Practical Lesson 2

Extracting Nematodes from Soil Samples

BAERMANN FUNNEL TECHNQUE

The Baermann funnel method (Fig. 3) is convenient for extracting active adult and juvenile nematodes from small amounts (30 to 100 cm^3) of finely crumbled soil and plant tissues. In this method, eggs will hatch while a sample is incubating, and second-stage juveniles will also be recovered. The method is not effective for extracting sluggish and sedentary nematodes (e.g., *Criconemella, Criconemoides, Hemicycliophora,* and *Xiphinema)* and nonvermiform, sedentary females of *Globodera, Heterodera, Meloidodera, Meloidogyne, Rotylenchulus,* and *Tylenchulus.*

The procedure requires some 12 h, or overnight, to as much as five days (typically three days). Most nematodes are recovered after 24 to 48 h, but the recovery will vary according to the sample size, temperature, time of storage, extraction method, and nematode species. Yields from compacted soils are lower than those from non-compacted soils.

1- Obtain the following equipment and supplies (Fig. 3 A):
   - A- wet-strength facial tissue containing no toxic substances or any other suitable materials (double-thickness muslin cheesecloth or nylon gauze, paper towels, cotton wool, and milk filters) which allow active vermiform nematodes to pass through these materials.
   - B- shallow, coarse, molded "basket," 17.5 cm in diameter, with a flat bottom made from stainless steel or plastic screen *(not* copper screen).
   - C- nonfluted glass funnel, 10-15 cm in diameter, with a 10 to 15-cm stem.
   - D- rubber or plastic tubing, about 8 cm long, fitting around the stem of the funnel.
   - E- funnel support or rack.
   - F- spring or screw clamp to close off the tubing 50- (or 100-) and 250-ml beakers.

2- Mount the funnel on the support, attach the tubing to the stem of the funnel, place the clamp near the free end of the tubing, and set the basket on top of the funnel (Fig. 3 B). Fill the funnel with water, open the clamp, tap the funnel stem, and drain off sufficient water to remove air bubbles trapped in the tube. Refill the funnel with water to the bottom of the basket.

3- Place double-thickness tissue (or other "filter" material) on top of the screen, being careful not to tear the tissue, and gently submerge the filter material in the water (Fig. 3 C). It is necessary to select a filter that retains as much debris as possible, with pores large enough for the vermiform nematodes to wiggle through. To extract large nematodes, such as *Anguina, Belonolaimus, Dolichodorus, Longidorus,* and *Xiphinema,* nylon gauze with apertures about 90 µm in diameter often provides a sufficiently clean extract.

4- Spread about 30 to 100 cm^3 of finely crumbled sailor washed plant tissue (e.g., small roots or chopped bulbs, corms, tubers, stems, leaves, or buds), cut into small pieces, uniformly over the filter (Fig. 3 D). Fold the edges of the filter over the sample. Gently

