rate of CO_2 evolution is dependent on the activity of the decarboxylases present within the seed homogenate. Thus the more carbon dioxide evolved per gramme of seed homogenate per hour the higher the viability of the accession. However, since the test has to be calibrated for each cultivar it is unlikely to be of use in gene banks.

X-radiography

Finally mention should be made of the use of X-ray techniques to provide rapid estimates of seed viability. These are discussed briefly in Chapter 8.

REFERENCES

The references to the abbreviations of the sources of the tetrazolium staining procedures given in Table 11.1 are provided below together with other useful sources of information.

A The authors of this handbook.

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CHAPTER 12. THE STATISTICS OF SAMPLING SEEDS FROM ACCESSIONS

The purpose of this chapter is to explain the principles upon which the techniques and recommendations in the three chapters which follow (Chapters 13, 14 and 15) are based. It is possible, however, to follow the advice in those chapters without reference to the explanations provided here. Although the examples used in this chapter refer mainly to germination tests designed to estimate the viability of accessions and/or decide whether regeneration is required, the same principles apply to other viability tests (e.g. the tetrazolium test, Chapter 11) and the sampling theory applies to samples withdrawn from accessions for any purpose - e.g. seed purity analysis (Chapter 8), seed health testing (Chapter 1), the estimation of seed moisture content and mean seed weight (Chapter 2), and the provision of sub-samples for regeneration, multiplication, evaluation or distribution.

SAMPLING

In earlier chapters it has been emphasised that an accession, or seed lot, is a population consisting of individual seeds some of which may be alive (viable) and some of which may be dead (non-viable). If we wished to determine the proportion of seeds in the accession which were viable with absolute precision, then we should have to test each and every seed for germination. Clearly this is a nonsense. To avoid this we accept something less than absolute precision and only test a small part of the accession for germination; that is a small sample of seeds is abstracted from the accession. The result of the test on this sample is thus an estimate of the viability of the accession as a whole. For commercial seed lots sampling can be a considerable problem for, in extreme cases, a single seed lot can weigh more than 40 tonnes! With accessions in gene banks, however, the process of sampling is physically easier since normally one expects to be sampling from a bulk of fewer than 20,000 seeds.

The problem of biased samples

In sampling one is attempting to produce a copy of the complete accession, but with fewer seeds. To do this the selection of individual seeds to comprise the sample must be random; that is without bias. Selection of seeds by eye to produce the sample would inevitably lead to some form of bias; for example, the selection of regular, whole, plump, shiny seeds in preference to broken, cracked, shrivelled, discoloured seeds. It is perhaps worth noting at this stage a further important reason for the effective random sampling of seed from accessions in gene banks for germination testing: unless random sampling is practised, not only may the estimate of whatever attribute of the accession is being tested for in the seed sample be in error, but also the seeds remaining in store in genetically heterogeneous accessions may have lost genes from the gene pool through preferential sampling of certain seed phenotypes. In addition, in both genetically homogeneous and heterogeneous accessions, the remaining seeds may show poorer agronomic features in the first generation - emergence, etc. - due to high proportions of broken, shrivelled, or discoloured seeds, etc. This may jeopardize the regeneration process and also might give breeders or evaluators of the accession a poorer, and false, impression of the genetic potential of the accession. It thus behoves gene banks to sample correctly (i.e. randomly) not only when sampling to monitor viability, but also when removing seeds from accessions for any other purpose.

There are a number of methods of sampling small numbers of seeds from larger bulks. Since accurate sampling is of such importance in gene banks, Chapter 13 discusses sampling equipment and procedures for those without any formal or practical training in sampling methods.

APPLICATION OF PROBABILITY THEORY TO ENSURE THAT SUB-SAMPLES OF GENETICALLY HETEROGENEOUS ACCESSIONS CONTAIN GENOTYPES WHICH REPRESENT A MINORITY OF THE ACCESSION

In genetically heterogeneous accessions staff of the gene bank must, to the best of their abilities, ensure that any one sub-sample derived from the accession for evaluation or distribution contains genes which are represented in only a minority of seeds within the population. Now classic probability theory states that the probability of an individual with a given character being selected is given by the proportion of individuals within the population which possess this character. Thus if a character is present within 5% of the seed population, then the probability that a seed with this character is selected when one seed is drawn at random from the population is also 5%, or 0.05. Similarly the probability of not selecting a seed with this character is 95%, or 0.95.

Probability of the presence of a character in a sample drawn from a genetically heterogeneous accession

Now, of course, no seed bank intends to supply breeders with sub-samples of accessions which contain only one seed. How then do we determine the probability that a sample from an accession of a given number of seeds does, or does not, contain at least one individual with the (minority) characteristic? If a second seed were sampled from the accession then the probability of this second seed showing the desired character is the same as the probability calculated for the first seed. The two samplings are independent of each other; the result of the first sampling does not affect the probability of the second sampling. (In a large accession we can ignore the fact that removing a single seed from the accession will change the frequency with which characters are represented in the remaining population, since the change will be imperceptible.) Now if the probability of the character not being present in the first seed is 0.95, and the probability of the character not being present in the second seed is also 0.95, what is the probability of the character not being present in either seed? The answer to this question is obtained by multiplying the two probabilities together. If we denote the probability of selecting seeds without the minority character by the symbol P(A), then if two seeds are selected the probability of the character not being present in both seeds is given by P(A) times P(A). In this example this gives 0.95 x 0.95 which equals 0.9025. This can now be extended by saying that if n seeds are sampled then the probability of the character not being represented by at least one seed in the sample is given by $[P(A)]^n$. Clearly the gene bank would wish if possible to provide sufficient seeds (that is increase the value of n) such that the probability of the minority character not being present in the sub-sample provided for evaluation or distribution is very low.

Based on these principles, Chapter 13 provides information on the relationships between the probability of a character being present in a sample, the frequency of the character in the accession, and sample size. Advice is also provided in Chapter 13 as to how to ensure adequate sample sizes when accessions also contain empty or dead seeds. No attempt is made here to suggest suitable sample sizes to ensure that the proportional composition of genes within the sub-sample accurately reflects the proportions of the various genes in the original accession but, if necessary, the equations provided in the remainder of this chapter could be used for this purpose. However their main function here is to determine the accuracy of germination test results.

THE BINOMIAL DISTRIBUTION OF ERROR RESULTING FROM PERFECT RANDOM SAMPLING

Throughout this chapter the term viability will be used to describe either the estimated or true proportion of live seeds in an accession. In the majority of cases it is envisaged that the tests applied to estimate viability will be germination tests in which, if necessary, appropriate techniques to break dormancy (see Chapter 5) have been applied. Inevitably we have decided to accept something less than absolute precision in our viability tests by only testing a sample of seeds from the accession - not the complete accession. But how precise is our estimate?

The variation resulting from random sampling error can be estimated theoretically from the binomial distribution. This distribution can be used if the following conditions are satisfied. (Statements in parenthesis give explicit meaning for seed testing application.)

1. The experiment consists of a fixed number of trials, denoted by the letter n. (n is the number of seeds sampled for each viability test.)
2. Each trial has only two possible outcomes, usually success or failure. (Each seed will prove either viable or non-viable.)
3. The outcome of any one trial is independent of the outcome of any other trial. (The result of the test on one seed does not influence the result of testing any other seed in the viability test.)
4. The probability of success, denoted by the letter p, is constant from trial to trial. (Each seed is a member of the same accession.)

The effect of viability on the accuracy of germination tests

For <u>percentage</u> viability the standard deviation (σ) resulting from random sampling error according to the theoretical binomial distribution can be calculated from the following equation

$$\sigma = \sqrt{[p(100-p)/n]} \qquad (12.1)$$

where p is percentage germination, (100-p) is the percentage of seeds not germinating and n is the number of seeds tested.

To consider the influence of percentage viability on the standard deviation let us ignore the value of n for the time being by setting it to the value 1 (the effect of sample size, n, on the accuracy of viability tests will be considered in the next section). Table 12.1 shows that as p moves from 50% towards either 0% or 100% the value $\sqrt{[p(100-p)/n]}$ declines.

Table 12.1 Relationship between p and $\sqrt{[p(100-p)/n]}$, where n is 1

p	0	5	10	15	20	25	30	35	40	45	
	or	or	or	or	or	or	or	or	or	or	50
p	100	95	90	85	80	75	70	65	60	55	
$\sqrt{[p(100-p)/1]}$	0	21.8	30.0	35.7	40	43.3	45.8	47.7	49.0	49.7	50.0

There are a number of points to note from Table 12.1.

1. The relationship between percentage viability and standard deviation is symmetrical about 50% viability.
2. The value $\sqrt{[p(100-p)/n]}$ is zero for 0% or 100% viability. This is because there can be no sampling error for these values: if every seed in the accession is dead then it is not possible to sample a live seed; if every seed in the accession is alive then it is not possible to sample a dead seed.
3. The standard deviation, for a fixed sample size, is greatest at 50% viability.
4. Between 100% and 50% viability, for a fixed sample size, the standard deviation increases quite rapidly at first but the increase declines progressively towards a plateau between 65% and 50%. (This can be seen more clearly in subsequent figures relating standard deviation, percentage viability and sample size.)

The practical implication of this for seed testing is that, between 50% and 100% viability, test results on seeds sampled from high viability accessions will more accurately estimate true accession viability than results from the same number of seeds sampled from accessions with poorer viability.

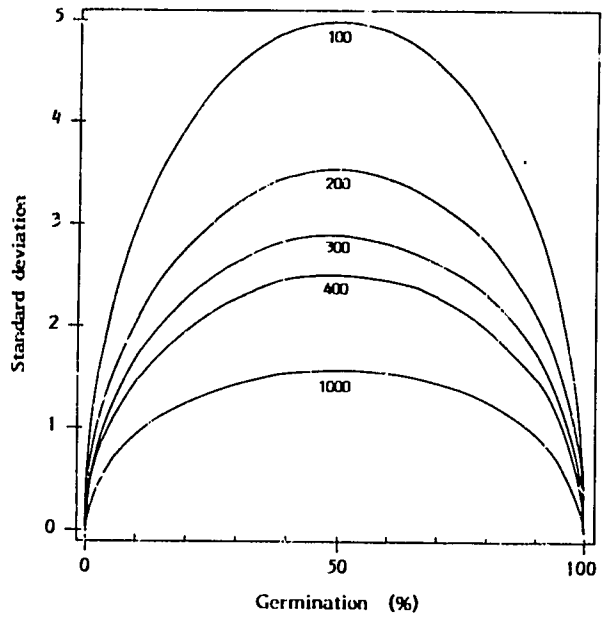
Practical limitations to theoretical considerations

All the points made above relate to the theoretical binomial distribution. Do the results of seed viability tests actually conform to this error distribution? The general form of the relationship between average actual standard deviation and percentage viability estimated from germination tests is very similar to that for the theoretical binomial distribution, but it is not identical. From our results with very many germination tests we have found that the agreement is very good between about 0% to 20% and 80% to 100% germination. Thus we can conclude that within these two ranges of percentage germination variation in test results of sampled seeds is solely due to the random selection of seeds. Between about 20% to 80% germination, however, the average actual standard deviations tend to be greater than the binomial distribution would predict. It is not difficult to suggest why this might be. We have already noted in Chapter 4 that the result of the germination test is much more vulnerable to slight differences in test conditions for seeds of accessions with intermediate levels of germination (particularly those accessions where viability is close to 50% than for accessions with very high germination. We can conclude then that between 20% to 80% viability variability in germination test results is brought about by both random sampling error and imperfect test conditions. In practice seed testers can ignore this discrepancy between the theoretical and actual error distributions, since the majority of decisions relating to germination test results will be for accessions with greater than 80% viability (because, as explained elsewhere, regeneration standards will normally be above this value). Thus for high viability accessions the binomial distribution of error provides an adequate basis for evaluating seed testing procedures and is applied in the following sections.

The effect of sample size on the accuracy of germination tests

Figure 12.1 plots the standard deviation predicted by the binomial distribution against percentage viability for several values of n, the number of seeds used for each test. Clearly the larger the sample size the lower the theoretical standard deviation. Note, however, the diminishing returns effect: the decrease in the standard deviation is not proportional to the increase in the number of seeds tested but to the increase in the square root of the number of seeds tested; for example increasing the size of n from 100 to 200 seeds reduces the standard deviation by about 30%, whereas a further increase in n by 100 to 300 seeds reduces the standard deviation further, but only by about 10%. Note also that the standard deviation for 100-seed tests at 95% viability is very similar to the value for 200-seed tests

Figure 12.1 Relationship between standard deviation and germination (%) for sample sizes of 100, 200, 300, 400 and 1000 seeds predicted by the binomial theorem.



at 90% viability, which in turn is very similar to that for 400-seed tests at 80% viability. This can be expressed in a slightly more useful way: as viability declines from 100% to 50% more seeds need to be sampled to maintain a constant degree of accuracy. This point will be returned to in a subsequent section of this chapter.

CONFIDENCE BOUNDS, LIMITS OR INTERVALS

Confidence statements are often used by seed physiologists when reporting results: for this reason an explanation of these terms is provided here. It should become clear, however, that confidence bounds, limits or intervals will have little or no practical application for decision-making procedures in gene banks. Consequently any reader who does not need an explanation of these terms can skip this section.

If we carry out a viability test on seeds sampled from an accession we will obtain a value for percentage viability (p in the equations which follow). We do not believe, however, that this estimate is the true value for the accession as a whole. But, we do hope that the values of the estimate and true viability are reasonably close. To derive some sort of range of confidence in our estimate of the true accession viability we can define what is known as a confidence interval. This is normally represented as c, d where c and d are two values of, in our case, percentage germination; c is the **lower confidence limit** (or bound), d the **upper confidence limit** (or bound) and the range of values between c and d the confidence interval. We must also give some idea of how likely true accession viability is to be within this confidence interval, or what is our degree of confidence in the confidence interval. The two most common values used for the degree of confidence are 95% and 99%, and the confidence interval of a viability test is reported as - the 95% confidence interval is c, d. If a is the degree of confidence

then the confidence interval is calculated as follows for percentage values,

$$p - (Z_{a/2}.\sigma + 50/n) \text{ to } p + (Z_{a/2}.\sigma + 50/n) \qquad (12.2)$$

where Z is the value obtained from standard normal tables. We can substitute equation (12.1) for the value of the standard deviation, σ, in the above equation and make this a little neater by writing as

$$p \pm [(Z_{a/2}.\sqrt{(p(100-p)/n)}) + 50/n] \qquad (12.3)$$

For a 95% degree confidence $Z_{a/2} = 1.96$ and for a 99% degree of confidence $Z_{a/2} = 2.575$. Thus for a 400 seed test result with percentage viability estimated at 90%, the 99% confidence interval is calculated as follows:

$$90 \pm [(2.575.\sqrt{(90 \times 10/400)}) + 50/400]$$

which gives $\quad 90 \pm ((3.8625) + 0.125)$

or $\quad 90 \pm 3.9875.$

Thus the 99% confidence interval is 86%, 94%. This of course is a theoretical confidence interval. In practice the value of σ used should be that obtained from variations between different viability tests. The value (50/n) is a correction factor enabling us to use the normal distribution to derive the value $Z_{a/2}$. In practice when the sample size is large, as in the example above, its value is negligible. A simplified version of equation (12.2) for large seed numbers is thus

$$p \pm (Z_{a/2}.\sigma) \qquad (12.4)$$

It is important to note that a confidence statement is not a probability statement. In the example above 'The 99% confidence interval is 86%, 94%' does not mean that there is a 99% probability of the true accession viability being between 86% and 94%. All this confidence statement means is that if we were to draw a large number of samples from the accession and find the confidence interval for each sample, then true accession viability would be within about 99% of these intervals. This is why confidence intervals are of no practical use in gene bank decision making procedures. In gene banks we wish to be able to make a decision after each and every viability test, and we wish to be able to attach probabilities of error to these decision-making procedures.

A SIGNIFICANT LOSS IN VIABILITY: THE TWO-TAILED TEST

One form of decision-making procedure of potential interest in gene banks is the following question. Has a significant loss in viability occurred since the accession was first placed in storage? To answer this question we must compare two values of percentage viability. The first value is an estimate of true accession viability when originally placed in store. The second value is an estimate of true accession viability at the present time. That is both values are estimates. Consequently both are subject to random sampling error.

The null hypothesis

In tests of significance we must first assume a null hypothesis. Here, to examine whether a difference between two percentages is significant, the null hypothesis that we test is that both values are equal to the mean of the two values, and we determine the probability that this hypothesis is true. The test is known as a two-tailed test because the two percentages p_1 and p_2 are located either side of the mean value $\bar{p} = (p_1 + p_2)/2$. To determine the probability of the null hypothesis being true we use the value of the normal deviate Z, where

$$Z = (p_1 - p_2)/\sqrt{\{\bar{p}(100 - \bar{p})(1/n_1 + 1/n_2)\}} \qquad (12.5)$$

and n_1 and n_2 are the number of seeds tested on each occasion.

Again a correction factor is applied to enable the normal distribution to be used in this analysis, where p_1 is not the initial estimate of percentage viability but 100 times the number of seeds initially estimated to be viable divided by the number of seeds tested, minus 0.5; similarly p_2 is not the percentage of seeds estimated to be viable after storage, but 100 times the number of seeds estimated to be viable after storage divided by the number of seeds tested, plus 0.5; and p_1 is greater than p_2.

As an example in the use of equation (12.5) let us consider that 400 seeds sampled from an accession when it was first put into store gave 95% viability and 400 seeds sampled from the accession after a period of storage showed 91% viability. Now $\bar{p} = 93\%$ and applying the correction factor gives $p_1 = 95.12 (= 100 \times 380/399.5)$ and $p_2 = 90.89 (= 100 \times 364/400.5)$.

Thus $Z = (95.12 - 90.89) / \sqrt{\{93 \times 7\ (1/400 + 1/400)\}} = 2.345$

Looking up Z in normal tables gives the two-tailed probability of 0.019; that is 2×0.0095 since this is a two-tailed test, p_1 and p_2 being both sides of $\bar{p}$. Thus we can reject the null hypothesis because there is a significant difference with the error probability less than 0.025 but greater than 0.01. Now the above is an average result, and we can attach a further probability to it. That is the probability of achieving this result is approximately 0.5. Suppose that the gene bank management decision procedure specified that it was desirable for accessions to be regenerated after a 4% loss in viability with an error probability of 0.05 and with a probability of 0.95 of achieving this desired result. How many seeds would we need to sample for each test of accession viability?

Number of seeds required to detect a significant loss in viability

This can be determined using the following formula

$$n = (Z_a + Z_b)^2.[p_1(100 - p_1) + p_2(100 - p_2)] / (p_2 - p_1)^2 \qquad (12.6)$$

where n is the number of seeds required for each germination test, Z_a is the normal deviate corresponding to the two-tailed significance required (a = 0.05 in this case) and Z_b is the two-tailed probability of obtaining the desired result (if the desired probability of achieving this result is 0.95 then the error probability is 1-0.95, and b = 2(1-0.95) = 0.10). If $p_1 = 95\%$ then, for a 4% loss in viability, p_2 is 91%.

Now $Z_a = 1.96$ and $Z_b = 1.645$. Thus $(Z_a + Z_b)^2 = 13.0$

and $n = 13(95 \times 5 + 91 \times 9) / (4)^2 = 1051.4$ seeds.

This example calculation emphasises that very large sample sizes are required to detect reliably a small loss in viability! Moreover, in addition to the requirement for large sample sizes, other considerations also suggest that such a two-tailed test is undesirable in gene banks. During the long period between accession viability monitoring tests there is the possibility of turnover in gene bank staff, equipment and even, perhaps, viability test procedures. Consequently the comparison of two test results on an accession in a two-tailed test may be subject to greater errors than would be predicted on the basis of random sampling error alone.

COMPARISON OF TEST RESULTS WITH A FIXED VALUE (e.g. THE REGENERATION STANDARD): THE ONE-TAILED TEST

What would happen if the gene bank compared the result of a viability test of seeds sampled from the accession after a period of storage with some fixed value (rather than with a previous test result)? We can call this fixed value the regeneration standard, this being the value of true accession viability at which we would like to ensure that regeneration is instituted: only if the test result is substantially higher than this value will we be reasonably confident that the true viability of the accession is above the regeneration standard. See both Chapters 14 and 15 for a further discussion of the regeneration standard and the probability of regeneration.

Number of seeds required for comparison with fixed value

For a one-tailed test the number of seeds required, n, is calculated from the following equation:

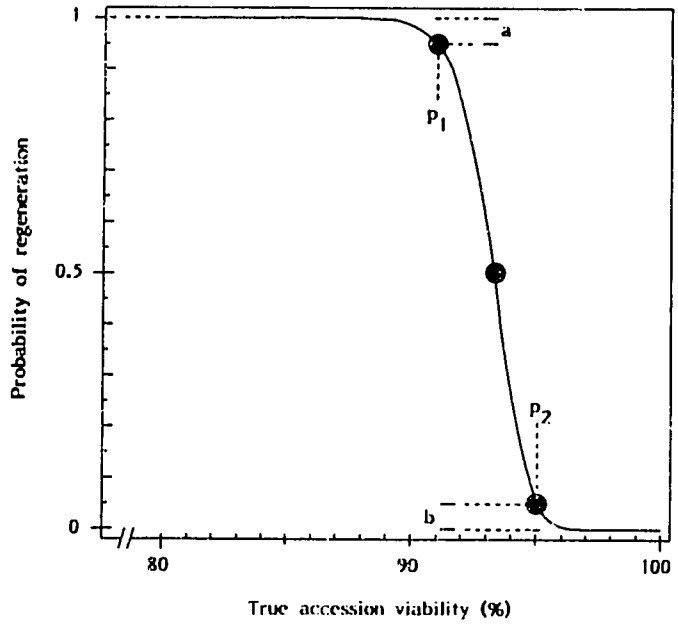
$$n = [(Z_a\sqrt{\{p_1(100-p_1)\}} + Z_b\sqrt{\{p_2(100-p_2)\}})/(p_2-p_1)]^2 \qquad (12.7)$$

where p_1 is the regeneration standard and Z_a the value of the normal deviate for the one-tailed significance level a where a is the probability of rejecting the null hypothesis when the true accession viability is equal to the regeneration standard, p_2 is a higher value at which we require a high probability of rejecting the null hypothesis, that is a low probability (b) of accepting the null hypothesis and Z_b is the value of the normal deviate for this error probability. Figure 12.2 provides further illustration of the meaning of these values.

If we return to the values used in our example of the two-tailed test we can demonstrate a considerable saving in the number of seeds required for each viability test if a one-tailed decision making procedure is applied.

Let p_1 = 91% (the regeneration standard) with the probability, a, of rejecting the null hypothesis set at 0.05, and let p_2 = 95%, the higher value at which the probability, b, of accepting the null hypothesis has also (though it need not) been set at 0.05.

Figure 12.2 Relationship between the probability of regeneration and true accession viability for fixed sample size germination tests of 430 seeds. The three closed circles on this curve show where the probability of regeneration is 0.95, 0.5 and 0.05. The probabilities a and b and the viabilities p_1 and p_2 are components of equation 12.7 and their values in this figure are the same as those in the example in the text below.



Thus, from equation 12.7, the number of seeds required in germination tests is given by

$$n = [(1.645\sqrt{(91 \times 9)} + 1.645\sqrt{(95 \times 5)}) / (95 - 91)]^2$$

$$= 429.8$$

i.e. a saving of almost 600 seeds.

Thus an earlier, provisional, recommendation of the IBPGR which suggested the regeneration of accessions after a significant loss of viability of approximately 5% had occurred implied two-tailed tests which require very large numbers of seeds for each viability test. The acceptance of a single regeneration standard for all accessions of a species (which is now recommended) enables a one-tailed test to be used which, as we have shown here, results in a considerable saving of seed.

