Laboratory Exercises

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Exercise 1— Seed Structure

Objective:

To learn basic seed structures and their function in important seed types.

Methods:

1. Presoak seed samples in tapwater at room temperature (or 27 °C) for 15 to 24 hours. The imbibition will soften tissues and facilitate dissection.
2. Using a knife, clippers, or a single-edged razor blade and depending on type of seeds, carefully cut the seeds in one of two different ways:
   a. cross section (transverse)
   b. lengthwise (longitudinal)

   Several cuts may be necessary to expose the embryo and other internal tissues.
3. Examine the tissues exposed by the cuts and label them on freehand sketches of the cut material. Determine which tissues are for embryo protection, storage of food reserves, etc. Look for abnormal structures, insect damage, etc.
4. On at least one seed of each species, try to remove the embryo without damage, sketch it, and label the parts.

Supplies:

Clippers (or knives), single-edged razor blades, dissecting needles, a small magnifying glass (or hand lens), pencil and paper, and seed samples of five tree species.

Exercise 2—Seed Crop Estimation

Objective:

To predict seed crops in advance of collection by estimating the number of:

1. Good seeds per fruit
2. Fruits per tree

Methods:

1. Good seeds per fruit
   a. Choose a multiple-seed fruit and collect 15 fruits prior to maturity.
   b. Cut fruits in half lengthwise and count good seeds visible on each half.
   c. Dry the fruit halves in an oven (40 to 50 °C) to extract seeds and to obtain actual counts of good seeds.
   d. Calculate regression equations to predict total seeds from fruit cross-section counts.
2. Fruits per tree —Visit nearby trees and estimate fruit crops by:
   a. Total count
   b. One-fourth crown count
   c. Sample branch count
   d. Any other known ways
3. Combine results of both methods to estimate size of the seed crop.

Supplies:

Cone cutters or sharp blades, an oven, drying containers, and binoculars.

Exercise 3a-Cone Drying and Seed Extraction (Central America)

Objective:

To learn how to calculate seed and fruit needs for a planting program.

Assumptions:

Area to plant-2,000 hectares (ha) at 1,700 trees per hectare
Species - *Pinus caribaea*

1. All moisture contents are percentage of wet weight.
2. There are 800 closed cones per hectoliter (hectoliter) (40 kg).
3. Moisture content of closed cones is 40 percent.
4. Cones double in size when open.
5. Yield averages 400 grams *(g)* of pure seeds per hectoliter of closed cones.
6. There are 68,200 seeds per kilogram (kg).
7. Laboratory germination is 80 percent; 50 percent of the germinated seeds produce plantable seedlings.
8. Cones are put into the kiln when they reach 25-percent moisture content.
9. Drying trays hold 0.5 hL of closed cones; each stack of eight drying trays holds 4.0 hL; eight stacks can fit in the kiln at once.
10. It takes 12 hours to dry a full charge to the 10-percent cone moisture needed for the cones to open fully.
11. It takes 700 kilocalories (Kcal) to heat 1 hL of cones for 1 hour.
12. Fuel value for wood of *Casuarina equisetifolia* is 4,950 Kcal per kilogram; for *P caribaea* cones, 4,500.
13. Open *P caribaea* cones weigh 104 g per liter (L).

Questions:

1. How many cones must be collected to meet the planting goal?
2. How much total moisture must be lost in predrying (prior to entering kiln)?
3. How many drying stacks will be needed to predry everything at once?
4. How many kiln charges will be needed?
5. How long will it take to open all cones?
6. How much fuel will be needed with *C. equisetifolia* wood? with *P. caribaea* cones?
7. Have enough cones been collected to heat the kiln?

Answers:

1. 312.5 hL

seeds required = $\frac{(1,700)}{(0.8)(0.5)}$ (2,000) = 8,500,000; or

$\frac{8,500,000}{68,200}$ = 125 kg

0.4 kg pure seed/hL: $\frac{125}{0.4}$ = 312.5

2. 2,500 kg of moisture (312.5) (8)

1 hL = 40 kg; 16 kg H_2O and 24 kg dry matter

At 25%, $\frac{x}{24 + x} = 0.25$

x = 8 kg

Since there were originally 16 kg, 8 kg must be lost.

