Nematological Techniques

11

It is difficult to detect a nematode damage merely on the basis of visible symptoms. Without a proper diagnosis, suitable nematode management schedule cannot be designed. Identifying a nematode problem comprises various steps, viz., drawing of suitable samples of soil and plant parts from the infested field, processing the samples to extract nematodes, separating and identifying specific parasitic species of the phytonematode based on morphological criteria, preparation of mounts, staining plant tissues to observe endoparasitic nematodes, etc. Some of the major techniques commonly adapted in plant nematology are furnished hereunder.

11.1 Sampling for Phytonematodes

It is essential to manage the nematode population in the field if it is above the economic threshold level (ETL). ETL is the population level at which nematodes cause economic damage to the crop. Therefore, nematode analysis is necessary before planting the crop to estimate possible damage and to help decide the suitable management options. The collection of soil samples and plants are the first step in the diagnosis of crop disorders caused by nematodes that attack root systems. An accurate diagnosis depends on proper collection and processing of samples. Improper collection and handling of samples may lead to the dismissal of nematodes as part of the problem; hence, any management strategy developed to alleviate the problem will be deficient. Information such as crop and cultivar, previous cropping history, history of other known or suspected problems, irrigation or rain fed, and previous applications of soil amendments (organic or any pesticides) are needed along with samples to assist in the diagnosis of the problem. The exact location of the samples is important. This information will permit comparisons with other problems reported previously from the region or indicate if the samples represent the first report of a nematode species from the area (Hafez and Pudasaini 2012).

Nematode sampling has become increasingly important in modern agriculture as the concepts of integrated pest management (IPM) and integrated crop production are developed and utilized. Scientists concerned with nematode populations have improved methods for their assay; however, data from the best extraction methods are of limited value if the sample is not representative of the area. Effective diagnostic sampling may involve rating plant roots (e.g., galls caused by *Meloidogyne* spp.), bioassays, or visually assessing aboveground growth for effects of foliar pathogens in addition to collecting soil and root samples for nematode counts. Since adoption of nematode management practices is need based, growers are advised to treat the soil only if they have identified the specific nematode that causes damage to that particular crop.

Nematode sampling is the basis for determining the occurrence and distribution of many plant-parasitic nematodes. Quarantine or phytosanitary

N.G. Ravichandra, *Horticultural Nematology*,
DOI 10.1007/978-81-322-1841-8_11, © Springer India 2014

regulations of many countries, or political subunits, require that planting materials be produced on land certified free from nematodes. Soil sampling for certification of widely distributed planting materials requires extreme precision for detection of quarantined pests. Although the objective of detection seems simple, a negative result does not necessarily prove absence of the pest, but only indicates that a nematode population is below the detection level. The fact that initial numbers of nematodes can be related to the yield of annual crops has enabled nematologists to develop functional advisory programs, even though relationships between nematode numbers and crop damage may be modified by environment. Because of the importance of reliable detection, most sampling for advisory purposes is conducted when population densities are near their maximum levels, often at the end of the growing season after harvest. Sampling at the time of planting, however, theoretically will give a better estimate of the initial nematode problem where population levels are high enough for detection. Follow-up sampling may be necessary with perennials because low, nondetectable populations sometimes increase over time to damaging levels.

Root and soil samples containing roots can be taken at any time as long as the soil is not frozen. During the active growing season, however, nematodes live and feed inside or along roots particularly during hot dry seasons. If nematodes are suspected of contributing to the decline of a particular area of a young crop during the growing season, collect entire root systems with surrounding soil separately from plants with symptoms and plants without symptoms. If the decline is noticed in a fruit tree orchard, vineyard, or other perennial crop, carefully dig and sample from the feeder root zone approximately 10–20 g fresh weight of roots from the infected plants and submit for analysis. Do not sample the roots from dead plants because the nematodes will have already died or moved away from dead roots into the soil. Place samples in a plastic bag out of direct sunlight and in a cool place during transportation to the diagnostic lab.

11.1.1 Suitable Time to Collect Soil Samples

The best time to sample soil for nematode population assessment is in the spring after the soil has warmed up or during the fall, soon after harvest. It is not advisable to take nematode samples when fields are very wet. Fields with a history of nematode problems may be sampled routinely to determine if the nematode population is approaching or has exceeded an economic threshold. Soil populations of most phytonematodes tend to be highest in September and October after crops have senesced and died. This is the best time of the year to sample for nematodes. Sampling in the early fall allows growers time to make decisions on whether to fumigate during the fall or spring or what crop should be planted the following spring. It also allows time to implement an integrated management strategy prior to growing a susceptible crop in that field. Sampling in the spring prior to planting a crop may also be reliable.

11.1.2 Where to Look for Sample?

Where to sample soil for nematode assessment depends on the purpose for taking the soil sample, the type of crop in the field, and the type of nematodes being sampled. The rhizosphere zone of a plant is the right place to collect soil and root samples. If the purpose of sampling soil for nematodes is to diagnose a problem during the growing season in a row crop, take 8–10 soil cores from areas where plants are unhealthy or near plants along the margin of a severely affected area. Sample another 8–10 soil cores separately from areas of healthy growing plants for comparison (Fig. 11.1). When sampling soil from row crops during the growing season, or from trees or perennial crops, it is very important to get the feeder roots of the crop in the soil sample, since this is where many nematodes live.

For individual fruit trees or ornamental shrubs suspected of being infested with nematodes, it is best to take soil samples from just below the drip

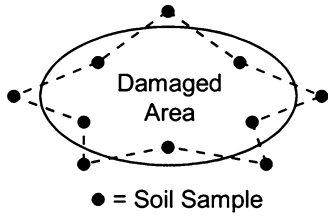


Fig. 11.1 Sampling pattern for damaged area or infected patch in a crop

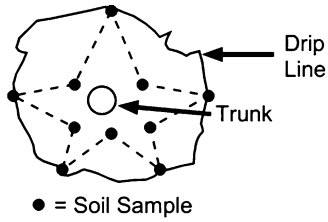


Fig. 11.2 Sampling pattern for individual tree or shrub

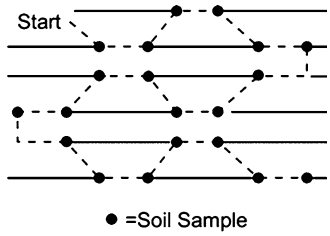


Fig. 11.3 Soil sampling pattern for row crops

line and in the area between the outer branch tips and the tree trunk (Fig. 11.2). If the purpose of sampling a field is to determine whether the nematode population has reached an economic threshold in a row crop, take soil cores within the row of actively growing plants to obtain samples that contain feeder roots (Fig. 11.3). When sampling from fallow fields, in the autumn after the crop has senesced or in the spring prior to planting, it is best to walk in a Z, W, or M pattern across the field (Fig. 11.4). The soil sample should represent not more than 2.5 ha.

11.1.3 How to Collect Soil Sample?

Nematodes are rarely distributed evenly throughout a field, and nematode populations fluctuate throughout the growing season. Soil should be

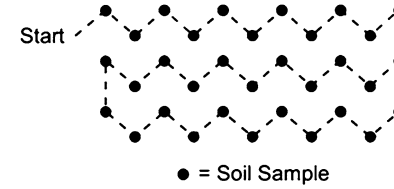


Fig. 11.4 Sampling pattern in the autumn after the crop has senesced or in the spring prior to planting. This pattern can also be used for fallow fields and crops that are not planted in rows

Table 11.1 Number of soil core samples/area required to estimate nematode populations

Area	Number of soil cores/sample
<500 m^2	8–10
500 m^2–0.5 ha	25–35
0.5–2.5 ha	50–60

sampled approximately 20 cm (8 in.) deep using a 2.5-cm (1-in.)-diameter soil core probe (Fig. 11.4). Alternatively, soil can be sampled with a narrow-bladed shovel or trowel; however, this method is less reliable than using a soil core probe. Extremely wet, dry, hot, or cool seasons can influence the population levels particularly in the top 2.5–5 cm (1–2 in.) of soil. Discard the top 2.5–5 cm (1–2 in.) of soil where nematodes would not usually live due to extreme environmental conditions. Collect soil cores in a clean bucket, mix the soil thoroughly but gently, and place in a labeled plastic bag or container. Never allow soil samples to heat up or dry out. Place soil samples in a cooler with ice until they can be stored in a fridge or analyzed for nematode populations.

11.1.4 How to Decide the Number of Soil Cores?

The number of soil core samples required to estimate nematode soil population levels depends on the size of the area under investigation (Table 11.1). The sample submitted to the laboratory should not represent more than 2.5 ha. Enough soil to give a good representation of the soil population is all that is necessary. The chart below is a guide of how many cores are necessary to

make up a representative sample. If soil type changes within the field, take separate samples from each soil type. Send the soil samples to a pest diagnostic clinic or laboratory that is qualified to isolate, identify, and enumerate nematodes.

While collecting soil samples, it is also best to collect root samples to aid in the diagnosis. When doing so, one should dig up the plant so as to obtain as many of the fine feeder roots as possible. If the plant is pulled from the ground, most of these feeder roots will be lost. If sampling a perennial crop, it is also important to collect feeder roots specifically from the current year's growth rather than larger and older roots. It will be difficult to make an accurate diagnosis from a sample that only contains large roots. Of course, some nematode parasites are rarely found in the soil or roots but are found primarily in the bulbs, corms, stems, or foliage. In such cases, care must be taken to collect the appropriate symptomatic tissues. Again, samples should not be taken from long dead plants, as the parasites may be difficult to detect in such samples. It is best to collect samples from a number of live plants that are exhibiting a range of symptoms. Extra effort in sampling plant tissues and soil is required to obtain accurate results. The accuracy in determining relative numbers and developmental stages of nematodes may be greatly affected by sample handling or extraction. Design and management for sampling may need to be modified for each specific type of study.

11.1.5 Sampling Tools

There are different tools to collect soil, such as soil probes, trowels, hoes, narrow-bladed spades, or shovels. However, they are collected most efficiently with sampling tools designed for the procedure, such as a standard Oakfield soil probe with a diameter of 1 in. If using a shovel or spade, it is best to collect only a narrow column of soil from each shovelful of soils to avoid excessive sample volumes. The multiple subsamples should be thoroughly mixed together in a large bag or bucket and a final sample of 1–2 L of soil placed in appropriately labeled plastic bags.

11.1.6 Care of Samples After Collection

The biological vitality of the sample should be preserved after the collection of plant and soil samples as the extraction and identification procedure needs live nematodes to achieve the best accuracy. Samples should be delivered or shipped to the diagnostic laboratory without delay. Samples should be protected from extremes of temperature, i.e., freezing (less than 32 °F) or temperatures above 95 °F. Thus, they should be packed in insulated containers and kept in a cool environment. Refrigeration (storage at 40 °F) is not required if the sample is being processed within a day or two but is helpful if the samples will be stored for a longer time period. It is usually not necessary to pack samples in ice for shipment, but shipping over a weekend or holiday period should be avoided. This will reduce the possibility of the samples being left unprotected on a loading dock or in a warehouse for several days. A good rule to follow is to treat the samples like perishable food that one wishes to consume in 3–4 days.

11.1.7 Considerations to Design Sampling Procedures

Various aspects are to be considered while designing sampling procedures, viz., influence of nematode distribution patterns on the results, the capacity of the nematode species to move or be moved by man or other carriers, presence of majority of nematodes in most annual cropping systems in the upper layer of soil (to 15 in.), influence of biology, feeding habits and environmental interactions of the nematode species involved, and the effect of crop rotation and cultural practices.

11.1.8 Field Mapping

The distribution of nematodes is seldom uniform or constant and changes may occur rapidly. Most of the time nematode distribution is patchy. For these reasons, the field to be sampled should

be mapped into subdivisions. Any observable variation in previous crop growth, soil texture, moisture and draining patterns, or cropping history will constitute a subdivision. An effective sampling map may then be constructed.

11.1.9 Sampling in Different Crops

11.1.9.1 Established Perennials

Several points are to be borne in mind while sampling in perennials, which include the following: collect separate samples for each plant species, collect from the feeder root zone of plants showing decline, and avoid sampling directly around dead plants; perennial crops will typically fluctuate during the year with the timing of the fluctuations varying by crop and location; it is important to always sample at the same time each year (because of population fluctuation); and if one wants to compare populations from year to year, it is important to leave some areas untreated for comparative sampling if treatments are applied to try to reduce nematode numbers. If this is not done and samples are taken several months after a treatment, populations may be either lower or higher than before treatment just because of normal population fluctuations.

11.1.9.2 Annual Crops

In this category, nematodes typically are at a low level during planting and increase toward harvest as long as roots are healthy enough to support nematode feeding. Following harvest, nematode numbers typically decrease until another susceptible crop is planted or weeds become available that will support reproduction. Under weed-free fallow conditions, for instance, root-knot nematode populations may drop as much as 85–90 % in a year's time, while sugar beet cyst nematode will only decrease by 20–60 % depending on the location. *This principle is illustrated in an example of population decrease of Heterodera schachtii following harvest of a susceptible crop until the planting of another susceptible crop (cabbage) during which time populations increase dramatically and then decrease once again during successive nonhost crops. Nematode populations are usually easier to detect at harvest when they are highest than several months following harvest. For example, after several months in a fallow or nonhost crop, populations of root-knot nematode may not be detectable through sampling but may still be high enough to cause significant damage when a host is planted.*

11.1.9.3 Ornamental Plantings

Population fluctuations on ornamental crops including turf is almost similar to other perennial crops. Those on ornamental plantings will likely vary depending on whether the planting is an annual or a perennial. Samples should be collected when soil is moist and one pint of soil should be collected for each sample. When sampling fields in row crops, samples should be collected to represent the top 8 in. of soil. When possible, sample directly in the root zone. For pastures, lawns, and other areas, take samples of only the top 5 in. of soil. For shrubs, each sample should be composed of soil taken from three or more places in the area. Even when the sample is to be collected from one shrub, take soil from three or more places around the plant. Collect the sample to represent the top 6 in. of soil.

11.1.9.4 Field Mapping

In general, the distribution of nematodes is seldom uniform or constant and changes may occur rapidly. Most of the time nematode distribution is patchy and not uniform. Hence, the field to be sampled should be mapped into subdivisions. Any observable variation in previous crop growth, soil texture, moisture and draining patterns, or cropping history will constitute a subdivision. An effective sampling map may then be constructed.

11.1.9.5 Sampling Nursery Stock

Strategies for individual nursery situations involve subjective on-site judgment. The greater the potential loss, the greater is the sampling intensity that can be justified. For routine monitoring of container grown plants to maintain plant health and product quality, extraction of nematodes from single pots may not adequately reflect the situation in a whole block. Removing

single cores from a series of pots and bulking them into a composite sample provide a sample representing more plants. A convenient sampling rate of 1- and 5-gal containers is one core per 100 containers. If the plant is particularly susceptible or if nematode problems are suspected, the block of containers should be divided into groups of 2,000, with each group represented by a single sample of 20 cores. For a more routine sampling, cores from the whole block may be composited into a bucket and mixed thoroughly and a 1-quarter sample removed to represent the block.

The proportionally larger sample taken from smaller containers by this approach will help detect earlier stages of nematode infestation on young plants. Core sampling is excessively destructive in containers smaller than 1 gal, but the same criteria can be applied by destructively sampling one container per hundred as representative of a single core. The number of cores and sampling pattern for nursery plants in raised beds depends on the value of the plants and potential magnitude of the problem. Representing each bed by one sampling of several cores provides information on the occurrence of nematode problems in individual beds and allows individual bed treatments. If a nematode problem is unlikely, a single sample may represent several beds, which may result in a need for subsequent sampling to identify distribution of a population.

11.1.9.6 Effects of Cropping History

Differences in cropping history of a field that has now uniformly planted crop susceptible to root-knot nematodes may lead to the irregularity of root-knot damage within that field. If a portion of that field had an established vineyard earlier, on which root-knot nematodes increased to high levels and the remainder of the field has a grassy pasture free of root-knot nematode, the pattern of plant damage due to root-knot nematode infection will correspond to the previous vineyard area where nematode population density is much higher.