FIXED SAMPLE SIZE TESTS

This term means simply that each time a test of viability is performed the same number of seeds are always sampled from each accession. For example, in seed testing stations 400 seeds are sampled from seed lots for germination tests (with the exception of certain tree species where the number of seeds tested is based on weight). Since this is a large number of seeds they cannot all be tested easily as one group, neither is it desirable to do so; instead they are divided into replicates of either 100, 50 or 25 seeds each. Now an interesting problem can arise when several replicates are used. We have already noted that the germination environment can be less than adequate. Suppose that in a fixed sample size test of 400 seeds one replicate suffered from an oxygen shortage. If we were to include the result from

this replicate in our estimate of percentage germination then we would expect this result to be less than the best estimate should be. If we were certain beyond reasonable doubt that this particular replicate had not been tested under the appropriate conditions (for example, if it had obviously dried out) then we could exclude the result of this replicate from our calculation of our estimate of accession germination.

Now such a decision would, to a certain extent, be subjective. If applied regularly it would be of doubtful validity because we might exclude replicates with lower percentage germination results when this was not justified and thus bias upwards estimates of accession germination. Consequently an objective method is required for deciding whether to include or exclude the results of a replicate giving lower percentage germination from the calculation of the estimate of accession germination.

Use of replicate results from a fixed sample size test

Such an objective method is provided by the use of tolerance tables. We have already discussed the binomial distribution of error. This distribution enables us to calculate, for a given probability, the expected variation between the results of replicates which is caused by random variation alone: if the result of one replicate is outside the range expected then we can, objectively now, assume that this is because of some factor other than random variation - that is an environmental factor - and exclude this result from our estimate of accession germination. Chapter 14 provides a wide range of tolerance tables which have been especially calculated for gene bank use, together with an explanation of their application. Tolerance tables can, of course, be applied to the replicate results of any other test of seeds sampled from an accession; e.g. the tetrazolium test for viability (Chapter 11), or a test of seed purity (Chapter 8).

THE SEQUENTIAL PROBABILITY RATIO TEST

The major drawback with fixed sample size tests is that sample size is dependent upon the worst possible situation in which a decision has to be made. Figure 12.1 demonstrated that a 100 seed test of an accession of 95% viability has approximately the same average standard deviation as a 200 seed test of an accession of 90% and that both values are similar to that for a 400 seed test of an accession of 80% viability. That is these three tests are approximately equally accurate: the higher percentage viability (between 50% and 100%) the fewer the number of seeds required to obtain the same degree of accuracy. Moreover there is a second factor to consider if the same regeneration standard is to be applied for all accessions of the same species regardless of initial viability. This second factor is the difference between true accession viability and the regeneration standard, the denominator in equation 12.7.

Consider a regeneration standard of 85% with a probability of rejecting the null hypothesis (i.e. deciding not to regenerate) of 0.05. Now if we wish there to be a 0.05 probability of accepting the null hypothesis (accession viability is less than or equal to the regeneration standard) when true accession viability is in fact 90% we will need to sample the following number of seeds for each test - calculated using equation 12.7 since it is a one-tailed test,

$$[\,(1.645\sqrt{\{85 \times 15\}} + 1.645\sqrt{\{90 \times 10\}})/(90-85)\,]^2 = 467 \text{ seeds}$$

Let this be our worst case. If a fixed sample size of 467 seeds is used for every test of viability on each accession then we can work out values of the probability of regenerating in error, b, at higher levels of accession viability. For example, substituting in equation 12.7 for a true accession viability

of 93% gives:

$$[(1.645\sqrt{(85 \times 15)} + Z_b\sqrt{(93 \times 7)})/(93-85)]^2 = 467$$

where Z_b has the value 4.47 and thus b, the probability of deciding to regenerate an accession in error, is less than 0.00003.

This means that if 467 seeds of each of 100,000 accessions, each with a true viability of 93%, were tested for viability then decisions to regenerate (in error) would only have been made for three of the accessions. For argument's sake let us just consider how many seeds would be necessary for the probability of regeneration to be 0.5 at 93% (of course this would be undesirable, but the reason for doing this should become clear later). If the probability of deciding to regenerate is 0.5 then the appropriate value of Z_b is 0, which makes the calculation much simpler.

In this case $n = [(1.645\sqrt{(85 \times 13)})/(93-85)]^2 = 53.9$, or 54 seeds. Table 12.2 shows how the value of n is affected by substituting for various values of b, the probability of regeneration, in equation 12.7 where p_2 has the value 93%, and also where p_2 has the value 90%.

Table 12.2. The relationship between the probability of regeneration, b, and the number of seeds tested, n, determined from equation 12.7 where p_1 = 85%, a 0.05 for values of p_2 of 90% or 93%.

	Probability of regeneration									
	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
p_2	Number of seeds, n									
90	467	377	282	221	175	138	105	74	45	17
93	158	130	100	81	66	54	43	32	22	11

Now it can be explained why the requirement for 467 seeds when true accession viability is 90% and the desired probability of regeneration (in error) is 0.05 represents the worst possible case in the previous example. Let us describe Table 12.2 a little differently. If we had carried out ten tests of viability with only 17 seeds per test on an accession of 90% true viability then in 9 of the tests we would have decided to regenerate the accession (in error). But on one occasion we would have decided to maintain the accession in store. Consequently for this one test if we had sampled 467 seeds (as required by our fixed sample size test) we would have wasted 450 seeds. As a further example, if true viability were 93% then in only 5% of tests would it have been necessary to use more than 158 seeds, but with the fixed sample size test 467 seeds must always be sampled and tested. Thus to cater for our worst case when p_2 = 90% and b = 0.05 entails using considerably more seeds than is necessary for the majority of decisions. What we need is an alternative to the fixed sample size procedure which uses only as many seeds as are necessary for each test to ensure that true accession viability is greater than the regeneration standard with an acceptably small probability of error.

Sample size not pre-determined

Sequential analysis is the name given to just such a procedure. Its essential feature is that the number of individual seeds required by the procedure is not pre-determined (that is, the sample size is not fixed) but depends on the results of all previous observations. Consequently, on average, a sequential test will require substantially fewer seeds than equally reliable fixed sample size tests. In the simplest form of sequential analysis a single seed would be sampled from an accession and tested for viability. The result will be either positive (the seed is viable) or negative (the seed is non-viable). Depending upon the result one of three decisions is made; to terminate the test and keep the accession in store until the next monitoring test is due; to terminate the test and regenerate the accession as soon as possible; or to continue the test by sampling a further seed from the accession and carrying out a further test. In the latter case the combined results of all tests are used in the subsequent decision making procedures.

The grouped sequential test procedure

In the majority of cases where germination tests are used to estimate viability this simple form of sequential analysis would be impossible because of the length of time between sampling an individual seed and obtaining the result from the germination test. However, a very worthwhile compromise can be achieved by adopting a grouped sequential test procedure. In this procedure a group of seeds (for example, 40 seeds) are sampled from the accession at a time. Depending upon the results from the first group of seeds it might be possible to reach a decision but, if not, a second group of, say 40, seeds may be tested. And so on. The grouped procedure does not save as many seeds as Table 12.2 might indicate, but the savings are still, nevertheless, quite substantial compared to equivalent, equally reliable, fixed-sample size procedures (Figure 12.3).

Number of seeds saved dependent on true accession viability

Figure 12.3 shows that the number of seeds saved by the grouped sequential test procedure depends on the true value of accession viability. In this example the savings resulting from the adoption of the sequential test procedure compared to the equivalent fixed sample size test are greatest at very high viability and decline until at 87.645% viability the saving is comparatively small. Below 87.645% the savings of the sequential procedure again increase. Why are the savings least at 87.645%? Substituting the value 87.645 for p_2 and 467 for n in equation 12.7 gives:

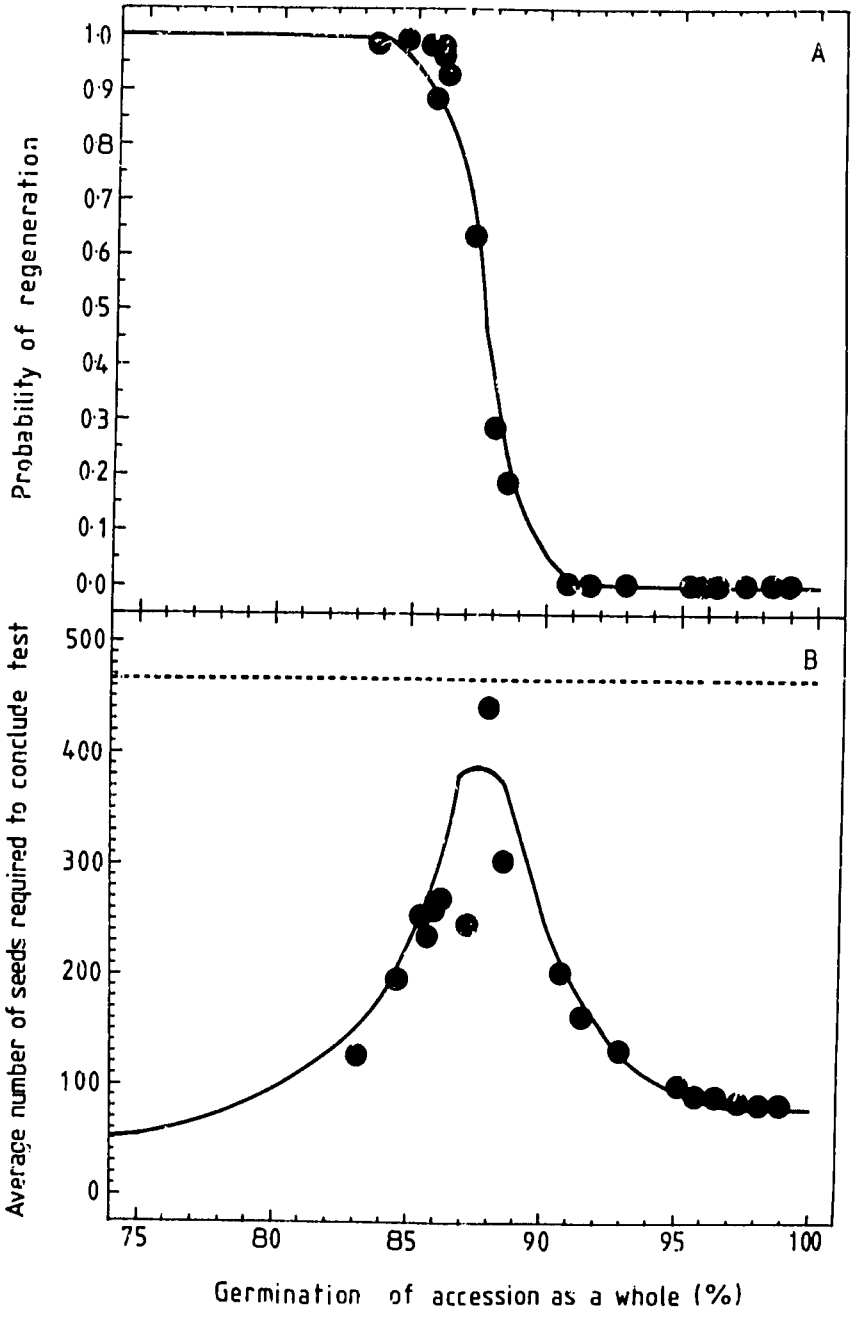
$$467 = [\,(1.645\sqrt{(85 \times 15)} + Z_b\sqrt{(87.645 \times 12.355)})\,/\,(87.645 - 85)\,]^2$$

and thus $Z_b = -0.0176$

and consequently $b = 0.52$

In other words there is, on average, no potential for saving seed when the viability of accessions is approximately midway between p_1 and p_2 because this is the point where it is the most difficult to make a decision either way. This can be seen more clearly by comparing the relationship between the probability of regeneration and true accession viability and the average number of seeds tested and true accession viability (Figure 12.3). The sequential test procedure requires most seeds when the probability of regeneration is 0.5. It might be thought that at this value the average number of seeds tested for the sequential procedure would equal the value (467) of the equivalent fixed-sample size test. However this is not so because the distribution of the number of seeds required for individual sequential

Figure 12.3 The operating characteristic (A) and economy (B) of a sequential germination test procedure. The example shown is for 40 seed groups using the sequential germination test plan shown in Table 15.5 (Chapter 15). The solid curves are the theoretical values, the solid circles the results of an experiment to test the validity of the theory. The broken horizontal line in B indicates the number of seeds required (467) for a fixed sample size test of equal reliability. (From Ellis, R.H. and Wetzel, M. (1983). Recent developments on applying sequential analysis to gene bank seed viability monitoring tests. Plant Genetic Resources Newsletter, 55, 2-19.)

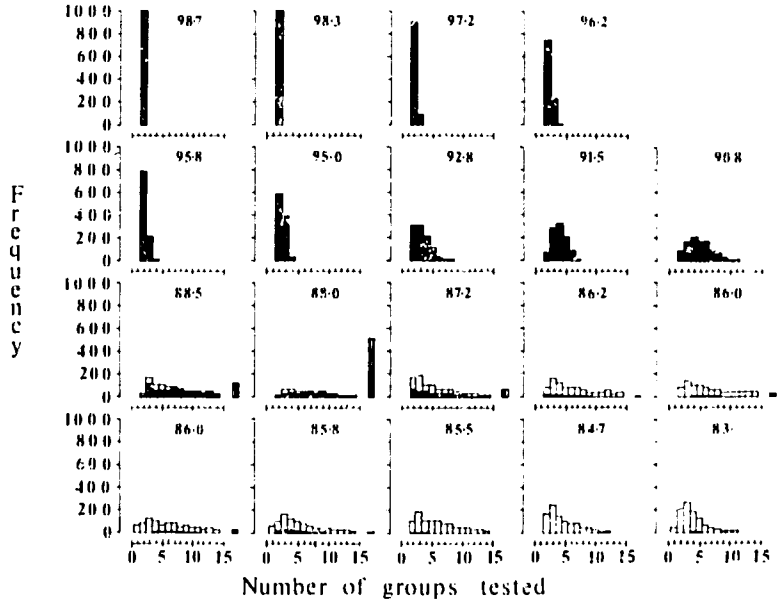


test procedures is not distributed uniformly. This is demonstrated for many different levels of accession viability in Figure 12.4. When true accession viability is high, and thus the probability of regeneration is very low, the average number of seeds tested is low and the distribution for many accessions at this level of viability is narrow. In contrast when the probability of regeneration is close to 0.5 the average number of seeds tested is much higher and the distribution much broader. In fact the distributions shown can be open-ended - particularly when p_2 is close to the value where $b = 0.5$ (Figure 12.4). In a few tests it may be impossible to conclude the sequential test procedure within a reasonable number of groups of seeds. Nevertheless this is not quite such a great problem as it may seem initially. If an excessive number of groups of seeds have been tested without a decision being reached then we can assume that p_2 is close to the value where $b = 0.5$. Since $b = 0.5$ by definition we do not mind whether this accession is regenerated or not but since seed numbers have been depleted by the monitoring test for viability we may as well regenerate and not test further groups of seeds. Chapter 15 gives further advice on truncating sequential tests before a decision is reached.

Much work has been done developing sequential test procedures that can be used with confidence in gene banks. Chapter 15 provides a number of sequential test plans for various regeneration standards (i.e. different values of p_1) and different numbers of seeds per test together with a clear explanation of how to apply sequential test procedures. It is recommended, for example by the IBPGR Seed Storage Committee, that gene banks adopt these procedures for routine monitoring of accession viability during long-term storage.

The major advantage of the sequential test approach to monitoring accession viability in long-term seed storage is that it minimises the waste of limited and valuable seed resources for monitoring tests early on in storage (that is when true accession viability is considerably greater than the regeneration

Figure 12.4 Frequency distributions of the number of 40 seed groups tested before concluding 1000 sequential germination tests at each of the 19 levels of percentage viability shown. Open and closed histograms indicate tests in which the decision was made to regenerate and not to regenerate the accession respectively. The histograms with vertical lines at certain levels of viability indicate the number of test sequences which were not concluded after 15 groups of 40 seeds had been tested. (Source as for Figure 12.3.)



standard) and yet at the same time is as reliable as more wasteful fixed sample tests to minimise the possibility of mistakenly regenerating the accession long before it is necessary. The actual saving on seed throughout the storage life of an accession which is obtained by using the sequential test approach is difficult to quantify. It will be very dependent upon the level of initial viability at the beginning of storage. The higher this is the greater the saving. An attempt has been made to estimate the saving in a practical experiment simulating long-term storage with a gradual decline in seed viability with period of storage. In this simulation - admittedly with a high viability accession - the saving of seeds was substantial - the sequential test only requiring one third the number of seeds as the equivalent fixed-sample size procedure. In practice then, it should be possible to achieve a saving of roughly 50% over equally reliable fixed sample size tests.

THE CUMULATIVE PROBABILITY OF DECIDING TO REGENERATE

It should be noted that, whether a sequential or fixed sample-size procedure is adopted, the probabilities of making each decision (keep in store or regenerate) apply to a single monitoring test alone. In practice it is likely that accessions will be monitored on a number of occasions before the decision is taken to regenerate. We should therefore consider the cumulative probability of regeneration throughout accessions' storage lives. It can be demonstrated very easily that this will be higher than the probabilities given so far.

For the sake of argument, assume that 100 accessions each have a true viability of 95% when placed in store and that we are using a test with the operating characteristic shown in Figure 12.3. (This could be either a sequential test or a fixed sample size test.) Further assume that each is monitored for viability on 4 occasions when true accession has fallen to 92.5%, 90%, 87.5% and 85%. Now from Figure 12.3 the probability of regeneration at 92.5% is only 0.001 and we can assume that all 100 accessions are kept in store until the next monitoring test. At 90% germination the probability of regeneration is now 0.05. Thus five of our accessions will be regenerated (prematurely) and only 95 be kept in store until the next monitoring test. At 87.5% the probability of regeneration is approximately 0.5. Thus half of the 95 accessions (say 48) are regenerated (slightly prematurely) and only 47 remain in store. At 85% the probability of regeneration is 0.95. Thus 45 of the 47 accessions are regenerated and only 2 remain in store. Thus the cumulative probabilities of regeneration since accession receipt at the subsequent monitoring tests would be 0.00, 0.05, 0.53, 0.98; not the values 0.00, 0.05, 0.50 and 0.95 which correspond to the probabilities for a single monitoring test.

The effect of frequent monitoring on the cumulative probability of regeneration

The differences between the two for gene banks which monitor accession viability infrequently (say on four occasions throughout accession storage life) are only slight and need not be of concern. But note that the frequent monitoring of accession viability would result in a considerable increase in the cumulative probability of regeneration and would, thus, need to be taken into account.

CHAPTER 13. PRACTICAL ASPECTS OF SAMPLING SEEDS FROM ACCESSIONS

In normal seed testing practice the term sampling describes procedures whereby a small sample of seeds is removed from a large seed lot. The various tests then performed in seed testing stations are carried out on working samples derived from the submitted sample by sub-sampling. Thus the sampling of seeds from accessions in gene banks for whatever purpose (laboratory tests of viability, germination, mean seed weight, purity, health, or moisture, or for regeneration, multiplication, evaluation or distribution) corresponds to sub-sampling in seed-testing stations.

The reasons why random sampling within gene banks is essential every time seeds are removed from an accession in store have been provided in Chapter 12: any test on an unrepresentative sample of seeds from an accession may be misleading; and on all occasions when seeds are removed from the accession those removed should be sampled randomly. The first part of this chapter provides information on the different methods which can be used to sample seed accessions randomly.

To sample seeds from an accession in storage will require the temporary removal of that accession from the coldroom. To avoid moisture from the laboratory atmosphere condensing on the very cold seeds it has been recommended that the opening of the container in which the accession is located be delayed until the seeds have warmed up - see the 1982 Report. Moreover, to avoid any increase in the moisture content of seeds in the accession which are returned to the coldroom after the sample has been removed, the 1982 Report recommends that the opening of and sampling from each container take place in a low temperature, low humidity environment similar to that maintained in the drying room. The second part of this chapter considers the sample sizes required for the regeneration, multiplication, evaluation or distribution of seeds from genetically heterogeneous accessions. The final part of this chapter considers the practice of storing a single accession in more than one container.

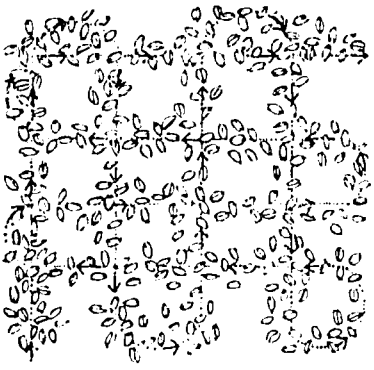
RANDOM SAMPLING PROCEDURES

Apart from the differences between individual seeds in viability (Chapter 4), dormancy (Chapter 5) and, in genetically heterogeneous accessions, genotype, seeds within accessions also differ in size and density. Further, broken or empty seeds (Chapter 8) and other plant or soil debris (Chapter 1) may be present. If any seed container is shaken, or repeatedly moved, then separation of the contents will occur: the denser material moving towards the bottom; the lighter material moving towards the top. Consequently within any container in store, or in the drying room, the seed bulk may not be homogeneous.

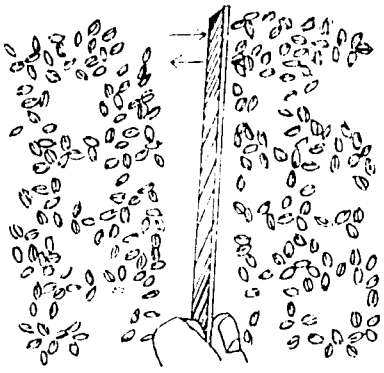
Mixing

There are two simple stages in random sampling from seed accessions. First, because the distribution of seeds within the container may not be homogeneous, the accession is thoroughly mixed. Then the required sample from the accession is obtained; either by repeated halving of the accession; or by abstracting many small portions of seeds from the accession at random and subsequently combining them.

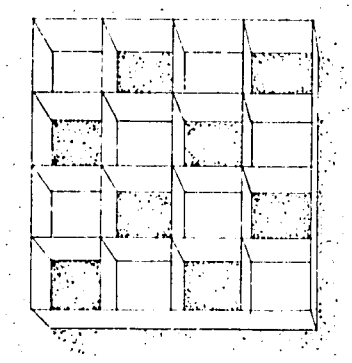
Figure 13.1 Illustration of simple sampling techniques.



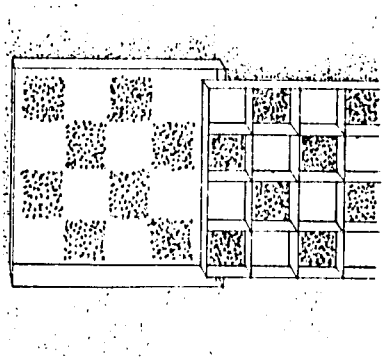
(i) Method of pouring seeds out on working surface (arrows indicate pouring movements).



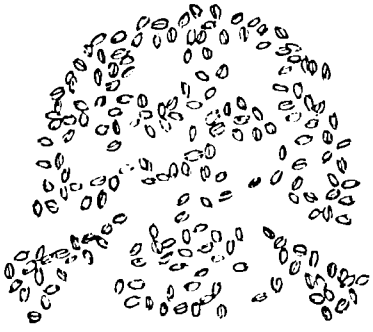
(ii) Using a ruler to halve a sample poured out (as (i)) onto a working surface.



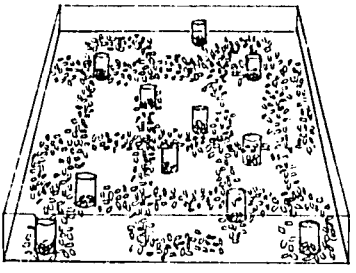
(iii) Use of a grid to halve a sample.