3. 78 +

$\frac{312.5}{4}$ = 78.12

4. 10

$\frac{78.12 \text{ stacks}}{8 \text{ stacks/charge}}$ = 9.8

5. 120 hr

(12 hr) (10 charges) = 120

6. *Casuarina equisetifolia* = **540 kg;**
Pinus caribaea = **600 kg**

Casuarina equisetifolia:

(32 hL/charge) (12 hr/charge) = 384 hL-hr
(384) (700 Kcal) = 268,800 Kcal/charge
$\frac{268{,}800}{4{,}950}$ = (54 kg/charge) (10 charges) = 540 kg

Pinus caribaea:

$\frac{268{,}800}{4{,}500}$ = (60 kg/charge) (10 charges) = 600 kg

7. **Yes**

Open cones weigh 10.4 kg/hL
At 2 x expansion, we have (312.5) (2) = 625 hL of open cones
(625) (10.4) = 6,500 kg

Exercise 3b — Cone Drying and Seed Extraction (India/Pakistan)

Assumptions:

Area to plant —2,000 ha at 1,700 trees per hectare
Species —*Pinus roxburghii*

1. All moisture contents are expressed as a percentage of wet weight.
2. There are 400 closed cones per hectoliter (hL) (40 kg).
3. Moisture content of closed cones is 40 percent.
4. Cones double in size when open.
5. Yield averages 1.2 kilograms (kg) pure seeds per hectoliter of closed cones.
6. There are 12,000 seeds per kilogram.
7. Laboratory germination is 80 percent; 50 percent of the germinated seeds produce plantable seedlings.
8. Cones are put into the kiln when they reach 25-percent moisture content.
9. Drying trays hold 0.5 hL of closed cones; each stack of eight drying trays holds 4.0 hL; eight stacks can fit in the kiln at once.
10. It takes 12 hours to dry a full charge to the 10-percent cone moisture needed for cones to open fully.
11. It takes 700 Kcal to heat 1 hL of cones for 1 hour.
12. Fuel value of wood of *Casuarina equisetifolia* is 4,950 Kcal per kilogram; for *P. roxburghii* cones, 4,500. Open *P roxburghii* cones weigh 110 g per liter **(L).**

Questions:

1. How many cones must be collected to meet the planting goal?
2. How much total moisture must be lost in predrying (prior to entering kiln)?

3. How many drying stacks will be needed to predry everything at once?
4. How many kiln charges will be needed?
5. How long will it take to open all cones?
6. How much fuel will be needed with *C. equisetifolia* wood? With *P roxburghii* cones?
7. Have enough cones been collected to heat the kiln?

Answers:

1. 590 hL

seeds required = $\frac{(1,700)}{(0.8)(0.5)}$ (2,000) = 8,500,000; or

or $\frac{8,500,000}{12,000}$ = 708 kg

1.2 kg pure seeds/hL: $\frac{708}{1.2}$ = 590 hL

2. 4,720 kg of moisture (590) (8)

1 hL = 40 kg; 16 kg H_2O and 24 kg dry matter
At 25%, $\frac{x}{24 + x} = 0.25$
x = 8 kg
Since there were originally 16 kg, 8 kg must be lost.

3. 147+

$\frac{590}{4}$ = 147.5

4. 19

$\frac{147.5 \text{ stacks}}{8 \text{ stacks/charge}}$ = 18.4

5. 228 hr

(12 hr) (19 charges) = 228

6. *Casuarina equisetifolia* = **1,026 kg;**
Pinus roxburghii = **1,140 kg**

Casuarina equisetifolia:

(32 hL/charge) (12 hr/charge) = 384 hL-hr
(384) (700 Kcal) = 268,800 Kcal/charge
$\frac{268,800}{4,950}$ = (54 kg/charge) (19 charges) = 1,026 kg

Pinus roxburghii:

$\frac{268,800}{4,500}$ = (60 kg/charge) (19 charges) = 1,140 kg

7. **Yes**

Open cones weigh 11.0 kg/hL
At 2 x expansion, we have (590) (2) = 1,180 hL of open cones
(1,180) (11.0) = 12,980 kg

Exercise 4-Storage Space Requirements

Objectives:

Once annual seed requirements are known, space requirements for cold storage must be calculated. A decision must also be made as to how many years' supply of seeds will be maintained as a safety margin: 1 year's? 3 years'?