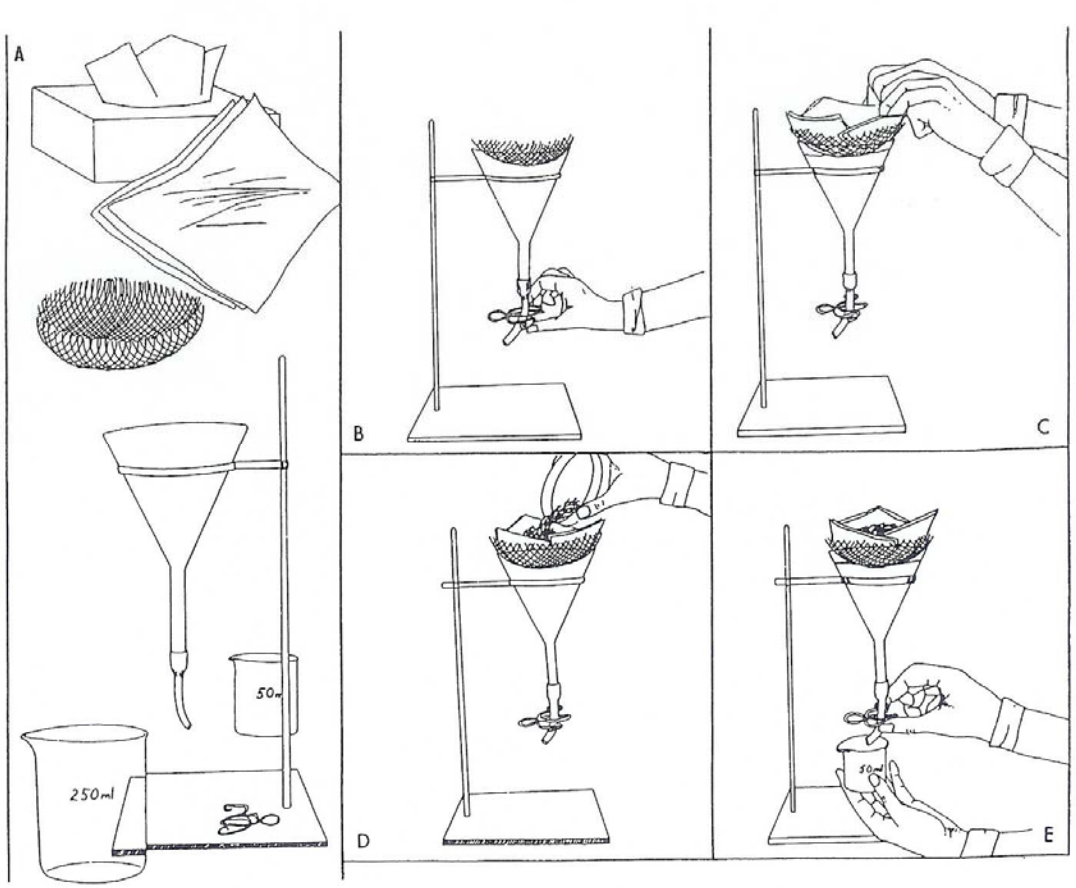

Fig 3. Extraction nematodes from soil and plant materials by the Bearmann Funnel Technique.

(Shurtleff *et al*., 2000)

pour water along the inner surface of the funnel until it just covers the lower surface of the soil.

CAUTION

The edges of the filter must not hang over the sides of the funnel, or water will be wicked out, and the sample will desiccate.

A- Do not overfill the funnel; the samples must not be completely submersed in water.

B- Do not pour water over the sample. Add it slowly down the inner surface of the funnel.

C- Check the apparatus periodically to see that the funnel is level, and replace any water lost by evaporation.

D- Label the funnel with the sample number and the date, and let it stand at a room temperature of 21-29°C, generally for two to five

days. *Do not allow the sample to dry out.* Cover to reduce evaporation. The motion of vermiform nematodes in the sample gradually carries them through the filter material and screen into the water in the funnel, where they settle to the bottom of the clamped tubing.

E- For soil samples, some workers use water containing methylene blue (2 ppm) and Separan NPlO (2 ppm) (Dow-Elanco Chemical Co.). For plant tissue samples, streptomycin sulfate (1,000 ppm) is added to reduce anaerobic conditions due to bacterial decay of organic matter in the sample.

5- At the end of the extraction period, fully open the clamp to rapidly withdraw 5 to 10 ml of water containing nematodes from the tube and transfer it to a shallow viewing dish (such as a Syracuse dish) or a 50-ml beaker (Fig. 3 E). Over 90% of the live nematodes are recovered in the first 5 to 8 ml of water drawn from the tubing. Only a few specimens of inactive species will be recovered. Samples may be stored at about 10-I6°C for examination within a few days. Lack of oxygen at the base of the funnel stem often inactivates nematodes, but they can usually be revived in fresh water.

MODIFICATIONS OF THE BAERMANN FUNNEL METHOD

A disadvantage of the Baermann funnel method is poor oxygenation, especially at the base of the funnel, where the nematodes settle, and some nematodes may lodge on the sloping sides of the funnel. Several modifications of this method overcome the problem of lack of oxygenation. One of them (Dish or tray apparatus) is described below.