(iv) Seeds remaining in cells with a bottom are removed to obtain the sample.



(v) Demonstration of pie method of sampling showing two slices being removed from the pie.



(vi) Demonstration of random cups method of sampling. Distribution of seeds shown to demonstrate method of pouring out seeds (as (i)). In practice seed distribution will be uniform.

Mixing of the accession is best achieved by using one of the halving procedures described below, but after halving both halves are then blended together, then halved again and, finally, the two halves blended together again. Occasionally a third halving/blending cycle may be required. Blending is achieved by slowly pouring the two halves together at the same time and with the same flow rate through a hopper or funnel into another container.

Number or weight of seeds sampled

To obtain sufficient seeds in the sub-sample the operator must first determine, or estimate, the appropriate number or weight of seeds required. (If the requirement is for a given number of seeds the mean seed weight can be used to calculate the required weight of seeds - see Chapter 2.) The operator should then aim to obtain a sample which is marginally heavier than the required weight - in order to ensure that sufficient seeds are present within the sample. However, it is important that the actual number (or weight) of seeds removed is recorded on the accession management file; otherwise the accession may become unwittingly depleted (see Chapter 2).

Repeated halving

To obtain a sample from the previously mixed accession by repeated halving entails dividing, without bias, the accession into two equal parts, and then repeatedly further dividing one part each time until the sub-sample has been reduced to the required size. Thus to obtain a sub-sample one-eighth the size of the accession requires three halvings ($2^3 = 8$) but to obtain a sub-sample only 1/256th of the accession (for example, to obtain a 40 seed sample from an accession containing slightly more than 10,000 seeds) requires eight halvings ($2^8 = 256$).

Halving

If no equipment is available, then the simplest method of halving is to pour - move from side-to-side and back-to-front, etc. as shown in Figure 13.1 - the mixed accession out onto a clean, flat, tray (with lips to prevent seeds falling off the tray) and then, with a ruler or other straight edge placed across the middle of the bulk, divide the bulk to the left and right. A similar procedure, but one which requires apparatus, is to fit upon the tray a grid of equal-sized cubical cells which are open at the top but where each alternate cell is closed at the bottom. An example of this apparatus is shown in Figure 13.1. Then pour the, previously mixed, accession uniformly over all the cells by moving from side-to-side and then front-to-back, etc. When the grid is lifted from the tray approximately half the sample remains on the tray - provided the total area of the grid is sufficient for the depth of the cubical cells to contain the accession.

Riffle divider

Probably the most suitable device for the repeated halving of seed accessions in gene banks is the riffle divider (also know as a soil divider or multiple slot divider). This consists of a hopper with attached channels or ducts supported on a frame, below which two receiving pans are positioned, and a pouring pan. The ducts alternate in the direction of seed flow, so that half the seed goes to the receiving pan on one side and half goes to the other receiving pan. A riffle divider is shown in Figure 13.2. We use a riffle divider with receiving pans each 280 mm long x 130 mm deep x 140 mm wide, a V-shaped hopper 110 mm wide (at the top) x 260 mm long supported on a frame such that the

top of the hopper is some 380 mm above the bench, with 20 ducts each 13 mm wide (20 x 13 = 260 mm, the length of the hopper) x 45 mm long (the V of the hopper being truncated at the bottom). When pouring the previously mixed accession into the hopper it is important to pour slowly, whilst moving from side-to-side across the top of the hopper. In other words use all of the hopper, not just the central part, and do not block the ducts below the hopper by pouring the seeds into the hopper too quickly.

Conical divider

The conical divider, also known as the Boerner divider, consists of an inverted metal cone below the spout from a hopper - see Figure 13.2. A valve below the hopper controls seed flow. The seeds fall over the cone: to achieve a uniform distribution of seeds over the cone it is important that the hopper outflow is positioned centrally above the apex of the cone. At the circumference of the metal cone is a series of baffles of equal width which - like the riffle divider - direct seeds into two outlets alternately, below which receiving pans are placed. For large seeds the appropriate conical divider contains 38 channels (19 to each spout) each 25.4 mm wide. For small seeds the appropriate conical divider has 44 channels (22 to each spout) each 7.9 mm wide. Cone diameters are 368 mm and 152 mm respectively.

Centrifugal divider

The centrifugal divider, also known as a Gamet Precision divider, requires electrical power to rotate (rapidly) a shallow rubber cup or spinner - see Figure 13.2. The seeds fall into this, directed from a hopper above, but are immediately thrown out again by centrifugal force and fall downwards. The area where the seeds fall is divided equally into two parts by a baffle and the seeds directed to two spouts.

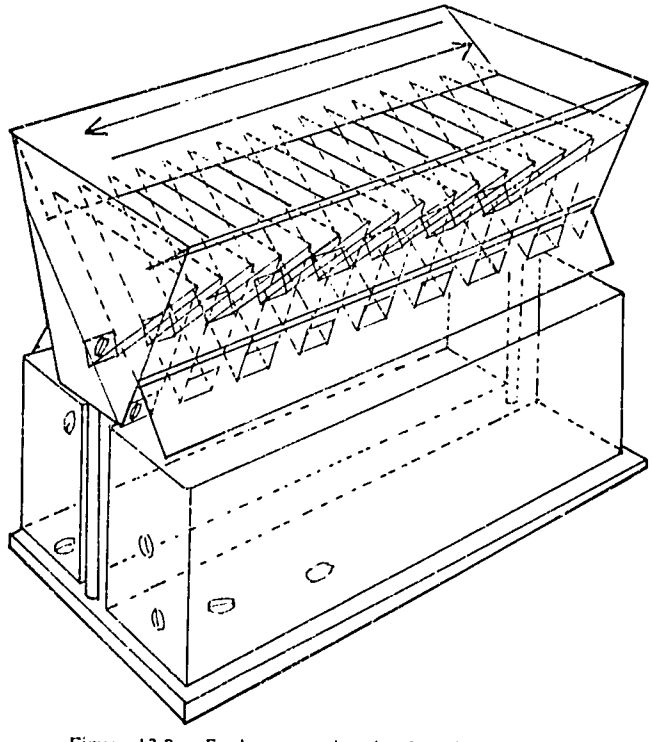


Figure 13.2a Explanatory sketch of a riffle divider.
The arrows indicate the repeated back and forth pouring motion across the hopper.

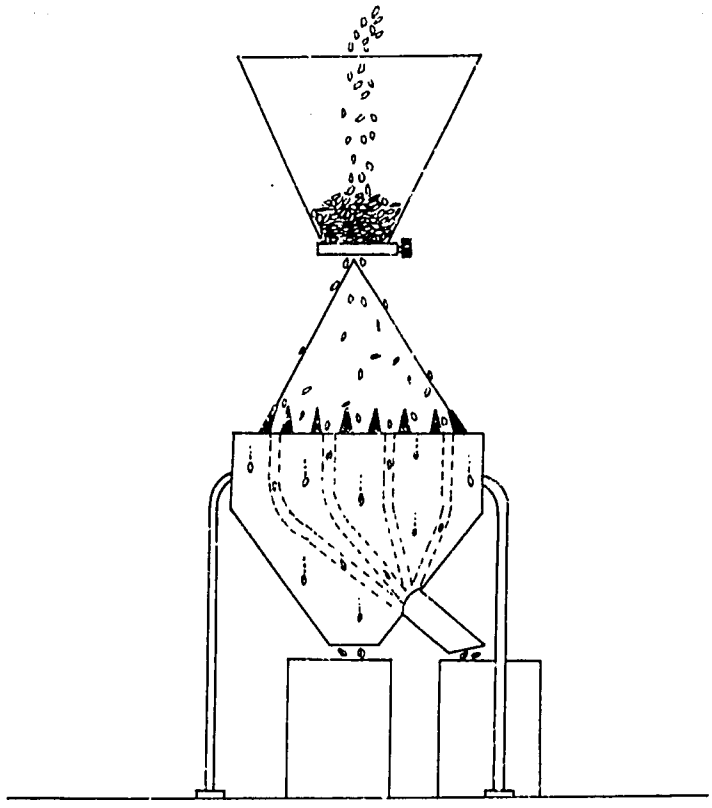
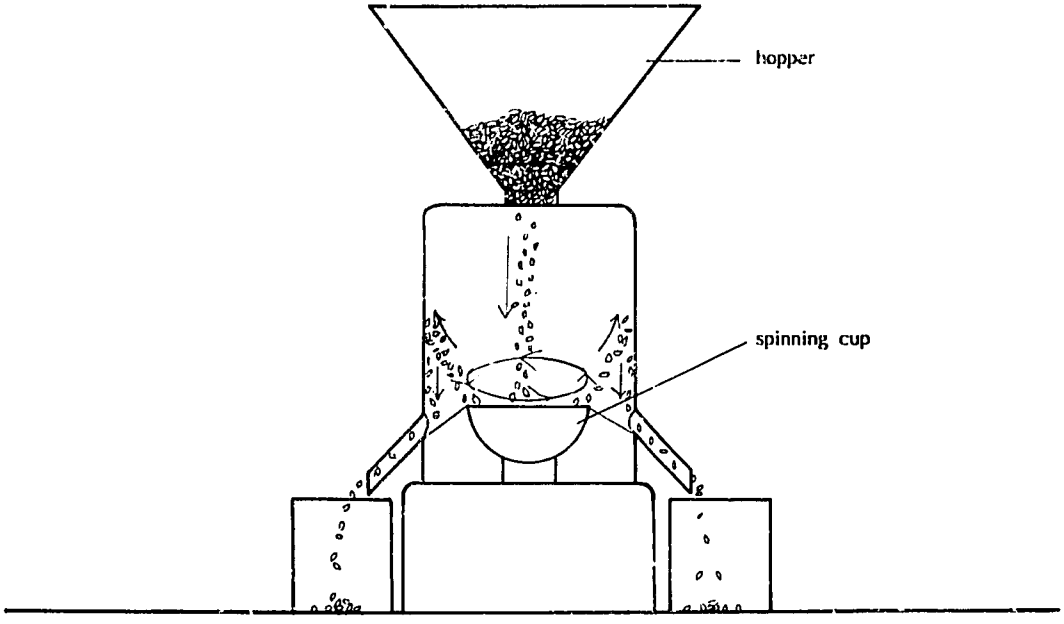


Figure 13.2b Explanatory sketch of a conical divider.

Figure 13.2c Explanatory sketch of a centrifugal divider.



The pie method of sampling

The simplest method of providing a sample of seeds from an accession by the abstraction of many small portions of seeds at random and then combining them is to use the pie method - see Figure 13.1. Spread the, mixed, accession on a clean, flat tray (with lips, to avoid loss of seeds by pouring from side-to-side and front-to-back, etc. Then divide the sample, using a ruler or other straight edge, into several narrow sections - as if cutting a pie. Select several, but not less than five, sections at random, remove these from the pie and combine to obtain the sub-sample.

The spoon method of sampling

A similar procedure, the spoon method, requires a spatula and a straight-edged spoon in addition to a tray. Instead of dividing the accession into portions as in a pie (above) small portions of seed are removed from not less than five random places on the tray by using the spoon in one hand and the spatula in the other and gripping the seeds between the two.

The random cups method of sampling

The final sampling procedure is known as the random cups method. Again a clean, flat tray with lips is required to form the working surface. Six to eight small cups or thimbles are placed on the tray at random positions within a square, marked, area of the tray - see Figure 13.1. The mixed accession is then slowly poured onto the area of the square within the tray in a uniform manner; again move from side-to-side and back-to-front alternately, but do not attempt to deliberately fill the cups/thimbles; instead aim to cover the square to a uniform depth. The sub-sample is then obtained by combining the seeds within the cups/thimbles. Obviously the size of the cups/thimbles and the area of the marked square will depend upon the total size of the accession, seed size, and the number of seeds required for the sample. The gene bank can easily determine these empirically, but the normal range of cup/thimble sizes is 5-20 mm for small seeded species within working squares of 100 x 100 mm to 150 x 150 mm. To avoid the cups/thimbles falling over they should be reasonably dense, e.g. made from brass, and no more than 20% taller than the diameter of their base.

Choice of sampling procedure and seed shape/size

To a certain extent the choice of sampling procedure and equipment will depend upon the nature of the accession being sampled. The riffle divider is suitable for most types and sizes of seeds provided the width of the ducts is suitable: if too narrow then large seeds - such as the grain legumes - may block the duct; if too wide then the sampling of small accessions of very small seed size may be unequal. It is suggested that two riffle dividers, one with narrow ducts for small seeds and one with wider ducts for large seeds, would be a sensible provision for a gene bank dealing with different species of contrasting seed sizes. Spherical-seeded species which tend to bounce or roll easily (for example, Brassica spp.) should not be sampled using those procedures which require the seed to be poured evenly over a working surface: if sampled in a riffle divider it may be necessary to use deeper receiving pans and/or add temporary lids to the receiving pans to avoid the seeds bouncing out after division. The spoon method can only be used for small-seeded species. Extremely chaffy seeds cannot be sampled using mechanical dividers or by the random cups method. Indeed with species such as cotton or sugar cane where lint or fuzz respectively prevent seed separation it is difficult to sample randomly. For such species it is necessary to divide by hand (large seeds) or by forceps (small seeds) to obtain a sample. This can be

extremely time-consuming, but it is important to be as cautious as possible and to make a conscious effort to avoid drawing a biased sample.

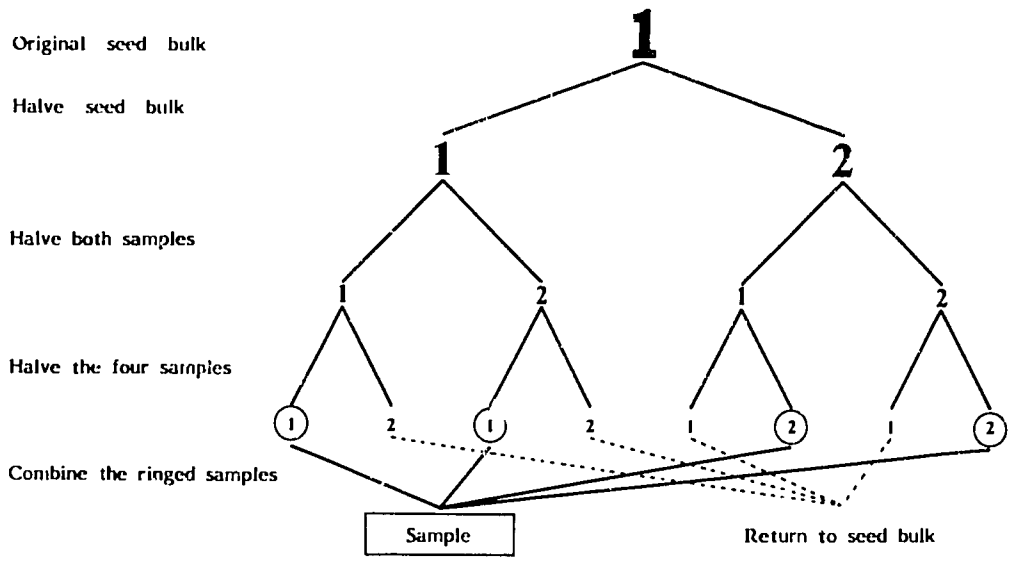
Constant halving method of sampling for chaffy seeds

Chaffy seeds (see Chapter 3) which do not flow easily (such as the pasture species within the Chloris, Cenchrus and Stylosanthes genera) can be sampled using a modified constant halving method where the sample is halved (e.g. with a ruler, Figure 13.1) three times into eight sub-samples but these eight sub-samples are then combined into either of two samples. Thus seven divisions are required to achieve each halving of sample size: Figure 13.3 provides a stylised explanation of this procedure.

Cleaning

When using any equipment to sample seeds it is vital that, for each accession, the equipment be cleaned before use. The cleaning of sampling equipment is essential since seeds will easily lodge in crevices, and on ledges, etc. The most effective method of removing seeds lodged in dividers is to dismantle the divider, sweep lodged seeds out with a soft brush, and then rebuild the divider. Some riffle dividers are designed to be easily dismantled, cleaned, and reconstructed. In addition to the cleaning of equipment being necessary to avoid a few seeds from one accession being mixed with those from another accession, it is important that no debris from one accession contaminates another accession; this might result in serious seed health problems. Thus dust, soil particles, etc. as well as lodged seeds must be removed. Disinfection of the equipment, followed by drying, may be required in certain cases.

Figure 13.3 Outline of the constant halving method to divide a sample of chaffy seeds.



Seed counters and sampling

Finally, it should be noted that equipment used to save time when setting up germination tests is not adequate to derive samples from the accession for germination tests, or any other purpose. Several types of equipment can be used to count or count and position seeds for germination tests, viz. automatic seed counters, vacuum counting heads, counting boards - see Chapter 9 for more information. All can result in biased samples if used to derive a sample of seeds from the total accession. Equipment such as vacuum counting heads and counting boards should only be used to position a known number of seeds from the sample of the accession (previously obtained by one of the methods described in this chapter) on to the germination test substrate.

SIZE OF SAMPLES REQUIRED FOR THE EVALUATION, REGENERATION, MULTIPLICATION, OR DISTRIBUTION OF GENETICALLY HETEROGENEOUS ACCESSIONS

Since plants produced by seeds sampled from genetically homogeneous accessions can be considered - by definition - to have identical genotypes (unless mutation has occurred), very few plants are required for use in each breeding programme, regeneration, multiplication cycle, or evaluation procedure. In this situation the number of seeds required to be sown is likely to be highest in the regeneration or multiplication cycle and will depend upon the number of seeds the gene bank wishes to produce for continued storage and/or distribution, the proportion of plants established to seeds sown and the seed multiplication factor for the crop (i.e. the number of seeds produced as a proportion of the number of plants grown). See Chapter 2 for a discussion of the number of seeds required for regeneration and the number of seeds at which regeneration is called for.

In a genetically heterogeneous accession, however, we are dealing with a population of genotypes, where certain genes may be present at low frequencies in the population. The question that arises is how many seeds must be sampled, randomly, from the accession in the long-term store to be reasonably confident that at least one of the plants grown from the sample sown out will contain a particular gene of low frequency in the accession as a whole? Figure 13.4 provides a nomograph showing the relationship between the number of seeds in the sample, the frequency of the gene in the accession as a whole, and the probability of this gene being present within at least one individual in the sample. An explanation of the relationship between these factors has been provided in Chapter 12. Figure 13.4 can be used to determine the value of any one of these factors from the values of the two remaining factors. It is suggested that the most useful calculation is that of the number of seeds required to satisfy a certain probability of at least one individual in the sample containing a gene of an assumed low frequency.

Field emergence and plant establishment

Before using Figure 13.4, however, the reader is asked to consider, and take into account, the following points. The assumed frequency of the gene in question is expressed as a proportion of the number of plants in the population which contain it. Not all seeds - or seed-like structures - sown out in the field or glasshouse will, however, produce a seedling. Moreover, some seedlings may not reach maturity. These losses must be taken into account when calculating the numbers of seeds required in samples.

What are the components of these losses? The first component is the proportion of empty seeds in the accession; the second the proportion of non-viable seeds; the third is the proportion of viable but

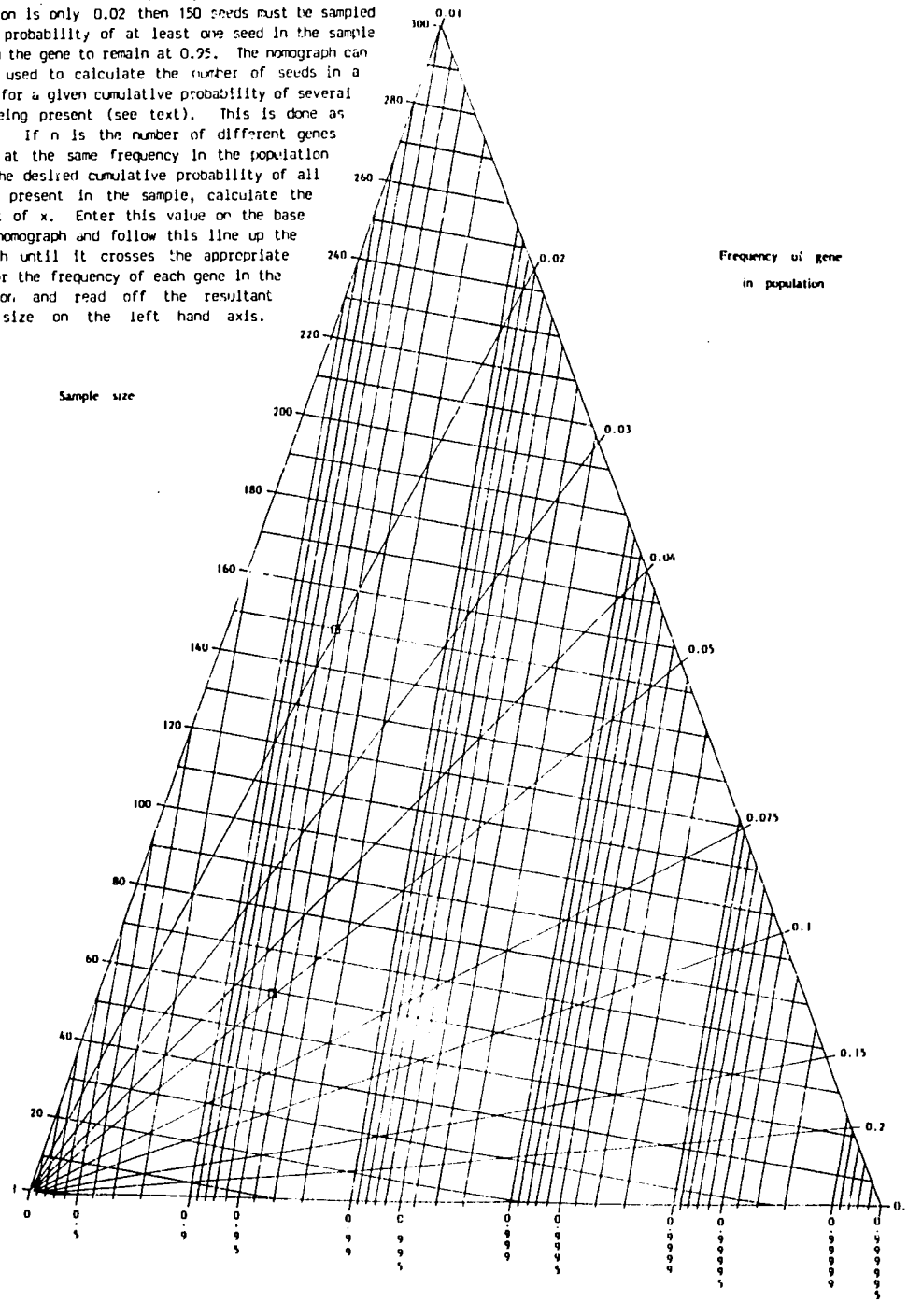
dormant seeds where germination cannot be easily promoted; the fourth is the proportion of viable, non-dormant seeds which, though they will germinate in a laboratory test under optimal environments, fail to germinate and emerge through the soil surface in field, nursery bed, or glasshouse sowings; and the final component is the proportion of emerged seedlings which fail to establish plants. The gene bank ought to be able to obtain reliable estimates of the proportions of seeds within each accession likely to be affected by the first three factors from the results of the tests carried out at accession receipt or soon after (see Chapter 1). The effect of the remaining two factors will normally have to be estimated from previous observations on relative field emergence and establishment (proportion of viable seeds which emerge and establish in the field) in the environment in which each sample is to be sown for other accessions of the same species.