Assumptions:

1. You must grow 2 million *Acacia nilotica* and 3 million *Pinus wallichiana* seedlings each year.
2. A 3-year supply of seeds will be stored.
3. Number of seeds per kilogram (kg) is 7,000 for *A. nilotica* and 26,000 for *R wallichiana.*
4. For every three seeds planted, only two will produce a plantable seedling.
5. The seeds will be stored in large plastic bottles that hold 10 kg each. The bottles are 80 centimeters (cm) tall and 40 cm in diameter.
6. Ten percent of the cold storage space is in aisles, etc.

Calculate:

1. How many kilograms of each species are to be stored?
2. How many bottles and cubic meters of storage space will be required?
3. Repeat calculation 2 if storage is in boxes 40 by 40 by 40 cm. Each box will hold 6.5 kg of seeds.
4. What are the minimum cold storage dimensions needed to store the seeds in calculations 2 and 3 above?

Answers:

1. *A. nilotica:* (2,000,000) (3) ± (0.67) = 8,955,224
   8,955,224 ± 7,000 = 1,279.3 kg
   R wallichiana: (3,000,000) (3) ± (0.67) = 13,432,836
   13,432,836 + 26,000 = 516.6 kg
2. *A. nilotica:* 128 bottles (127. 93)
   Bottle volume = (0.1257) (0.8) = 0.10 m^3,
   Space needed = (0.4) (0.4) (0.8) (128) = 16.4 m^3
   P *wallichiana:* 52 bottles (51.7)
   Space needed = 6.6 m^3
3. A. *nilotica:* 197 boxes (196.8)
   Space needed = (0.4) (0.4) (0.4) (197) = 12.6 m^3
   P wallichiana: 80 boxes (79.5)
   Space needed = 5.1 m^3
   But square boxes need air space. A 10-cm space on four sides increases space needed to 19.7 m^3 and 8.0 m^3.
4. Bottles: (16.4 + 6.6). ± 0.9 = 25.6 m^3
   Boxes: (12.6 + 5.1) ± 0.9 = 19.7 m^3; 30.8 m^3 with spaces.

Exercise 5-Sampling

Objective:

To learn the basic methods of sampling bulk lots and some special applications for tree seeds.

Methods:

1. Mix each lot thoroughly either with a mechanical mixer or by hand. To do the latter, spread the seeds out on a smooth surface and mix by scooping from side to side. Then pour back and forth between two containers.

2. Determine the proper size of the submitted sample (twice the working sample).
3. Draw samples using the following equipment/methods:
   a. Seed trier
   b. Mechanical divider
   c. Division
   d. Extended hand
4. Weigh each sample to the nearest gram, place in a plastic bag, and label.
5. Save these bagged samples for later measurements of purity, weight, and moisture.

Supplies:

A seed trier, a mechanical divider, a spatula, a spoon, plastic bags (15 by 15 cm), marking pens, and laboratory balances.

Exercise 6—Moisture, Purity, and Weight

Objective:

To carry out the basic steps in measuring moisture, purity, and weight of a submitted sample.