11.1.9.7 Soil Sampling Strategies

There are mainly three sampling strategies, viz., scouting, diagnostic, and predictive.

Scouting Sampling: When sampling for scouting purposes, soil should be collected from areas that are likely to be first infested with the nematode, called “high risk” areas. These areas include spots of the field where equipment enter along fence lines where windblown soil accumulates, in low spots of the field where surface water accumulates, or in areas of the field where unthrifty soybean growth had been observed in the past. However, if collection of soil samples for the purpose of scouting for soybean cyst nematode is not feasible, soil samples collected for soil fertility analysis also can be assayed for the nematode.

Diagnostic Sampling: Diagnostic soil sampling is performed when the soybean crop is in the field and the plants are showing obvious aboveground symptoms. Two separate samples have to be collected, one from the infected spot and another from a nearby spot which does not appear to be affected. Soil should be collected from near plants showing the most dramatic symptoms as well as near some that are not as severely affected.

Predictive Sampling: Predictive soil sampling is performed to gain information on the severity of a known soybean cyst nematode infestation for use in making management decisions for the upcoming growing season. It is done after the crops have been harvested, or in early spring prior to planting. If sampling for predictive purposes, collect the soil in a systematic, zigzag pattern within the area. Limit the area sampled to no more than 15–20 acres; if a larger field is to be sampled, divide the field into 15–20-acre parts and collect separate samples from each part. Define the part of land to be sampled based on agronomic parameters such as soil type, pH, drainage, elevation, or prior cropping history. The fewer the number of acres represented in each sample, the more accurate and representative the results will be.

Nematode Sampling Data Sheet: This Sheet Should Always Accompany the Sample

1. Collector: _____
2. Sample number: _____
3. Date of collection: _____
4. Locality/village: _____ Taluk: _____
   District: _____ State: _____
5. Precise location: _____
   Grower's name and address: _____
6. Host: _____ Variety: _____ Crop stage/age: _____
7. Condition of crop (healthy/diseased): _____
8. Previous crop/intercrop: _____ Soil type: _____
9. Rain fed: _____ Irrigated: _____ Water stable: _____
10. Symptoms observed:
    i. Above ground/part:
       Stunting [ ] Yellowing [ ] Necrosis [ ] Wilting [ ] Slow decline [ ]
    ii Below ground/root system:
       Galls [ ] Cysts [ ] Decay [ ] Lesions [ ] Rotting [ ]
11. Severity/disease index: _____
12. Others:
    i. Distribution of affected plants:
       General [ ] Localized areas [ ] Scattered plants [ ]
    ii. Percent of planting affected: _____
    iii. Acre age (size of the field):
       <½ ac [ ] ½ ac [ ] 1 ac [ ] 5 ac [ ] 10 ac [ ] >10 ac [ ]
    iv. Stand of the crop:
       Very good [ ] Good [ ] Fair [ ] Poor [ ]
    v. Comments: _____
    vi. Remarks, if any:_____

11.2 Techniques of Nematode Extraction

Once the samples, either soil or plant part, are collected, they need to be processed in the laboratory for extracting nematodes for further studies. Extraction is a fundamental and the most essential technique in nematological research (Ravichandra 2010). Plant-parasitic nematodes can be extracted from soil and plant parts by using different techniques. Some techniques are more effective than others for particular types of nematodes or for special kinds of plant materials. Some techniques are better adapted than others to specific purposes such as nursery stock inspection or quarantine enforcement. Others require expensive equipment or are too laborious to be practical except in extraction research work. Plant-parasitic nematodes can be extracted from both soil and plant parts. The technique depends upon the type of nematodes or kind of plant materials. Processing of samples consists of separating the nematodes from soil or plant materials in order to count them. Some techniques of processing samples are adapted to specific purposes. Nematode extraction techniques can be categorized into major groups like extraction from soil and plant material and cyst extraction.

Extraction of Phytonematodes from Soil: For smaller sample size, the soil may be directly placed in the funnel and processed by the mist extraction of Baermann's funnel technique. Larger ones may be processed first by the combined screening-funnel technique or by the gravity-screening technique followed by the mist extraction.

Extraction of Cyst Nematodes from Soil: The centrifugal floatation technique or the special water floatation technique is appropriate.

Extraction of Phytonematodes from Roots: The roots are to be cut into small bits, processed by mist extraction, blending, or Baermann's funnel technique or by gently washing and subjecting to jar or plastic bag incubation. The mist chamber is more effective for the extraction of both endoparasitic and ectoparasitic nematodes from both roots and soil samples. Root samples from nursery stock are processed by jar incubation to recover migratory endoparasites only. Direct examination can be done if the roots show galling or other clear evidence of nematode damage.

Extraction of Nematodes from Other Plant Parts: Other plant parts may include tubers, bulbs, stems, leaves, crowns, etc. Baermann's funnel and mist extractions are the most effective techniques.

Extraction of Sluggish Nematodes: Sluggish nematodes like *Hemicycliophora* and *Criconemoides* are difficult to extract by funnel method but can be extracted using centrifugal floatation technique.

11.2.1 Techniques of Nematode Extraction from Soil

These techniques are adapted to extract phytonematodes from soil, particularly from rhizosphere by using various techniques. Plant-parasitic nematodes can be extracted from soil surrounding the roots on which they feed, in several ways. The following are some of the most commonly followed ones:

1. Petri plate technique
2. Baermann's funnel technique
3. Cobb's sieving/gravitation technique
4. Combined Cobb's and Baermann's funnel technique
5. Mist chamber technique/Mistifier
6. Elutriation technique
7. Centrifugation technique
8. Fenwick can technique

11.2.1.1 Petri Plate Technique

This is a very fundamental technique to extract nematodes from a small quantity of soil sample.

Procedure Take a Petri plate filled with water; above that place a wire gauge and over that place tissue paper; water level per plate should be in touch with the wire mesh; place the soil sample above tissue paper. Leave this for 24–48 h; take out the Petri plate and observe for nematodes present in it under a stereo binocular dissecting microscope.

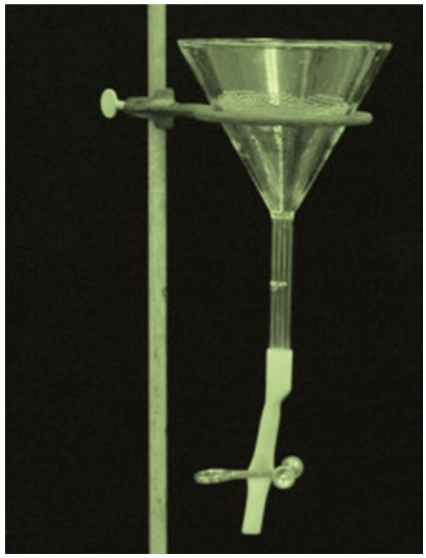


Plate 11.1 Baermann's funnel apparatus

This is a very simple, basic, less expensive, and quick technique. It can be used to extract nematodes from both soil and plant tissues. However, it is not very precise because the Petri plate cannot hold large amounts of water. It can hold only a small quantity of soil sample. Since there is not much space in the Petri plate, there may be suffocation.

11.2.1.2 Baermann's Funnel Technique

This technique is an excellent system of separating nematodes from soil and also plant parts including roots and condensing them for examination. This technique utilizes a long-necked funnel that has been named after its inventor, "Baermann" (Plate 11.1).

Procedure Attach a 10-cm length of rubber tubing to the funnel stem and clamp the tubing; mount the funnel on the ring stand; fill the funnel two-thirds full with water; place the wire-mesh basket on top of the funnel and use it to support tissue; mix the soil sample and remove plant debris, stones, inert matter, etc.; spread the soil subsample (50–200 cm^3) evenly on tissue; fold the edges of the tissue paper without allowing them to face downward, extending outside the wire mesh. This is to avoid trickling of water drops from the edges which might carry nematodes. Fill the funnel completely with water so that the water level is about 5 mm above the wire mesh; do not let the water and soil lose contact during the extraction period; add water as needed; maintain a temperature at 22–25 °C so that it is conducive to nematode movement; nematodes move through tissue and settle in the funnel; only active stages are recovered; after 24–48 h, recover the extracted nematodes by releasing 20 ml of water from the stem of the funnel into a counting dish/beaker.

In this technique, most nematodes are recovered after 24–48 h. However, nematode yield may vary depending on several factors including the size of the sample, temperature, time of storage and extraction, and nematode species.

This technique is simple and easy to set up, and the equipment is inexpensive. Nematodes from a small quantity of soil sample can be effectively recovered. However, lack of aeration in the water may reduce nematode movement, hindering recovery. Only active nematodes can be recovered; sluggish/inactive forms cannot be extracted. Recovery of active nematodes from large samples is poor. The funnel capacity is small; hence it may be too small to be a representative. Frequent care needs to be taken to check whether the wire gauze is in touch with the water surface.

11.2.1.3 Cobb's Sieving and Gravity Method (Decanting and Sieving Method)

This is the most basic technique that consists of mixing soil (the volume varies) with a large volume of water (normally three- to five-folds), allowing a brief time for heavy particles to settle, and then pouring the mixture through one or more sieves of a mesh size expected to retain large debris or nematodes. The sizes of screens used vary depending on the type of nematodes expected to be recovered and soil characteristics. Some begin with a course sieve of 10–20 mesh/in. which will catch large debris but allows nematodes to pass through. The solution is then passed through sieves of 60–500 mesh/in. to catch nematodes. Nematodes and soil particles caught on the sieves are "backwashed" into containers.

If not too murky, this solution can then be viewed under a microscope or subjected to an additional technique to further purify the sample. A set of three or four sieves is used here. Although the size of the sieves may vary slightly, big-, medium-, and small pore-size sieves are employed.

The following is a list that gives the different sieve sizes (British Standard Sieve Series) (Southey 1986).

Sl. No.	Mesh/in.	Pore aperture (μm)
1	4	4,000 (=4 mm)
2	6	2,800 (=2.8 mm)
3	8	2,000 (=2 mm)
4	12	1,400 (=1.4 mm)
5	16	1,000 (=1 mm)
6	20	840
7	22	710
8	30	500
9	44	355
10	60	250
11	72	210
12	85	180
13	100	150
14	120	125
15	150	105
16	170	90
17	200	75
18	240	63
19	300	53
20	350	45

Most adults of large dorylaimids are caught on a 250 μm-aperture sieve, adults of average-size nematodes on a 90 μm-aperture, and many larvae and small adults on a 63 μm-aperture sieve. A 45 μm-aperture sieve is necessary to recover small larvae. Only a proportion of the nematodes are caught when a suspension is poured once through even the finest sieve (65 % of nematodes), 500 μm long or 25 % of those 250 μm long when the suspension is poured once through a 50 μm-aperture sieve. It is therefore advisable to use a bank of sieves to pour the suspension three or four times through the finest sieve in use, collecting the residue off the sieve each tine. The diameter of the sieve, the quantity of water used, and the amount of debris collected on the sieve will affect the number of nematodes retained.

Plate 11.2 Set of sieves

Procedure Mix soil sample and pass through coarse sieve to remove debris, inert matter, roots, etc.; take a 200-cm^3 subsample of soil, pack lightly into beaker for uniformity; place soil in one of the buckets or pans; mix water in the ratio of 1:3 (soil:water); sieving and decanting process (various combinations of the following) (Plate 11.2): mix soil and water by stirring with hand or paddle; allow to stand until water almost stops swirling; pour all but heavy sediment through a 20-mesh sieve into the second bucket; discard residue in the first bucket; discard material caught on sieve (mostly it contains inert matter, saprophytic nematode forms, etc.); stir material in the second bucket; allow to stand until water almost stops swirling; pour all but heavy sediment through a 200-mesh sieve into the first bucket; discard residue in the second bucket; backwash material caught on the 200-mesh sieve (which includes large nematodes) into a 250-ml beaker; stir material in the first bucket; allow to stand until water almost stops swirling; pour all but heavy sediment through a 325-mesh sieve into the second bucket; discard residue in the first bucket; backwash material caught on the 325-mesh sieve (which includes small- to midsized nematodes and silty material) into a 250-ml beaker; sample in the 250-ml beaker will probably be too dirty with fine soil particles for direct viewing; sample may be placed on Baermann's funnel or subjected to sucrose centrifugation; the combined procedure allows extraction of nematodes from larger volumes of soil.

Soil particles may block the aperture of fine sieves before all the suspension had passed through. Blocking can be avoided by pouring the suspension carefully onto sieves inclined at about 30° above the horizontal and by ensuring (by mean of small spacers) that airtight joints forming air locks do not occur between sieves in a bank. Should a blockage occur, tapping the frame or gently stroking the underside of the gauze with the fingers may clear it; alternatively, the sieve may be partly immersed in water and gently shaken until the mesh clear.

The technique is not dependent on nematode movement; hence, sluggish nematodes can also be recovered. It allows recovery of most nematodes from large soil samples. Nematodes are available for direct examination in less than half an hour. Nematodes can be differenced based on their size. However, the technique requires expensive sieves and experienced workers. The suspension may not be very clear. Difficulty may be encountered in observing nematodes because of fine soil particles. It cannot be used for nematode extraction from plant tissues. Careful handling is required as mesh is delicate.

11.2.1.4 Combined Cobb's and Baermann's Funnel Technique

This technique avoids the disadvantages of both techniques. Both active and sluggish nematodes can be extracted by this combined technique.

Procedure Nematodes may be separated from soil particles after sieving process by the use of sieving and gravitation technique; put the sieved soil in the tissue paper on top of the wire mesh in the funnel; fill the funnel with water up to the rim; nematodes will pass through settling at the bottom of the funnel; collect 5–10 ml after 24–48 h.

This method allows recovery of most nematodes from large soil samples. The resultant sample contains less silt and debris compared to Cobb's sieving method, and it is easier to examine under a dissecting microscope. However, sluggish nematodes recovered during the sieving part of the technique may fail to pass through the tissue in the funnel, although recovery of mobile nematodes is good. Processing takes longer and requires considerable equipment.

11.2.1.5 Mist Chamber Technique/ Mistifier

This technique is a modification of Baermann's funnel technique and can be used for both soil and plant samples. A continuous fine mist of water is sprayed over soil samples. Active nematodes emerge which can be recovered from the water which collects below. Nematodes recovered by this method are often more active than those extracted by some of the above methods because oxygenation is better and sap and toxic decomposition products are washed away (Seinhorst 1950).

Procedure Before operating, make sure the equipment is functioning properly. The nozzle should deliver a fine, fairly uniform mist at the proper temperature (70–75 °F) and be on the correct time cycle (1.5 min on, 8.5 min off). When the nozzle valve shuts off, do not allow water from the nozzles to drip into the funnels; place one funnel for each sample into the funnel rack. Cover the inside of the stainless steel wire basket with a double thickness of tissue. Avoid puncturing or tearing the tissue. Carefully place the material for extraction into the tissue-lined basket, then set the basket in the funnel; samples may include moist soil (1/4 cup), small root bits (1/4–1/2 in.), bulb, corm, sucker, or tuber tissues cut into bits; label the culture tubes with sample number and date. Insert the stem of each funnel into the corresponding labeled culture tube; insert the loaded funnel rack into the mist chamber; incubate samples under the mist for 3–5 days (2 days for foliar and bud samples); the water should gradually fill the culture tubes and overflow slowly enough so that the nematodes remain at the bottom of the tubes; carefully remove each culture tube from its funnel without disturbing the contents; place the labeled tubes in a test tube rack; with a tube attached to an aspirator or with a large pipette, draw off the water from each tube to within 1 in. of the bottom; this must be done very carefully to avoid stirring up of nematodes which have settled to

the bottom; the inside and outside of the aspirator tube or the pipette must be rinsed between samples or a fresh pipette used for each sample to avoid contamination; the nematode suspension remaining in each culture tube is now ready for examination or prepared for shipment to the laboratory.

By this technique, more nematodes can be recovered from a given quantity of soil sample or plant material than by Baermann's funnel. It prevents the loss of nematodes which occurs in the jar incubation technique when rinsed water is poured through the sieves. There is no accumulation of toxic materials during the incubation period. However, this technique involves relatively expensive specialized equipment.