DISH OR TRAY APPARATUS

A shallow evaporating dish, Petri dish, or tray can be substituted for the funnel.

1- Spread a thin layer of soil, residue from sieving, or chopped infected tissue on a double layer of cheesecloth, nylon gauze, or paper placed on a flat-bottomed plastic or stainless steel screen about 2 cm deep.

2- Place the screen, together with the soil or plant material, on supports (glass rods or other nontoxic materials) in an evaporating dish, Petri dish, or large tray (e.g., 30 x 35 cm) containing just enough water to wet the lower surface of the sample material. Maintain the water at this level.

CAUTION: Do not cover the sample material with water.

3- Place a circle of cheesecloth or paper tissue on top of the material to keep it moist and prevent plant material from floating. Add water as needed, by carefully pouring it between the cheesecloth or paper and the edge of the dish or tray.

4- After 12 to 24 h, gently remove the screen, and examine the water for nematodes. For further extraction, re-immerse the screen in fresh water for another 12 to 24 h.

COBB'S SIEVING AND GRAVITY METHOD

Cobb's sieving and gravity method is rapid (taking less than 30 min) and permits the recovery of active, sluggish, and some dead nematodes from large soil samples (1.5 kg). Eggs and cysts can also be recovered by this procedure. However, the centrifugal flotation method) is better for recovering dead nematodes.

Sieves

Sieves of accurately graded sizes are available from scientific supply houses but are expensive. Stainless steel sieves are preferred. Inexpensive substitutes can be made from sections of plastic pipe and nylon mesh cloth of known mesh size. These are fitted over pipe-like embroidery hoops. Plastic cups cut across in half nest together for constructing sieves.

The fineness of sieves is designated by a mesh number, which is the number of openings per inch of surface. For example, a 325-mesh sieve has 105,625 openings (44 µm in diameter, each) per square inch.

Usually only three or four of a set of sieves are used for a particular soil or plant sample. Sieves are selected to match the size of the nematode that is expected to be extracted and the type of soil in the sample. Most adults of large nematodes (e.g., *Anguina, Belonolaimus, Dolichodorus, Longidorus,* and *Xiphinema)* are caught on a 60-mesh sieve; adults of average-sized nematodes (e.g., *Aphelenchoides, Ditylenchus,* and *Hemicycliophora)* on a 150- or 170-mesh screen; adults of small nematodes (e.g., *Criconemella, Paratrichodorus, Paratylenchus, Pratylenchus,* and *Radopholus)* and juveniles of many genera on a 200- or 240-mesh screen; and small juveniles on a 325- or 350 mesh screen. The diameter of the sieve, the quantity of water used, and the amount of debris collected on the sieve affect the number of nematodes retained.

The number and fineness of sieves used in Cobb's method can be varied to better handle different nematode species and soil types. For example, a series of 20-, 100-, 200-, 325-, 500-, and 625-mesh sieves may be appropriate for fine-textured soils. For some soils and nematodes, the suspension is poured through a 60-mesh sieve, and the nematodes are collected on a 325-mesh sieve. To recover cyst nematodes, use a 60- or 80-mesh sieve last in the series, and backwash it into a beaker.

Some soils that contain high levels of organic matter or fine soil particles (e.g., some highly oxidized clayey tropical soils) tend to clog the openings in sieves, making screening difficult. Clogging can be partially avoided by inclining the sieve at an angle of about 30 to 35° to the horizontal while a suspension is poured over it. Gently patting the underside of the sieve into the bucket below and lifting it in and out a few times helps to clear it. In extracting nematodes from such soils, it may be necessary to reduce the size of the sample. In some cases, soil particles must occasionally be dislodged by sonication.

Generally, use screens singly. Never stack them and attempt to work a sample through them all simultaneously, as this may reduce the efficiency of recovery.

Sieves are expensive and will frequently need replacement if they are not handled properly. Thoroughly wash all sieves and pans in hot water, preferably using a strong spray from a nozzle. *Do not use soap or detergent,* which tends to plug the openings of fine sieves. Thorough rinsing is essential.