Methods:

1. Moisture
   a. Use submitted samples drawn in the sampling exercise.
   b. Use a spoon or spatula to draw two subsamples of 4 to 5 g each. Place samples in drying cans. Follow guidelines in Bonner (1981b).
   c. Weigh to the nearest 0.01 g and dry in ovens for 17 hours at 103 °C.
   d. Cool in desiccators and reweigh. If desiccators are not available, use rapid-weigh techniques to obtain dry weight.
   e. Calculate moisture as a percentage of wet weight:

$$\text{percent moisture} = \frac{\text{wet wt.} - \text{dry wt.}}{\text{wet wt.}} (100)$$

2. Purity
   a. Reduce the remainder of the submitted sample to the proper working sample size. To determine the proper size, take at least 2,500 seeds up to a maximum of 1,000 g. Table 19 no equivalent table in Student Outline] may be used to determine sample size also.
   b. Weigh the working sample (see 3.5.1.A in ISTA 1985).
   c. Divide the sample into the following components:
      (1) Pure seeds
      (2) Other seeds (other species)
      (3) Inert matter (includes seed parts)
   d. Weigh each component and express as a percentage of the working sample weight:

$$\text{percent pure seed} = \frac{\text{wt. of pure seed}}{\text{wt. of entire sample}} (100)$$

3. Weight
   a. Use the pure seed component from the purity test.
   b. Either weigh and count the entire pure seed component or use smaller replicates (the usual method).
   c. Replicate method:
      (1) Randomly count out 8 replicates of 100 seeds each.
      (2) Weigh each replicate to the same number of decimal places used in the purity determination.
      (3) Obtain the mean weight of 100 seeds and multiply by 10 for 1,000-seed weight.

(4) Convert to pure seeds per kilogram as follows:

$$\frac{1{,}000{,}000}{\text{wt. of 1,000 seeds}} = \text{seeds per kg}$$

d. In official testing, variation would be estimated as follows:

(1) Variance = $\frac{n(Ix^2)\ (//x)^2}{n(n — /)}$

(2) Standard deviation (u) = ✓variance

(3) Coefficient of variation (CV) = x) (100)
(X = mean wt. of 100 seeds, see 3.c above)

(4) If CV is 4.0 or less, the answer in "3.c" above is acceptable. If CV is more than 4.0, take 8 more replicates and repeat the process, using all 16 replicates in the calculations.

Supplies:

A spatula, a spoon, laboratory balances, an oven, drying cans, desiccators, forceps, and pencil and paper.

Exercise 7—Calibration of Electric Moisture Meters

Objective:

To demonstrate a simple method for developing calibration charts for electric moisture meters. These methods will work with any type of meter.

Methods:

1. Draw 4 to 5 kg of seeds from a bulk lot of the desired species; mix well.
2. Separate into 10 random samples of about 400 g each.
3. Adjust moisture in these samples to span the range of moisture content that will be encountered (approximately 5 to 20 percent). Do this by drying several samples (vary drying conditions) and by adding water to others (vary the amount of water).
4. Place each sample in a plastic bag and place the bag in a cooler for 1 week to allow complete moisture equilibration.
5. After 1 week, remove the samples and let them come to room temperature (2 to 3 hours).
6. Take a meter reading on the driest lot according to the manufacturer's instructions. Record the value and immediately draw two 5-g subsamples for oven determinations of moisture content. Follow previous instructions.
7. Repeat step 6 with the other samples in the order of ascending moisture content.
8. Plot data on a graph: oven moisture percentage vs. meter reading. Use this curve to relate future meter readings to actual moisture content for this particular species only.
9. For more accurate calibration, fit a regression curve (oven moisture percentage on meter readings) and calculate values for a calibration table. More than 10 observations should be available for a regression, so another 10 samples should be drawn for a repeat of the entire process.
10. This procedure must be done separately for each species to be tested.

Supplies:

An electric meter, a spoon, laboratory balances, an oven, weighing dishes, desiccators, plastic bags, and graph paper.

Exercise 8 — Germination Tests

Objective:

To learn the basic steps of a germination test and to carry out simple tests on some important species. Because of the length of this course, a full test may not be possible. By starting a few samples before talking about testing at length, some germination should occur and be available for evaluation.