11.2.1.6 Sieving and Sucrose-Centrifugation Technique

This technique is also useful for the nematode extraction both from the soil and plant parts. Some difficulty may be encountered with fine clay soil. Only a small quantity of the sample can be used. It is also useful to isolate cysts and juveniles of *Heterodera* and *Globodera* species (Barker 1985).

Procedure Prepare sucrose solution; add deionized water to 454 g sugar to bring total volume to 1 L; stir until sugar is completely dissolved; mix soil sample and pass through coarse sieve to remove inert matter, roots, etc.; collect a 100-cm^3 subsample of soil. Pack lightly into beaker for uniformity; remove inert and organic material; mix soil subsample in 500 ml water by pouring between beakers ten times; rinse residues in the second beaker into beaker with sample; swirl beaker with sample; allow to stand for 15 s (for settling of sand); pour supernatant through 20/500-mesh stacked sieves. Tap gently the side of 500-mesh sieve to facilitate drainage.

Larger particles will remain in the beaker; organic debris is caught on the 20-mesh sieve; nematodes and silt are retained on the 500-mesh sieve. Using the coarse-spray water bottle, gently wash nematodes into one sector of the 500-mesh sieve. Using the fine spray water bottle, wash sample into a centrifuge tube.

Add water to centrifuge tubes to equalize volumes; place tubes in centrifuge in balanced pairs; spin at 1,700 rpm (810 g) for 5 min without using the brake; allow to settle for 5 min; aspirate the supernatant to approximately 1 cm above the pellet; fill tubes with sucrose solution at room temperature; stir with a spatula to break up the pellet (must be completely dispersed). Spin the sample and bring the centrifuge up to 1,000 rpm (280 g) in 30 s and then apply brake.

Nematodes and clay are suspended in sucrose supernatant; silt and larger particles are in the pellet. Pour supernatant through a 635-mesh sieve. Rinse gently with water and transfer to labeled vials using the fine spray water bottle.

11.2.1.7 Elutriation Techniques

In this technique, a measured flow of water in an upward direction will support nematodes in a given range of specific gravities but will allow heavier soil debris to pass downward so that nematodes can be collected in a relatively clean state. There are two major types of elutriation system which differ mainly with respect to the equipment and the processing methods.

Advantages: Nematodes are separated according to their size.

Disadvantages:

(a) Time-consuming and cumbersome. It normally takes about 35–40 min per sample.
(b) May be expensive in case of any breakages.
(c) Major glass units, if broken, are difficult to repair.

The Oostenbrink Elutriation Technique

Procedure Fill Oostenbrink elutriation the apparatus with clear water till the outlet of the funnel (up to level 1) by a constant water stream of 1,000 ml/min through a perforated pipe from the bottom of the can (Oostenbrink 1960); thoroughly mix the soil. Place the moist soil sample (100–500 ml) in the 1-mm-pore-size top sieve; wash the sample into the can via the funnel by means of a nozzle delivering about 700 ml/min until two-thirds of the column is filled up (up to level 2); turn off the top nozzle. Reduce the constant water stream from the

bottom to 600 ml/min until the water reaches level 3; pour the suspension into 4 sieves of 325 or 400 mesh sizes, 30 cm diameter, placed on top of one another; the catch is immediately washed into a 250-ml beaker; nematode suspension is poured onto a wire gauge sieve containing two layers of tissue paper; the wire gauge with tissue paper is placed in a funnel holding sufficient water to remain in contact with the bottom of the wire gauge. Leave the funnel overnight. The final suspension containing the nematodes is ready for analyzing at the bottom of the funnel system.

Seinhorst's Elutriation Technique

Procedure Stir the soil sample (500 cm^3) in 750 ml water and strain through a coarse sieve into a 2-l Erlenmeyer flask (A) provided with a funnel cap (C) and a rubber plug (O) (Seinhorst 1962); close the flask with the cap and place in such a way that only the outlet protrudes into the funnel C1 + C2, which is connected with a second funnel of similar shape but smaller in size (D1 + D2); C1 has an overflow pipe at the upper end which discharges into a small funnel; C2 and D2 have outlet pipes provided with rubber sleeves and stopcocks; the end of funnel D2 discharges into funnel E1; the outlet pipe (I) is fitted with a clamp; a feed pipe (N) is connected to the upper end of funnel E1; E1 discharges into E2 which is a straight tube; the lower end of E2 is closed with a plug provided with a device (M). A cable connects E1 and E2 and a free plug is used to close the passage between them if E2 has to be emptied; the floatation apparatus is filled with tap water up to the overflow pipe. The Erlenmeyer flask is opened and the sample is poured through a funnel using a length of wire. By regulating the counterflow of water, the nematodes and small-sized soil particles are maintained in a suspended state or settle slowly, while the heavier particles of soil settle relatively more rapidly than in container E2; regulate the flow of water so that the rate of rise in D2 is 975 or 380 cm/h in C2. The reserve tank (P) ensures the constant flow of water through an outlet pipe located above the overflow pipe (F) and connected by a hose with syringe (G); the small-sized nematodes with a settling rate below 380 cm/h remain in the funnel C1 + C2, whereas particles above 50 μ size settle to the bottom; in the funnel D1 + D2 the nematodes with a settling rate of 380–975 cm/h are separated from particles over 100 μ in size. The large nematodes (above 2 mm) settle within 7–9 min in E1 and the largest soil particles are collected in E2; particles of more than 50 μ size will settle within 20 min in sandy soil type, 30 min in loamy soil type, from the flask and may be removed; 10–15 min later, the clamp may be opened and the contents of C1 + C2 are poured into vessel 1. Later, D1 + D2 are emptied into vessel 2 and E1 into vessel 3; empty E2 and the whole apparatus is washed; contents of vessel 2 is sieved through seven 100 μm-aperture sieves of 10 cm diameter, washing the residues into vessel 1; sieve the contents of vessel 1 through seven 50 μm-aperture sieves of 20 cm diameter, collecting the residues in smallest quantity of water; the contents of vessel 3 are poured through 250 μm-aperture sieves; wash all the residues and concentrate their suspension by pouring through 1 50 μm-aperture sieve; wash the residues into a beaker small amount of water.

Using this technique, nematodes are separated according to their size. However, it is time-consuming and cumbersome and normally takes about 35–40 min per sample. It is expensive in case of any breakages and major glass units, if broken, are difficult to repair.

11.2.1.8 Sugar Floatation Technique

Preparation of Sugar Flocculant Solution Add 908 g (2 lb) sugar to 1 L warm water and stir for 20 min or until dissolved. To this, add 25 ml of 0.1 % flocculant solution (1 g Super Floc 16 added slowly to 1 L of warm water and stirred).

Procedure Pour sample to pan A, add an equal amount of water to the sample, and mix soil and water, breaking up soil clods; pour surface liquid in pan A through the 20-mesh sieve, held over pan B until the soil begins to flow onto the sieve. Still holding the 20-mesh sieve over pan B, wash the soil through the sieve with the fogger

nozzle; discard soil retained on the 20-mesh sieve and in pan A. Pour the material in pan B through 100-mesh sieve, held over pan A until soil begins to flow onto the sieve. Still holding the 100-mesh sieve over pan A, wash the soil through sieve with the fogger nozzle; backwash the material retained on the 100-mesh sieve into a 1,000-ml beaker. Pour the material in pan A through the 200-mesh sieve, held over pan B until soil begins to flow onto the sieve. Still holding the 200-mesh sieve over pan B, wash the material through the sieve with the fogger nozzle; backwash the material retained on the 200-mesh sieve into a 1,000-ml beaker. Pour it through the 500-mesh sieve, held over the sink, until soil begins to flow onto the sieve. Backwash the material retained on the 500-mesh sieve into a 1,000-ml beaker. Add water to the1,000-ml beaker containing the sieved material, raising the total volume of liquid and residue to 300 or 400 ml; add an equal volume of sugar flocculant solution to the contents of the 1,000-ml beaker; with the high-speed stirrer, stir for 1 min at high speed. Allow the suspension to settle for 1 min; wash sugar off the residue on the 500-mesh sieve with the fogger nozzle. Backwash the residue on the 500-mesh sieve into a 250-ml beaker; keep the final volume in the beaker to an absolute minimum (50 ml maximum). Label the beaker and examine the sample.

Nematodes are recovered quickly, making timely identification possible. But this technique needs some relatively expensive equipment. Sugar, if left on the sample too long, may distort nematodes, making identification difficult.

11.2.1.9 Floatation-Modified Fenwick Can Technique

This technique is useful for extracting cysts from dry soil although centrifugation with 50 % sucrose (by weight) and other methods are also popular (Fenwick 1940).

Procedure Mix the soil thoroughly; fill the modified Fenwick can with water; place the sample (100 cm^3) in the top sieve (20.5 cm diameter, 18- or 24-mesh sieves); wash the sample into the apparatus via the funnel (Plate 11.3). The coarse material is retained on the top sieve, heavy soil particles such as sand sink to the bottom of the apparatus, and the floating cysts are carried off over the overflow collar; cysts, root debris, and other particles are collected on a 20-cm-diameter sieve (60–80 mesh).

Plate 11.3 Fenwick can apparatus

Particles of 175 μm or smaller pass with water through the sieve; after washing, dry the debris at room temperature. Transfer the somewhat dried debris retained on the sieve to a 250-ml capacity flask; pour technical acetone or a mixture of three parts acetone and one part carbon tetrachloride into a volumetric flask up to the neck of the flask. Shake the flask and fill it completely (use an exhaust hood); one minute later, decant the floating cysts and debris through a filter paper (18.5 cm diameter) in a glass funnel into a volumetric or Erlenmeyer flask while rotating the original flask; the acetone passes through the filter; place the filter in a Petri dish and view it through a stereoscopic microscope (50× magnification) with overhead light; pick up the cysts with a camel hairbrush and transfer them to a small watch glass containing moist filter paper. Identify the cysts under the dissection microscope using an overhead light; transfer the cysts of desired species with a camel hairbrush into a small drop of water in the glass tube of the homogenizer. Place the piston in the tube and

carefully rotate it by hand; pour the eggs and juveniles that were released from the cysts into a bottle; fill the bottle with water up to 100 ml; mix the suspension carefully using compressed air; pipette out two 100-ml aliquots and place in dishes for counting.

11.2.2 Techniques to Extract Cyst Nematodes

These techniques are necessary to extract cysts of cyst nematodes (*Heterodera* and *Globodera* spp.) from soil. Efficient assays for cyst nematodes (*Globodera* and *Heterodera* species) must include numbers of cysts, eggs, and juveniles. The use of a reliable technique for determination of numbers of eggs in cysts, in egg masses if present, is essential for the evaluation of efficacy of nematicides and other management practices on *Heterodera* or *Globodera* species. A glass house homogenizer is enough for this purpose. The sodium hypochlorite (NaOCl) technique is also a good method in dissolving cysts and releasing eggs. The NaOCl concentration should be two to three times more for egg masses of root-knot nematodes. The following are the most commonly used cyst extraction techniques.

11.2.2.1 Centrifugation Technique with Heavy Sugar

This technique is useful for isolation of cysts and juveniles of *Heterodera* and *Globodera* species. Some difficulty may be encountered with fine clay soils. Sugar flotation and centrifugation utilize a concentrated sugar solution to float nematodes away from soil particles. Typically, these procedures are used following a sieving-type procedure (e.g., elutriation–sugar centrifugation). The concentration of the sugar solution varies from and can be adjusted to facilitate recovery of different-sized nematodes. The length and speed of the centrifugation also vary. A typical procedure consists of elutriation, followed by centrifugation of the material retained on the screen, after which the pellet is suspended in a sugar solution, recentrifuged during which nematodes float and soil particles sink. The supernatant is poured through a sieve and retained nematodes are "backwashed" and saved for identification. This procedure greatly increases the recovery of ring nematode relative to Baermann funnels and works well for smaller nematodes such as lesion and juveniles of root-knot and cyst nematode. The recovery of larger nematodes such as dagger and needle is typically lower with this technique than Baermann funnels lined with cheesecloth. Increasing the sugar concentration or adjusting the length and speed of centrifugation can increase the recovery of larger nematodes, but there may be a trade-off with respect to nematode survival or identification because of the increased osmosis.

Procedure Wash 100-cm^3 soil through a 25-mesh sieve and collect it in a beaker (use 1-l water); mix the suspension thoroughly and allow it to settle for 5 s; pour the supernatant through a 100-mesh screen; add a 400-mesh sieve for juveniles; wash any residue from the screen into a centrifuge tube or tubes with 1.8 M sucrose solution; centrifuge at 420 g for 2.5 min. Collect the supernatant on a 100-mesh screen; add a 400-mesh sieve for juveniles; rinse thoroughly; wash the sample into a beaker, using about 20 ml of water; crush the cysts with a homogenizer or dissolve them with NaOCl as described earlier and count the eggs and juveniles.

11.2.2.2 Semiautomatic Elutriator Technique

This technique includes an elutriator similar to Oostenbrink along with a sample splitter and sieve shaker. It may be used in combination with Baermann's trays or centrifuge (Oostenbrink 1960).

Procedure Add 500-cm^3 non-mixed soil to the elutriator, with air and water flowing at desired rates; run the elutriator for 3–4 min, catching roots on the 35-mesh sieve over a sample splitter and free nematodes on the 400-mesh sieve on the motorized shaker; rinse the sieves; cysts of *Heterodera* and *Globodera* may be collected on the 60-mesh sieve under 10- or

20-mesh sieves; the eggs from the cysts may be extracted by the NaOCl method or with a glass tissue grinder.

11.2.2.3 Sodium Hypochlorite (NaOCl) Extraction Technique

This technique is very commonly used to extract cysts of *Heterodera* and *Globodera* and also for dissolving egg masses of *Meloidogyne* species. Care should be taken to minimize the exposure to the NaOCl. Even with normal precautions, only about 20 % of the eggs extracted with NaOCl produce infective juveniles (Barker 1985).

Procedure Collect and cut 6–10-week-old infected roots into 1–2-cm segments; shake root segments in 200 ml of a 0.5–1.0 % a.i. NaOCl solution for 8–10 min (for cyst extraction) and 1–4 min (for egg extraction); pass NaOCl solution through a 200-mesh (75 μm) sieve, nested over a 500-mesh sieve to collect freed eggs/cysts; quickly place the 500-mesh sieve with eggs under a stream of cold water to remove residual NaOCl (rinse for several minutes); rinse remaining roots with water to remove additional eggs/cysts and then collect them by sieving; for maximum precision in experiments, the eggs should be placed on 500-mesh nylon hatching sieves in 1–2-cm-deep chlorine-free water; tap water allowed to sit in the laboratory for 2–4 days before use is practically chlorine-free due to evaporation; the hatching juveniles are then collected and used as inoculum.

11.2.3 Technique to Enhance Extraction of Nematodes from Clayey Soils

The extraction of nematodes from clay soil is difficult using floatation sieving techniques of soil water suspensions. Aggregated soil particles trap nematodes and prevent them from passing through the top coarse sieves during decanting. In addition, the soil aggregates clog the sieves which slow the decanting operation. Clay particles are difficult to clean from centrifuge tubes that are used in the centrifugal floatation extraction process. Hence, this technique helps to extract nematodes from clay soil samples by replacing exchangeable calcium with sodium on soil particles to induce deflocculation/dispersal of the clay soil. In this technique, there is no need for soaking and agitating the clay soil during extraction (Wehunt 1973).

Procedure Collect the soil sample, pass twice though a sieve with 1.3-mm openings to mix and remove large clods and other debris; divide the soil into 150-ml subsamples and impose the following treatments: a soaking in 150 ml water for 2, 12, and 24 h; a shaking in 150 ml water at three reciprocations/sec on a wrist action shaker for 2, 12, and 24 h; a soaking in 20 ml Electrasol solution (a detergent containing sodium metasilicate, sodium carbonate, and sodium tripolyphosphate) (454 g in 3 L water) plus 150 ml water for 5 min, 30 min, and 8 h; a soaking in 150 ml water for 24 h plus Electrasol solution for 8 h; and a shaking in 150 ml water for 24 h plus Electrasol solution for 8 h.