To combine the relative frequency of the gene in the (plant) population and the proportion of seeds which are likely to emerge at a sowing in order to determine the appropriate value of the relative frequency of the gene in the accession to be used in Figure 13.4 is easy; the two are multiplied together. Thus if the relative frequency in the plant population is 0.05 (or 5%) and the expected field emergence of all seeds sown is 0.6 (60%) then the appropriate value for use in Figure 13.4 is 0.03 (0.05 x 0.6). This change greatly increases the number of seeds required for the sample: the number of seeds required in the sample to achieve a probability of 0.95 that the sample when grown out will contain at least one individual of the gene in question, for example, is increased from 59 (Figure 13.4) if the relative frequency is 0.05 and all seeds sown are expected to produce plants, to 99 (Figure 13.4) if the relative frequency is 0.05 but only 60% of seeds sown are expected to produce plants and thus the value 0.03 applied as being appropriate for the relative frequency of the gene in the accession. Of course these calculations assume that the seeds possessing the gene in question will show the same proportional emergence in the field as the accession as a whole. In reality the performance of this sector of the population may be either better or worse than the accession as a whole. Either way this suggests that it is important for seeds from genetically heterogeneous accessions to be sown and grown out in the most suitable environments available in order to avoid selection of genotypes during seedling emergence. The other way to calculate the number of seeds required in the sample for the above example is to use the relative frequency of 0.05 in Figure 13.4, but to describe the result, 59, as the number of seeds likely to produce plants. Now if we expect 60% field emergence of the seeds sown then we need to sample 59 x 100/60, that is roughly 100, seeds.

Field emergence and the regeneration standard of genetically heterogeneous accessions

Note, in passing, that the calculations of the type provided in Figure 13.4 have an important role to play in decisions concerning appropriate regeneration standards; particularly since the relative field emergence of low viability accessions can be extremely poor. For example, from our work typical results for field emergence in good conditions for a cereal might be 60% field emergence for an accession 90% viable but only 15% for an accession only 50% viable. If the relative frequency is 0.05 and the desired probability 0.95 that the sample when grown out will contain at least one individual of the genotype in question, then 99 seeds need to be sampled and sown out for the high viability accession, but 398 seeds for the low viability accession. The simplest way of calculating the latter value is to say that the proportion of seeds establishing plants in the latter case is only one quarter that in the former case; therefore 4 times as many seeds are required (4 x 99 equals 396). The other method is to derive the relative frequency of plants containing the gene in question expressed as the proportions of seeds or seed-like structures in the samples, 0.0075 (0.05 x 0.15). Then $(1-0.0075)^{398}$ gives the cumulative probability of 0.05 of not a single plant exhibiting the genotype in question, that is a probability of 0.95 that at

Figure 13.4 Nomograph showing the relationship between the size of samples from genetically heterogeneous accessions, the frequency of the gene in the accession and the probability of at least one seed of those sampled carrying the gene. Two example calculations are shown by the squares where the probability of at least one seed in the sample carrying the gene is 0.95: in the lower square it will be seen that if the frequency of the gene in the population is 0.05 then a sample size of just under 60 seeds is required; in the upper square, however, it will be seen that if the frequency of the gene in the population is only 0.02 then 150 seeds must be sampled for the probability of at least one seed in the sample carrying the gene to remain at 0.95. The nomograph can also be used to calculate the number of seeds in a sample for a given cumulative probability of several genes being present (see text). This is done as follows. If n is the number of different genes present at the same frequency in the population and x the desired cumulative probability of all n being present in the sample, calculate the nth root of x. Enter this value on the base of the nomograph and follow this line up the nomograph until it crosses the appropriate value for the frequency of each gene in the population and read off the resultant sample size on the left hand axis.



least one plant does. Of course, in addition to this great increase in the number of seeds required for each sample, such a low regeneration standard makes it likely that short-lived genotypes in the accession would be lost completely.

Probability of a given number of genes being present in a sample from a genetically heterogeneous accession

So far we have only considered the probability of a single gene from the genetically heterogeneous accession being present within a sample: perhaps more important is the probability that each of a given number of different genes are all present within the sample. This probability is obtained by multiplying together the probabilities that each gene alone is present - assuming that the characters are not linked. Thus, if, for example, we consider the case of a gene with a relative frequency of 0.02, a 150-seed sample provides a probability of 0.952 (Figure 13.4) that at least one individual contains the gene; in the same sample another gene with a relative frequency of 0.05 has a probability of 0.99954 of being present in a 150-seed sample (Figure 13.4). The probability of both genes being present in a 150-seed sample is thus 0.952 x 0.99954 = 0.951, presumably an acceptable value for most gene banks.

An extreme case would be to consider 20 different and independent genes within a genetically heterogeneous accession, each with a relative frequency of 0.05. The probability of all 20 genes being present is the product of all the probabilities of each gene being present. Thus, if we wish to ensure that our sample has a probability of 0.95 of containing all 20 genes, we need to find the probability, p, of an individual gene being present when $p^{20} = 0.95$, i.e. when $p = \sqrt[20]{0.95}$ the value of which is 0.9974.

The next stage is to consult Figure 13.4 in order to find the number of seeds required in the sample to ensure, at a probability of 0.9974, that it contains a gene which is present in the population at a frequency of 0.05. Inspection shows that 117 seeds are required.

However in practice, more than 117 seeds will be required since not all the seeds that are sown will be likely to emerge. If the gene bank is using a relatively high regeneration standard, as recommended by IBPGR, the seed should be of good quality, and it would be reasonable to expect that 75% of the seeds that are sown will emerge and produce plants. In which case 156 seeds should be distributed. (In practice, say 160.) This would ensure, at a probability of 0.95, that all 20 genes would be present in the progeny.

THE STORAGE OF EACH ACCESSION WITHIN MORE THAN ONE CONTAINER

Some gene banks store each accession in several containers, rather than within a single container. Their purpose in so doing is to avoid - once the accession has been placed in the long term store - repeatedly moving the whole accession from store, sampling the required number of seeds from this accession and then returning the remainder of the accession to the store whenever a sample is required. Instead a single container is removed from storage and not returned.

Two observations on this practice are relevant here. First it should be apparent that it is essential for the accession to be divided randomly, without bias, before sealing in the various containers. Secondly the subsequent removal of any one container from the coldroom after a period of storage for any test of accession quality which may have altered during storage (germination, viability, dormancy, seed health)

will not, strictly speaking, provide any estimate (of whichever attribute has been measured) for those samples of the accession which have been stored in different containers and remain in storage. The use of such values to estimate accession quality after a period of storage will require the assumption that the sub-populations of seeds in each container remaining in storage have experienced exactly the same environment as those in the sampled container, and responded equally to it. In well-managed gene banks operating at the preferred conditions (hermetic storage at -20°C with 5 ± 1% moisture content) with reliable hermetic containers this assumption is not unreasonable, but it remains an assumption nonetheless.

CHAPTER 14. OPERATING CHARACTERISTICS AND TOLERANCE TABLES FOR FIXED SAMPLE SIZE VIABILITY TESTS

A fixed sample size germination, or viability, test, is one in which a standard (pre-determined) number of seeds are sampled randomly from a seed population (accession) and subjected to a germination or viability test. Fixed sample size tests are widely applied in seed testing work. For example, in most tests of germination according to the ISTA or AOSA rules the number of seeds sampled and tested is 400. The advantages and disadvantages of fixed sample size tests have been considered elsewhere (Chapter 12). The purpose of this chapter is to provide Tolerance tables for various fixed sample test sizes which may be used in gene banks, together with information on the associated operating characteristics. The information on operating characteristics should be consulted before deciding upon the particular fixed sample size test to be used, since the operating characteristic describes the reliability of the procedure to be used. In contrast the tolerance tables should be consulted when the germination (or viability) test results are known; since this will indicate whether the variation observed in results between replicates within a single test is no greater than expected and, thus, whether the mean value of the results of all replicates can be accepted.

OPERATING CHARACTERISTICS

Since germination, or viability, tests are carried out on a sample of seeds drawn from a larger bulk (the accession as a whole) the result obtained is not the germination, or viability, of the accession as a whole, but only an estimate of this. This estimate may be above, below or identical to the (unknown) true value for the accession as a whole. Since this estimate is used to decide whether regeneration is required (yes if the estimate is below the regeneration standard, no otherwise) it is possible for errors to

Table 14.1 Accuracy of estimates of viability and errors in the decision-making procedure

true = true viability of accession

est. = estimate of accession viability derived from test on random sample of seeds

reg. = regeneration standard for the species

	Accuracy of estimate of viability	Decision	Error?
true > est. > reg.	underestimate	keep in store	no
est. > true > reg.	overestimate	keep in store	no
est. > reg. > true	overestimate	keep in store	yes
true > reg. > est.	underestimate	regenerate	yes
reg. > est. > true	overestimate	regenerate	no
reg. > true > est.	underestimate	regenerate	no

Figure 14.1 Operating characteristics of 100, 200, 400 and 1000 seed fixed sample size germination tests where the decision boundary is 65% (i.e. if the test result is below 65% the decision is made to regenerate the accession).

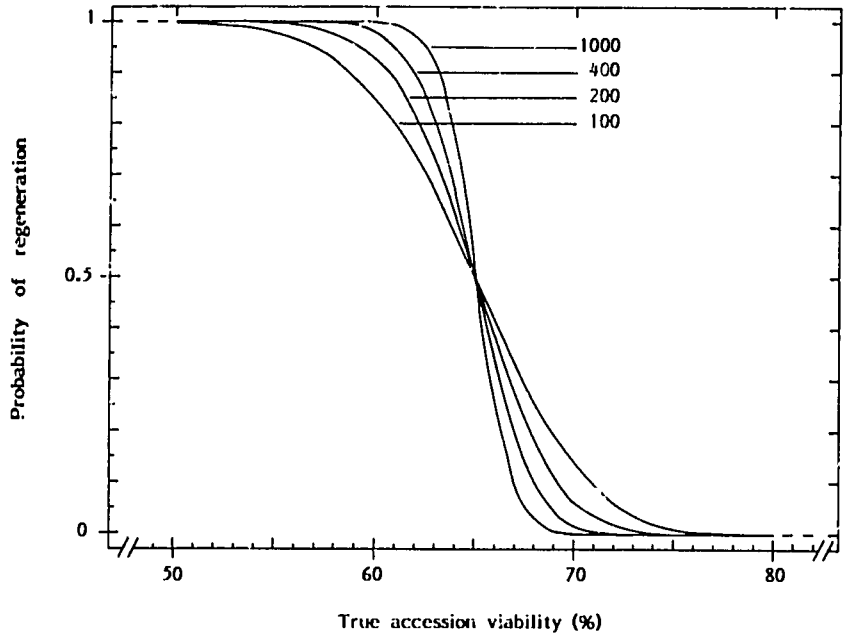


Figure 14.2 Operating characteristics of 100, 200, 400 and 1000 seed fixed sample size germination tests where the decision boundary is 70% (i.e. if the test result is below 70% the decision is made to regenerate the accession).

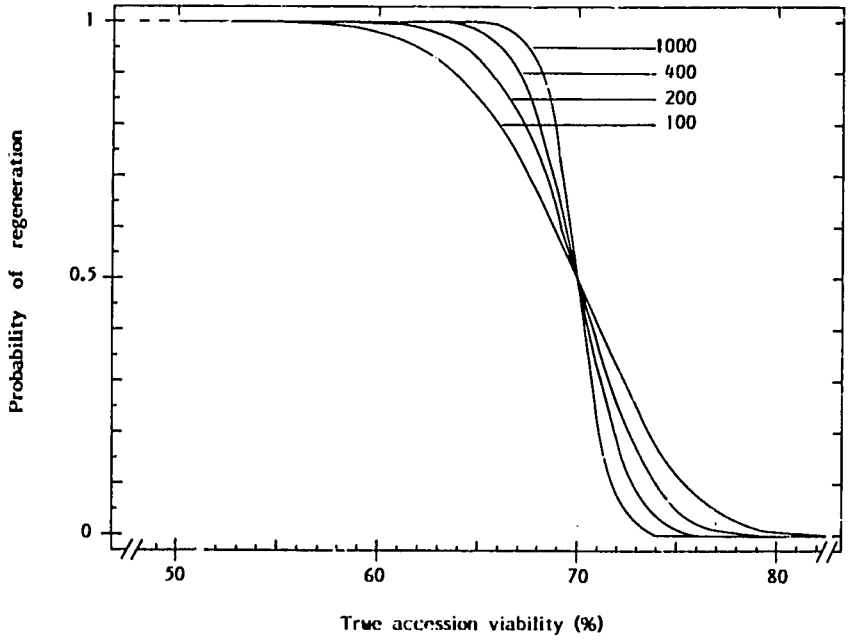


Figure 14.3 Operating characteristics of 100, 200, 400 and 1000 seed fixed sample size germination tests where the decision boundary is 75% (i.e. if the test result is below 75% the decision is made to regenerate the accession).

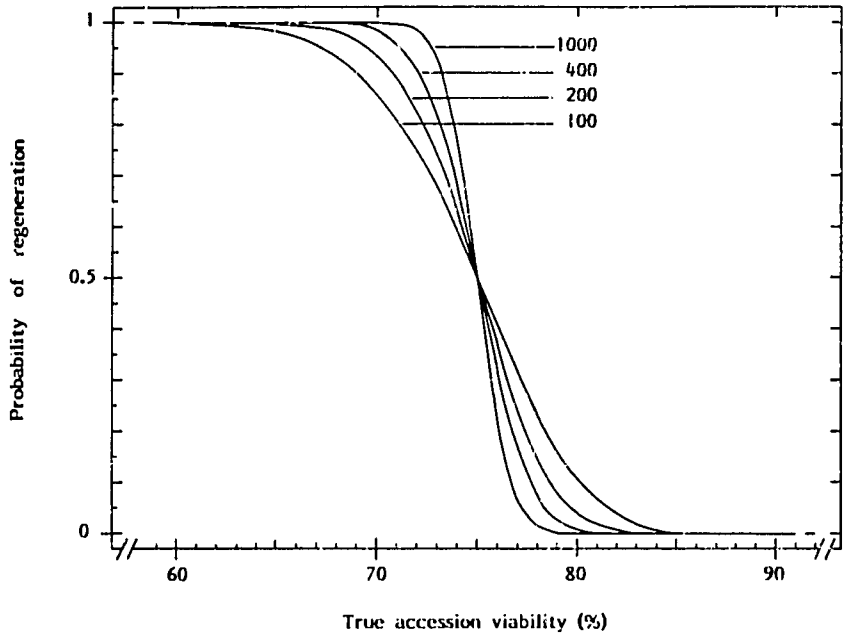


Figure 14.4 Operating characteristics of 100, 200, 400 and 1000 seed fixed sample size germination tests where the decision boundary is 80% (i.e. if the test result is below 80% the decision is made to regenerate the accession).

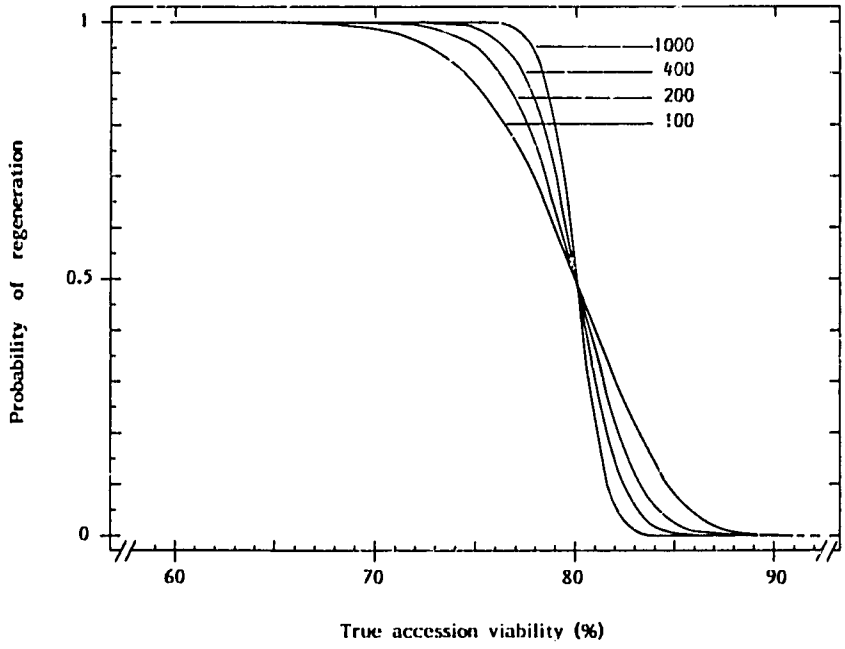


Figure 14.5 Operating characteristics of 100, 200, 400 and 1000 seed fixed sample size germination tests where the decision boundary is 85% (i.e. if the test result is below 85% the decision is made to regenerate the accession).

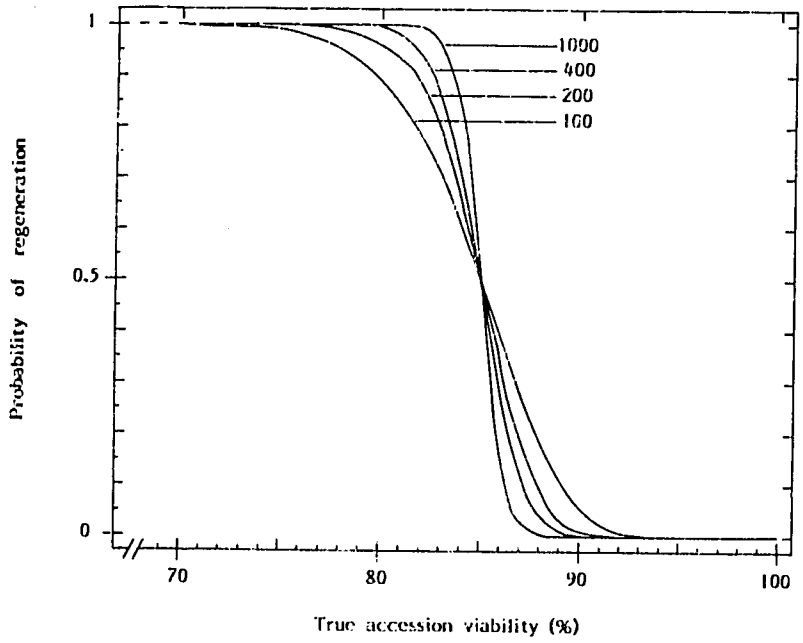
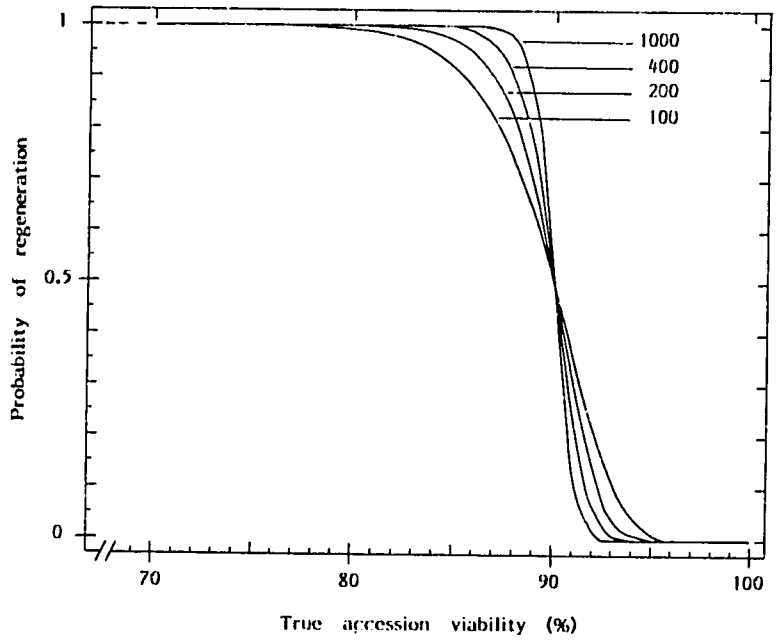


Figure 14.6 Operating characteristics of 100, 200, 400 and 1000 seed fixed sample size germination tests where the decision boundary is 90% (i.e. if the test result is below 90% the decision is made to regenerate the accession).



be made in this decision-making procedure. See Table 14.1 for a summary of the circumstances where the inability to determine accession viability as a whole affect this decision making procedure. Note that an inaccurate estimate of accession viability does not necessarily cause an error in the decision making procedure (Table 14.1).

Operating characteristics (the relationship between the probability of regeneration and true accession viability) are provided for fixed sample size tests of 100, 200, 400 and 1000 seeds with the decision point set at 65, 70, 75, 80, 85 and 90% viability (Figures 14.1 to 14.6 respectively). For a fixed sample size test it seems sensible to define the regeneration standard as the decision point of the test: if the test result is above this value then the accession is maintained in store until the next monitoring test is due; if the test result is below this value then the accession is regenerated as soon as possible. In this case, then, the regeneration standard is the level of true accession viability where the probability of regeneration is 0.5. The probability of making a decision in error is thus 0.5 if true accession viability equals the regeneration standard in these examples. Where true accession viability is greater than the regeneration standard then the probability of making a decision in error is the probability of regeneration shown in Figures 14.1 to 14.6. Where true accession viability is lower than the regeneration standard then the probability of making a decision in error is 1 minus the probability of regeneration shown in Figures 14.1 to 14.6. Although errors in the decision making procedure are less likely the larger the size of the fixed sample, where true accession viability is considerably above or below the regeneration standard the sample size is of little importance (Figures 14.1 to 14.6).

Although at first sight it seems logical to define the regeneration standard to the same value as the decision point of a fixed sample size monitoring test, this does infer that we are prepared for half of the accessions to be regenerated after their viability has fallen below this standard. In some cases true accession viability may be substantially below the regeneration standard if it is defined in this way: for example, where the decision point of the fixed sample size test is set at 65% some accessions would not be regenerated until true accession viability had fallen below 50% (Figure 14.1). In other words in this situation the regeneration standard is not a minimum standard of accession quality. See Chapter 15 for a further discussion of this point and a preferred alternative definition of the regeneration standard where the regeneration standard is described as a minimum standard of accession quality.

TOLERANCE TABLES

Fixed sample size germination tests are normally replicated, that is seeds sampled for the germination test are divided into smaller groups of seeds and tested for germination in these smaller groups. The major reason for doing this is to prevent overcrowding of germination tests which might result, for example, in serious cross infection from decayed seeds to other, otherwise healthy, seeds. Replication also minimises the chance of an incorrect result being recorded because of exceptional circumstances which might occur by accident in a single dish or towel, or because of other errors. The result of the germination test is obtained by averaging the results of the replicates. However, the results from the individual replicates will differ. The cause of this variation may be one or more of the following.

(1) Random sampling variation.
(2) Non-random distribution of seeds between replicates.
(3) Poor equipment (for example, variation of environment within the germinator/incubator).

(4) Poor method (for example, the test environment is not optimal - in which case even minor differences in environment can have large effects on germination).
(5) Poor technique (for example, inadequate regulation of substrate moisture content).
(6) Errors in counting seeds, inconsistency when classifying seedlings and remaining seeds, or errors when recording the results.
(7) Contamination of a replicate by bacteria, fungi, or chemicals (for example by failing to clean germination test containers).
(8) Replicates failing to represent the same seed lot (for example, one of the replicates may have been incorrectly labelled).

The purpose of providing tolerance tables here is to enable gene bank staff to detect variation between replicates of fixed sample size tests which is outside the range likely to result from random sampling error alone. If the variation between the replicates is outside the tolerated range then the estimate provided by the germination test will be unreliable.