1- Obtain the following equipment (Fig. 4 A):

A- several buckets or stainless steel pans (2-3 L).
B- 500-ml Erlenmeyer flask.
C- 50, 250, and 600-ml beakers.
D- Syracuse dishes, 50-mm Petri plates, or pillboxes wash bottle.
E- series of 20 cm diam. Sieves.
F- 10 cm diam. sieve, 500- or 625-mesh.

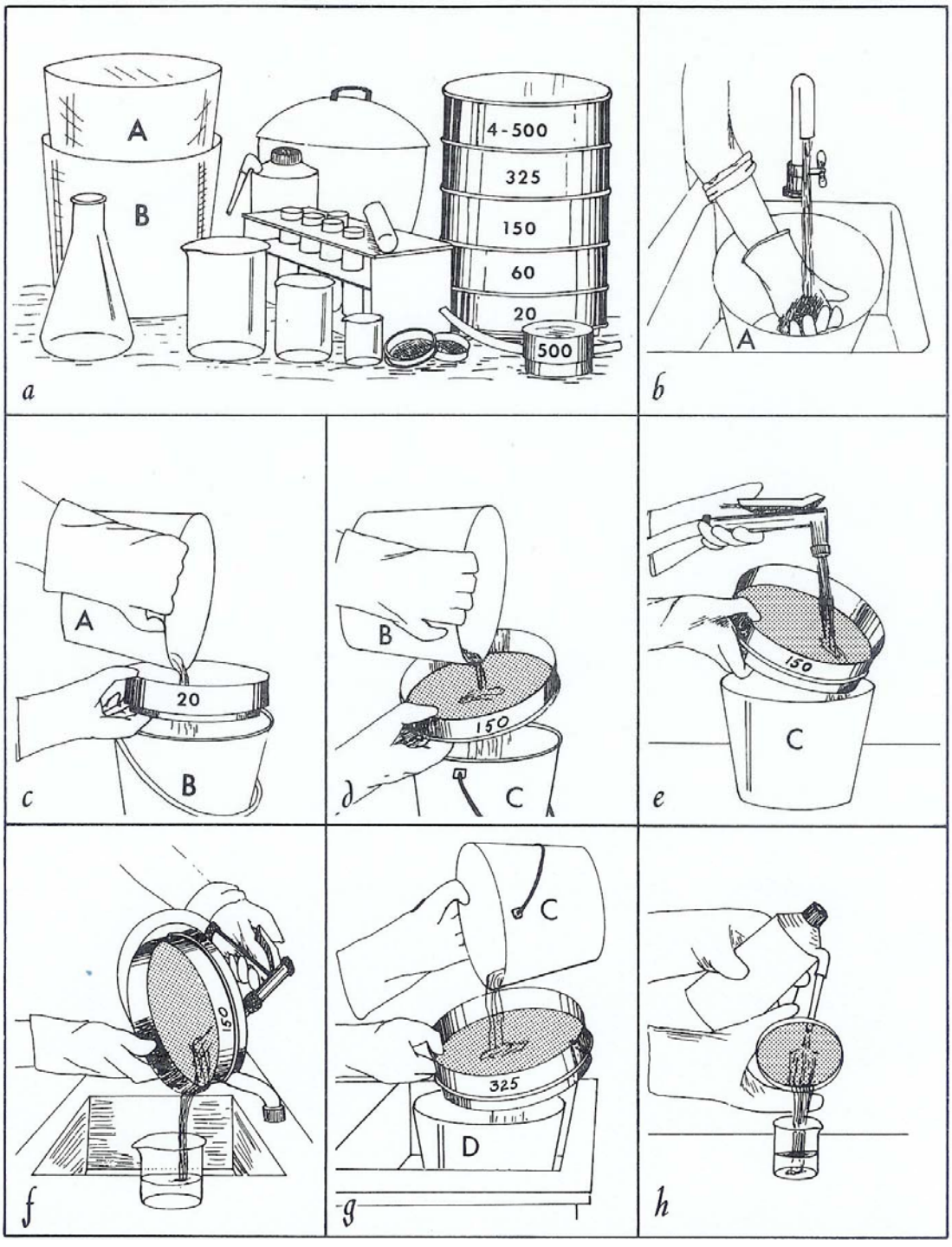

Fig 4. Extracting nematodes from soil by Cobb's sieving gravity method.
(Shurtleff *et al.*, 2000)