11.2.4 Techniques of Extraction of Nematodes from Plant Tissues

Plant material containing nematodes should be kept cool and moist and examined at the earliest. Whole plants are stored free from soil. Shoots often decompose more quickly than roots and hence should be kept in separate bags if to be stored for more than a day or two. Polyethylene bags are better containers for samples. Many nematode species will survive for several days, sometimes weeks, in samples stored at 5 °C, but it is safer to store samples from the tropic at about 10 °C.

11.2.4.1 Direct Examination

This is the most conclusive and direct technique to diagnose a nematode infecting plant material is the microscopic examination (Hooper 1970). Small amounts of plant tissue can usually be examined directly for nematodes under a stereomicroscope at magnifications from

15 to 50×, using transmitted and/or incident light. For root-knot nematode and cyst nematode this is the best method since swollen females can be directly observed. Careful examination of roots/plant material can reveal the presence of nematodes, i.e., galls or swellings on roots, tubers, or suckers/rhizomes; white, yellow, or brown pinhead-size bodies adhering to the roots; swollen or malformed leaf, stem, or other tissue; and root lesions or unusual root proliferation. If nematodes are located, they can be dissected out from the plant tissue.

11.2.4.2 Dissecting Technique

Procedure The infected plant tissue is washed and thoroughly cut into small bits with a scissor, and a few of them are placed in a Petri plate or Syracuse dish containing water; the pieces are sliced/dissected/shred with dissecting needles under stereo binocular dissecting microscope; if nematodes are not traced out in the tissues, keep aside with proper labeling and reexamine after an hour for nematodes; if nematodes are traced out, pick them up with a fine needle or a bamboo splinter; transfer them into a Syracuse dish for identification.

11.2.4.3 Baermann's Funnel Technique

This technique is an excellent system of separating nematodes in plant material and also soil and condensing them for examination.

Procedure Attach a 10-cm length of rubber tubing to the funnel stem and clamp tubing; mount the funnel on the ring stand; fill the funnel two-thirds full with water; place a wire-mesh basket on top of the funnel and use it to support tissue; mix soil sample and pass through coarse sieve to remove rocks, roots, etc.; spread soil subsample (50 cm^3 soil) evenly on tissue; fold in edges of tissue; complete filling funnel with water so that water level is about 5 mm above the wire mesh; do not let the water and soil lose contact during extraction period – add water as needed; maintain temperature at 22–25 °C so that it is conducive to nematode movement.

Plate 11.4 Waring blender

Nematodes move through tissue and settle in the funnel; only active stages are recovered. After 48 h, recover extracted nematodes by releasing 20 ml of water from the stem of the funnel into a counting dish.

11.2.4.4 Waring Blender Technique or Maceration Technique

This technique is quicker, popular, and more efficient to extract migratory and sedentary endoparasitic nematodes from various plant parts like roots, foliage, and stems. A similar technique may be used for recovery and estimation of stem and leaf endoparasites and for detecting *Anguina* larvae in grass-seed samples. This is commonly used for extracting *Meloidogyne* eggs in roots to be used for inoculation.

Procedure This is a quick and useful method of examining roots for the presence of endoparasitic nematodes including *Pratylenchus, Helicotylenchus, Hirschmanniella,* and Radopholus; wash the roots gently to remove soil particles; cut 10 g of roots into short pieces (1–2 cm) with a scissor and place 50 ml water in the blending jar (Plate 11.4); run the motor for 10–30 s intermittently for three times; pour the mixture through the coarse sieve into a plastic pail; wash the macerated tissue with water and discard the material on the sieve; stir the mixture until the residue is all in suspension; pour gently through the

200-mesh sieve into the pail; stir the mixture in the pail and gently through a 325-mesh sieve; wash gently to transfer residue into the 100-ml beaker or set up in a Baermann funnel (it is optional, to be followed to get a clear suspension, free of soil particles) for 48 h prior to observation and counting under the dissecting microscope.

11.2.4.5 Root Incubation Technique

Most plant-parasitic nematodes are basically aquatic in nature. When roots are stored moist or immersed in water, migratory endoparasites tend to leave them. This method extracts potential root endoparasites such as *Pratylenchus* and *Radopholus* and also the immature stages and males of sedentary parasites.

Procedure Wash the roots gently to remove soil particles; place roots while still wet in a glass jar and add a small amount of water and close the jar lid loosely; leave the sample 24–48 h at 27 °C; add 50–100 ml water, shake gently, and invert the jar several times to wash off nematodes; pour water through the coarse sieve mesh size 100 on top of a very fine sieve mesh size 325; collect residues from very fine sieve in a beaker; place suspension in Syracuse watch glass or counting dish and count the nematodes with the use of a dissecting microscope.

11.2.4.6 Mist Chamber Technique/ Mistifier

The details and the procedure of this technique has been mentioned under Sect. 11.2.1.5.

11.2.4.7 Maceration–Sedimentation Technique

This technique is effective in extracting plant-parasitic nematodes like coconut red ring nematode, *Rhadinaphelenchus cocophilus*, from coconut palm tissue. These nematodes are extremely thin and active and remain suspended in water for several hours.

Procedure The material is chopped and well macerated and the suspension transferred to a 2-l conical flask, which is filled with water; allow to stand for 30 min; shake the flask and invert with its neck in a vessel of water and sedimented for 30 min; the sediment which has passed from the flask to the lower vessel is discarded; the flask contents are poured four times through a sieve of 63 μm aperture or less, washing off the residue each time; collect it in a beaker.

11.2.5 Techniques for the Extraction of Eggs from Cysts

The procedure comprises three stages: extracting the cysts from the soil, crushing the cysts to extract the eggs, and microscopic observation of the suspension of eggs for counting (Southey 1970).

Extraction of Cysts from Soil Cysts are recovered from soil through a combination of wet sieving and decanting. The technique is a modification of the Cobb (1918) sifting and gravity technique.

Procedure Combine a well-mixed 100-cm^3 soil sample (approx. 1/2 cup) in a bucket with two (2) quarts of water; break clumps, if any, with fingers and mix the soil suspension well for 15 s; pour the soil suspension through an 8-in.-diameter #20 (850 mm pore) sieve into another bucket; briefly rinse the debris caught on the 20-mesh sieve; pour the soil suspension in the second bucket through a #60 (250 mm pore) sieve; backwash the debris caught on the 60-mesh screen into a pan; repour the suspension through the 60-mesh screen; hold the screen at an angle to concentrate the cysts and debris; backwash into a pan using a minimal (<250 ml) amount of water. Pour the cysts and debris into a 250-ml beaker. Discard the heavier material that quickly settles to the bottom of the buckets/pans during the above sieving process.

11.2.5.1 Extraction of Eggs from the Cysts

The above technique will result in a suspension of cysts, along with organic debris and sediments similar in size to the cysts. The cysts in this

suspension could be counted using a simple dissecting microscope. Egg content of cysts is highly variable and will not yield reliable counts of the population in the sample.

Procedure Allow cysts/debris to settle for 30 min in the 250-ml beakers. Pour off excess water, resuspend sediments, and transfer to 50-ml beakers; allow cysts to settle in the 50-ml beakers; pour off excess water (30 ml) and transfer the cyst/debris suspension to a 55-ml Wheaton Potter–Elvehjem tissue grinder; grind at 7,500 RPM for 10 s; rinse pestle into grinding tube; after grinding, pour the suspension in the tube through an 8-in.-diameter #200 (75 mm pore) sieve over a stainless steel #500 (25 mm pore) sieve; rinse the tube several times with tap water, each time pouring the contents through the sieves; discard sediments caught on the #200 sieve. Carefully wash sediments and eggs caught on the #500 sieve into a clean beaker with as little water as possible.

11.2.5.2 Technique to Estimate the Egg Content of Cysts in *Heterodera* spp.

Several techniques are available to open the cysts to release the eggs. A cyst is cut open individually with needles of an oculist's scalpel. This routinely followed method is tedious and time-consuming and is of importance only in assessing the proportion of cysts with contents or "full cysts," in a population. A more rapid and convenient technique has been given below (Reid 1955).

Procedure Squash cysts on a channeled aluminum slide, 7.5×2.5 cm (3×1 in.); the channel is 15 mm (0.6 in.) wide and 0.05 mm (0.002 in.) deep; draw off the water in which the cysts have been soaking with a Pasteur pipette; provided that the end of the pipette is straight and is pressed against the bottom of the staining block, no cysts will be sucked up; transfer this water to the marked boiling tube or graduated cylinder to which the rest of the cyst contents will later also be transferred; draw the cysts to the edge of the staining block with a dissecting needle, the end of which has been bent round in a loop; transfer the batch of cysts with this needle to the slide; place them in a thin line down the center of the channel and with the pipette draw off most of the water, leaving a thin film around the cysts; roll a glass rod backward and forward about three times over the slide, applying gentle pressure; the raised edges of the slide prevent damage to the eggs; if the aluminum slide is not available, cysts can, with care, be squashed between two glass slides; wash the squashed sample from the slide and the glass rod, with a liter of water, into the marked container; agitate the suspension with a electric stirrer for about 30 s to separate the eggs. Make up to a known and convenient volume, viz., 25 or 50 ml.

11.3 Precautions to Be Taken While Extracting Phytonematodes

Certain hazards prevail for some of the techniques of nematode extractions. Some precautions to be considered and safety measures to be followed are listed below (Barker et al. 1978).

- When using the NaOCl extraction procedure for eggs of *Meloidogyne* spp. and other nematodes that produce external egg masses, work should be done in a fume hood to avoid inhaling the vapors.
- Soil samples should be collected within 14 days after application of highly toxic nematicides like organic carbamates or phosphates.
- When soil assays are carried out within 2–4 weeks after applying chemicals at any residual concentration, appropriate precautions (rubber or plastic gloves) should be taken in handling and mixing soil.
- When selecting techniques for extracting different nematode species, the biology and population dynamics of the nematodes should be considered to choose the most suitable method for each sampling time.
- Root samples are best suited for endoparasitic nematodes like and semi-endoparasites in evaluating chemical soil treatments, because a significant portion of these nematode populations may exist in the roots.

- Root sampling may not be necessary shortly after a chemical treatment, because the fractions of the populations in the soil reflect the relative efficacy of the test material.
- Many sedentary ectoparasites can be extracted only by floatation techniques.
- For soil samples collected within 1–3 weeks after chemical soil treatments, techniques that yield only motile nematodes are the best.
- Vital stains like Phloxine B and new Blue R may be used with procedures like the centrifugal floatation method, which yields dead and live worms.
- Many non-fumigant nematicides act over a period of 6 weeks or more, causing nematode starvation and slow disappearance from the soil.
- Many factors may affect the efficiency of specific extraction techniques.
- Certain problems/difficulties like losing nematodes through sieve openings occur with numerous techniques.
- Some procedures and major potential difficulties are listed below (Barker et al. 1978).

11.4 Techniques of Handling, Killing, Fixing, Staining, and Mounting of Phytonematodes

Handling of nematodes includes several steps: picking nematodes and transferring them from one solution to another, observing under a stereo binocular dissecting microscope with a range of magnifications (10–100×), and counting the number of nematodes present in a population.

11.4.1 Examination of Nematode Suspensions

Procedure Place all or part of the suspension in an open counting dish and examine under microscope; Petri dish or flat bottomed Syracuse watch glasses or dishes are used for counting and a grid is etched or scratched with a marking diamond, on the inside of the base to act as a guide when searching; Doncaster's circular, rotating, perspex dish which has concentric channels is very convenient; the space between the grid lines should be a little less than the field width of the microscope at the magnification used to be sure of searching over the whole area of the dish; a sample being searched for large nematodes would be examined at about 15× magnification in a dish with guide lines about 1 cm apart, whereas an extract containing average-sized nematodes would be examined at about 50× in a dish with lines about 3 mm apart; a hand tally counter or a bank of counters is required for counting nematodes.

11.4.2 Transferring Nematodes

There is a need to transfer individual nematodes from one dish or vial to another. It can be done by two ways, viz., the traditional method relies on a picking device such as a handle equipped with a fine needle, eyelash, nylon fiber, platinum wire, etc.; it usually requires a good deal of exercise before you will get the hang of it. The other type is pipetting, which is usually much easier but requires that the nematodes are more or less suspended in liquid.

11.4.3 Technique of Picking Nematodes

The objective of the technique are to pick nematodes from a suspension. Practicing of picking nematodes is a basic and essential to all those who want to work with nematodes.

Procedure Picking devices are sharpened to a fine point under a stereomicroscope and mounted later in a needle holder or stuck on to the end of a mounted steel needle or a perspex rod; a hair or a fine wire loop secured to the end of a mounted needle or mounted at the end of a glass pipette may also be used; for smaller nematodes, an eyebrow hair stuck onto the end of the mounted needle is very useful. It has the following advantages: it has very little damage to the nematodes, is highly

durable, and does not split. It is also useful for handling nematodes in glycerol or lactophenol; a quill toothpick with a spatulate end is suitable for handling short and plump specimens like insect-parasitizing nematodes.

Handpicking This is carried out with any one of the above mentioned picking devices; picking worms out of a suspension is a common step in almost all techniques. Selecting a good picking device is a very important basic step, e.g., a fine and rigid insect needle tapped against the table to bend its tip to a minute hook or a handpicked hair from the brushy mustache. Mount one of these on a handle and use as a nematode pick; choose a particular nematode to pick in a suspension taken in a container like Petri dish, cavity dish, and Syracuse dish; lift the nematode from the bottom with a pick and slowly get it up to the surface of the suspension by placing the pick at the middle of the body and also changing the fine adjustment knob of the microscope so that while lifting the nematode does not go out of the focus; once the pick along with the nematode on it (mostly coiled around the pick due to the viscosity and surface tension of the liquid) reaches the surface of the solution, suddenly lift the pick out of the surface of the solution and place it in the drop of water/any liquid on a glass slide; check the slide for the presence of the nematode.

11.4.4 Technique of Anesthetizing Nematodes

Live and stationary nematodes are more suitable than dead ones or fixed or processed nematode specimens in order to observe certain structures (Hoper 1970). It is, therefore, desirable to prepare temporary mounts of anesthetized nematodes in the following manner.

Procedure Mount nematodes in a 0.5–1.0 % solution of propylene phenoxetol in tap water (a weak solution of dichlorodiethyl ether can also be used); at the lower strength nematode juveniles can be immobilized within 30 min which can survive for about 2 h.; add two drops of the ether to 50 ml of water in a small stoppered bottle; shake well and allow it to clear; ether dissolves to make a solution which will immobilize nematodes mounted in it; nematodes will recover when placed in fresh water, or they may be killed and fixed for subsequent processing and mounting.

11.4.5 Technique of Sterilization and Inoculation of Nematodes

Procedure Transfer a mono-population of the nematodes with a handling needle in a small drop of water in a glass tube; fill the tube with a mixture of 0.02 % ethoxy-ethyl-mercury chloride (Aretan) and 0.1 % di-hydro-streptomycin sulfate; shake the closed tube for about 1 min; allow the nematodes to settle; remove carefully the supernatant with a standard pipette until a small droplet of the nematode suspension remains in the tube; transfer this nematode suspension over a cotton wool filter which is placed on top of a glass tube drawn to a fine point and closed at the tip, which is filled with Aretan–streptomycin sulfate mixture; the nematode will pass the filter; allow the nematodes to settle; after about 60 min pour the nematodes in a silicone watch glass by opening the tip of the glass tube; remove the excess water with a micropipette; sterilize a chosen area of the foam bag with 70 % ethanol; make a slit and transfer the sterilized nematodes with a needle in an air space of the agar bag; seal the slit; inject sterile water through a hypodermic needle since about 2 weeks after planting, the seedlings have used up the water in the agar bag; sterilize the puncture area with 70 % ethanol before injecting; seal the hole made by the hypodermic needle with adhesive tape.