Methods:

1. Presoak seed samples in tapwater at room temperature (27 °C) for 15 to 24 hours.
2. Divide samples into 2 replicates of 20 to 50 seeds each, depending on the species. If *Eucalyptus* spp. seeds are used, weigh out two replicates according to **ISTA** (1985) rules.
3. Half the class will surface-sterilize their samples with a 10-percent chlorine bleach solution, and the other half will not treat theirs. Treatment will consist of a 5- to 10-minute soak, followed by rinsing in running tapwater.
4. Hard-seeded species (e.g., *Acacia)* will be scarified with a knife, file, or sandpaper on the radicle end as determined in Exercise 1.
5. Place replicates in glass or plastic dishes on moist filter paper or other suitable media. Paper should be moist, but not moist enough to leave "free water" in a depression made by mashing down on the paper with a finger. Put dish covers on; if there are no covers, use plastic wrap.
6. Label all dishes and place them in a germinator or constant temperature room if available. If these facilities are not available, place the dishes on a table under lights in the center of the room. If good lights are not available, place the dishes near windows that allow good natural light.
7. Check dishes every day for moisture; add water if they dry out. Germination may become evident in about 7 days. Record normal germination, abnormal germination, and evidence of insect or disease problems.

Supplies:

A knife, small file, or sandpaper for scarification, chlorine bleach, germination blotters, dishes (10 per student), a glass-marking pen, and laboratory balances. A germinator or constant temperature room is desirable but not necessary.

Suggested species:

Pinus, Acacia or another legume, *Eucalyptus,* and two indigenous species.

Exercise 9—Scarification

Objective:

To demonstrate the relative effectiveness of simple scarification techniques that can be used in seed testing.

Methods:

1. Count out 120 seeds of a hard-seeded species and divide them into 8 samples of 15 seeds each.
2. Scarify 2 replicates of 15 by each of the following procedures:
   a. Rub hand files or similar abrasive devices across the seedcoat enough to cut a notch in the seed.
   b. Use hand clippers, shears, or a knife to cut through the seedcoat along one side.
   c. Sandpaper the seed enough to cut through the seedcoat on the radicle end.
   d. The other two samples will be the untreated controls.

3. Place the scarified samples on moist blotters in dishes and cover as in the germination test. Place all dishes in the germinator if there is space. If not, place them on a table with good lighting and leave them for observation through the rest of the course.
4. Periodically count the number of germinating seeds and the number of swollen seeds. This latter condition confirms that water uptake has occurred, but something else may be blocking germination. Report both conditions as a percentage of the total number of seeds in the test.

Supplies:

Four glass or plastic dishes, germination blotters, a hand file, clippers or shears, rough sandpaper, and marking pens.

Exercise 10 — Rapid Test: Tetrazolium Staining

Objective:

To learn basic techniques of the tetrazolium (TZ) stain test for viability.

Methods:

1. Draw two 50-seed samples from seeds that have been soaking in tapwater for 24 hours.
2. Prepare a 1-percent solution of TZ by dissolving 10 g of a TZ salt (chloride or bromide) in 1,000 mL of distilled water (pH 6.5 to 7.0). If the pH of the water is outside this range, a buffered solution must be prepared as follows:
   a. Prepare two solutions:
      (1) Solution 1—Dissolve 9.078 g KH_2PO_4 in 1,000 mL of water.
      (2) Solution 2—Dissolve 11.876 g $Na_2HPO4 \cdot 2H_20$ in 1,000 mL of water.
   b. Mix two parts of solution 1 with three parts of solution 2.
   c. Dissolve 10 g of TZ salt in 1,000 mL of the buffer solution to make a 1-percent solution.
3. Carefully cut open the imbibed seeds to fully expose the embryo. The embryo may be completely removed, as in the excised embryo test.
4. Completely immerse the embryos in TZ solution in dishes and incubate them in the dark at 30 °C for 15 to 24 hours (depending on the species and seed condition).
5. For evaluation, decant the TZ solution, rinse seeds in water, and examine the embryos on a wet surface. Moderate red staining generally indicates viable tissues, heavy red staining indicates damaged tissues, and the absence of any staining indicates nonviable tissues. Stain interpretations may vary by species. See ISTA (1985) for guidelines.
6. Compare results with other rapid test results or germination test results.

Supplies:

Dissecting equipment, dishes, tetrazolium salt, buffers (if necessary), and a constant-temperature dark incubator.