2- Mix soil samples thoroughly by gently tumbling them in plastic bags or on a large sheet of paper or clean plastic. Place a known volume of mixed soil (e.g., 100 to 500 cm^3) and about 1.5 L of water in a plastic bucket (bucket A) or a stainless steel pan. Gently mix the soil with tap water, stirring by hand or with a stick to break up all clods, free nematodes from the soil, and suspend them in the water (Fig. 4 B). Work the mass into a thick, muddy mixture. Most of the nematodes will now be suspended in the water. Dry soils should be soaked for several hours. Some workers use water containing a flocculating agent, such as Separan NP10 (12.5 µg/ml), to help break up soil aggregates and release nematodes from the sample.

3- Let the mixture settle for 30 sec to 1 min. Then decant the muddy water through a 10- or 20-mesh sieve into a second bucket (bucket B) or pan, to eliminate trash and heavy soil particles (Fig. 4 C). Avoid pouring the sediment; discard it in a receptacle for disposal. For greater recovery of nematodes, repeat steps 2 and 3 by resuspending the sediment in bucket A in an additional 1 L of water and pouring it again through the 10- or 20-mesh sieve into bucket B. Discard the sediment and thoroughly clean the sieve and bucket A. Most of the nematodes in the soil sample will now be in bucket B.

4- Resuspend the nematodes in bucket B by stirring or agitating the water. Let the bucket stand for about 1 min, and then pour its contents through a 150- or 200-mesh sieve into a clean bucket (bucket C or bucket A after it has been cleaned) (Fig. 4 D). Stop pouring *before* the sediment begins to move from the bucket into the sieve.

5- Hold the sieve at a steep angle (35 to 40°), and direct a gentle stream of water onto its upper side, to move the nematodes to the edge of the screen, and collect the water in bucket C (Fig. 4 E). Small nematodes and eggs will be washed through the sieve and will be recovered later.

6- Collect large nematodes on the edge of the upper surface of the sieve by directing a gentle stream of water (about 25 ml) onto the lower surface of the sieve (back washing), and collect the water in a 250-ml beaker (Fig. 4 F).

7- Repeat steps 5 and 6, using a 325-mesh and then a 500-mesh sieve (Fig. 4 G). Recover the nematodes adhering to the sieves, and backwash them into the 250-ml beaker.

8- Resuspend the small nematodes and eggs collected in bucket C in step 5. Concentrate them on the 325-mesh sieve, and backwash them into the 250-ml beaker.

9- Adult nematodes, juveniles, and eggs in the 250-ml beaker can be concentrated by pouring the contents through a 500-mesh sieve and backwashing with small quantities of water into a viewing dish or 50-ml beaker (Fig. 4 H). A sample can also be concentrated by permitting the nematodes to settle to the bottom of the beaker and then very carefully decanting all but the last 10 to 20 ml of water. An experienced technician can recover a large percentage of the nematodes in a soil sample in about 20 min.

The procedure must be carefully followed. At the end of the process, if the nematodes are mixed with an excessive amount of debris, they can be recovered by centrifugal flotation in a sugar solution (as described in the following section), or by permitting them to migrate through well-shrunk fine-mesh cloth or paper over an inverted glass or a funnel (Fig. 4).

The material collected from sieves may be examined directly, or it may be placed in a Baermann funnel for additional separation of nematodes from soil particles (as described in the preceding section). Most nematodes pass through the "filter" in a

Baermann funnel in 24 h and can be collected almost free of debris.

For direct examination, allow all the material in the 250-ml beaker to settle to the bottom. Slowly pour off the excess water into a sink, leaving about 50 ml in the beaker. Resuspend the material in the beaker, and gently pour 5 to 15 ml into a Syracuse dish, for examination under a dissecting microscope. Finally, isolate nematodes on a glass slide, and observe their morphological and anatomical features with a compound microscope for identification of the genus and species.