11.4.6 Techniques of Killing and Fixing Nematodes

Nematode killing is an essential part of most studies since live nematodes get distorted and spoiled when they are treated with cold fixatives.

There are several ways to kill nematodes. Nematodes must be killed instantaneously, either by using hot fixative or by heat-killing them prior to adding fixative. Both procedures result in "heat relaxation," relying on a knock-out heat shock to instantly relax the musculature. Another method is cooling the nematodes prior to adding hot fixative on melting ice, but this is not fail-safe. It may sometimes be advisable to starve the nematodes for a few days prior to killing and fixing, because well-fed specimens can contain so many intestinal granules that other organs remain obscured even after transfer to glycerin.

However, one of the best ways to kill live nematodes that are collected in a small volume of water (e.g., from an extraction tray or an agar plate) is to transfer them to a glass vial and plunge this in a 70–90 °C water bath. Stir the vial for 20–30 s and check under the stereomicroscope that they are all motionless and stretched out. Make sure they are not boiled – this messes up the cellular structure. After heat-killing, it is usually best to fix with hot fixative because this will be more chemically active.

11.4.6.1 Seinhorst's Technique of Killing

Procedure Collect specimens in a small drop of water in a glass staining block or similar deep concave vessel (Seinhorst 1966); heat formalin acetic acid fixative 4:1 (FAA) to 100 °C and an excess 3–4 ml is added to the nematodes; the fixative can be heated in a special dropper, whose orifice can be cleaned by an internal rod; sufficient fixative is picked up with the dropper from a stock bottle, and the dropper is later stood in boiling water for 3–4 min; the fixative can be heated in a small tube stood in boiling water and then poured on to the nematodes; if a pipette is used, it should have a wide aperture to allow a quick discharge and be stood in, and heated with, the fixative; this technique fixes glands and gonads and also nuclei are clearly visible; in place of acetic acid in killing propionic acid may also be used.

11.4.6.2 Flame Killing

Procedure Transfer a few nematodes to a drop of water on a plain or cavity slide which is heated over a small flame for 4–6 s until the nematodes suddenly assume straight in some genera and curved or spiral in a few; remove the slide as soon as nematodes appear in this state since overheating will spoil the nematodes. On the other hand if they are not properly heated, they begin to twitch; transfer them immediately to fixative or fix on the slide by adding double-strength fixative.

11.4.7 Technique for Killing and Preserving Nematodes in Soil Samples Before Extraction

This technique involves killing and preserving phytonematodes present in soil samples using chemicals and microwave energy, prior to routine extraction (Barker et al. 1972). The most uniform and reliable results can be obtained by adding chemical preservatives directly to the soil. In addition to providing identifiable specimens when extracted by sugar floatation techniques, the addition of 2 % NaN_3, 5 % formalin, or FAA + picric acid results in increased recoveries of nematodes as compared to nontreated controls. The increased recovery may be due to individual dead specimens having an increased buoyancy when placed in sugar solution. Sometimes adding chemicals like formalin directly to the soil may cause slight distortion of the nematode, but still they may be identified easily. These chemicals affect the morphological characters of the nematodes less than exposure to methyl bromide. The use of hot chemicals reduces distortion of the nematodes.

Procedure

Soil Preparation and Assay Procedures: Premix soil in either a concrete mixer or a sample splitter; collect 50 cm^3 soil sample for treatment.

Direct Addition of Chemical Preservatives: Add fixatives and other toxic or preservative chemicals to the soil directly, viz., 2 % NaN_2, 5 % formalin, and FAA + picric acid (15–20 ppm

concentration); place the samples in closed containers, stored at room temperature; extraction of nematodes can be made weekly or monthly intervals after treatment; most nematodes get killed by these chemicals.

Microwave Energy: A microwave oven model that emits 2,450 MHz (6.30w) has to be used; at a specific exposure duration, killing of nematodes is greatly influenced by the volume of soil and type of container; all juveniles of *M. incognita* can be killed by 3 min exposure of 150 cm^3 of soil, 2 cm deep in sealed 700-cm^3 cellophane bags, approximately 25 cm from the emitter; soil moisture often influences the effects of microwave treatments and rates of nematode killing. Nematode killing was observed at soil moisture levels ranging from 4.5 % (oven dry weight basis) to saturated soil; a 5-min exposure to microwave energy can kill all stages of soybean cyst nematode (*Heterodera glycines*). This nematode is resistant to microwave energy compared to root-knot nematode. This is mainly due to the basic structure of the cyst. They are resistant to ultrasonics and gamma radiation also; pretreatment saturation of the soil with water can greatly increase the killing effect of microwave energy, but incubation at 15 °C can slightly enhance the effectiveness.

11.4.8 Fixatives and Preservatives Used for Nematode Fixing

The main objective of this technique is to prepare different fixatives and preservatives for fixing and preserving plant-parasitic nematodes in order to study the various structures of nematode body (Hooper 1970). A wide range of fixatives are used for preserving nematodes.

FA 4:1 (FA 4:10): Formalin (40 % formaldehyde), 10 ml; glacial acetic acid, 1 or 10 ml; and distilled water, up to 100 ml

Specimens are not usually distorted in these fixatives but they may become brown and the posterior half of tylenchid stylets transparent after more than a few days, especially in FA 4:10. Several observations on the swelling of the cuticle of nematodes like *Trichodorus pachydermis* in FA 4:10 have been reported.

TAF (Triethanolamine Fixative): Formalin (40 % formaldehyde), 7 ml; triethylamine, 2 ml; and distilled water, 91 ml. After fixation, nematodes appear lifelike. The solution remains stable for a long time and the TAF neutralizes any free formic acid, and being hygroscopic, it prevents specimens from drying even if the fixative evaporates.

Formalin–Glacial Fixative (3 % Formaldehyde Solution + 2 % Glycerol): Formalin (40 % formaldehyde), 8 ml; glycerol, 2 ml; and distilled water, 90 ml. Directly from this fixative, nematodes can be brought to glycerol by slow evaporation. Another advantage here is that the specimens will not dry if vials are not properly sealed, but remain finally in glycerol.

Ditlevsen's Fixative or FAA: 95 % ethanol, 20 ml; formalin (40 % formaldehyde), 6 ml; glacial acetic acid, 1 ml, and distilled water, 40 ml. In this fixative, shrinkage of the specimens may be observed since it has ethanol. This fixative is useful while preparing plain like structures as incisures and annulations.

Formalin Fixative: Dilute solutions of 2–4 % formaldehyde (5–10 % formalin) are used for fixing dead nematodes although in this fixative, nematodes tend to appear dark and granular. By adding a little quantity of calcium carbonate granulation may be prevented as it neutralizes free formic acid. By adding hot formalin, muscles in the killed nematodes can be clearly seen. However, 4 % formaldehyde may also be used for fixing nematodes killed by formalin propionic acid 4:1 (FP 4:1).

11.4.9 Formalin–Glycerin Technique (FG Fixation) for Fixing Nematodes

Procedure Prepare double-strength formalin–glycerin (FG) fixative containing 8 % formalin and 2 % glycerin in distilled water; transfer live nematodes to a small glass vial and allow them to settle to the bottom; draw off surplus water until they are left in about 2 ml water; kill the nematodes

by stirring the vial for 20–30 s in a 70–90 °C water bath, check if they are all dead and stretched, and then add an equal volume of 65–70 °C fixative; stir and then leave the vial alone for a day to allow the fixative to penetrate and act on all tissues; take a clean jam jar with airtight-sealable cap; prepare a "weck pot" by adding ethanol to the jam jar until there is a layer of about 5–10 mm on the bottom; place a small platform (a small inverted Petri dish or cavity block) on the bottom of the jam jar so that its top surface is raised above the ethanol; take the vial with FG-fixed worms – if this has a narrow opening, transfer the worms to a cavity block; draw off as much fixative as possible without losing nematodes and then fill the vial or block with 5 % glycerin–95 % ethanol solution (to the brim if in a cavity block, to about 5 mm high if in a vial); place the block or vial on the platform inside the jam jar and seal this; leave the "weck pot" overnight in an incubator at 35–40 °C.

This will allow all water in the suspension with the nematodes to be replaced with ethanol; the next day, take the vial or block out of the weck pot and leave it open in the 35–40 °C incubator for 2–3 h, to evaporate about half of the ethanol (if necessary, cover partly to prevent complete evaporation); refill with 5 % glycerin–95 % ethanol solution, leave for another 2–3 h, and refill one last time before leaving the vial or block overnight in the incubator at 35–40 °C. After 24 h, the nematodes will be impregnated in pure glycerin and ready for mounting in slides, or for stocking without fear of desiccation; the entire FG/ethanol procedure takes only 3 days and usually results in well-fixed worms that will not decay for decades; transferring through ethanol dissolves cuticular lipids, however, and may result in a finely wrinkled cuticle that will show up as such under the scanning electron microscope. To avoid this, the following slightly slower technique may be followed.

11.4.10 Techniques of Staining Nematodes

For clarity in observation and detailed investigations of the internal structures of the nematode body, staining phytonematodes is an essential technique (Ravichandra 2010). A wide range of stains have been used by several nematologists. Some are specific in their function and some are nonspecific. Important stains include carmine propionic acid, acetic orcein, propionic orcein, gold chloride, silver nitrate, vital stains, new Blue R, chrysoidin, aqueous Phloxine B, alcoholic eosin-Y, acridine orange, pronase, collagenase, polychrome blue, methyl red pH indicator dyes, redox indicator, 1-naphthol-2-sodium sulfonate-iodine, acid fuchsin–lactophenol, acid fuchsin, Johansen's quadruple stain, Sass safranin, fast green, triarch quadruple stain, etc.

Acetic Orcein: This is useful mainly to study the reproductive system in nematodes. The composition of this stain is glacial acetic acid, 1 part; absolute ethanol, 6 parts; and chloroform, 3 parts.

Procedure Kill nematodes by gentle heat and fix in Carnoy for 10–20 min; stain the nematodes in 1 % acetic orcein for 1 h; mount in 45 % acetic acid.

Gold Chloride: This is more suitable to stain gonads, esophageal glands, and nerve ring.

Procedure Wash the fixed nematodes in distilled water; place in an aqueous solution of 0.1 % gold chloride and 0.1 % mercuric chloride until they are light yellow; after washing the nematodes for 19 min in distilled water, place them in 1 % formic acid; expose them to the light of a photoflood lamp which causes breakdown of the gold chloride to colloidal gold which stains organs bright red. Nematodes can be processed to glycerol and mounted permanently.

Silver Nitrate: This technique is used on live nematodes and to stain the peripheral nervous system of nematodes.

Procedure Place the live nematodes in a hypertonic solution of 10 % sodium nitrate for 5 min, which lessens their size by exosmosis; place in 0.5 % silver nitrate for 15 s and finally in distilled water; endosmosis occurs in the hypotonic silver nitrate which gets flushed into the tissues; this treatment kills the nematodes, but after illumination the peripheral nervous system becomes apparent

due to the deposition of colloidal silver; it remains visible in nematodes subsequently processed to glycerol.

Vital Staining: Since cuticle of nematodes is almost impermeable and few nematodes ingest liquid in vitro, it is difficult to use vital stains on nematodes. Methyl red redox indicator, 1-naphthol-2-sodium sulfonate-indole, and neutral red pH indicator dyes have been shown to be ingested by rhabditids with their food in vitro. The dyes are absorbed in different concentrations by various regions on the intestinal wall.

11.4.11 Technique of Mounting Nematodes

The objective of this technique is to mount processed plant-parasitic nematodes either temporarily or permanently for future studies. In nematodes that are fixed, internal structures like gonads may be obscured by the granular appearance of the intestine. Nematodes may be cleared by processing to lactophenol or glycerols which are also suitable mountants (Hooper 1990). Phenol is supposed to be a dangerous poison so lactophenol is a better option which should be used with caution. Lactogylcerin also can be used which is quicker than glycerol in preparation of mounts in lactophenol/lactogylcerol, which if well sealed may last for several years. Aqueous solution of dimethyl formaldehyde resin 70 % (w/v), 30 % solution of Bovine Ox Plasma fraction V, immersion oil, saturated aqueous picric acid, araldite, etc., have also been used by several nematologists. If a stain is used, some features are more readily observed than in glycerol.

Lactophenol: Phenol (liquid), 500 ml; lactic acid, 500 ml; glycerol, 1,000 ml; and distilled water, 500 ml. A stain, usually cotton blue, is used in water before mixing the ingredients.

Glycerol: Ethanol 96 %, 20 parts; glycerol, 1 part; and distilled water, 79 parts

Rapid Technique to Glycerol: Fixed nematodes are cleared in lactophenol (with cotton blue added if required) and then processed through a series of solutions, all kept at 55 °C; the solutions are kept in a series of numbered dropping bottles fitted into holes of wooden box. Their composition is as follows (in parts) (Franklin and Goody 1949):

Solution	1	2	3	4	5
Glycerol	55	70	82	90	100
Lactic acid	15	10	05	2.5	0
Phenol (crystals)	10	05	05	2.5	0
Formalin (40 % formaldehyde)	05	05	03	2.5	0

From lactophenol transfer nematodes in a series of solutions, allowing a minimum of 10 min in each in oven; after the last solution, i.e., glycerol, mount immediately in pure anhydrous glycerol; any tendency for cotton blue stain to come out during processing can be counteracted by using glycerol containing 0.0025 % cotton blue as the last solution and for final mounting.

11.4.12 Mounting Nematodes in Temporary Slides

11.4.12.1 Technique of Mounting Dead Nematodes

By preparing temporary slides of killed nematodes, many observations can be made on refractive structures like spear, head skeleton, lumen of esophagus, excretory pore, and spicules.

Procedure Place a few nematodes in a drop of water on the glass slide; place three pieces (1 mm) of glass fiber around; check that nematodes are at the center of the drop and not floating; put the cover slip; seal the edges with nail polish or wax or recently extinguished candle; a mixture of eight parts paraffin wax to three parts petroleum jelly is better than ordinary wax for sealing cover slips (Doncaster 1962).

11.4.12.2 Technique of Mounting Live Nematodes

If the detailed morphology of live nematodes to be studied, a temporary slide with live nematodes can be prepared on a thin layer of agar.

Procedure Administer one or two drops of hot 4–5 % agar on a glass slide; flatten this agar with another glass slide provided with spacer strips of thick plastic tape; carefully remove the top slide when the agar has set; add a drop of water on the agar, transfer the nematode to it, and put a cover slip on top; the pressure between the cover slip and the hard agar will slow down the nematode sufficiently making it observable with oil immersion magnification; if nematode has to be immobilized, smearing some vaseline on the rims of the cover slip may be done; place it on top of the agar and nematode, and carefully press down the rims of the cover slip until the nematode is trapped but not squashed; a complete vaseline seal will also prevent desiccation.

11.4.13 Technique of Mounting Nematodes in Permanent Slides

Preparing permanent slides is easy once nematodes have been fixed and transferred to glycerin. Various types of slides and mounting aids exist, but glass slides using paraffin as seal and separator is more common. The most luxurious and safe mount for microscopic nematodes is a "Cobb slide" which consists of an aluminum carrier supporting two cover slips between which the nematodes are sandwiched and sealed. Unlike glass slides, Cobb slides will not break when dropped and they can be turned over and observed from both sides at high power. Several techniques are available to mount nematodes in permanent slides.

11.4.13.1 Glycerin Technique

Procedure

Preparing a Glass Slide: Fill a Petri dish with paraffin granules, melt them at about 60 °C, and allow the paraffin to set into a solid layer (Daykin and Hussey 1985); take a 10-cm-long cross-cut metal tube with smooth, thin rim and slightly smaller diameter than the cover slips (e.g., a 16-mm-diameter tube for 18-mm-diameter cover slips); heat one end in a flame; when the other end of the tube is beginning to get hot in your hand, push the heated end down vertically in the paraffin so that it gets covered by melting paraffin; press this end down vertically on the middle of a glass slide; lift the tube, and a complete 3–4-mm-thick ring of setting paraffin should be left behind; transfer a small drop of pure glycerin to the center of this wax ring, with a thin glass rod, leaving a spot of 4–5 mm on the slide; repeat this for as many slides required to prepare; getting the proportions of wax and glycerin right is important because too little paraffin and too much glycerin will result in an incomplete seal and too much wax and too little glycerin will result in nematodes being covered or trapped by paraffin.