It is important to examine germination test replicates critically in all cases, but particularly when the tolerated range is exceeded. Often the tolerated range will be exceeded because of a specific deficiency of the test environment in one replicate (or possibly more) which will result in a lower proportion of seeds germinating. Where a deficiency of test environment is detected remedial action should be taken to prevent this deficiency recurring. This may require changes in the way germination tests are set up. Note that such factors may be reducing germination in other germination tests, but will not be detected from the use of the tolerance tables if all replicates are affected equally.

In a seed testing laboratory germination tests are repeated if the replicate variation is greater than the tolerated range. Strictly speaking it could be argued that gene bank staff should also repeat the test. However, the circumstances are different in gene banks since the seed is precious and, if it appears obvious that the excessive replicate variation is caused by an identified deficiency in the test environment for one replicate, then the result of this replicate could be ignored. Provided the variation between the remaining replicates is within the tolerated range for this (reduced) number of replicates the estimate for the accession could be determined as the mean result of the remaining replicates. An example of how to use the tables in this way is provided below (Example 2).

How to use the tolerance tables

At first sight Tables 14.2-14.6 may present a bewildering display of information. The reason for presenting so much is to enable as many gene banks as possible to use tolerance tables, irrespective of the details of the procedures adopted. Consequently tolerance ranges are provided for between two and up to ten replicates for between 20 and 100 seeds per replicate and with probabilities of 0.001-0.05 for the tolerated ranges being exceeded as a result of random sampling error alone.

The choice of which table to use depends on the number of seeds in each replicate: for 100 seed replicates use Table 14.2; for 50 seed replicates use Table 14.3; for 40 seed replicates use Table 14.4; for 25 seed replicates use Table 14.5; and for 20 seed replicates use Table 14.6.

The choice of which column to use depends upon the number of replicates of the germination test and the probability the user wishes to define for the rare chance that the tolerated range will be exceeded by random sampling error alone. Unless a gene bank has some special reason for adopting a

TABLE 14.2 Maximum tolerated difference between the numbers of seeds within any category which is rarely exceeded by chance alone (probabilities of 5%, 2.5%, 1%, or 0.1%) for four, three, or two 100-seed replicates of a germination test

Average germination (%) of all replicates		Number of replicates											
		4				3				2			
		Probability (%)											
		5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1
99	2	4	5	5	6	4	4	5	6	3	4	4	5
98	3	5	6	7	8	5	5	6	8	4	5	5	7
97	4	6	7	8	9	6	6	7	9	5	6	6	8
96	5	7	8	9	11	7	7	8	10	5	6	7	9
95	6	8	9	10	12	7	8	9	11	6	7	8	10
94	7	9	10	11	13	8	9	10	12	7	8	9	11
93	8	9	10	11	14	8	9	11	13	7	8	9	12
92	9	10	11	12	15	9	10	11	14	7	9	10	13
91	10	10	11	13	15	9	11	12	15	8	9	10	13
90	11	11	12	13	16	10	11	12	15	8	9	11	14
89	12	11	12	14	17	10	11	13	16	9	10	11	15
88	13	12	13	14	17	11	12	13	16	9	10	12	15
87	14	12	13	15	18	11	12	14	17	9	11	12	16
86	15	13	14	15	18	11	13	14	18	9	11	13	16
85	16	13	14	16	19	12	13	15	18	10	11	13	17
84	17	13	15	16	19	12	13	15	19	10	11	13	17
83	18	14	15	16	20	12	14	15	19	10	12	14	17
82	19	14	15	17	20	13	14	16	19	10	12	14	18
81	20	14	16	17	21	13	14	16	20	11	12	14	18
80	21	14	16	18	21	13	15	16	20	11	13	14	19
79	22	15	16	18	22	13	15	17	21	11	13	15	19
78	23	15	16	18	22	14	15	17	21	11	13	15	19
77	24	15	17	18	22	14	15	17	21	11	13	15	19
76	25	15	17	19	23	14	16	17	22	12	13	15	20
75	26	16	17	19	23	14	16	18	22	12	14	16	20
74	27	16	17	19	23	14	16	18	22	12	14	16	20
73	28	16	18	19	23	15	16	18	22	12	14	16	21
72	29	16	18	20	24	15	16	18	23	12	14	16	21
71	30	16	18	20	24	15	17	19	23	12	14	16	21
70	31	16	18	20	24	15	17	19	23	12	14	17	21
69	32	17	18	20	24	15	17	19	23	13	14	17	21
68	33	17	18	20	25	15	17	19	23	13	15	17	22
67	34	17	19	21	25	15	17	19	24	13	15	17	22
66	35	17	19	21	25	15	17	19	24	13	15	17	22
65	36	17	19	21	25	16	17	19	24	13	15	17	22
64	37	17	19	21	25	16	17	20	24	13	15	17	22
63	38	17	19	21	25	16	18	20	24	13	15	17	22
62	39	17	19	21	26	16	18	20	24	13	15	17	22
61	40	18	19	21	26	16	18	20	25	13	15	18	23
60	41	18	19	21	26	16	18	20	25	13	15	18	23
59	42	18	19	21	26	16	18	20	25	13	15	18	23
58	43	18	19	22	26	16	18	20	25	13	15	18	23
57	44	18	20	22	26	16	18	20	25	13	15	18	23
56	45	18	20	22	26	16	18	20	25	14	16	18	23
55	46	18	20	22	26	16	18	20	25	14	16	18	23
54	47	18	20	22	26	16	18	20	25	14	16	18	23
53	48	18	20	22	26	16	18	20	25	14	16	18	23
52	49	18	20	22	26	16	18	20	25	14	16	18	23
51	50	18	20	22	26	16	18	20	25	14	16	18	23

TABLE 14.3 Maximum tolerated difference between the numbers of seeds within any category which is rarely exceeded by chance alone (probabilities of 5%, 2.5%, 1%, or 0.1%) for eight, seven, six, five, four, three, or two 50-seed replicates of a germination test

Average germination (%) of all replicates		Number of replicates																											
		8				7				6				5				4				3				2			
		Probability (%)																											
		5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1
99	2	3	4	4	5	3	4	4	5	3	3	4	5	3	3	4	4	3	3	3	4	3	3	3	4	2	2	3	4
98	3	4	5	5	6	4	5	5	6	4	5	5	6	4	4	5	6	4	4	5	6	3	4	4	5	3	3	4	5
97	4	5	6	6	7	5	6	6	7	5	5	6	7	5	5	6	7	4	5	5	7	4	4	5	6	3	4	4	6
96	5	6	6	7	8	6	6	7	8	6	6	7	8	5	6	6	8	5	6	6	8	5	5	6	7	4	4	5	7
95	6	7	7	8	9	6	7	8	9	6	7	7	9	6	6	7	9	6	6	7	8	5	6	6	8	4	5	6	7
94	7	7	8	8	10	7	8	8	10	7	7	8	10	6	7	8	9	6	7	7	9	5	6	7	9	5	5	6	8
93	8	8	8	9	11	7	8	9	10	7	8	9	10	7	8	8	10	6	7	8	10	6	7	7	9	5	6	6	8
92	9	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	11	7	8	8	10	6	7	8	10	5	6	7	9
91	10	9	9	10	12	8	9	10	12	8	9	10	11	8	8	9	11	7	8	9	11	7	7	8	10	5	6	7	9
90	11	9	10	11	12	9	9	10	12	8	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
89	12	9	10	11	13	9	10	11	13	9	10	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
88	13	10	10	11	13	9	10	11	13	9	10	11	13	9	10	10	13	8	9	10	12	7	8	9	12	6	7	8	11
87	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	13	8	9	10	12	6	7	9	11
86	15	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
85	16	11	12	13	15	10	11	12	14	10	11	12	14	10	10	12	14	9	10	11	13	8	9	10	13	7	8	9	12
84	17	11	12	13	15	11	12	13	15	10	11	12	14	10	11	12	14	9	10	11	14	8	9	11	13	7	8	9	12
83	18	11	12	13	15	11	12	13	15	11	11	13	15	10	11	12	14	9	10	12	14	9	10	11	13	7	8	10	12
82	19	12	12	13	16	11	12	13	15	11	12	13	15	10	11	12	15	10	11	12	14	9	10	11	14	7	8	10	13
81	20	12	13	14	16	11	12	13	16	11	12	13	15	11	11	13	15	10	11	12	15	9	10	11	14	7	9	10	13
80	21	12	13	14	16	12	13	14	16	11	12	13	16	11	12	13	15	10	11	12	15	9	10	11	14	8	9	10	13
79	22	12	13	14	17	12	13	14	16	11	12	14	16	11	12	13	16	10	11	13	15	9	10	12	14	8	9	10	13
78	23	12	13	14	17	12	13	14	17	12	13	14	16	11	12	13	16	10	11	13	15	10	11	12	15	8	9	10	13
77	24	13	14	15	17	12	13	14	17	12	13	14	17	11	12	14	16	11	12	13	16	10	11	12	15	8	9	11	14
76	25	13	14	15	17	12	13	15	17	12	13	14	17	11	13	14	16	11	12	13	16	10	11	12	15	8	9	11	14
75	26	13	14	15	18	13	14	15	17	12	13	14	17	12	13	14	17	11	12	13	16	10	11	12	15	8	9	11	14
74	27	13	14	15	18	13	14	15	18	12	13	15	17	12	13	14	17	11	12	13	16	10	11	13	16	8	10	11	14
73	28	13	14	16	18	13	14	15	18	12	14	15	17	12	13	14	17	11	12	14	17	10	11	13	16	8	10	11	14
72	29	13	14	16	18	13	14	15	18	13	14	15	18	12	13	14	17	11	12	14	17	10	11	13	16	9	10	11	15
71	30	14	15	16	19	13	14	15	18	13	14	15	18	12	13	15	17	11	13	14	17	10	12	13	16	9	10	11	15
70	31	14	15	16	19	13	14	16	18	13	14	15	18	12	13	15	18	12	13	14	17	11	12	13	16	9	10	12	15
69	32	14	15	16	19	13	14	16	19	13	14	15	18	12	14	15	18	12	13	14	17	11	12	13	16	9	10	12	15
68	33	14	15	16	19	14	15	16	19	13	14	16	18	13	14	15	18	12	13	14	17	11	12	13	17	9	10	12	15
67	34	14	15	16	19	14	15	16	19	13	14	16	19	13	14	15	18	12	13	14	17	11	12	13	17	9	10	12	15
66	35	14	15	17	19	14	15	16	19	13	14	16	19	13	14	15	18	12	13	15	18	11	12	14	17	9	10	12	15
65	36	14	15	17	19	14	15	16	19	13	15	16	19	13	14	15	18	12	13	15	18	11	12	14	17	9	10	12	15
64	37	14	15	17	20	14	15	16	19	13	15	16	19	13	14	15	18	12	13	15	18	11	12	14	17	9	11	12	16
63	38	14	16	17	20	14	15	16	19	14	15	16	19	13	14	16	19	12	13	15	18	11	12	14	17	9	11	12	16
62	39	15	16	17	20	14	15	17	19	14	15	16	19	13	14	16	19	12	13	15	18	11	12	14	17	9	11	12	16
61	40	15	16	17	20	14	15	17	20	14	15	16	19	13	14	16	19	12	14	15	18	11	12	14	17	9	11	12	16
60	41	15	16	17	20	14	15	17	20	14	15	16	19	13	14	16	19	12	14	15	18	11	13	14	17	9	11	12	16
59	42	15	16	17	20	14	15	17	20	14	15	16	19	13	14	16	19	12	14	15	18	11	13	14	17	9	11	12	16
58	43	15	16	17	20	14	15	17	20	14	15	16	19	13	14	16	19	12	14	15	18	11	13	14	17	9	11	12	16
57	44	15	16	17	20	14	16	17	20	14	15	16	19	13	14	16	19	12	14	15	18	11	13	14	18	9	11	13	16
56	45	15	16	17	20	14	16	17	20	14	15	16	20	13	15	16	19	13	14	15	18	11	13	14	18	9	11	13	16
55	46	15	16	17	20	14	16	17	20	14	15	17	20	13	15	16	19	13	14	15	18	11	13	14	18	9	11	13	16
54	47	15	16	17	20	14	16	17	20	14	15	17	20	13	15	16	19	13	14	15	19	11	13	14	18	10	11	13	16
53	48	15	16	17	20	14	16	17	20	14	15	17	20	13	15	16	19	13	14	15	19	11	13	14	18	10	11	13	16
52	49	15	16	17	20	15	16	17	20	14	15	17	20	13	15	16	19	13	14	15	19	11	13	14	18	10	11	13	16
51	50	15	16	17	20	15	16	17	20	14	15	17	20	13	15	16	19	13	14	15	19	11	13	14	18	10	11	13	16

TABLE 14.4 Maximum tolerated difference between the numbers of seeds within any category which is rarely exceeded by chance alone (probabilities of 5%, 2.5%, 1%, or 0.1%) for ten, nine, eight, seven, six, five, four, three, or two 40-seed replicates of a germination test

Average germination (%) of all replicates		Number of replicates: 10				9				8				7				6				5				4				3				2			
	Probability (%)	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1
99	2	3	3	4	4	3	3	4	4	3	3	4	4	3	3	3	4	3	3	3	4	3	3	3	4	2	3	3	4	2	3	3	4	2	2	3	3
98	3	4	4	5	6	4	4	5	6	4	4	5	5	4	4	5	5	4	4	4	5	4	4	4	5	3	4	4	5	3	3	4	5	2	3	3	4
97	4	5	5	6	7	5	5	6	7	5	5	6	6	5	5	5	6	4	5	5	6	4	5	5	6	4	4	5	6	4	4	4	6	3	3	4	5
96	5	6	6	6	8	5	6	6	7	5	6	6	7	5	6	6	7	5	5	6	7	5	5	6	7	4	5	5	7	4	5	5	6	3	4	4	6
95	6	6	7	7	8	6	6	7	8	6	6	7	8	6	6	7	8	6	6	7	8	5	6	6	8	5	5	6	7	4	5	6	7	4	4	5	6
94	7	7	7	8	9	7	7	8	9	6	7	7	9	6	7	7	9	6	7	7	8	6	6	7	8	5	6	7	8	5	5	6	8	4	5	5	7
93	8	7	8	8	10	7	8	8	10	7	7	8	9	7	7	8	9	6	7	8	9	6	7	7	9	6	6	7	9	5	6	7	8	4	5	6	7
92	9	8	8	9	10	7	8	9	10	7	8	9	10	7	8	8	10	7	7	8	10	7	7	8	9	6	7	7	9	6	6	7	9	5	5	6	8
91	10	8	9	9	11	8	8	9	11	8	8	9	11	7	8	9	10	7	8	9	10	7	8	8	10	6	7	8	10	6	7	7	9	5	6	6	8
90	11	8	9	10	11	8	9	10	11	8	9	9	11	8	8	9	11	8	8	9	11	7	8	[illegible]	10	7	7	8	10	6	7	8	10	5	6	7	9
89	12	9	9	10	12	9	9	10	12	8	9	10	11	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	10	6	7	8	10	5	6	7	9
88	13	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	12	8	9	10	11	8	9	9	11	7	8	9	11	7	7	8	10	6	6	7	9
87	14	9	10	11	13	9	10	11	12	9	10	11	12	9	9	10	12	8	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
86	15	10	10	11	13	10	10	11	13	9	10	11	13	9	10	11	12	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
85	16	10	11	12	13	10	10	11	13	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
84	17	10	11	12	14	10	11	12	14	10	11	11	13	10	10	11	13	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	12	6	7	8	11
83	18	10	11	12	14	10	11	12	14	10	11	12	14	10	11	11	14	9	10	11	13	9	10	11	13	8	9	10	12	8	9	10	12	6	7	8	11
82	19	11	11	12	14	11	11	12	14	10	11	12	14	10	11	12	14	10	10	11	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
81	20	11	12	13	15	11	12	12	15	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	[illegible]	7	8	9	11
80	21	11	12	13	15	11	12	13	15	11	11	12	15	10	11	12	14	10	11	12	14	10	10	11	14	9	10	11	13	8	9	10	13	7	8	9	12
79	22	11	12	13	15	11	12	13	15	11	12	13	15	11	11	12	15	10	11	12	14	10	11	12	14	9	10	11	14	8	9	10	13	7	8	9	12
78	23	12	12	13	16	11	12	13	15	11	12	13	15	11	12	13	15	10	11	12	15	10	11	12	14	9	10	11	14	8	9	11	13	7	8	9	12
77	24	12	13	14	16	12	12	13	16	11	12	13	15	11	12	13	15	11	11	13	15	10	11	12	14	9	10	12	14	9	10	11	13	7	8	9	12
76	25	12	13	14	16	12	13	14	16	11	12	13	16	11	12	13	15	11	12	13	15	10	11	12	15	10	11	12	14	9	10	11	14	7	8	10	12
75	26	12	13	14	16	12	13	14	16	12	12	13	16	11	12	13	16	11	12	13	15	10	11	12	15	10	11	12	14	9	10	11	14	7	8	10	13
74	27	12	13	14	16	12	13	14	16	12	13	14	16	11	12	13	16	11	12	13	15	11	11	13	15	10	11	12	15	9	10	11	14	7	9	10	13
73	28	12	13	14	17	12	13	14	16	12	13	14	16	12	12	14	16	11	12	13	16	11	12	13	15	10	11	12	15	9	10	11	14	8	9	10	13
72	29	13	13	14	17	12	13	14	17	12	13	14	16	12	13	14	16	11	12	13	16	11	12	13	15	10	11	12	15	9	10	11	14	8	9	10	13
71	30	13	14	15	17	12	13	14	17	12	13	14	17	12	13	14	16	11	12	13	16	11	12	13	16	10	11	12	15	9	10	12	14	8	9	10	13
70	31	13	14	15	17	13	13	15	17	12	13	14	17	12	13	14	16	11	12	14	16	11	12	13	16	10	11	13	15	9	10	12	14	8	9	10	13
69	32	13	14	15	17	13	14	15	17	12	13	14	17	12	13	14	17	12	13	14	16	11	12	13	16	10	11	13	15	9	11	12	15	8	9	10	13
68	33	13	14	15	17	13	14	15	17	12	13	15	17	12	13	14	17	12	13	14	16	11	12	13	16	10	12	13	15	10	11	12	15	8	9	11	14
67	34	13	14	15	18	13	14	15	17	13	13	15	17	12	13	14	17	12	13	14	17	11	12	13	16	11	12	13	16	10	11	12	15	8	9	11	14
66	35	13	14	15	18	13	14	15	17	13	14	15	17	12	13	14	17	12	13	14	17	11	12	14	16	11	12	13	16	10	11	12	15	8	9	11	14
65	36	13	14	15	18	13	14	15	18	13	14	15	17	12	13	15	17	12	13	14	17	11	12	14	16	11	12	13	16	10	11	12	15	8	9	11	14
64	37	13	14	15	18	13	14	15	18	13	14	15	17	12	13	15	17	12	13	14	17	11	13	14	16	11	12	13	16	10	11	12	15	8	9	11	14
63	38	13	14	16	18	13	14	15	18	13	14	15	18	13	13	15	17	12	13	14	17	12	13	14	17	11	12	13	16	10	11	12	15	8	9	11	14
62	39	14	14	16	18	13	14	15	18	13	14	15	18	13	14	15	17	12	13	14	17	12	13	14	17	11	12	13	16	10	11	12	15	8	9	11	14
61	40	14	15	16	18	13	14	15	18	13	14	15	18	13	14	15	17	12	13	14	17	12	13	14	17	11	12	13	16	10	11	12	15	8	10	11	14
60	41	14	15	16	18	13	14	16	18	13	14	15	18	13	14	15	18	12	13	15	17	12	13	14	17	11	12	13	16	10	11	13	15	8	10	11	14
59	42	14	15	16	18	13	14	16	18	13	14	15	18	13	14	15	18	12	13	15	17	12	13	14	17	11	12	13	16	10	11	13	16	8	10	11	14
58	43	14	15	16	18	13	14	16	18	13	14	15	18	13	14	15	18	12	13	15	17	12	13	14	17	11	12	14	16	10	11	13	16	8	10	11	14
57	44	14	15	16	18	14	14	16	18	13	14	15	18	13	14	15	18	12	13	15	17	12	13	14	17	11	12	14	16	10	11	13	16	8	10	11	14
56	45	14	15	16	19	14	15	16	18	13	14	15	18	13	14	15	18	12	13	15	17	12	13	14	17	11	12	14	16	10	11	13	15	8	10	11	14
55	46	14	15	16	19	14	15	16	18	13	14	15	18	13	14	15	18	12	13	15	17	12	13	14	17	11	12	14	16	10	11	13	16	8	10	11	14
54	47	14	15	16	19	14	15	16	18	13	14	16	18	13	14	15	18	12	14	15	18	12	13	14	17	11	12	14	17	10	11	13	15	8	10	11	14
53	48	14	15	16	19	14	15	16	18	13	14	16	18	13	14	15	18	12	14	15	18	12	13	14	17	11	12	14	17	10	11	13	16	8	10	11	14
52	49	14	15	16	19	14	15	16	18	13	14	16	18	13	14	15	18	12	14	15	18	12	13	14	17	11	12	14	17	10	11	13	16	8	10	11	14
51	50	14	15	16	19	14	15	16	18	13	14	16	18	13	14	15	18	12	14	15	18	12	13	14	17	11	12	14	17	10	11	13	16	8	10	11	14

TABLE 14.5 Maximum tolerated difference between the numbers of seeds within any category which is rarely exceeded by chance alone (probabilities of 5%, 2.5%, 1%, or 0.1%) for eight, seven, six, five, four, three, or two 25-seed replicates of a germination test