CENTRIFUGAL FLOTATION

In the centrifugal flotation method (Fig. 5), nematodes are separated from soil and suspended in water, and the suspension is centrifuged to concentrate the nematodes, which are then centrifuged again in a sucrose solution of specific gravity sufficient to cause them to float to the top. The procedure takes only a few minutes and can be used to process large soil samples. However, it is *appropriate only for soil samples.*

Centrifugal flotation recovers most nematodes from soil, including both active and inactive types. It is an excellent method for the recovery of sluggish and sedentary nematodes, such as *Criconemella* and *Hemicycliophora,* and it readily recovers dead, molting, or fixed nematodes and eggs. Recovery of very long nematodes, such as *Longidorus* and *Xiphinema,* or cyst nematodes requires a more dense sucrose solution than that used for standard analysis.

This procedure is generally more efficient for extracting nematodes than the Baermann funnel method or Cobb's sieving and gravity technique. Centrifugal flotation is often used to clean extracts obtained by sieving.

1- Obtain the following equipment (Fig. 4 A):

A- equipment needed for Cobb's sieving and gravity method (as described in the preceding section).

B- centrifuge.

C- centrifuge tubes and rack.

2- Separate the nematodes from a soil sample and suspend them in water as described in steps 1-7 of Cobb's sieving and gravity method (Fig. 4). Concentrate the nematodes in about 20 to 30 ml of water in a beaker, and then pour the contents equally into four or six 50-ml round-bottomed plastic centrifuge tubes (Fig. 5 A).

3- Fill each tube to within 0.5 cm of the top; fresh water may be carefully added to the centrifuge tubes from a wash bottle or pipette to balance them (Fig. 5 B).

4- Load the centrifuge properly, according to the manufacturer's instructions (Fig. 5 C). Tubes on opposite sides of the centrifuge rotor must weigh the same. If an odd number of samples is being processed, prepare a water blank as a counterweight for the rotor. The centrifuge should have a horizontal rotor head with swinging buckets. The *g* force of the rotor is related to its speed (in rpm) as follows: $g = 0.00001118 \times (\text{radius of the rotor, in cm}) \times (\text{rpm})^2$.

CAUTION:

Always follow the directions for use and the safety instructions provided by the centrifuge manufacturer.