Transferring Nematodes: Pick out the specimens with a needle and transfer them to the glycerin drop in the center of a wax-ringed glass slide; up to ten of them per slide can be mounted; more may result in nematode overlapping or ending up in paraffin; after transferring the required number to a slide, put it under the stereomicroscope; push all nematodes to the bottom of the glycerin drop with the pick, making sure none overlap with one another.

Sealing and Shuffling: Drop a cover slip over the wax ring and glycerin drop; put the slide on a moderately hot plate or a mesh or metal plate above a small flame; make sure one end of the slide sticks out over the rim of the plate; allow the paraffin to melt around the glycerin drop and allow all air to escape from under the cover slip; then put the slide back under the stereomicroscope and check that no nematodes are overlapping; if so, gently push the cover slip in the required direction to dislodge one of the overlapping nematodes; if the paraffin has set by now, return the slide to the hot plate; reheating can also be done and gently push the cover slip sideways to turn nematodes over; once set, the paraffin will act both as a seal and a separating layer between the cover slip and the glass slide, and the slide will contain just a small circular central area with glycerin and nematodes; if some nematodes are covered by smudges of paraffin under the cover slip, and/or the paraffin is too thick to observe specimens

with high power objectives, put the slide back on the hot plate and allow the wax to heat and spread out further so that it forms a thinner layer; if specimens are to be picked for transferring to another slide or for use in scanning electron microscope or cross sections, gently raise the cover slip open with a scalpel or thin needle while keeping track of nematodes under the stereomicroscope.

11.4.13.2 Seinhorst's Technique

Procedure Kill nematodes by pouring in an equal volume of hot (80 °C) 1.0 acetic acid to create 0.5 %. Alternatively, kill in hot FA 4:1 fixative; fix for 48 h in FA 4:1 fixative; rinse specimens in distilled water; place in Seinhorst I solution (ethanol–glycerin mix) in watch glass; add 1–2 drops of saturated aqueous picric acid to provide a yellow stain and prevent clearing of stylet; place this open watch glass in a larger container (desiccator) surrounded by 95 % ethanol and incubate at 35–40 °C for 12 h; add Seinhorst II solution (ethanol–glycerin mix) and store in a partly closed container (covered Petri dish) at 40 °C; transfer to pure glycerin.

FA 4:1: 10 parts formalin (40 % formaldehyde), 1 part glacial acetic acid, and 89 parts distilled water

Seinhorst I Solution: 20 parts 95 % ethanol, 1 part glycerin, and 79 parts water

Seinhorst II Solution: 95 parts 95 % ethanol and 5 parts glycerine

11.4.14 Major Steps for Preparation of Nematode Mounts

There are three basic steps in preparation of nematode mounts, viz., killing and fixing nematodes, processing nematodes to glycerin, and mounting nematodes.

1. *Killing and Fixing Nematodes*: Collect live nematode specimens in distilled or deionized water in a small beaker or watch glass; concentrate the nematodes in a minimal volume of water and add equal volume of hot (90 °C) fixative solution, buffered formalin, to it. Nematodes may be killed with heat before adding fixative although adding hot fixative directly is also effective. Buffered formalin provides very good fixation; leave the specimens in the fixative for 1–2 days. Nematodes may be stored in buffered formalin indefinitely, it does not clear characters
2. *Buffered Formalin Solution*: Formalin (ca 40 % formaldehyde), 10.0 ml; water, 90.0 ml; sodium acid phosphate, 0.4 g; and anhydrous disodium phosphate, 0.65 g
3. *Processing Specimens to Glycerin*: Prepare Seinhorst I and II solutions (procedure as mentioned above) and keep them at room temperature. Place fixed nematodes in a dish; draw off excessive fixative and concentrate the nematodes in a small volume; add ca 6–8 ml of Seinhorst solution I to the nematode suspension; place the dish in a larger closed glass container (desiccator) with 95 % ethanol at the bottom and place in oven at 35–40 °C for at least 12 h. This removes most of the water in the dish; remove dishes from oven and draw off excess Seinhorst solution I from the dish, using a pipette under a dissecting microscope to avoid loss of specimens; add Seinhorst solution II to the dish; place it in a partially covered Petri dish and return to oven at 40 °C; several hours (at least 3 h) later, draw off excess solution from the dish; keep the dishes in oven until all the alcohol has evaporated (at least 3 h) and nematodes are in pure glycerin.

11.4.15 Mounting Nematodes

11.4.15.1 Temporary Mounts

Procedure Place a small drop of the fixative in the center of a clean glass slide; using a "nematode pick" under a dissecting microscope, pick up the desired specimens and place them in the fixative on the center of the slide; place the slide under the dissecting microscope; arrange the nematodes in the center of the slide and bottom of the drop; place glass wool (about 5 mm in length) or glass

micro beads in a triangular position near the edge of the drop; place a cover glass (18 mm wide) gently over the drop using a forceps or supporting it with a needle; draw off excess fixative carefully using filter paper; apply zut, glyceel, or nail polish with a small brush to the edge of the cover glass, to seal it; after the sealant has dried, the slides can be observed under microscope.

11.4.15.2 Semipermanent Mounts in Lactophenol

Procedure Collect the nematodes and kill them as explained before; transfer them to a dish containing fixative; cover with cover slip and leave it overnight; transfer the nematodes into a drop of warm, vaporizing lactophenol and allow to cool.

Lactophenol Liquid phenol, 94 ml; lactic acid, 83 ml; glycerine, 160 ml; and distilled water, 100 ml

Heat the lactophenol (or lactophenol with a little 0.1 % cotton blue in it) on a cavity slide with a depression above a small flame on a brass heating table; mount the nematodes in a small drop of lactophenol on a final glass slide; apply a cover slip and ring with glyceel.

11.4.15.3 Permanent Mounts

Procedure Fix a clean cover glass (25 mm wide) in the center of a Cobb aluminum slide by supporting with appropriate-sized white cardboard pieces; place a small drop of anhydrous glycerin in the center of the cover glass in the aluminum slide; pick up nematodes from the fixative and place them in the drop of glycerin; arrange the nematodes in the center of the slide and place glass wool; carefully place a cover glass (18 mm wide) over the drop and seal the edges of the cover glass; after the sealant has dried, a second coat of sealant may be added. Allow to dry, label the slides on the white cardboard, and examine under a compound microscope; excess glycerin on the slide is difficult to remove and can cause smudges, which interferes with the sealing process; store the slides in a flat position to avoid settling of nematodes toward the edge of the cover glass; use of aluminum slides enables viewing of the nematodes from both sides of the slide.

11.4.16 Technique to Prepare Permanent Mounts of Nematodes Within Plant Tissues Stained with Cotton Blue or Acid Fuchsin

The major objective of this technique is to prepare permanent mounts of nematodes within plant tissue. The selected portions of the tissues containing well-stained nematodes have to be cut out and further processed by following techniques (Hooper 1970).

Procedure

Glycerol Mounts: Transfer 5–10 % glycerol in water; set aside in a desiccator until the glycerol is concentrated; mount in pure glycerol; seal the cover slip with zut/glyceel.

Canada Balsam Mounts: Pass the plant tissue through graded ethanol series to absolute; clear in clove oil; mount in Canada balsam.

"Euparal" Mounts: Pass the plant tissue through 30, 50–70 % ethanol; pass through isobutanol; mount in "Euparal."

11.4.17 Technique of Remounting

Sometimes, mounts prepared long back may get spoiled due to some reasons. Such damaged or partially damaged specimens can be remounted using this technique (Hooper 1970).

Procedure Peel-off the sealing ring as much as possible; add excess mounting fluid around the edge of the cover slip; carefully lift the cover slip with a fine needle. While doing so, avoid it from slipping laterally by holding another needle vertically against the far edge; place the cover slip upside down on a clean slide and add excess mounting fluid and to the old slide; if nematodes are stuck to the glass, dislodge them by gently stroking with a mounted eyebrow hair or place them in an oven at 50 °C for 3–4 h in excess

mounting fluid; deteriorated nematodes will improve if put into hot cotton blue lactophenol and then reprocessed to glycerol.

11.4.18 Techniques of Preservation and Staining of Nematodes in Plant Tissues

These techniques are very important to investigate host–parasitic relationships involving endoparasitic nematodes, which requires techniques that facilitate observation of nematodes inside the plant tissues. Preserving and staining of nematodes in plant tissues will be useful and essential in many studies, particularly, in order to properly understand host–parasitic relationships. The studies on the penetration and development of endoparasitic nematodes within intact plant tissues are of importance in nematological research.

The most suitable technique of preserving plant tissue containing nematodes depends on the ultimate treatment of the plant tissue.

11.4.18.1 Lactophenol/FAA/Technique

Procedure Immerse the cleanly washed nematode-infected plant material (roots or whole plants) in cold 5–6 % formaldehyde solution or FAA, in appropriate airtight plastic or glass jars (Hooper 1970); display the jars or keep for museum purposes; better nematode specimens will often be obtained if the infested tissue is immersed in hot fixative (70–80 °C); if the infested tissue is immersed in lactophenol, tissues are softened and nematodes are easily dissected from them.

11.4.19 Technique for Retaining Green Color of the Plant Material

Procedure Dissolve 14 g copper sulfate in 2.5 L tap water; slowly bubble sulfur dioxide through the solution until cloudiness has cleared; fresh plants are introduced and are stored or displayed in the same solution; the color of the plants fades at first but retains after some time; if the container is not airtight, some more quantity of sulfur dioxide may be added.

11.4.20 Technique to Preserve Juveniles of *Meloidogyne* spp.

Procedure Incubate *M. graminicola* juveniles in 10 % ethylene glycol at 37 °C for 15 min (Bridge 1985); incubate later in cold 40 % ethylene glycol for 30 or 45 min before freezing in liquid nitrogen at −196 °C; the treatment reduces number of active nematodes, but those that recovered after thawing infected rice roots produce large populations of females and eggs after 40 days.

11.4.21 Technique to Preserve Infected Roots

Procedure The roots are carefully washed, air dried, and fully labeled; wrap the roots in paper bags or sheets; in this condition they may be kept well for prolonged periods; the roots may be soaked for 30 min in warm water and later may be cut into bits of 1–1.5 cm for examination; in case of cyst infected cereal roots by *Heterodera avenae*, seedling roots collected 3–4 weeks after beginning of growth clearly show white females and those examined at 6–8 weeks show brownish cysts and males.

11.4.22 Technique for Killing and Preserving Nematodes in Soil Samples Before Extraction

The objective here is to kill and preserve plant-parasitic nematodes present in soil samples using chemicals and microwave energy, prior to routine extraction (Barker et al. 1972). The most uniform and reliable results can be obtained by adding chemical preservatives directly to the soil. In addition to providing identifiable specimens when extracted by sugar floatation techniques, the addition of 2 % NaN_3, 5 % formalin, or FAA + picric

acid results in increased recoveries of nematodes as compared to nontreated controls. The increased recovery may be due to individual dead specimens having an increased buoyancy when placed in sugar solution. Sometimes adding chemicals like formalin directly to the soil may cause slight distortion of the nematode, but still they may be identified easily. These chemicals affect the morphological characters of the nematodes less than exposure to methyl bromide. The use of hot chemicals reduces distortion of the nematodes.

Procedure

Soil Preparation and Assay: Premix soil in either a concrete mixer or a sample splitter; collect 50 cm^3 soil sample for treatment.

Direct Addition of Chemical Preservatives: Add fixatives and other toxic or preservative chemicals to the soil directly, viz., 2 % NaN_2, 5 % formalin, and FAA + picric acid (15–20 ppm concentration); place the samples in closed containers, stored at room temperature; extraction of nematodes can be made weekly or monthly intervals after treatment; most nematodes get killed by these chemicals. If needed, potato dextrose agar, water agar, and nutrient agar dilution plates (1:10 to 1:1,000) of soils from each treatment/chemical can be made at monthly intervals to determine the relative rates of fungal and bacterial killing. In order to kill all soil-inhabiting fungi and bacteria in addition to nematodes, it is important to increase the concentration to 2 %.

Microwave Energy: A microwave oven model that emits 2,450 MHz (6.30w) has to be used. At a specific exposure duration, killing of nematodes is greatly influenced by the volume of soil and type of container. All juveniles of *M. incognita* can be killed by 3 min exposure of 150 cm^3 of soil, 2 cm deep in sealed 700-cm^3 cellophane bags, approximately 25 cm from the emitter. Soil moisture often influences the effects of microwave treatments and rates of nematode killing. Nematode killing was observed at soil moisture levels ranging from 4.5 % (oven dry weight basis) to saturated soil. A 5-min exposure to microwave energy can kill all stages of soybean cyst nematode (*Heterodera glycines*). This nematode is resistant to microwave energy compared to root-knot nematode. This is mainly due to the basic structure of the cyst. They are resistant to ultrasonics and gamma radiation also. Pretreatment saturation of the soil with water can greatly increase the killing effect of microwave energy, but incubation at 15 °C can slightly enhance the effectiveness.

11.4.23 Staining Techniques

In order to observe endoparasitic nematodes present inside the tissue, it is important to clear and stain the nematode-infected plant material. A wide range of staining techniques have been reported depending upon the different types of plant tissues (Daykin and Hussey 1985). It includes the most common technique, the acid fuchsin–lactophenol technique that was developed by Mc Beth et al. (1941). In general, for staining dense, bulky material is not suitable, unless sliced thinly, because it will not transmit sufficient light even when cleared. Cotton blue or acid fuchsin, in lactophenol, methylene blue is useful for routine assessment of nematodes in fresh or preserved plant tissue.

11.4.23.1 Sodium Hypochlorite–Acid Fuchsin Technique

For clearing and staining nematode-infected root tissues, this technique is most suitable which has the following advantages over other techniques (Byrd et al. 1983). It eliminates exposure of personnel to toxic compounds like phenol, which are utilized in other techniques. Since plant tissue is cleared with NaOCl prior to staining with acid fuchsin, it does not become heavily stained. Hence, the time required for destaining is reduced; frequent destaining is not required. This is also a very good technique to stain endoparasitic nematodes in cotton and soybean roots, in particular.

Procedure Place the washed infected plant tissue (e.g., roots) in a 150-ml beaker. Cut the plant tissue into small bits and stain. Add 50 ml of tap water to clear the tissue along with an appropriate quantity of chlorine bleach (5.25 %

NaOCl). The quantity depends on the age of the plant material as indicated below.

(a) Young aged: 10 ml 5.25 % NaOCl
(b) Medium aged: 20 ml 5.25 % NaOCl
(c) Older or more ligneous: 30 ml 5.25 % NaOCl

Soak the roots in the NaOCl solution for 4 min and agitate occasionally; rinse roots for 45 s in running tap water and soak in tap water for 15 min to remove any residual NaOCl which may affect staining with acid fuchsin; drain the water and transfer the roots into a beaker with 30–50 ml of tap water; add one ml of stock acid fuchsin stain solution to the water (stock solution is prepared by dissolving 3.5 g acid fuchsin in 250 ml acetic acid and 730 ml distilled water); boil the solution for about 30 s on a hot plate or in a microwave oven; cool the solution to room temperature, drain it from the roots, and rinse the roots in running water; place the roots in 20–30 ml of glycerin acidified with a few drops of 5 N HCl and heat to boiling for destaining; roots may be stored in acidified glycerin with little change in contrast between nematodes and root tissue.

11.4.23.2 McBryde Technique

This technique does not require heating and destaining. It is comparatively a rapid technique and can be easily regulated (Southards 1965).

Procedure Fix and stain infected plant tissue/roots for 6–24 h in a solution of equal parts of 95 % ethanol and glacial acetic acid, containing 0.0175 mg/ml acid fuchsin (17.5 mg/l); destain root tissue by soaking in a saturated solution of chloral hydrate (4.5 g/ml of water) for 12–24 h; rinse roots with stained nematodes in tap water and store in acidified glycerine. The nematode color, if it fades with time, the roots may be stained again by this technique after being rinsed in tap water.