Average germination (%) of all replicates		Number of replicates																											
		8				7				6				5				4				3				2			
		Probability (%)																											
		5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1
99	2	2	3	3	3	2	2	3	3	2	2	3	3	2	2	3	3	2	2	2	3	2	2	2	3	1	2	2	3
98	3	3	3	4	4	3	3	4	4	3	3	3	4	3	3	3	4	3	3	3	4	2	3	3	4	2	2	3	3
97	4	4	4	4	5	4	4	4	5	3	4	4	5	3	4	4	5	3	3	4	5	3	3	3	4	2	3	3	4
96	5	4	4	5	6	4	4	5	6	4	4	5	6	4	4	4	5	3	4	4	5	3	4	4	5	3	3	3	5
95	6	5	5	5	6	4	5	5	6	4	5	5	6	4	4	5	6	4	4	5	6	3	4	4	5	3	3	4	5
94	7	5	5	6	7	5	5	6	7	5	5	6	7	4	5	5	6	4	5	5	6	4	4	5	6	3	4	4	5
93	8	5	6	6	7	5	6	6	7	5	5	6	7	5	5	6	7	4	5	6	7	4	5	5	6	3	4	5	6
92	9	6	6	7	8	6	6	7	8	5	6	6	8	5	6	6	7	5	5	6	7	4	5	5	7	4	4	5	6
91	10	6	6	7	8	6	6	7	8	6	6	7	8	5	6	6	8	5	6	6	8	5	5	6	7	4	4	5	7
90	11	6	7	7	9	6	7	7	9	[illegible]	6	7	8	6	6	7	8	5	6	6	8	5	5	6	7	4	5	5	7
89	12	7	7	8	9	6	7	8	9	6	7	7	9	6	6	7	8	6	6	7	8	5	6	6	8	4	5	6	7
88	13	7	7	8	9	7	7	8	9	6	7	8	9	6	7	7	9	6	6	7	9	5	6	7	8	4	5	6	7
87	14	7	8	8	10	7	7	8	10	7	7	8	9	6	7	8	9	6	7	7	9	5	6	7	8	4	5	6	8
86	15	7	8	9	10	7	8	8	10	7	7	8	10	7	7	8	9	6	7	7	5	6	6	7	9	5	5	6	8
85	16	7	8	9	10	7	8	9	10	7	8	8	10	7	7	8	10	6	7	8	9	6	6	7	9	5	5	6	8
84	17	8	8	9	11	7	8	9	10	7	8	9	10	7	8	8	10	6	7	8	10	6	7	7	9	5	6	6	8
83	18	8	8	9	11	8	8	9	11	7	8	9	10	7	8	8	10	7	7	8	10	6	7	8	9	5	6	7	9
82	19	8	9	9	11	8	8	9	11	8	8	9	11	7	8	9	10	7	7	8	10	6	7	8	10	5	6	7	9
81	20	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	11	7	8	8	10	6	7	8	10	5	6	7	9
80	21	8	9	10	11	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	10	6	7	8	10	5	6	7	9
79	22	9	9	10	12	8	9	10	12	8	9	10	11	8	8	9	11	7	8	9	11	7	7	8	10	5	6	7	9
78	23	9	9	10	12	8	9	10	12	8	9	10	11	8	8	9	11	7	8	9	11	7	7	8	10	6	6	7	9
77	24	9	9	10	12	9	9	10	12	8	9	10	12	8	9	9	11	7	8	9	11	7	8	8	10	6	6	7	10
76	25	9	10	10	12	9	9	10	12	8	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
75	26	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
74	27	9	10	11	13	9	10	11	12	9	9	10	12	8	9	10	12	8	9	9	11	7	8	9	11	6	7	8	10
73	28	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
72	29	9	10	11	13	9	10	11	13	9	10	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
71	30	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
70	31	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
69	32	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	7	8	9	11	6	7	8	11
68	33	10	11	11	13	9	10	11	13	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	12	6	7	8	11
67	34	10	11	12	13	10	10	11	13	9	10	11	13	9	10	11	13	8	9	10	12	8	8	9	12	6	7	8	11
66	35	10	11	12	14	10	10	11	13	9	10	11	13	9	10	11	13	8	9	10	12	8	8	10	12	6	7	8	11
65	36	10	11	12	14	10	10	11	13	9	10	11	13	9	10	11	13	8	9	10	12	8	9	10	12	6	7	8	11
64	37	10	11	12	14	10	11	11	14	9	10	11	13	9	10	11	13	8	9	10	13	8	9	10	12	6	7	8	11
63	38	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	9	9	10	13	8	9	10	12	6	7	9	11
62	39	10	11	12	14	10	11	12	14	10	10	11	13	9	10	11	13	9	9	10	13	8	9	10	12	6	7	9	11
61	40	10	11	12	14	10	11	12	14	10	10	11	13	9	10	11	13	9	9	10	13	8	9	10	12	6	7	9	11
60	41	10	11	12	14	10	11	12	14	10	10	11	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
59	42	10	11	12	14	10	11	12	14	10	10	11	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
58	43	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
57	44	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
56	45	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
55	46	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
54	47	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
53	48	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
52	49	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11
51	50	10	11	12	14	10	11	12	14	10	11	12	14	9	10	11	13	9	10	11	13	8	9	10	12	7	8	9	11

TABLE 14.6 Maximum tolerated difference between the numbers of seeds within any category which is rarely exceeded by chance alone (probabilities of 5%, 2.5%, 1%, or 0.1%) for ten, nine, eight, seven, six, five, four, three, or two 20-seed replicates of a germination test

Average germination (%) of all replicates		Number of replicates: 10				9				8				7				6				5				4				3				2			
	Probability (%):	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1	5	2.5	1	0.1
99	2	2	2	3	3	2	2	2	3	2	2	2	3	2	2	2	3	2	2	2	3	2	2	2	3	2	2	2	3	2	2	2	2	1	1	2	2
98	3	3	3	3	4	3	3	3	4	3	3	3	4	3	3	3	4	3	3	3	4	2	3	3	4	2	2	3	3	2	2	3	3	2	2	2	3
97	4	3	4	4	5	3	4	4	5	3	3	4	4	3	3	4	4	3	3	4	4	3	3	3	4	3	3	3	4	2	3	3	4	2	2	3	4
96	5	4	4	4	5	4	4	4	5	4	4	4	5	4	4	4	5	3	4	4	5	3	4	4	5	3	3	4	5	3	3	4	4	2	3	3	4
95	6	4	5	5	6	4	5	5	6	4	4	5	6	4	4	5	6	4	4	5	5	4	4	4	5	3	4	4	5	3	3	4	5	3	3	3	4
94	7	5	5	5	6	5	5	5	6	4	5	5	6	4	5	5	6	4	5	5	6	4	4	5	6	4	4	5	6	3	4	4	5	3	3	4	5
93	8	5	5	6	7	5	5	6	7	5	5	6	7	5	5	5	6	4	5	5	6	4	5	5	6	4	4	5	6	4	4	5	6	3	3	4	5
92	9	5	6	6	7	5	6	6	7	5	5	6	7	5	5	6	7	5	5	6	7	5	5	5	7	4	5	5	6	4	4	5	6	3	4	4	6
91	10	6	6	6	8	5	6	6	7	5	6	6	7	5	6	6	7	5	5	6	7	5	5	6	7	4	5	5	7	4	5	5	6	3	4	4	6
90	11	6	6	7	8	6	6	7	8	6	6	7	8	5	6	6	8	5	6	6	7	5	5	6	7	5	5	6	7	4	5	5	7	4	4	5	6
89	12	6	7	7	8	6	6	7	8	6	6	7	8	6	6	7	8	5	6	7	8	5	6	6	8	5	5	6	[illegible]	4	5	6	7	4	4	5	6
88	13	6	7	7	9	6	7	7	8	6	7	7	8	6	6	7	8	6	6	7	8	5	6	7	8	5	6	6	9	5	5	6	7	4	4	5	7
87	14	7	7	8	9	6	7	7	9	6	7	7	9	6	7	7	8	6	6	7	8	6	6	7	8	5	6	6	8	5	5	6	7	4	5	5	7
86	15	7	7	8	9	7	7	8	9	6	7	8	9	6	7	7	9	6	7	7	9	6	6	7	8	5	6	7	8	5	6	6	8	4	5	5	7
85	16	7	7	8	9	7	7	8	9	7	7	8	9	6	7	8	9	6	7	7	9	6	7	7	9	6	6	7	8	5	6	6	8	4	5	6	7
84	17	7	8	8	10	7	8	8	10	7	7	8	9	7	7	8	9	6	7	8	9	6	7	7	9	6	6	7	9	5	6	7	8	4	5	6	7
83	18	7	8	8	10	7	8	8	10	7	8	8	10	7	7	8	9	7	7	8	9	6	7	8	9	6	6	7	9	5	6	7	8	4	5	6	8
82	19	7	8	9	10	7	8	9	10	7	8	8	10	7	8	8	10	7	7	8	9	6	7	8	9	6	7	7	9	5	6	7	9	5	5	6	8
81	20	8	8	9	10	8	8	9	10	7	8	9	10	7	8	8	10	7	7	8	10	7	7	8	9	6	7	8	9	6	6	7	9	5	5	6	8
80	21	8	8	9	11	8	8	9	10	7	8	9	10	7	8	9	10	7	8	8	10	7	7	8	10	6	7	8	9	6	6	7	9	5	5	6	8
79	22	8	9	9	11	8	8	9	11	8	8	9	10	7	8	9	10	7	8	8	10	7	7	8	10	6	7	8	9	6	6	7	9	5	6	6	8
78	23	8	9	9	11	8	9	9	11	8	8	9	11	8	8	9	10	7	8	9	10	7	8	8	10	6	7	8	10	6	7	7	9	5	6	7	8
77	24	8	9	10	11	8	9	9	11	8	8	9	11	8	8	9	11	7	8	9	10	7	8	8	10	7	7	8	10	6	7	8	9	5	6	7	9
76	25	8	9	10	11	8	9	9	11	8	9	9	11	8	8	9	11	7	8	9	11	7	8	9	10	7	7	8	10	6	7	8	9	5	6	7	9
75	26	8	9	10	11	8	9	10	11	8	9	9	11	8	8	9	11	8	8	9	11	7	8	9	10	7	7	8	10	6	7	8	10	5	6	7	9
74	27	9	9	10	12	8	9	10	11	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	11	7	8	8	10	6	7	8	10	5	6	7	9
73	28	9	9	10	12	8	9	10	12	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	11	7	8	9	10	6	7	8	10	5	6	7	9
72	29	9	9	10	12	9	9	10	12	8	9	10	11	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	10	6	7	8	10	5	6	7	9
71	30	9	9	10	12	9	9	10	12	8	9	10	12	8	9	10	11	8	9	9	11	8	8	9	11	7	8	9	11	6	7	8	10	5	6	7	9
70	31	9	10	10	12	9	9	10	12	9	9	10	12	8	9	10	12	8	9	10	11	8	8	9	11	7	8	9	11	7	7	8	10	5	6	7	9
69	32	9	10	10	12	9	9	10	12	9	9	10	12	8	9	10	17	8	9	10	11	8	8	9	11	7	8	9	11	7	7	8	10	5	6	7	9
68	33	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	17	8	9	10	12	8	9	9	11	7	8	9	11	7	7	8	10	6	6	7	9
67	34	9	10	11	12	9	10	10	12	9	9	10	12	9	9	10	12	8	9	10	12	8	9	9	11	7	8	9	11	7	7	8	10	6	6	7	10
66	35	9	10	11	12	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	12	8	9	10	11	7	8	9	11	7	8	8	10	6	6	7	10
65	36	9	10	11	13	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	12	8	9	10	11	7	8	9	11	7	8	9	11	6	7	8	10
64	37	9	10	11	13	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
63	38	9	10	11	13	9	10	11	13	9	10	11	12	9	9	10	12	8	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
62	39	9	10	11	13	9	10	11	13	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	5	7	8	10
61	40	10	10	11	13	9	10	11	13	9	10	11	12	9	10	10	12	9	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
60	41	10	10	11	13	9	10	11	13	9	10	11	13	9	10	10	12	9	9	10	12	8	9	10	12	8	8	9	11	7	8	9	11	6	7	8	10
59	42	10	10	11	13	9	10	11	13	9	10	11	13	9	10	10	12	9	9	10	12	8	9	10	12	8	9	9	11	7	8	9	11	6	7	8	10
58	43	10	10	11	13	9	10	11	13	9	10	11	13	9	10	11	12	9	9	10	12	8	9	10	.2	8	9	9	11	7	8	9	11	6	7	8	10
57	44	10	10	11	13	9	10	11	13	9	10	11	13	9	10	11	12	9	9	10	12	8	9	10	12	8	9	9	12	7	8	9	11	6	7	8	10
56	45	10	10	11	13	9	10	11	13	9	10	11	13	9	10	11	12	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
55	46	10	10	11	13	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
54	47	10	10	11	13	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
53	48	10	10	11	13	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
52	49	10	10	11	13	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10
51	50	10	10	11	13	10	10	11	13	9	10	11	13	9	10	11	13	9	9	10	12	8	9	10	12	8	9	10	12	7	8	9	11	6	7	8	10

higher or lower probability we suggest that a probability of 0.025 is a suitable compromise (and conforms with that adopted by ISTA and AOSA). For example, if the germination test comprises four 100 seed replicates and we wish to determine the maximum tolerated range for a probability of 0.025 then we would use the fourth column of Table 14.2.

The choice of row is dependent upon the mean result of the germination test. Values for 51% mean germination percentage or more are given in the first column. Values for 50% or less are given in the second column. Note that the maximum tolerated ranges for 99% to 51% are the same as those for 2% to 50% respectively. The mean result obtained from all the replicates should be corrected to the nearest one percent. Where the fraction is exactly 0.5 the result should be corrected to the next larger whole number. For example, if the mean result of four replicates of 100 seeds were 95.5% then the fourth row (96%) of Table 14.2 should be used.

Example 1. Eighty-nine, 95, 90 and 87 seeds germinated in the four 100-seed replicates of a germination test. The mean percentage germination is thus ((89+95+90+87)/4=) 90.25 which is rounded down to 90%. From Table 14.2 the maximum tolerated range for four 100 seed replicates at a probability of 0.025 is 8 at 96%. The actual range of replicate results is 95-87, which is also 8. Since the appropriate value in Table 14.2 has not been exceeded we conclude that the replicate variation is no greater than would be expected by random sampling error, and the viability estimate of 90% can be accepted.

Example 2. Thirty-seven, 39, 38, 36 and 25 seeds germinated in the five 40-seed replicates of a germination test. The mean percentage germination is ((37+39+38+36+25)/2=) 87.5 which is rounded up to 88%. From Table 14.4 the maximum tolerated range for five 40 seed replicates at a probability of 0.025 is 9 at 88%. The actual range between the number of seeds germinating is 39-25; that is 14. Now this is greater than the maximum tolerated range. Thus we conclude that the variation observed between replicates is likely to be greater than that due to random sampling error alone. Consequently we should ignore this test and repeat the germination test on a further sample of seeds from the accession. However, suppose that the constraints of time or the number of seeds within this accession make a further test undesirable. In addition further suppose that we have observed a deficiency (e.g. excess moisture) in the test environment for the replicate which gave the lowest response. If we ignore the result for this replicate the mean percentage germination of the remaining four replicates is ((37+39+38+36)x5/8=) 93.75 which is rounded up to 94%. From Table 14.4 the maximum tolerated range for four (remember this has been reduced) 40 seed replicates at a probability of 0.025 is 6 at 94%. The actual range between the numbers of seeds germinating is 39-36, that is 3, which is less than the maximum tolerated range. Consequently we could accept 94% as the best estimate of accession germination we can obtain given limited resources which make it difficult to repeat germination tests.

Probability

The probabilities given in Tables 14.2 to 14.6 are the relative frequencies of the maximum tolerated range being exceeded due to chance alone. Returning to Example 1 the maximum tolerated range for four 100 seed replicates at a probability of 0.025 is 8 at 96%. What this means is that if we carried out 200 germination tests in which the mean result was 96% and in all 200 tests the range between replicates is 9 then in slightly less than 5 of these tests (5/200 = 0.025), this range between replicates would have been caused by random sampling error (item 1 in the previous list), but in slightly more than 195 of the tests the cause would have been something else (items 2 to 8 in the previous list).

Note that in practice whichever probability is used fewer than this proportion of decisions to reject the test result are actually wrong. In many cases the replicate range will be considerably greater than the values provided in the appropriate table.

The 1976 ISTA rules used a probability of 0.025 in their tolerance test for germination. In fact those ISTA values are the same as those in Table 14.2 for four 100 seed replicates and a probability of 0.025. Tolerances are provided here for four levels of probability in order to enable users to apply the levels considered most suitable for their requirements.

When deciding on a probability level it should be remembered that there is an unstated negative argument. If the level of probability used is reduced, then as well as reducing the probability that we would decide that the variation is not due to random sampling error when in fact it is due to random sampling error, we would at the same time be increasing the unstated probability that the variation is due to one or more factors other than random sampling error whilst we decide that the variation results from random sampling error alone. What does this mean in practice?

Well suppose we wanted to use the tolerance tables when commissioning a new germinator. One common problem with such equipment is that the variation of temperature within the room or cabinet can be considerable once the volume is occupied by germination tests. We might test the seeds at a temperature where germination was known to be very sensitive to small differences in temperature and place the replicates as far apart from one another within the germinator. When it came to using Tables 14.2 to 14.6 we might use the values at 5% rather than lower values because we would wish to maximise the probability of detecting variation between one replicate and another. In contrast under normal use we would wish to minimise the probability of deciding that some factor other than random sampling error is causing variation when in fact only random sampling error is responsible for the observed variation. In this case we would use the probabilities lower than 5% for which tolerances are provided in Tables 14.2 to 14.6. For most purposes, unless there are other special reasons, it is suggested that a probability of 0.025 is adopted as standard.

Classification of response

In the examples provided the variation of the numbers of seeds germinating between replicates was the response which was compared to the maximum tolerated ranges in Tables 14.2 to 14.6. However, the tables can be used for any category of seed response in the germination test, or any combination of categories. These categories include normal seedlings, abnormal seedlings, fresh seeds, dead seeds, hard seeds and of course any viability test results; although it will usually be the variation in the number of seeds producing normal seedlings which will be compared with the tolerance tables. Finally it should be noted that the use of these tables need not be limited to results from germination tests. Results from all viability tests (e.g. tetrazolium tests), or seed health or purity tests can be used with these tables if the tests are replicated.

Calculation of the tolerance tables

The tolerance tables presented here assume random variation only according to the binomial distribution where the standard deviation = $\sqrt{(p.q/n)}$ where **p** is the percentage of seeds responding, **q** is the percentage of seeds not responding (i.e. 100-p) and **n** is the number of seeds per replicate.

For values of p above 50% the standard deviation was calculated after first subtracting 0.5 from p. For values below 50% the standard deviation was calculated after adding 0.5 to p. This was done because in practice mean germination percent is rounded to the nearest whole per cent. Thus the value calculated for the standard deviation was the highest possible within the range of results that would give the value given for p in Tables 14.2 to 14.6. The significant range was calculated by multiplying the standard deviation by a factor obtained from a table showing "The percentage points of the distribution of the range" (Pearson, E.S. and Hartley, H.O. (1966). Biometrika Tables for Statisticians, Volume 1 (3rd ed.), 264 pp., Cambridge University Press). The value of this factor varies with the number of tests being compared and probability.

The maximum tolerated ranges in Tables 14.2 to 14.6 are less than this significant range and are the whole number next smaller than the significant range with one exception. The values for the percentage points of the distribution of the range calculated by Pearson and Hartley assume a normal distribution whereas of course the binomial distribution is not normal (although the two distributions are similar). Consequently the correction factor suggested by S.R. Miles (Handbook of Tolerances and of Measures of Precision for Seed Testing. Proceedings of the International Seed Testing Association, 28 (1963), 525-686) was used. Namely, the tolerated range provided in Tables 14.2 to 14.6 is the smaller whole number than the significant range except where the fraction of the significant range is 0.8 or above, in which case the maximum tolerated range is the larger whole number than the significant range.

CHAPTER 15. SEQUENTIAL GERMINATION TEST PLANS AND SUMMARY OF PREFERRED GERMINATION TEST PROCEDURES

Seed accessions are stored in gene banks under conditions of low temperature and low moisture in order to maintain viability, and genetic integrity which is associated with high viability, for a substantial period. It is necessary to estimate viability when each accession is placed in store and to reassess viability at regular, though infrequent, intervals during storage. Traditionally this monitoring of accession viability has been carried out using germination tests of fixed sample size (see Chapters 12 and 14). But IBPGR now recommends an alternative procedure for monitoring viability which is described in this chapter. It has the advantage of utilizing, on average, less seeds than a fixed sample size test for a similar degree of accuracy. This procedure is not based on a pre-determined sample size which is the same for all tests: instead the sample size of each test depends on the results obtained during its progress. The test used is known as the sequential probability ratio test and it is described in detail in the references at the end of this chapter. A simpler type of sequential test, in which the sample size can be either 200 or 400 seeds (and in that sense is not fixed), is also described in this chapter. The chapter is concluded by a summary of preferred procedures for germination tests.

THE SEQUENTIAL PROBABILITY RATIO TEST

Monitoring tests carried out on samples withdrawn from accessions during storage are essentially decision making procedures. At the end of a test one of two decisions is possible: either maintain the accession in store until the next monitoring test is due; or regenerate the accession as soon as practicable. In fixed sample size tests the decision boundary is a single test result; the regeneration standard. If this result is obtained or exceeded then the accession is maintained in store. If the result is less than this value then the accession is regenerated.

The decision boundaries

In sequential analysis, however, each test proceeds in stages and at the end of each stage there are two decision boundaries between which there is an intermediate zone where no decision is possible and consequently the test must be continued to the next stage. (The test is continued by sampling and testing another group of seeds.) In the simplest form of sequential analysis a single article (e.g. one seed) is sampled from a batch (accession) and tested (for viability in a germination test). The result will be either positive (the seed is viable) or negative (the seed is non-viable). Depending upon the result a decision is made to accept the batch (maintain in store until the next monitoring test); or to reject the batch (regenerate the accession as soon as possible); or to continue the sequential test (sample another seed from the accession and test). In a gene bank it would not be feasible to sample seeds one at a time because of the time delay between sampling and obtaining the result. To overcome this difficulty observations can be grouped, that is a certain number of seeds can be sampled from the accession at one time and tested. Depending on the results obtained the sequential test can then either be concluded or a further group of seeds sampled and tested.

Figure 15.1 Diagram of sequential analysis monitoring procedure showing the three regions delineated by the two decision boundaries for the sequential germination test plan provided in Table 15.5. The broken horizontal line corresponds to 85% germination. The asterisk shows the decision point of an equally reliable fixed sample size procedure (see text). The sequential test continues until $G_m - 34m \geqslant 1.058m + 5.166$ or $G_m - 34m \leqslant 1.058m - 5.166$ where m is the number of groups of seeds tested (40 seeds per group), and G_m is the number of germinated seeds. The horizontal line corresponds to 85 percent germination (i.e. when $G_m/100 = 34/40 = 85\%$). In this example seeds are tested in groups of 40. When the response of the first group has been determined, the number of seeds germinated minus 40 x 0.85 can be plotted against 40 seeds or 1 group on the diagram. After m groups have been tested, the number of seeds germinated minus 40 x 0.85m is plotted against 40m seeds or m groups. If the plotted point lies on or above the upper boundary then the accession will be retained in store and this monitoring test will be discontinued; after the next monitoring interval, a further test will be conducted. If the plotted point lies on or below the lower boundary then the accession will be regenerated. If the plotted point lies between the boundaries, then a further group of 40 seeds will be tested. Examples of sequential test results are shown for three accessions (○, ●, ■). The results from which these plots are derived are shown in Table 15.1.

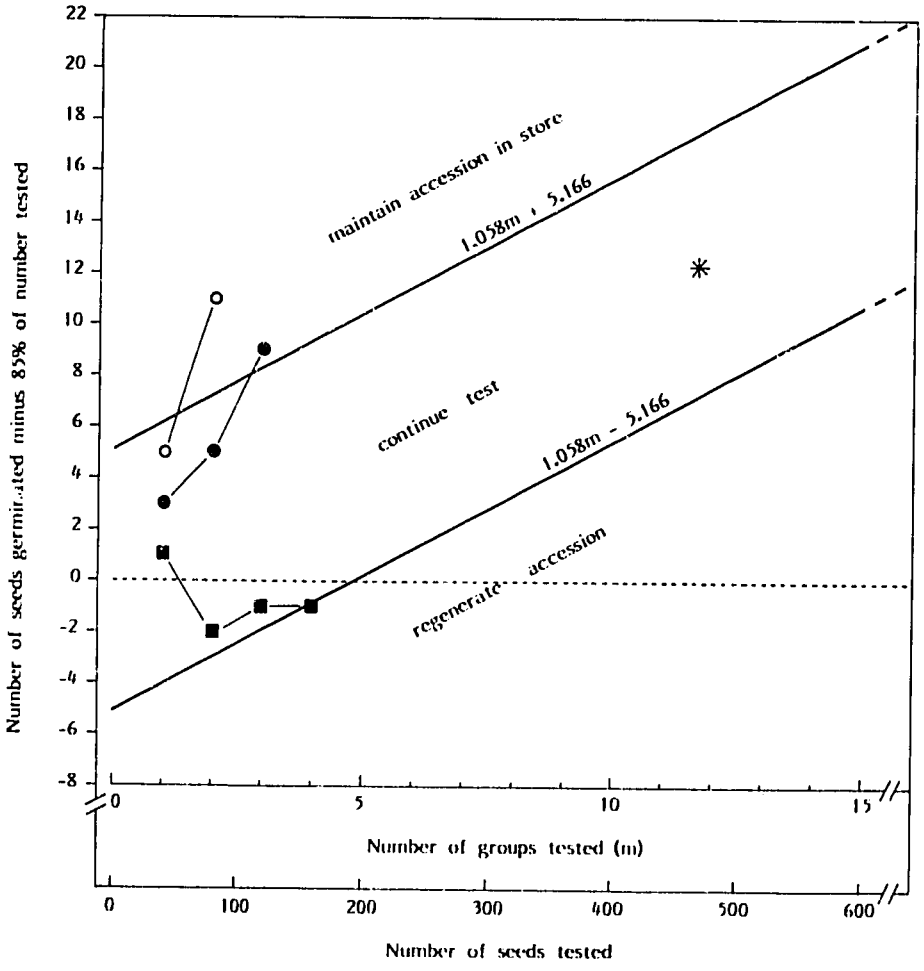


Figure 15.1 illustrates the two decision boundaries, between which lies the intermediate zone wherein tests cannot be concluded, for one sequential probability ratio test. The zone above (and including) the upper boundary is that for the decision to maintain the accession in storage. The zone below (and including) the lower boundary is that for the decision to regenerate the accession. An example of the results obtained from testing successive groups of seeds (each line representing the same accession after different periods of storage) are superimposed on these zones.