Exercise 11 — Rapid Tests: Cutting and Excised Embryo

Objective:

To learn techniques of the cutting test for viability estimation and of embryo removal for the embryo excision test.

Methods:

1. Cutting Test
   a. Draw samples of 50 seeds from each of several seedlots and divide them into sublots of 25 seeds each.
   b. Cut the seeds in half, using a transverse cut through the center of the seeds. Categorize seeds as either viable, damaged by insects or disease, or empty. Average the results from the sublots.
2. Excised Embryo Test
   a. Draw samples of 50 seeds from each of several seedlots that have been soaking in tapwater for 24 to 48 hours at room temperature and divide into sublots of 25 as before.
   b. Using razor blades or scalpels, carefully cut through each seedcoat and endosperm (if present), and expose the embryo.
   c. Carefully "tease" the embryo out of the surrounding tissues with dissecting needles or other sharp-pointed instruments. Avoid damaging the embryo.
   d. Carefully place the excised embryo on moist filter paper in a covered dish, such as a petri dish. Maintain at 20 °C in light until an evaluation can be made (usually within 14 days).
   e. Diseased or damaged embryos should not be placed in the dishes. Empty seeds should be categorized as such and not replaced in the test.
   f. The working surface and all instruments should be disinfected to reduce mold infections with a 50-percent ethanol solution. Instruments should be "dipped" between each dissection.
   g. Embryos should be categorized within 14 days as follows:
      (1) Viable
         (a) germinating embryos
         (b) embryos with one or more cotyledons exhibiting growth or greening
         (c) embryos remaining firm, slightly enlarged, and either white or yellow according to species
      (2) Nonviable
         (a) embryos that rapidly develop severe mold, deteriorate, and decay
         (b) degenerated embryos
         (c) embryos exhibiting extreme brown or black discoloration, an off-gray color, or white watery appearance
         (d) seeds in which the embryo is dead, missing, or deformed
   h. Compare your results with the cutting test results.

Supplies:

Single-edged razor blades, scalpels, dissecting needles, dishes, filter paper, and ethanol.

Exercise 12—Seed Health Testing

Objective:

To learn basic techniques of seed health testing.

Background:

Health testing of seeds is important for three reasons:

1. Seed-borne inoculum may cause diseases in the field.
2. Imported seedlots may introduce new diseases, so tests to meet quarantine regulations may be required.
3. Seed health testing may aid in seedling evaluation and help determine causes for poor germination or field establishment. It supplements the germination test.

Seed Health refers primarily to the presence or absence of disease-causing organisms (e.g., fungi, bacteria, and viruses) and animal pests (e.g., eelworms and insects). However, physiological conditions such as trace element deficiency may be involved.

Incubation maintains seeds in an environment favorable to the development of pathogens or symptoms.

Pretreatment is any physical or chemical laboratory treatment of the working sample preceding incubation that is done solely to facilitate testing.
Treatment is any process, physical or chemical, to which a seedlot is submitted.

Sample:

1. Entire submitted sample may be the working sample, depending on the test.
2. The working sample is normally 400 pure seeds or an equivalent weight.
3. Sampling rules are followed.
4. Replicates containing a specified number of seeds, if required, are taken at random for a subsample after thorough mixing.

General directions:

1. Use different methods of testing depending on factors such as pathogen or condition being investigated, species of seeds, and purpose of test. See ISTA (1966, 1985).
2. Examine the working sample with or without incubation.
    a. Examine without incubation. (This method provides no indication of the viability of the pathogen.)
        (1) Examine the sample with a stereomicroscope for general evidence of diseases or pests.
        (2) Examine imbibed seeds. Immerse the working sample to make fruiting bodies, symptoms, or pests more easily visible and to encourage the release of spores. Examine with stereomicroscope after imbibition.
        (3) Examine organisms removed by washing. Immerse the working sample in water with a wetting agent, or in alcohol, and shake to remove spores, hyphae, nematodes, etc. Examine the excess liquid with a compound microscope.
    b. Examine after incubation.
        (1) After a specific period of incubation, examine the working sample. Note the presence of disease organisms or pests on or in seeds or seedlings. Use blotters, sand, or agar for incubation media.
        (2) Use blotters when required to grow the pathogens from the seeds or to examine the seedlings. Seeds may or may not be pretreated. Space widely to avoid secondary spread of organisms. Use light as necessary to stimulate sporulation. Examine with a microscope.
        (3) Sand or artificial composts can be used for certain pathogens. Seeds are not usually pretreated, but they are widely spaced on the medium. Incubation is favorable for symptom expression.
        (4) Use agar plates to obtain identifiable growth of organisms from seeds.
            (a) Sterility is required; seeds are normally pretreated and spaced.
            (b) Identify characteristic colonies and spores by microscopy.
            (c) Use lighting and germination inhibitors.
3. Examine growing plants. Grow plants from seeds and examine them for disease symptoms to determine the presence of bacteria, fungi, or viruses. Use inoculum from the test seedlot to test for infection of healthy seedlings.

Calculations and Expression of Results:

1. Express results as a percentage of seeds affected or as number of organisms in the weight of sample examined.
2. Report results on the ISTA certificate.
    a. Report test method.
    b. Report pretreatments.
    c. Absence of health test does not imply satisfactory health condition.

Specific Test Example — Pitch Canker Fungus:

1. Adapted from Anderson (1986b).
2. Blotter Method, 400-seed sample.

a. Pentachloronitrobenzene (PCNB) Broth
   Combine peptone, 15 g; $MgSO_4$ $7H_20$, 5 g; KH_2PO_4, 1 g; terraclor, 1 g with 1 L of distilled H_2O. Stir well using magnetic stirrer. Autoclave for 15 minutes. After autoclaving, place flask on magnetic stirrer and stir slowly until solution cools to room temperature or slightly warmer. Add 1 g of streptomycin sulfate and 1 to 2 g of neomycin sulfate under sterile conditions, and stir.
b. Place 25 seeds on blue blotter paper in plastic containers. Crush the seeds with a sterilized piece of plastic cut to fit the plastic box opening. Spray seeds and blotter paper with PCNB broth.
c. Incubate 14 days at 20 °C or until colonies are 2 cm in diameter.
d. Inspect all seeds for slow-growing, granular white colonies. Check each suspected colony using a light microscope at 100 to 400 magnification for microconidia and polyphialids. Select fungus from seed surface, not the blotter surface. Split the seeds into 4 groups of 100 for reporting purposes.

3. Agar Method
   a. Prepare fresh potato dextrose agar (PDA) (makes 1 L)
      (1) Clean and dice one medium-sized potato.
      (2) Put diced potato in beaker with 500 mL of distilled H_2O. Run through autoclave.
      (3) In flask, add 20 g of dextrose and 17 g of agar to 500 mL of distilled H_2O.
      (4) Put dextrose/agar solution on magnetic stirrer and low heat.
      (5) Strain cooked potatoes through two layers of cheesecloth to obtain at least 200 mL of slurry.
      (6) Note amount of slurry, and pour slurry in flask with dextrose/agar solution.
      (7) Add enough distilled H_2O to make total slurry solution amount to 500 mL (i.e., if there are 200 mL of slurry, add 300 mL of distilled H_2O).
      (8) Put solution in autoclave and run for 15 minutes. To acidify media, add 20 drops of 50 percent lactic acid to obtain a pH of 4.7.
   b. Isolating external seed fungi, 25-seed sample
      (1) Place the whole seeds on acidified PDA (pH 4.7).
      (2) Incubate 14 days at 20 °C.
      (3) If possible, observe fungal growth daily and identify the fungi.
   c. Isolating internal seed fungi, 25-seed sample
      (1) Surface sterilize the whole seeds in 70 percent ethanol for 10 minutes. Stir the seeds every 2 minutes.
      (2) Under sterile conditions, cut each seed open and remove half the center material.
      (3) Place the seed half (center material) on acidified PDA (pH 4.7) using sterile technique.
      (4) Incubate 14 days at 20 °C.
      (5) If possible, observe fungal growth daily and identify the fungi.