11.4.23.3 Lactophenol Technique

This one is the very commonly adopted technique to stain nematodes in plant tissues. Nematodes in the infected roots of either young plants or small roots of older plants can be well stained by this technique (Mc Beth et al. 1941). This technique may not suit well for roots with a high fat content like most perennials as their roots will retain more stain. The destaining step cannot be easily regulated and may take many days. By keeping stained roots in clear lactophenol and autoclaved for 10 min at 15 lb pressure, the procedure can be accelerated.

Procedure Prepare lactophenol (by mixing liquid phenol, 500 ml; lactic acid, 500 ml; glycerine, 100 ml; distilled water, 500 ml); prepare stain solution by adding 5 ml of a 1 % stock solution of acid fuchsin or cotton blue per 100 ml of lactophenol. The concentration of stain may require variation according to the age of the tissue; stain is added to a beaker and brought to a boil on a hot plate; immerse infected roots in boiling water; destain in clear lactophenol solution until maximum contrast between the nematodes and root tissue is observed; heat the solution in a water bath (100 °C) or an oven (70 °C) for about 90–120 min in lieu of direct boiling of the stain; destaining time may vary from a few hours to several days.

11.4.23.4 Sodium Hypochlorite–Acetic Acid Technique

This technique is most suited for fresh roots or roots fixed in any of the common fixatives (formalin, alcohol, FAA, etc.). It is important that the roots be either stained or fixed as soon as possible after collection since nematodes may migrate from the roots (Byrd et al. 1983).

Procedure Wash soil from roots with tap water; place roots in a 250-ml conical flask with approximately 70 ml of 1.5 % sodium hypochlorite (or half strength household bleach) and bleach for 5 min with occasional stirring; rinse roots with water and soak for 15 min in 1 % acetic acid. This acid rinse step is critical for consistent staining, especially of fixed roots; drain off acid solution and place roots in 30 ml distilled water to which 1 ml stain has been added; heat over a low flame until boiling; boil gently for 30 s and then allow to cool for 30 min at room temperature; remove excess stain by rinsing with water; place roots

in 20 ml acidified glycerol and heat to boiling; remove from heat immediately as boiling commences and cool quickly by standing flask in shallow water; pour roots in glycerol into a Petri dish; gently tease apart and mount on a microscope slide; nematodes are stained red.

11.4.23.5 Cold Staining Technique

This technique provides better differentiation between nematodes and plant tissues.

Procedure Wash plant material and quickly dry it on a filter paper; immerse it in boiling lactophenol for 2 min (without stain); remove and allow it to cool in the air and immerse it in cold lactophenol containing 0.001 % cotton blue. By using this technique, *Meloidogyne* stains well in about 10 days; *Scutellonema* in yam tubers takes 1–2 days. The contrast remains as long as the material is left in the stain solution.

11.4.23.6 Technique to Stain Nematodes in Leaf Litter

This is a technique to determine the number of nematodes left in forest litter (viz., oak) (Minderman 1956). This technique helps in staining nematodes in litter, after removing most of them by modified Baermann's technique.

Procedure Bleach the dead leaves for 24 h in the mixture (NH_4OH, 2 parts; H_2O_2, 5 parts; H_2O, 11 parts); wash the litter in water and pour on hot (65 °C) tinted lactophenol; leave for 5 min and pour off the stain; wash out the excess with clear lactophenol until little dye remains in the washing fluid; mount in lactophenol.

11.4.23.7 Flemming's Technique

Staining infected plant material in Flemming's solution helps in proper detection of nematodes. The osmic acid of Flemming's solution has been reported to blacken fats within nematodes, due to its reduction, causing the nematodes to show clearly against the comparatively unstained plant tissues (Godfrey 1929).

Procedure Immerse the nematode-infected plant material in Flemming's solution in a suitable container, for 4–6 h; nematodes are stained in black, while tissue remains unstained. Observe the nematodes under a stereo binocular dissecting microscope.

11.4.23.8 Technique to Stain Green Shoot Material

This is a modified technique of Flemming's solution technique that avoids the darkening of the tissues due to rapid reduction of osmic acid (Godfrey 1935).

Procedure Green shoots/leaves are treated with hot 80 % acetone before soaking them in Flemming's solution; pour sufficient 80 % acetone into a small conical flask; place it in a large beaker of water heated over a small flame (the boiling point of 80 % acetone is about 63 °C); drop into the boiling acetone small pieces of shoot or leaf material containing nematodes; allow to boil for a few minutes; leave the material in the slowly cooling acetone for 3–4 h or until the green color is removed; pour off the acetone and wash in 2–3 changes of water; stain as described for roots.

11.4.23.9 Techniques to Stain Root Material

Flemming's Solution Technique

Procedure Wash the roots free from soil and plunge them into hot water (70–80 °C) for 2–3 min to kill nematodes present within; transfer the roots to strong Flemming's solution for 5 min; control the depth of staining by examining under the stereomicroscope; a closed container should be used to protect the eyes from osmic acid vapor; wash the stained roots in running water for a few hours to overnight; pass them through graded ethanol solutions to absolute ethanol; clear in clove oil and mount in Canada balsam.

Modified Aceto-osmium Technique

This technique is helpful to stain endoparasitic nematodes present in more opaque roots.

Procedure Fix and stain washed roots for 2 h at 52 °C in the solution (prepare the solution by

mixing acetic acid 10 %, 10 parts; aqueous osmic acid 2 %, 2 parts; distilled water, 16 parts); wash in running water for 1 h; bleach in 10–30 % hydrogen peroxide at 30 °C till the darkened tissues lighten perceptibly; wash several times in water; pass through a graded series of ethanol solutions half an hour in each at 52 °C: 30, 50, 70, 95, and 100 %; for nematodes within roots, start at 70 %, but for ectoparasitic ones start at 30 %; clear in methyl salicylate at 52 °C for half an hour or in clove oil; mount in Canada balsam.

11.4.23.10 Technique to Stain Fresh/ Preserved Shoot Material

This technique is used to stain fresh or preserved shoot material to observe the stained (bright red) endoparasitic nematodes and their stages against a green background of the shoot (Hooper 1970).

Procedure Wash the material, if fresh, to remove dirt and plunge it into hot water (70–80 °C) for 2–3 min to kill nematodes; preserved material if used should be brought to water and passed through 30, 50, and 70 % ethanol, giving one or two changes in the last; transfer the material to a specimen tube; fill the tube with a saturated solution of scarlet R in 70 % ethanol with 2 % acetone added; cork the tube and leave overnight or for several days; overstaining should not occur; wash the stained material in 70 % ethanol and then transfer it to isobutanol which displaces ethanol; control the steps under a stereomicroscope; mount in "Euparal"; nematodes are stained bright red against a green background of the shoot material.

11.4.23.11 Techniques for Counting Nematodes in Root Tissue

This is a useful technique to count the stained endoparasitic nematodes, either directly from intact root tissue or following root maceration technique (Daykin and Hussey 1985).

Direct Counting

Procedure Distribute the roots in a small amount of glycerin on a Petri dish cover (glass or plastic); press against the cover with the Petri dish bottom; mark a grid on the Petri dish that aids in counting the nematodes under a stereoscopic microscope at 40× magnification.

Root Maceration Technique

Objective of this technique is to free nematodes from the roots by maceration technique and to remove a subsample for counting as considerable time is required for direct counting nematodes inside large root systems.

Procedure Macerate the roots in a Waring blender or laboratory mixer as explained earlier; separate the nematodes from the root tissue by sieving; take care to ensure that nematodes are not ruptured or distained during maceration.

11.5 Histopathological and Histochemical Techniques (Microtome Techniques/ Microtomy)

A microtome is a mechanical instrument that is used to cut biological specimens including nematodes into transparent thin sections for microscopic examination. It is used for cutting histological sections from tissue block. A microtome consists of steel, glass, or diamond blades depending upon the specimen being sliced and the desired thickness of the sections being cut. Steel blades are used to prepare sections of animal or plant tissues for light microscopy histology. Glass knives are used to slice sections for light and to slice very thin sections for electron microscopy. Industrial-grade diamond knives are used to slice hard materials like bone, teeth, and plant matter for both light microscopy and electron microscopy. Gem-quality diamond knives are used for slicing thin sections for electron microscopy (Zacheo 1987). The microtome mechanically holds a definite thickness of tissue between the undersurface of a transparent plate and the advancing edge of a thin, flat razor blade. Multiple slices can be made with a minimum trauma and with considerable reproducibility.

11.5.1 Types of Sections

11.5.1.1 Traditional Histological Sections

In this technique, tissues are hardened by replacing water with paraffin. The tissue is cut using the microtome at the thickness varying from 2 to 25 μm. The tissue is mounted on a microscope slide, stained with appropriate aqueous dyes after prior removal of the paraffin, and examined using a light microscope.

11.5.1.2 Cryosection

This technique involves hardening of water-rich tissues by freezing. They are cut in the frozen state with a freezing microtome or microtome. Cryostat sections are stained and examined with a light microscope. This technique is much faster than traditional histology (5 min vs. 16 h). This technique can be used in conjunction with medical procedures to achieve a quick diagnosis. Cryosections are also used in immunohistochemistry since freezing tissue stops degradation of tissue faster than using a fixative and does not alter or mask its chemical composition as much.

11.5.1.3 Electron Microscopic Sections

After embedding tissues in epoxy resin, a microtome equipped with a glass or gem-grade diamond knife is used to cut very thin sections (typically 60–100 nm). Sections are stained with an aqueous solution of an appropriate heavy metal salt and examined with a transmission electron microscope (TEM). This instrument is often called an ultramicrotome. This is also used with its glass knife or an industrial-grade diamond knife to cut survey sections prior to thin sectioning. These survey sections are generally 0.5–1.0 μm in thickness. They are mounted on a glass slide, which are stained to locate areas of interest under a light microscope prior to thin sectioning for the TEM. Thin sectioning for TEM is done with a gem-quality diamond knife.

11.5.1.4 Botanical Microtomy Sections

Sledge microtome is required for hard materials like wood, bone, and leather. These microtomes have heavier blades and cannot cut as thin as a regular microtome.

11.5.2 Spectroscopic Sections

Infrared spectroscope, in particular, requires thin polymer sections as the infrared beam penetrates the samples. Samples are cut into a thickness of 20–100 μm.

11.5.2.1 Laser Microtomy Sections

Sample is cut with a femtosecond laser instead of a mechanical knife. This technique is contact-free and does not require sample preparation techniques. It has the ability to slice almost every tissue in its native state. Thicknesses of 10–100 μm are feasible depending on the material in consideration.

11.5.3 Microtechniques in Phytonematology

Microtechniques allow a complete analysis of the tissue which includes samples of both healthy and infected root tissue and collection of the infected tissues at different stages of nematode development (Ravichandra 2010). Study and comparison of reactions of tissues to nematodes and treatments is possible. A matrix of either paraffin or plastic provides easy sectioning and handling of the tissues. The paraffin-embedded tissue sectioning needs sophisticated equipment which provides a rapid examination of a large quantity of tissue. It is useful to enable greater cytological details with the help of thinner sections by using plastic-embedded tissue, and to prepare the infected plant material for histopathological and histochemical investigations, the following procedure has to be followed (Daykin and Hussey 1985).

11.5.3.1 Selection and Preparation of the Plant Tissue

Always include tissues of both healthy and nematode-infected plant material; it is advisable to have infected tissue at various stages of nema-

Table 11.2 Tertiary butyl alcohol dehydration schedule

			Quantity (ml) needed for solution			
Step	% alcohol	Time	Distilled water	95 % ethanol	100 % ethanol	100 % TBA
1	50	2 h or more	50	40	0	10
2	70	Overnight	30	50	0	20
3	85	1–2 h	15	50	0	35
4	95	1–2 h	0	45	0	55
5	100	1–3 h	0	0	25	75
6	100	1–3 h	0	0	0	100
7	100	1–3 h	0	0	0	100
8	100	Overnight	0	0	0	100

Note: It is important to keep the tertiary butyl alcohol changes in a warm place as the chemical solidifies at 25.5 °C

tode development; collect the roots carefully and wash gently and thoroughly under running tap water to remove all soil particles because presence of a small soil particle may come in the way of the sharpness of the microtome knife while sectioning; cut the tissue into small pieces; place them into fixative immediately; give longitudinal cuts to the roots, small roots into 1 cm and larger roots and stems into more than 1 cm diameter.

Table 11.3 Isopropyl alcohol dehydration schedule

			Quantity (ml) needed for solution	
Step	% alcohol	Time	Distilled water	100 % IPA
1	70	1 day–1 week	30	70
2	90	1 day–1 week	10	90
3	100	1 day–1 week	0	100
4	100	1 day–1 week	0	100

11.5.3.2 Fixation

The objective is to kill and harden the tissue and also to preserve the cellular structures. In fixation, the tissue is submerged in a volume of fixative at least ten times greater than that of the volume of the tissue to ensure that the fixative does not become overly diluted by water from the tissue. If pieces do not sink rapidly after they are put into the fixative, the container of fixative maybe placed under a mild vacuum to draw air out of the tissue more quickly. Tissue must remain in the fixative for a minimum of 24 h to several days depending on its thickness. Material may also be stored indefinitely in the fixative. One of the best and most common fixatives is formalin-aceto-alcohol (FAA): 90 ml of 50 % ethanol, 5 ml of glacial acetic acid, and 5 ml of 37 % formaldehyde. Another fixative which is also good is formalin-propiono-propanol (FPP): 90 ml of 50 % isopropyl alcohol, 5 ml of propionic acid, and 5 ml of 37 % formaldehyde. The proportions of reagents given for FAA and FPP are satisfactory for most tissues, but the concentrations of acids and formaldehyde may be varied for certain types of material if poor results are obtained with the standard concentrations.

11.5.3.3 Dehydration

The purpose of dehydration is to remove water from the tissue. Water must be removed gradually if plasmolysis is to be avoided. Hence, dehydration is accomplished by moving the tissue stepwise through increasingly higher concentrations of alcohols. When FAA is used as the fixative, the tertiary butyl alcohol (TBA) dehydration schedule should be followed (Table 11.2). If the material has been fixed in FPP, the isopropyl alcohol (IPA) dehydration schedule should be followed (Table 11.3).

When solutions in the dehydration schedule are changed, the liquid is drained from the container holding the tissue and then the tissue is covered immediately with the next solution. Care must be taken that the solution is never allowed to desiccate. The time the material is allowed to remain in the various dehydrating solutions depends on its thickness. Fine roots

may need the minimum length of time whereas thick woody material will require the maximum length of time. Material allowed to remain too long in the higher concentrations of alcohols may become brittle.

11.5.3.4 Infiltration

In this step, alcohols in the tissue are replaced by paraffin so that the tissue is saturated with a pure solution of paraffin. When the TBA dehydration schedule has been followed, the 100 % TBA solution is first replaced with a 1:1 mixture of 100 % TBA and paraffin oil. The tissue is allowed to remain in this solution for 1 h or more, depending on its thickness. Shortly before the next step, another container is 3/4 filled with melted paraffin and the paraffin is allowed to solidify slightly. The tissue in the TBA–paraffin oil mixture is then placed on top of the solidified paraffin and is covered with a layer of the TBA–paraffin oil solution. This container is placed uncovered in an oven that is set slightly above the melting point of the paraffin. The tissue sinks to the bottom of the container as the paraffin melts. After 1–3 h, the TBA–paraffin oil–paraffin mixture is poured off and is placed with pure melted paraffin. The uncovered container is placed back in the oven for about 3 h. This step should be repeated at least once more. The melted paraffin is replaced by a specialized type of melted paraffin, like paraplast or tissueprep, which is made especially for histopathological use. Tissue should remain in the oven in this paraffin overnight. After one more exchange with fresh melted paraplast or tissueprep for about 1 h, the tissue is ready for embedding. The infiltration procedure to be followed when the IPA dehydration schedule has been used is much simpler but requires a greater length of time.