The decision boundaries shown in Figure 15.1 are those of a sequential test plan which was designed (and has been confirmed) to be equally reliable as a fixed sample size test of 467 seeds. The decision point of this equivalent fixed sample size test is shown as an asterisk in Figure 15.1. The major advantage of sequential germination test plans should now be apparent. At any given level of accession viability the sequential germination test plan, on average, requires fewer seeds to make a decision than equally reliable fixed sample size tests. The difference is least when true accession viability is close to the decision point of the equivalent fixed sample size test. At this level of viability the probability of regeneration is 0.5, that is the decision could go either way. The saving of seed increases as the difference between true accession viability and this point increase. This is particularly useful for gene banks since the saving in seed is consequently substantial at very high levels of viability where it would be particularly undesirable to have to regenerate an accession due to seed depletion occurring as a result of monitoring tests.

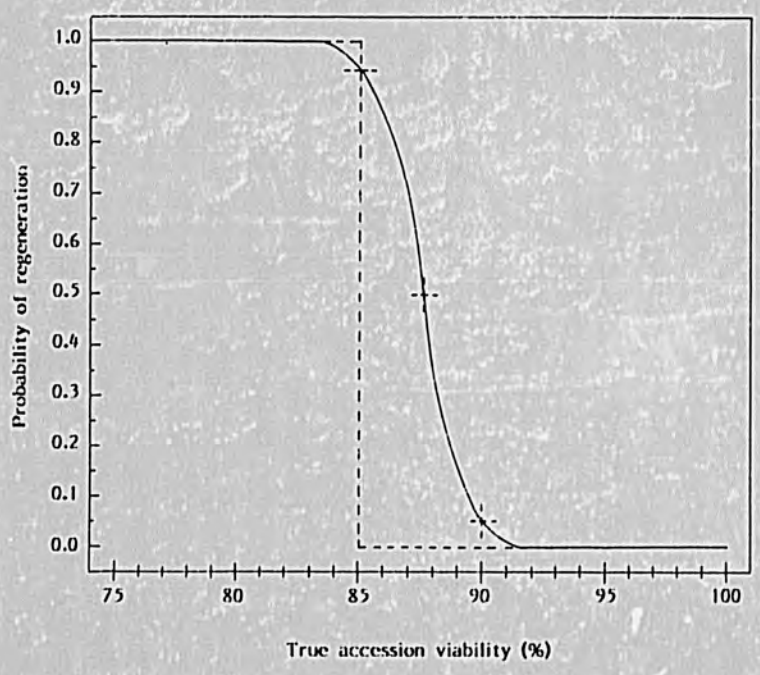
Figure 15.1 also illustrates a potential problem of sequential analysis. Since the decision boundaries are parallel the test is potentially open-ended, that is it is possible for tests not to be concluded. Experience has shown, however, that this problem only arises when the level of true accession viability is such that the probability of regeneration is close to 0.5. A solution to this problem is discussed later in this chapter.

A series of sequential germination test plans (Tables 15.3 to 15.27) have been calculated for this publication. Table 15.2 provides a summary of these sequential test plans together with details of their operating characteristics, economy and equivalent fixed sample size tests. These sequential test plans have a number of attributes which require explanation.

The Operating Characteristic

In any decision making procedure based on sampling there is a possibility of error. For our purposes there are two kinds of possible error. Either we might decide to regenerate an accession when its viability was above the regeneration standard, or we might decide to retain it in storage when in fact its true viability was below the regeneration standard. (The same problem occurs with fixed sample size tests; see Table 14.1, Chapter 14). Such errors cannot be completely avoided; but they can be quantified. One way of doing this is to plot the probability of deciding that regeneration is necessary against true accession viability. This relationship, known as the operating characteristic (or OC), is curved (Figure 15.2). Three points on this OC curve are highlighted; the probabilities of regeneration of 0.95, 0.5 and 0.05, where true accession viability is 85%, 87.645% and 90% respectively. The ideal OC function would be rectangular in which the probability of regeneration is zero above the regeneration standard and one (i.e. a certainty) at, or below, the regeneration standard - as shown by the dotted line in Figure 15.2. Such an OC function is impossible to achieve although it can be approached if very large numbers of seeds are to be tested. The choice of the OC function is thus a compromise between the level of accuracy required and the number of seeds that can be sacrificed in monitoring tests.

Figure 15.2 Comparison of ideal and practical operating characteristics (relationship between probability of regeneration and true accession viability). The ideal operating characteristic is shown by a broken line: in this example all accessions are regenerated if true accession viability is below 85%, but maintained in store otherwise; thus 85% is the regeneration standard. A practical operating characteristic (of the sequential germination test plan shown in Table 15.5) is shown by the solid curve: here accessions are regenerated at different values of true accession viability; the majority are regenerated when true accession viability is between 85 and 90%, but a minority are regenerated above 90% and a minority not regenerated below 85%. Three points are marked on this solid curve; where the probability of regeneration is 0.05, 0.5 and 0.95. For this operating characteristic these are at true accession viabilities of 85%, 87.645% and 90% respectively. For other sequential germination test plans these three levels of true accession viability are shown in columns 1, 2 and 3 of Table 15.2. For sequential germination tests we describe the regeneration standard as the level of true accession viability where the probability of regeneration is 0.95 (85% in this example) since only a minority of accessions will not be regenerated if their viability is below the regeneration standard. (Note, in passing, that for practical reasons the regeneration standard of a fixed sample size test is described differently - see Chapter 14.)



The compromise we have chosen are OC functions similar to those for 400 seed fixed sample size tests. For example, the OC function shown in Figure 15.2 is identical to that of a fixed sample size test of 467 seeds where the decision point of the fixed samle size test is set at 37.645%.

The Regeneration Standard

If rectangular OC functions were possible then the regeneration standard would be well defined, being the value of true accession viability where the probability of regeneration changes from zero to one. In practice, however, the value of true accession viability below which regeneration is instituted varies between accessions (e.g. Figure 15.2).

In Chapter 14 it was noted that where the regeneration standard is defined as the level of true accession viability at which the probability of regeneration is 0.5 then the regeneration standard does not describe minimum accession quality. Consequently it is suggested here that the regeneration standard be described as a minimum level of accession quality and defined as the value of true accession viability where the probability of regeneration is 0.95. In other words where the probability of not regenerating the accession is only 0.05. The reason for this is to minimise, within reasonable bounds, the probability of an accession falling below the regeneration standard. The regeneration standard defined in this way is shown in column 1 of Table 15.2 for the various sequential germination test plans shown in Tables 15.3 to 15.27. Note that the decision point of equally reliable fixed sample size germination test procedures (column 7, Table 15.2) is the viability level where the probability of regeneration is 0.5 (column 2, Table 15.2).

The value chosen for the regeneration standard is unlikely to be the same for all species since there will have to be a compromise between, on the one hand, the desire for accessions of high viability and associated high genetic integrity and, on the other, the relative difficulty in obtaining accessions of initial high viability. For example, for crops with uniform maturity dates (such as the cereals), most gene banks will agree they should have a higher regeneration standard than for crops with non-uniform maturity characteristics (such as carrot). For these reasons sequential germination test plans are provided here for five regeneration standards, viz. 65%, 70%, 75%, 80% and 85% viability.

Group size

On average the smaller the group size (the number of seeds sampled and tested at a time) the greater the saving of seed. To save seeds, then, one would ideally utilise a small group size. However, the smaller the group size the greater the number of sampling and testing operations required to complete the sequential test. The proportion of seeds saved by using small group sizes when true accession viability is close to the regeneration standard is, however, small, whereas the proportion saved at higher (or indeed lower) levels of viability is much greater.

The most suitable group size is likely to be that most suited to the germination test. For example, if pleated papers are used (see Chapter 9) then 50 seeds would be a convenient group size (since there are 50 pleats in a strip).

Since each monitoring test after different periods of storage will be statistically independent there is no need for each monitoring test sequence to utilise the same number of seeds per test or even the same method, provided they are equally accurate (i.e. have the same OC function). For example, the initial

test for accession viability at receipt is more suited to a fixed sample size test. First the result may be required reasonably quickly. Secondly an estimate of accession viability needs to be derived which will be used for purposes other than just deciding whether regeneration is immediately called for (e.g. the result may be used to determine the monitoring interval, i.e. the storage period between subsequent monitoring tests). This fixed sample size test should be as accurate as the sequential test plan which is to be used subsequently. (In practice 400 seeds rather than the exact equivalent size provided in Table 15.2 are likely to be used, but the difference in accuracy will only be very slight and practically unimportant.)

In subsequent, sequential, monitoring tests the group size may be, say, 40 or 50 seeds if viability is expected to be substantially higher than the regeneration standard, but, say, 100 seeds if viability is estimated (on the basis of previous monitoring test results) to be approaching the value where the probability of regeneration is 0.5 (the second column of Table 15.2). We suggest that gene banks use a sequential procedure with a group size of 100 seeds if:

the previous estimate of viability was 89% or lower where the OC function in Tables 15.3 to 15.7 is applied;
the previous estimate of viability was 85% or lower if the OC function in Tables 15.8 to 15.12 is applied;
the previous estimate of viability was 80% or lower if the OC function in Tables 15.13 to 15.17 is applied;
the previous estimate of viability was 75% or lower if the OC function in Tables 15.18 to 15.22 is applied;
the previous estimate of viability was 70% or lower if the OC function in Tables 15.23 to 15.27 is applied.

This would minimise the number of separate sampling and testing operations required without greatly increasing the average number of seeds tested. Another alternative is to use, say, the 40 seed sequential test plan but to sample and test more than one group of 40 seeds at a time and examine the results sequentially.

Sequential germination test plans are provided for group sizes of 20, 25, 40, 50 or 100 seeds. This range should provide sufficient flexibility for most needs. Strictly speaking a group size of 100 seeds is rather excessive. However, sequential germination test plans are provided for this group size to encourage maximal application of sequential test plans in gene banks where, for some reason, it is found inconvenient to use a smaller group size. In particular those gene banks currently using fixed sample size tests of only 100 seeds might wish to modify these to sequential tests utilising groups of 100 seeds.

How to use the sequential germination test plans

Just as a diagram of the sequential test boundaries shows three zones (e.g. Figure 15.1) so the sequential germination test plans are divided into three columns. The left hand column gives the regeneration decision boundary. The right hand column gives the maintain in store decision boundary. If the cumulative group test result lies in either of these zones the monitoring test is concluded. The centre column is the intermediate zone wherein no decision is possible. If the cumulative group test result lies in this central zone the monitoring test is continued: that is a further group of seeds is sampled and tested.

Table 15.1 provides examples of results obtained from three separate monitoring tests. The sequential germination test plan used was that shown in Table 15.5 for groups of 40 seeds and a

regeneration standard (probability of regeneration - 0.95) of 85%. After the first group of 40 seeds had been tested in the first sequence shown no decision was possible since the result lay in the centre column of Table 15.5. Consequently another group of 40 seeds were sampled from the accession and tested for germination. The combined result when the result of this test on the second group of 40 seeds was added to the result of the test of the first group of 40 seeds lay in the right hand column of Table 15.5; that is the maintain in store decision boundary was crossed. Thus this sequential test was concluded after only two groups of 40 seeds had been tested. The second monitoring test sequence was concluded after three groups of 40 seeds had been tested, the decision being made to maintain the accession in storage (Table 15.1). In the third monitoring sequence, however, four groups of 40 seeds were tested before the sequential test was concluded - the decision being to regenerate the accession (Table 15.1).

Table 15.1 Results (numbers of seeds germinated) of three different monitoring test sequences carried out on three different accessions according to the sequential germination test plan shown in Table 15.5.

Number of groups of seeds	Sequence					
	1		2		3	
	a	b	a	b	a	b
1	39	39	37	37	35	35
2	40	79	36	73	31	66
3			38	111	35	101
4					34	135
Decision	Maintain in store		Maintain in store		Regenerate	

a individual group test result on 40 seeds

b cumulative result of group test results

Truncating a sequential germination test plan

It is remotely possible that a sequential germination test of an accession might be continued almost indefinitely, i.e. until no seeds remain in store. Clearly this would be a nonsense. Accordingly it is necessary to have a practical procedure for reaching an arbitrary decision if a decision has not automatically been reached after a reasonable number of groups of seeds have been tested.

Two questions arise if a sequential test is to be truncated arbitrarily. First, how many seeds should be tested before abandoning a sequential test? Secondly, which decision should be made: regenerate or maintain in store until the date of the next monitoring test?

The sequential germination test plans shown in Tables 15.3 to 15.27 show the decision boundaries until 600 seeds have been tested. Our experience suggests that only very rarely will no decision have been reached once 600 seeds have been tested. Consequently we suggest that sequential germination tests be terminated after 600 seeds have been tested. The subsequent decision can be based upon the cumulative result from the truncated sequential test plan: regenerate if the proportion of viable seeds

Table 15.2 Operating characteristics, economy and decision boundaries of various sequential tests with group sizes between 20 and 100 seeds and regeneration standards between 65% and 85% and sample sizes of equivalent fixed sample size tests. (The single decision boundary of the equivalent fixed sample size test is the value of percentage viability provided when the probability of regeneration is 0.5.)

True accession viability, %, (& average no. of seeds tested) when probability of regeneration is			Number of seeds per group	Decision boundaries		Sample size of equivalent fixed sample size test	Table showing test plan
0.95	0.5	0.05		Regenerate if no. of seeds germinating is equal to or less than	Do not regenerate if number of seeds germinating is equal to or greater than		
85(221)	87.645(381)	90(248)	20	17.529m* - 5.521	17.529m + 5.521	467	15.3
85(222)	87.645(382)	90(250)	25	21.911m - 5.420	21.911m + 5.420	467	15.4
85(226)	87.645(386)	90(254)	40	35.058m - 5.166	35.058m + 5.166	467	15.5
85(228)	87.645(388)	90(256)	50	43.823m - 5.023	43.823m + 5.023	467	15.6
85(240)	87.645(401)	90(269)	100	87.645m - 4.461	87.645m + 4.461	467	15.7
80(201)	83.142(343)	86(221)	20	16.629m - 5.901	16.629m + 5.901	419	15.8
80(203)	83.142(344)	86(223)	25	20.786m - 5.786	20.786m + 5.786	419	15.9
80(206)	83.142(348)	86(227)	40	33.257m - 5.497	33.257m + 5.497	419	15.10
80(209)	83.142(350)	86(229)	50	41.572m - 5.334	41.572m + 5.334	419	15.11
80(220)	83.142(363)	86(242)	100	83.144m - 4.695	83.144m + 4.695	419	15.12
75(209)	78.372(352)	81.5(225)	20	15.673m - 6.599	15.673m + 6.599	432	15.13
75(211)	78.372(354)	81.5(226)	25	19.592m - 6.472	19.592m + 6.472	432	15.14
75(214)	78.372(357)	81.5(230)	40	31.347m - 6.154	31.347m + 6.154	432	15.15
75(217)	78.372(360)	81.5(233)	50	39.183m - 5.975	39.183m + 5.975	432	15.16
75(229)	78.372(372)	81.5(246)	100	78.366m - 5.272	78.366m + 5.272	432	15.17
70(209)	73.6(348)	77(221)	20	14.720m - 7.019	14.720m + 7.019	427	15.18
70(210)	73.6(350)	77(222)	25	18.400m - 6.883	18.400m + 6.883	427	15.19
70(214)	73.6(353)	77(226)	40	29.440m - 6.543	29.440m + 6.543	427	15.20
70(216)	73.6(356)	77(229)	50	36.800m - 6.351	36.800m + 6.351	427	15.21
70(228)	73.6(368)	77(242)	100	73.600m - 5.598	73.600m + 5.598	427	15.22
65(202)	68.832(335)	72.5(211)	20	13.767m - 7.202	13.767m + 7.202	410	15.23
65(203)	68.832(336)	72.5(212)	25	17.208m - 7.060	17.208m + 7.060	410	15.24
65(207)	68.832(340)	72.5(216)	40	27.534m - 6.702	27.534m + 6.702	410	15.25
65(209)	68.832(342)	72.5(219)	50	34.417m - 6.501	34.417m + 6.501	410	15.26
65(222)	68.832(355)	72.5(232)	100	68.834m - 5.710	68.834m + 5.710	410	15.27

*m = number of groups tested

TABLE 15.3 Sequential germination test plan for 85 percent regeneration standard when testing seeds for germination in groups of 20

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 20 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	20	12	13 - 20	
2	40	29	30 - 40	
3	60	47	48 - 58	59
4	80	64	65 - 75	76
5	100	82	83 - 93	94
6	120	99	100 - 110	111
7	140	117	118 - 128	129
8	160	134	135 - 145	146
9	180	152	153 - 163	164
10	200	169	170 - 180	181
11	220	187	188 - 198	199
12	240	204	205 - 215	216
13	260	222	223 - 233	234
14	280	239	240 - 250	251
15	300	257	258 - 268	269
16	320	274	275 - 285	286
17	340	292	293 - 303	304
18	360	310	311 - 321	322
19	380	327	328 - 338	339
20	400	345	346 - 356	357
21	420	362	363 - 373	374
22	440	380	381 - 391	392
23	460	397	398 - 408	409
24	480	415	416 - 426	427
25	500	432	433 - 443	444
26	520	450	451 - 461	462
27	540	467	468 - 478	479
28	560	485	486 - 496	497
29	580	502	503 - 513	514
30	600	520	521 - 531	532

TABLE 15.4 Sequential germination test plan for 85 percent regeneration standard when testing seeds for germination in groups of 25

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 25 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	25	16	17 - 25	
2	50	38	39 - 49	50
3	75	60	61 - 71	72
4	100	82	83 - 93	94
5	125	104	105 - 114	115
6	150	126	127 - 136	137
7	175	147	148 - 158	159
8	200	169	170 - 180	181
9	225	191	192 202	203
10	250	213	214 - 224	225
11	275	235	236 - 246	247
12	300	257	258 - 268	269
13	325	279	280 - 290	291
14	350	301	302 - 312	313
15	375	323	324 - 334	335
16	400	345	346 - 356	357
17	425	367	368 - 377	378
18	450	388	389 - 399	400
19	475	410	411 - 421	422
20	500	432	433 - 443	444
21	525	454	455 - 465	466
22	550	476	477 - 487	488
23	575	498	499 - 509	510
24	600	520	521 - 531	532

TABLE 15.5 Sequential germination test plan for 85 percent regeneration standard when testing seeds for germination in groups of 40

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 40 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	40	29	30 - 40	
2	80	64	65 - 75	76
3	120	100	101 - 110	111
4	160	135	136 - 145	146
5	200	170	171 - 180	181
6	240	205	206 - 215	216
7	280	240	241 - 250	251
8	320	275	276 - 285	286
9	360	310	311 - 320	321
10	400	345	346 - 355	356
11	440	380	381 - 390	391
12	480	415	416 - 425	426
13	520	450	451 - 460	461
14	560	485	486 - 495	496
15	600	520	521 - 531	532

TABLE 15.6 Sequential germination test plan for 85 percent regeneration standard when testing seeds for germination in groups of 50

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 50 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	50	38	39 - 48	49
2	100	82	83 - 92	93
3	150	126	127 - 136	137
4	200	170	171 - 180	181
5	250	214	215 - 224	225
6	300	257	258 - 267	268
7	350	301	302 - 311	312
8	400	345	346 - 355	356
9	450	389	390 - 399	400
10	500	433	434 - 443	444
11	550	477	478 - 487	488
12	600	520	521 - 530	531

TABLE 15.7 Sequential germination test plan for 85 percent regeneration standard when testing seeds for germination in groups of 100

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 100 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	100	83	84 - 92	93
2	200	170	171 - 179	180
3	300	258	259 - 267	268
4	400	346	347 - 355	356
5	500	433	434 - 442	443
6	600	521	522 - 530	531

TAPLE 15.8 Sequential germination test plan for 80 percent regeneration standard when testing seeds for germination in groups of 20

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 20 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	20	10	11 - 20	
2	40	27	28 - 39	40
3	60	43	44 - 55	56
4	80	60	61 - 72	73
5	100	77	78 - 89	90
6	120	93	94 - 105	106
7	140	110	111 - 122	123
8	160	127	128 - 138	139
9	180	143	144 - 155	156
10	200	160	161 - 172	173
11	220	177	178 - 188	189
12	240	193	194 - 205	206
13	260	210	211 - 222	223
14	280	226	227 - 238	239
15	300	243	244 - 255	256
16	320	260	261 - 271	272
17	340	276	277 - 288	289
18	360	293	294 - 305	306
19	380	310	311 - 321	322
20	400	326	327 - 338	339
21	420	343	344 - 355	356
22	440	359	360 - 371	372
23	460	376	377 - 388	389
24	480	393	394 - 404	405
25	500	409	410 - 421	422
26	520	426	427 - 438	439
27	540	443	444 - 454	455
28	560	459	460 - 471	472
29	580	476	477 - 488	489
30	600	492	493 - 504	505

TABLE 15.9 Sequential germination test plan for 80 percent regeneration standard when testing seeds for germination in groups of 25

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 25 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	25	14	15 - 25	
2	50	35	36 - 47	48
3	75	56	57 - 68	69
4	100	77	78 - 88	89
5	125	98	99 - 109	110
6	150	118	119 - 130	131
7	175	139	140 - 151	152
8	200	160	161 - 172	173
9	225	181	182 - 192	193
10	250	202	203 - 213	214
11	275	222	223 - 234	235
12	300	243	244 - 255	256
13	325	264	265 - 276	277
14	350	285	286 - 296	297
15	375	306	307 - 317	318
16	400	326	327 - 338	339
17	425	347	348 - 359	360
18	450	368	369 - 379	380
19	475	389	390 - 400	401
20	500	409	410 - 421	422
21	525	430	431 - 442	443
22	550	451	452 - 463	464
23	575	472	473 - 483	484
24	600	493	494 504	505

TABLE 15.10 Sequential germination test plan for 80 percent regeneration standard when testing seeds for germination in groups of 40

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 40 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	40	27	28 - 38	39
2	80	61	62 - 72	73
3	120	94	95 - 105	106
4	160	127	128 - 138	139
5	200	160	161 - 171	172
6	240	194	195 - 205	206
7	280	227	228 - 238	239
8	320	260	261 - 271	272
9	360	293	294 - 304	305
10	400	327	328 - 338	339
11	440	360	361 - 371	372
12	480	393	394 - 404	405
13	520	426	427 - 437	438
14	560	460	461 - 471	472
15	600	493	494 - 504	505

TABLE 15.11 Sequential germination test plan for 80 percent regeneration standard when testing seeds for germination in groups of 50