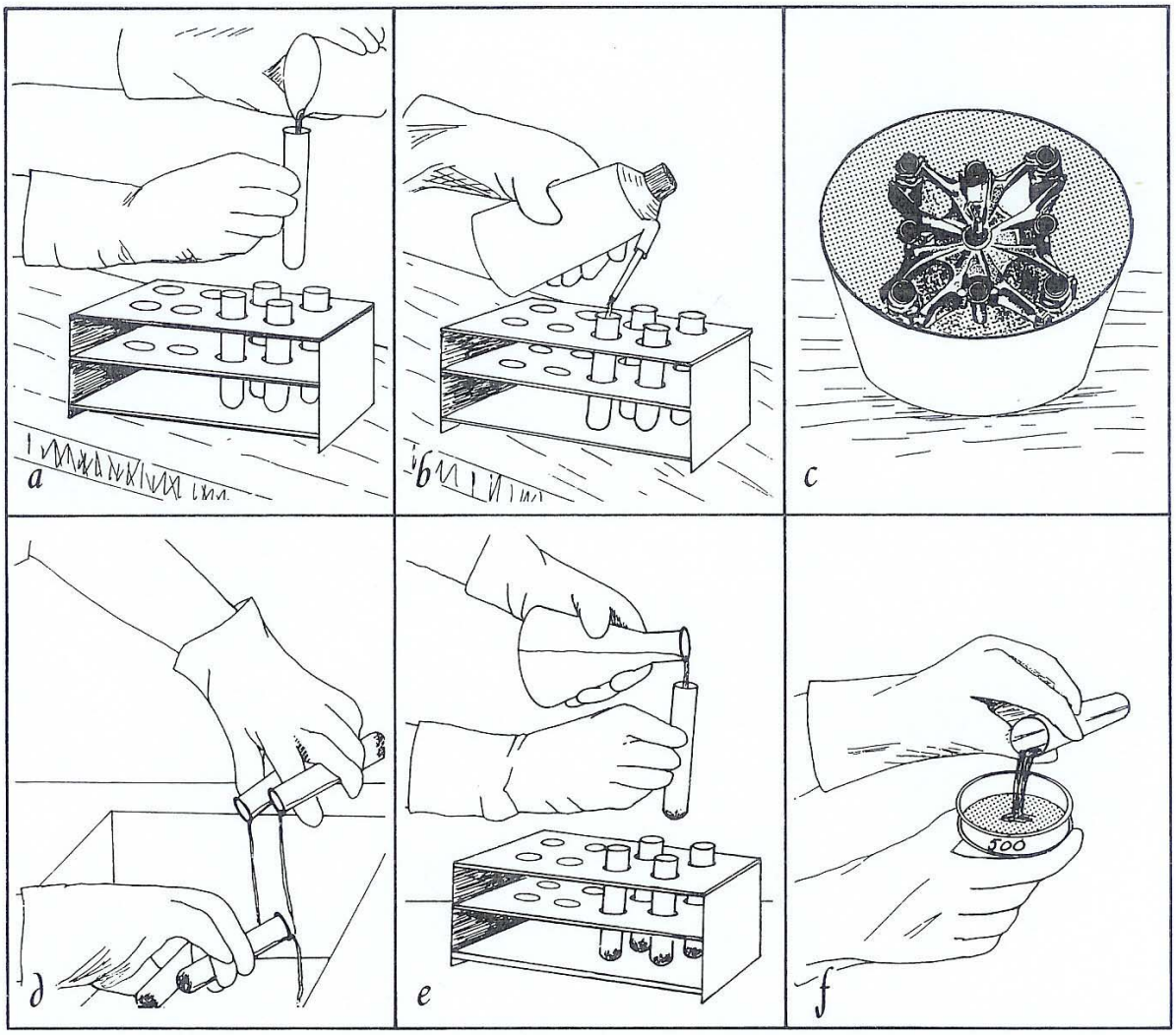

Fig 5. Extracting nematode from soil samples by the centrifugal floatation method. (Shurtleff *et al.*, 2000)

5- Centrifuge for 4 to 5 min at 2,900 x *g* to extract vermiform nematodes, 1,150 x *g* to extract cyst nematodes, and 420 x *g* to extract *Criconemella.* Let the rotor come to a rest without braking. The nematodes and eggs will be concentrated in a pellet at the bottom of the tube.

6- Remove the tubes carefully to avoid resuspending the nematodes, and decant the supernatant (Fig. 5 D).

7- Refill the tubes halfway to the top with sucrose solution (Fig. 5 E):

   a. To suspend vermiform nematodes, dissolve 673 g of white sugar in 1.0 L of water, to obtain a specific gravity of 1.18.

   b. To suspend cyst nematodes, dissolve 1,210 g of white sugar in 1.0 L of water, to obtain a specific gravity of 1.25.

8- Resuspend the nematodes in the sucrose solution by thoroughly mixing with a stirring rod or vibrator mixer.

9- Load the centrifuge as previously described. Centrifuge for 60 sec at 2,900 x *g* to extract vermiform nematodes and 1,150 x *g* to extract cyst nematodes. The nematodes will be at the top of the container.

10- Collect the nematodes by pouring the supernatant through a 300- to 625-mesh sieve (Fig. 5 F). Backwash the nematodes on the sieve into a 50-ml beaker or viewing dish.

CAUTION:

Do not permit nematodes to remain in the sucrose solution longer than 2 min. The osmotic pressure of the solution may distort or even kill them if they are left in it longer, making identification difficult.

STORAGE OF NEMATODES IN SUSPENSION

Many nematodes remain active and in good condition for several days when stored in shallow, fresh tap water at about 5-10 °C. Contaminating bacteria can be kept down by adding three drops of a 5% solution of streptomycin sulfate for each 5 ml of nematode suspension.

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