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 50 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	50	36	37 - 46	47
2	100	77	78 - 88	89
3	150	119	120 - 130	131
4	200	160	161 - 171	172
5	250	202	203 - 213	214
6	300	244	245 - 254	255
7	350	285	286 - 296	297
8	400	327	328 - 337	338
9	450	368	369 - 379	380
10	500	410	411 - 421	422
11	550	451	452 - 462	463
12	600	493	494 - 504	505

TABLE 15.12 Sequential germination test plan for 80 percent regeneration standard when testing seeds for germination in groups of 100

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 100 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	100	78	79 - 87	88
2	200	161	162 - 170	171
3	300	244	245 - 254	255
4	400	327	328 - 337	338
5	500	411	412 - 420	421
6	600	494	495 - 503	504

TABLE 15.13 Sequential germination test plan for 75 percent regeneration standard when testing seeds for germination in groups of 20

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 20 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	20	9	10 - 20	
2	40	24	25 - 37	38
3	60	40	41 - 53	54
4	80	56	57 - 69	70
5	100	71	72 - 84	85
6	120	87	88 - 100	101
7	140	103	104 - 116	117
8	160	118	119 - 131	132
9	180	134	135 - 147	148
10	200	150	151 - 163	164
11	220	165	166 - 179	180
12	240	181	182 - 194	195
13	260	197	198 - 210	211
14	280	212	213 - 226	227
15	300	228	229 - 241	242
16	320	244	245 - 257	258
17	340	259	260 - 273	274
18	360	275	276 - 288	289
19	380	291	292 - 304	305
20	400	306	307 - 320	321
21	420	322	323 - 335	336
22	440	338	339 - 351	352
23	460	353	354 - 367	368
24	480	369	370 - 382	383
25	500	385	386 - 398	399
26	520	400	401 - 414	415
27	540	416	417 - 429	430
28	560	432	433 - 445	446
29	580	447	448 - 461	462
30	600	463	464 - 476	477

TABLE 15.14 Sequential germination test plan for 75 percent regeneration standard when testing seeds for germination in groups of 25

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 25 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	25	13	14 - 25	
2	50	32	33 - 45	46
3	75	52	53 - 65	66
4	100	71	72 - 84	85
5	125	91	92 - 104	105
6	150	111	112 - 124	125
7	175	130	131 - 143	144
8	200	150	151 - 163	164
9	225	169	170 - 182	183
10	250	189	190 - 202	203
11	275	209	210 - 221	222
12	300	228	229 - 241	242
13	325	248	249 - 261	262
14	350	267	268 - 280	281
15	375	287	288 - 300	301
16	400	306	307 - 319	320
17	425	326	327 - 339	340
18	450	346	347 - 359	360
19	475	365	366 - 378	379
20	500	385	386 - 398	399
21	525	404	405 - 417	418
22	550	424	425 - 437	438
23	575	444	445 - 457	458
24	600	463	464 - 476	477

TABLE 15.15 Sequential germination test plan for 75 percent regeneration standard when testing seeds for germination in groups of 40

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 40 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	40	25	26 - 37	38
2	80	56	57 - 68	69
3	120	87	88 - 100	101
4	160	119	120 - 131	132
5	200	150	151 - 162	163
6	240	181	182 - 194	195
7	280	213	214 - 225	226
8	320	244	245 - 256	257
9	360	275	276 - 288	289
10	400	307	308 - 319	320
11	440	338	339 - 350	351
12	480	370	371 - 382	383
13	520	401	402 - 413	414
14	560	432	433 - 445	446
15	600	464	465 - 476	477

TABLE 15.16 Sequential germination test plan for 75 percent regeneration standard when testing seeds for germination in groups of 50

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 50 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	50	33	34 - 45	46
2	100	72	73 - 84	85
3	150	111	112 - 123	124
4	200	150	151 - 162	163
5	250	189	190 - 201	202
6	300	229	230 - 241	242
7	350	268	269 - 280	281
8	400	307	308 - 319	320
9	450	346	347 - 358	359
10	500	385	386 - 397	398
11	550	425	426 - 436	437
12	600	464	465 - 476	477

TABLE 15.17 Sequential germination test plan for 75 percent regeneration standard when testing seeds for germination in groups of 100

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 100 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	100	73	74 - 83	84
2	200	151	152 - 162	163
3	300	229	230 - 240	241
4	400	308	309 - 318	319
5	500	386	387 - 397	398
6	600	464	465 - 475	476

TABLE 15.18 Sequential germination test plan for 70 percent regeneration standard when testing seeds for germination in groups of 20

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 20 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	20	7	8 - 20	
2	40	22	23 - 36	37
3	60	37	38 - 51	52
4	80	51	52 - 65	66
5	100	66	67 - 80	81
6	120	81	82 - 95	96
7	140	96	97 - 110	111
8	160	110	111 - 124	125
9	180	125	126 - 139	140
10	200	140	141 - 154	155
11	220	154	155 - 168	169
12	240	169	170 - 183	184
13	260	184	185 - 198	199
14	280	199	200 - 213	214
15	300	213	214 - 227	228
16	320	228	229 - 242	243
17	340	243	244 - 257	258
18	360	257	258 - 271	272
19	380	272	273 - 286	287
20	400	287	288 - 301	302
21	420	302	303 - 316	317
22	440	316	317 - 330	331
23	460	331	332 - 345	346
24	480	346	347 - 360	361
25	500	360	361 - 375	376
26	520	375	376 - 389	390
27	540	390	391 - 404	405
28	560	405	406 - 419	420
29	580	419	420 - 433	434
30	600	434	435 - 448	449

TABLE 15.19 Sequential germination test plan for 70 percent regeneration standard when testing seeds for germination in groups of 25

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 25 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	25	11	12 - 25	
2	50	29	30 - 43	44
3	75	48	49 - 62	63
4	100	66	67 - 80	81
5	125	85	86 - 98	99
6	150	103	104 - 117	118
7	175	121	122 - 135	136
8	200	140	141 - 154	155
9	225	158	159 - 172	173
10	250	177	178 - 190	191
11	275	195	196 - 209	210
12	300	213	214 - 227	228
13	325	232	233 - 246	247
14	350	250	251 - 264	265
15	375	269	270 - 282	283
16	400	287	288 - 301	302
17	425	305	306 - 319	320
18	450	324	325 - 338	339
19	475	342	343 - 356	357
20	500	361	362 - 374	375
21	525	379	380 - 393	394
22	550	397	398 - 411	412
23	575	416	417 - 430	431
24	600	434	435 - 448	449

TABLE 15.20 Sequential germination test plan for 70 percent regeneration standard when testing seeds for germination in groups of 40

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 40 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	40	22	23 - 35	36
2	80	52	53 - 65	66
3	120	81	82 - 94	95
4	160	111	112 - 124	125
5	200	140	141 - 153	154
6	240	170	171 - 183	184
7	280	199	200 - 212	213
8	320	228	229 - 242	243
9	360	258	259 - 271	272
10	400	287	288 - 300	301
11	440	317	318 - 330	331
12	480	346	347 - 359	360
13	520	376	377 - 389	390
14	560	405	406 - 418	419
15	600	435	436 - 448	449

TABLE 15.21 Sequential germination test plan for 70 percent regeneration standard when testing seeds for germination in groups of 50

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 50 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	50	30	31 - 43	44
2	100	67	68 - 79	80
3	150	104	105 - 116	117
4	200	140	141 - 153	154
5	250	177	178 - 190	191
6	300	214	215 - 227	228
7	350	251	252 - 263	264
8	400	288	289 - 300	301
9	450	324	325 - 337	338
10	500	361	362 - 374	375
11	550	398	399 - 411	412
12	600	435	436 - 447	448

TABLE 15.22 Sequential germination test plan for 70 percent regeneration standard when testing seeds for germination in groups of 100

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 100 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	100	68	69 - 79	80
2	200	141	142 - 152	153
3	300	215	216 - 226	227
4	400	288	289 - 299	300
5	500	362	363 - 373	374
6	600	436	437 - 447	448

TABLE 15.23 Sequential germination test plan for 65 percent regeneration standard when testing seeds for germination in groups of 20

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 20 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	20	6	7 - 20	
2	40	20	21 - 34	35
3	60	34	35 - 48	49
4	80	47	48 - 62	63
5	100	61	62 - 76	77
6	120	75	76 - 89	90
7	140	89	90 - 103	104
8	160	103	103 - 117	118
9	180	116	117 - 131	132
10	200	130	131 - 144	145
11	220	144	145 - 158	159
12	240	157	158 - 172	173
13	260	171	172 - 186	187
14	280	185	186 - 199	200
15	300	199	200 - 213	214
16	320	213	214 - 227	228
17	340	226	227 - 241	242
18	360	240	241 - 255	256
19	380	254	255 - 268	269
20	400	268	269 - 282	283
21	420	281	282 - 296	297
22	440	295	296 - 310	311
23	460	309	310 - 323	324
24	480	323	324 - 337	338
25	500	336	337 - 351	352
26	520	350	351 - 365	366
27	540	364	365 - 378	379
28	560	378	379 - 392	393
29	580	392	393 - 406	407
30	600	405	406 - 420	421

TABLE 15.24 Sequential germination test plan for 65 percent regeneration standard when testing seeds for germination in groups of 25

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 25 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	25	10	11 - 24	25
2	50	27	28 - 41	42
3	75	44	45 - 58	59
4	100	61	62 - 75	76
5	125	78	79 - 93	94
6	150	96	97 - 110	111
7	175	113	114 - 127	128
8	200	130	131 - 144	145
9	225	147	148 - 161	162
10	250	165	166 - 179	180
11	275	182	183 - 196	1[illegible]
12	300	199	200 - 213	214
13	325	216	217 - 230	231
14	350	233	234 - 247	248
15	375	251	252 - 265	266
16	400	268	269 - 282	283
17	425	285	286 - 299	300
18	450	302	303 - 316	317
19	475	319	320 - 334	335
20	500	337	338 - 351	352
21	525	354	355 - 368	369
22	550	371	372 - 385	386
23	575	388	389 - 402	403
24	600	405	406 - 420	421

TABLE 15.25 Sequential germination test plan for 65 percent regeneration standard when testing seeds for germination in groups of 40

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 40 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	40	20	21 - 34	35
2	80	48	49 - 61	62
3	120	75	76 - 89	90
4	160	103	104 - 116	117
5	200	130	131 - 144	145
6	240	158	159 - 171	172
7	280	186	187 - 199	200
8	320	213	214 - 226	227
9	360	241	242 - 254	255
10	400	268	269 - 282	283
11	440	296	297 - 309	310
12	480	323	324 - 337	338
13	520	351	352 - 364	365
14	560	378	379 - 392	393
15	600	406	407 - 419	420

TABLE 15.26 Sequential germination test plan for 65 percent regeneration standard when testing seeds for germination in groups of 50

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 50 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	50	27	28 - 40	41
2	100	62	63 - 75	76
3	150	96	97 - 109	110
4	200	131	132 - 144	145
5	250	165	166 - 178	179
6	300	200	201 - 213	214
7	350	234	235 - 247	248
8	400	268	269 - 281	282
9	450	303	304 - 316	317
10	500	337	338 - 350	351
11	550	372	373 - 385	386
12	600	406	407 - 419	420

TABLE 15.27 Sequential germination test plan for 65 percent regeneration standard when testing seeds for germination in groups of 100

Number of groups of seeds tested	Cumulative number of seeds tested	Regenerate if the cumulative number of seeds germinated is less than or equal to:	Retest a further group of 100 seeds if between:	Maintain in store if the cumulative number of seeds germinated is equal to or greater than:
1	100	63	64 - 74	75
2	200	131	132 - 143	144
3	300	200	201 - 212	213
4	400	269	270 - 281	282
5	500	338	339 - 349	350
6	600	407	408 - 418	419

is below the decision boundary of the equivalent fixed sample size test (the values provided in the second column of Table 15.2); otherwise maintain the accession in store until the next monitoring test is due. These suggestions are arbitrary, the consequence (a marginal change in the operating characteristic) is not a serious practical problem and is preferable to severe depletion of the precious seeds.

A SEQUENTIAL TEST PROCEDURE BASED ON A MAXIMUM SEQUENCE OF ONLY TWO GROUPS OF SEEDS

The major reason for advocating the use of sequential probability ratio test plans in gene banks, such as those shown in Tables 15.3 to 15.27, is to avoid wasting precious seeds - particularly where accession viability is high. Such tests are, however, more complicated to carry out than the simpler fixed sample size tests.

One compromise suggested by the IBPGR Seed Storage Committee for gene banks who, for logistic reasons, feel unable to take full advantage of this procedure is to carry out a germination test on 200 seeds sampled from the accession. If 180 or more seeds germinate then the test is terminated and the decision made to maintain the accession in store until the next monitoring test date. If, however, fewer than 180 of the 200 seeds tested germinate then a further 200 seeds are sampled from the accession and tested for germination. At the end of this second test the results of the first and second tests are summed. If 340 or more of the 400 seeds tested germinated then the accession is maintained in store until the next monitoring test date. If, however, fewer than 340 seeds germinated then the decision is made to regenerate the accession as soon as this is practicable.

The economy and reliability of this two-test procedure are shown in Figures 15.3 and 15.4 respectively. The regeneration standard of this procedure (where the probability of regeneration is 0.95) is 81.8% (Figure 15.4). It will be seen that the operating characteristic of this procedure differs only slightly from a fixed sample size test on 400 seeds where the decision boundary is set at 85% (Figure 15.4), but offers some saving in seeds where true accession viability is in excess of the regeneration standard (Figure 15.3). However, the saving in seeds is not as great as for sequential probability ratio tests with similar operating characteristics (Table 15.2).

A SUMMARY OF PREFERRED PROCEDURES FOR GERMINATION TESTS

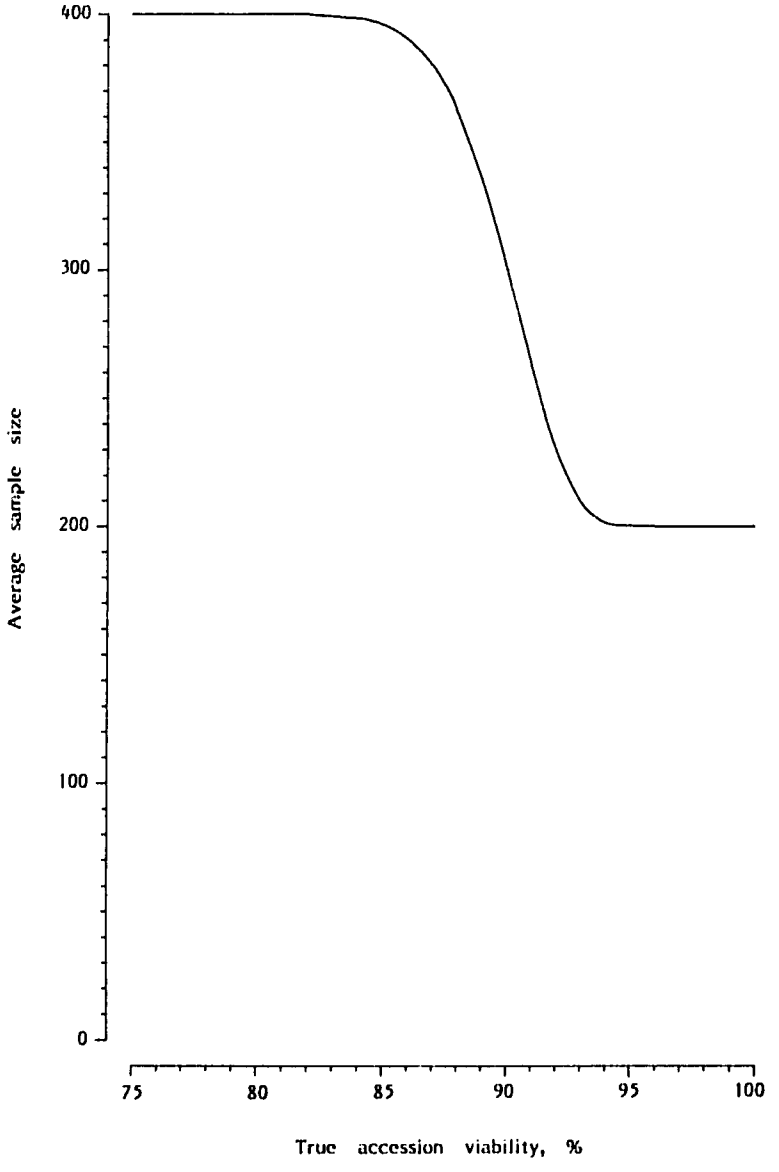
This handbook has recognised the need for flexibility in the practices adopted by different gene banks. So that practices can be adapted to meet local problems and constraints, a number of alternative procedures have often been suggested. However, some practices are preferred to others and this section summarises these.

General guidance

(1) The purpose of germination tests in gene banks is to estimate the seed viability of accessions from time to time in order that steps may be taken to regenerate accessions when viability has fallen to the regeneration standard. Unless there are special circumstances the IBPGR Seed Storage Committee has recommended 85% viability as the normal regeneration standard.

(2) In order that the results of germination tests reflect true viability steps must be taken to sample seeds correctly (Chapters 12 and 13), to remove as much of the empty seed fraction as possible where

Figure 15.3 Economy (relationship between the average number of seeds required to conclude a monitoring test and true accession viability) for the two test procedure described in the text in which 200 seeds are first tested for germination with the decision boundary set at 180 seeds germinating; if fewer than 180 seeds germinate then a further 200 seeds are tested for germination, the results of both tests added together, and the combined result tested against an 85% decision boundary.



necessary and determine the empty seed fraction remaining so that viability is expressed as a proportion of true seeds (Chapter 8), to provide optimal germination test conditions (Chapter 4 and Volume II) to remove dormancy (Chapters 5, 6, 7 and Volume II) and hardseededness (Chapter 7), to evaluate the results of tests (Chapter 10) and, where necessary, to use additional methods for assessing the viability of ungerminated seeds (Chapter 11).

(3) It is convenient to divide germination tests used in gene banks into two categories: (i) the initial germination test, which is designed to check that initial viability is above the regeneration standard and also to estimate the initial percentage viability of the accession; (ii) the monitoring germination tests, which are carried out subsequently from time to time to check whether or not the viability of the accession is still above the regeneration standard. It will be seen that the objectives of the two types of test are not identical: the main difference being that a special emphasis is placed on estimating initial viability (not merely that the viability is above the regeneration standard). The reason for this is that initial percentage viability reflects the seed quality of the accession and, when sufficient information for a species is available, initial viability can be used as a guide to how long the first monitoring interval should be (i.e. the time between the initial germination test and the first monitoring test after storage). Because of the different objectives, the preferred recommendations for initial germination tests and monitoring germination tests are also different.

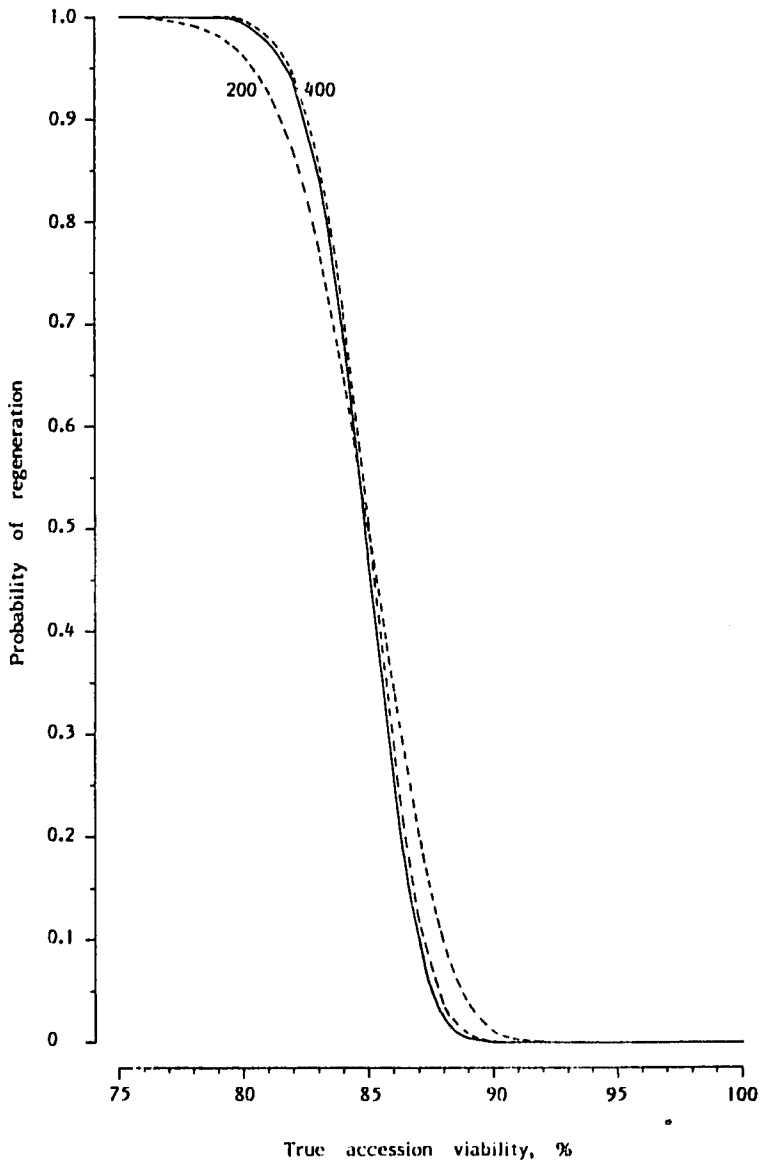
The initial germination test

(4) The initial germination test should be either (i) a fixed sample size test carried out on 400 seeds using 4 or more replicates or (ii) a sequential test on up to 2 groups of seeds, each of 200 seeds, using 4 or more replicates per group. In either method (i or ii) the results of individual replicates should be checked against the appropriate tolerance table in Chapter 14 at a probability of 0.025. The estimate of initial viability should be based on at least 200 seeds so that in the case of method (ii) if the value of one replicate in the first group falls outside the tolerated range, a further sample of 200 seeds should be tested. In the case of method (i) where the seeds are in short supply, it is suggested that if the results of one replicate falls outside the tolerated range, that replicate is ignored and the estimated initial viability is taken as the mean of the other replicates. If the seed supply is plentiful it is preferable to repeat the test.

Monitoring germination tests

(5) The IBPGR Seed Storage Committee recommends a sequential test procedure for germination tests designed to monitor accession viability. Ideally a group size of 40 or 50 seeds (the sequential germination test plan shown in Table 15.5 or Table 15.6) should be chosen, as is most convenient, if viability is expected to be well above the regeneration standard of 85%. This could be adopted as a standard procedure for all accessions and would result in maximum saving of seeds. But, if preferred, the work load could be reduced with little penalty in seed wastage if a group size of, say, 100 (the sequential germination test plan shown in Table 15.7) is adopted when it is expected that the viability of the accession is approaching the regeneration standard - say 89% or below. When small group sizes (e.g. 40 or 50 seeds) are used, replication is not necessary. But if large group sizes are used (e.g. 100 seeds) then there is some advantage in using replicates which can be checked against the tolerance tables (Chapter 14) as a check on satisfactory technique. Whatever the group size continue until a decision is reached according to Tables 15.5, 15.6 or 15.7, but if no decision has been automatically reached after 600 seeds have been tested the decision is then taken to end the test and regenerate the accession

Figure 15.4 Operating characteristic of the two test procedure described in the text and in the caption to Figure 15.3 (solid curve). The broken curves provide operating characteristics for fixed sample size tests of 200 or 400 seeds where the decision boundary is 85% for comparison. Note that the operating characteristic of the two test procedure differs only marginally from that for the 400 seed fixed sample size test whilst requiring fewer seeds (Figure 15.3).



if 525 or fewer seeds germinated in the sequential procedure or maintain in store until the next monitoring test if 526 or more seeds germinated.

FURTHER READING

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