To start with, part of the 100 % IPA in the last dehydration step is poured off so that the tissue in the bottom of the container remains covered; the container is then filled with chips of paraplast or tissueprep and is placed uncovered in an oven set at 59–60 °C; once the chips have melted, the paraffin–IPA mixture is poured off and replaced with pure melted paraplast or tissueprep; this paraffin should be exchanged for freshly melted paraplast or tissueprep at least twice more at 3–4-day intervals; small, fine roots should be ready to embed 1 week after beginning the infiltration procedure, while thicker tissues may take 1–2 weeks longer.

11.5.3.5 Embedding

The tissue is positioned in cooling paraffin so that it can be sectioned after hardening. Molds for embedding may either be constructed in the lab out of folded paper, or metal base colds and embedding rings designed especially for histology may be purchased. Molds should first be coated with a thin layer of glycerin. The tissue sample is then poured or carefully lifted into the mold with heated forceps and additional melted paraffin is added to fill the mold. This step may be done on a hot plate set at 60 °C. However, an embedding table, consisting of a rectangular metal plate which has a heat source at one end and which becomes progressively cooler toward the other end, gives increased control of the embedding procedure. The filled mold is next moved to a cooler surface either on the laboratory bench or on the embedding table. As soon as the paraffin begins to solidify on the bottom of the mold, the tissue is rapidly oriented in the desired fashion with a heated dissecting needle. Once the paraffin begins to solidify over the top of the mold, the mold is plunged into ice water and left there until the paraffin is completely solidified. After hardening, the paraffin is removed from the mold and may be cut into smaller blocks which can either be mounted on wooden blocks with melted paraffin or inserted directly into the microtome. Samples of tissue may be stored in these blocks indefinitely if kept in a cool place.

11.5.3.6 Sectioning

Paraffin block sectioning is carried out on a rotary microtome equipped with a knife or disposable razor blade. A knife must be used when the tissue is tough or woody. However, for other types of tissue, razor blades will cut sections equal in quality to the knife-cut sections. Because razor blades can be frequently replaced with fresh blades, they have the advantage of not requiring sharpening and therefore are useful when working with root tissue, which often carries soil particles that can rapidly dull a cutting edge. Excess paraffin

surrounding the tissue should be trimmed away before sectioning, leaving at least 1 mm around the tissue. Care should be taken so that opposite edges of the trimmed block face are parallel. The block is then cooled in ice water for at least 5 min, inserted into the microtome clamp, and one edge of its face aligned parallel to the knife edge. Sections 8–12 µm in thickness are usually cut for histological studies. Tissue that is especially tough or woody will section more easily if the trimmed block is first soaked overnight in the refrigerator in a softening solution consisting of 90 ml of 1 % sodium lauryl sulfate and 10 ml of glycerin. The excess paraffin on the face of the block must first be trimmed away exposing the tissue so that the softening solution can penetrate. As the sections are cut, the edge of each section should adhere to the previous section to form a ribbon.

A sharp knife edge and proper knife angle are most important in obtaining a ribbon; the back of the knife edge should also be checked frequently and cleaned if necessary as paraffin buildup there will adversely affect ribbon formation; as the ribbon increases in length, it should be held away from the microtome with a dissecting needle or brush; it is then removed from the knife edge with a second needle and transferred, shiny side down, to a clean, flat surface; the ribbon should never be touched with hands; the ribbons can either be mounted immediately or stored in a cool, dust-free place for several weeks, if necessary.

11.5.3.7 Ribbon Mounting

Ribbon mounting adheres sections to glass microscope slides so that they can be stained. Ribbons must first be cut into shorter lengths so that they can be fit onto the slides. Slides may be labeled with a diamond pencil or, if the glass on one end is frosted, with a lead pencil. The surface of the slides is then coated with a small amount of Haupt's adhesive.

Preparation Dissolve the gelatin in the water at 30 °C; add the phenol and glycerin and filter the solution; before the adhesive dries on the slides, the slides are flooded with a 2–3 % formalin solution, which should be made fresh each day; the flooded slides are then placed on a warming tray held at 35–40 °C, and segments of the ribbon are floated on the slides; as the slides warm up, the ribbon will flatten out and the liquid will evaporate after several hours, when the slides are completely dry; remove and dry them indefinitely.

11.5.3.8 Staining

The process of staining removes the paraffin from the sections and increases the contrast in the tissues. The staining procedures that have been most used for nematode-infected root material are Johansen's quadruple stain (Table 11.4), Sass safranin and fast green stain (Table 11.5), and triarch quadruple stain (13) (Table 11.6). Solutions made from dry, powdered stains should always be filtered before they are first used. If only a few slides are being stained, the alcohols and staining solutions may be kept in Coplin jars and the

Table 11.4 Johansen's quadruple stain

Step	Solution	Time
1	Xylene	5 min
2	Xylene–absolute ethanol (1:1)	5 min
3	95 % ethanol	5 min
4	70 % ethanol	5 min
5	Safranin O solution[a]	6–24 h
6	Rinse in tap water	
7	1 % aqueous methyl violet 2B	10–15 min
8	Rinse in tap water	
9	95 % ethanol-methyl cellosolve-tertiary butyl alcohol (1:1:1)	15 s
10	Fast green FCF solution[b]	10–15 min
11	95 % ethanol-tertiary butyl alcohol (1) plus 0.5 % glacial acetic acid	15 s
12	Orange G solution[c]	3 min
13	Clove oil–methyl cellosolve–95 % ethanol (1:1:1)	15 s
14	Clove oil–absolute ethanol–xylene (1:1:1)	15 s
15	Xylene	5 min
16	Xylene	5 min or longer

[a]The Safranin O solution is prepared by dissolving 4 g Safranin O in 200 ml methyl cellosolve. When the safranin is dissolved, add 100 ml 95 % ethanol and 100 ml distilled water. Finally, add 4 g sodium acetate and 8 ml formalin

[b]The fast green FCF solution is prepared by adding 0.25 g fast green FCF to 50 ml of a solution composed of methyl cellosolve and clove oil (1:1). After the fast green has dissolved, 150 ml 95 % ethanol, 150 ml tertiary butyl alcohol, and 3.5 ml glacial acetic acid are added

[c]The orange G solution is prepared by dissolving 1 g orange G in 200 ml methyl cellosolve and then adding 100 ml 95 % ethanol

Table 11.5 Sass safranin and fast green stain

Step	Solution	Time
1	Xylene	5 min
2	Absolute ethanol	5 min
3	95 % ethanol	5 min
4	70 % ethanol	5 min
5	50 % ethanol	5 min
6	30 % ethanol	5 min
7	1 % aqueous Safranin O	1–12 h
8	Rinse in tap water	
9	30 % ethanol	3 min
10	50 % ethanol	3 min
11	70 % ethanol	3 min
12	95 % ethanol	3 min
13	0.1 % fast green FCF in 95 % ethanol	5–30 s
14	Absolute ethanol	15 s
15	Absolute ethanol	3 min
16	Xylene–absolute ethanol (1:1)	5 min
17	Xylene	5 min
18	Xylene	5 min or longer

Table 11.6 Triarch quadruple stain

Step	Solution	Time
1	Xylene	5 min
2	Xylene	5 min
3	Xylene–absolute ethanol (1:1)	5 min
4	95 % ethanol	5 min
5	70 % ethanol	5 min
6	1 % Safranin O in 50 % ethanol	5–15 min
7	Rinse in distilled water	
8	1 % aqueous crystal violet	1–2 min
9	Rinse in distilled water	
10	Absolute ethanol	30 s
11	Absolute ethanol	30 s
12	Orange G[a]–fast green[b] (135 ml–15 ml)	3 min
13	Orange G–fast green (145 ml–5 ml)	2 min
14	Orange G–fast green (148 ml–2 ml)	2 min
15	Orange G	2 min
16	Absolute ethanol	1 min
17	Xylene	5 min
18	Xylene	5 min or longer

[a]Orange G is prepared by dissolving 0.4 g orange G in 100 ml clove oil

[b]Fast green is prepared by dissolving 1 g fast green FCF in 100 ml absolute ethanol

slides moved individually after each time period. However, larger containers and racks that hold 25 or 50 slides are much more convenient when staining larger quantities. Stains and alcohols that are not being used over long periods should be stored in tightly capped bottles. In these three staining schedules (Tables 11.4, 11.5, and 11.6), containers holding water rinses should be emptied and refilled with fresh water after each group of slides moves through. Rinses containing alcohols should be changed when the liquid becomes heavily stained. Staining solutions and xylene require replacement less often. After completion of the staining procedure, cover slips are mounted with a few drops of either balsam or permount.

Slides are first removed from the xylene, which is always the final step in a staining procedure and laid on a flat, absorbent surface; the mounting medium is then applied to the surface of the slide before the xylene evaporates, and a cover slip is lowered gradually over the slide; a minimum of mounting medium should be used, as any excess will run out over the surface of the cover slip; finished slides should be left flat to dry for at least 24 h at room temperature. However, the medium will harden better if the slides are held on a 60 °C, warming tray overnight.

The safranin in the previously discussed stains turns lignified or cutinized cell walls red, whereas the fast green generally turns cellulose walls greenish. Starch grains stain purple by methyl violet and crystal violet. Nematodes in tissue vary from brownish to red in color. Triarch quadruple stain gives the best contrast consistently and takes the least amount of time. Also, a wide variety of tissue types may be stained without altering the staining time. However, since it requires a large amount of clove oil, it is the most expensive stain to prepare. Staining times in the Johansen's quadruple stain and Sass safranin and fast green stain may need to be adjusted for the best contrast between cell types to be obtained.

11.5.3.9 Plastic Embedding of Plant Tissue for Light Microscopy

The previous sections presented information on preparation of histological sections by the traditional embedding method in which paraffin is used. Plastic-embedded material, however,

provides superior preservation of cell structure. Plastic-embedded material will not entirely replace paraffin-embedded material, which still can be used for initial examination of infected tissue. However, critical tissue evaluation is enhanced with plastic-embedded material due to the added resolution obtainable with thin sections. The cytological detail seen in 3-µm-thick sections of plastic-embedded material is superior to that which can be obtained in paraffin-embedded sections. Several procedures for plastic embedding are available. The most commonly followed procedure is given below (Feder and O'Brien 1968).

Procedure Fix small pieces of tissue in 4 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2; rinse twice in buffer and dehydrate in a graded, acetone series; infiltration is done by embedding in soft Spurr's resin; Sections 3 µm thick are cut from a carefully trimmed block on a Sorvall MT-2 ultramicrotome on a glass knife and floated on distilled water on a gelatin-coated slide at 60'C; sections are stained with toluidine blue and permanently mounted with polymount resin; excellent results have been obtained with plastic-embedded tissue that is infected with nematodes.

11.6 Techniques to Evaluate Botanicals Against Phytonematodes

11.6.1 Technique to Screen Plants for Their Efficacy Against Nematodes

Procedure Prepare leaf extract by grinding 2 g of fresh leaves of a botanical plant (Table 11.7) in 5 ml of distilled water using pestle and mortar (Bhatti 1988); filter the water extract through a four-ply muslin cloth to remove the plant debris; centrifuge for 5 min at 4,000 rpm; filter through Whatman's filter paper No.1; preserve the extract in plastic bottles in refrigerator for 24 h and use it as stock solution for further evaluation against nematodes.

Table 11.7 List of some plants possessing nematicidal properties

Sl. No.	Common name of the plant	Botanical name of the plant
1	Neem	*Azadirachta indica*
2	Castor	*Ricinus communis*
3	Datura	*Datura stramonium*
4	Pudina	*Mentha piperita*
5	Subabul	*Leucaena leucocephala*
6	Bhang	*Cannabis stramonium*
7	Aak	*Calotropis procera*
8	Bathua	*Chenopodium album*
9	Safeda	*Eucalyptus naundina*
10	Paper flower	*Bougainvillea spectabilis*
11	Kandal	*Cirsium arvense*

11.6.2 Effect on Nematode Mortality

Procedure Pour 10 ml of larval suspension into each of 5-cm-diameter Petri dishes; add measured volumes of stock solution to these dishes to make the resultant dilutions of 1:5, 1:10, 1:20, 1:40, and 1:80; water alone can be taken as control; replicate each treatment three times; place the Petri dishes with leaf extracts and larval suspension in BOD incubator at 28 ± 1 °C for *Tylenchulus semipenetrans*, *Heterodera cajani*, and *Meloidogyne javanica* at 18 ± 1 °C for *H. avenae* and *Anguina tritici*; after 48 h, record the observations on larval mortality by counting living and dead second-stage juveniles under the stereo binocular microscope; calculate the percent mortality, and data obtained is subjected to angular transformation and statistically analyzed by applying complete randomized design.

11.6.3 Effect on Egg Hatching

Procedure Fit Petri dishes of 5 cm diameter with molded pieces of wire gauges and upon each of them place two-ply filter paper; prepare two dilutions from stock solution of

plant extract (viz., 1:5 and 1:20); pour 10 ml from each dilution into each Petri dish containing 4–5 cysts of *H. avenae, H. cajani*, and 5 egg masses of *M. javanica* separately; replicate each treatment thrice; water can be used as control; place the Petri plates containing cysts of *H. avenae* in BOD incubator at 18 ± 1 °C; place the Petri plates containing egg masses of *M. javanica* and cysts of *H. cajani* at 28 ± 1 °C; count the number of larvae emerging from cysts or egg masses at 3, 6, and 9 days interval; subject the data to square root n or square root $n+1$ transformation and analyze the data by applying complete randomized design.

11.6.4 Techniques to Study the Effects of Few Physicochemical Factors on the Nematicidal Property of Botanicals

11.6.4.1 Effect of Temperature on the Efficacy of Leaf Extracts

Procedure Expose freshly prepared leaf extracts (stock solutions) to varied temperatures, viz., 40, 50, and 60 °C, and boiling temperature for 5 min each; prepare two dilutions, viz., 1:5 and 1:20, from the stock solutions by adding the required volume of distilled water; replicate each dilution three times; use water alone as control; place the Petri dishes containing leaf extracts and larval suspension in incubator at 27 ± 1 °C for *M. javanica*, *T. semipenetrans*, or *H. cajani* and 18 ± 1 °C for *A. tritici*; record the data on mortality after 48 h by observing under the stereo binocular microscope.

11.6.4.2 Effect of Longevity/ Persistence on the Efficacy of Leaf Extracts

Procedure Fill the beakers (100 ml) with sterilized river sand; add leaf extracts in dilutions of 1:5 and 1:20 into these beakers to drench the river sand on the same day; add the 100 larvae/beaker at 0, 1, 2, 4, and 6 weeks after drenching the river sand; replicate each dilution three times; in control, drench the river sand with sterile water; keep the river sand moist during the period of experiment; place the beakers under shade; wash the river sand of beakers carefully with water, 72 h after liberation of larvae; pass through a series of sieves (10 and 300 mesh size); subject the suspension to Baermann's funnel extraction technique; count those larvae which migrate down as alive and calculate the percent recovery in each extract.

11.6.4.3 Effect of Ageing on the Efficacy of Leaf Extract

Procedure Prepare the stock solutions of the fresh plant leaf extracts and keep in corked plastic bottles for 5, 10, 15, and 45 days at room temperature; prepare two dilutions, viz., 1:5 and 1:20, from this stock solution; test the efficacy of these at 27 ± 1 °C for 48 h against *M. javanica*, *T. semipenetrans,* or *H. cajani* and at 18 ± 1 °C against *A. tritici* for 48 h; replicate each dilution three times; use water alone as control; record the data on mortality by observing under the stereo binocular microscope.

11.6.4.4 Effect of pH on the Efficacy of Leaf Extracts

Procedure Maintain different pH range of the extract from 4 to 10 (i.e., 4, 8, and 10) by adding 1 N HCl or 6 N NaOH; note down the actual pH of the extract; place these extracts at room temperature for 1 h for final check of the pH; prepare two dilutions (1:5 and 1:20) from the stock solution by adding the needed volume of distilled water; replicate each dilution three times; use water alone as control; place the Petri plates containing leaf extracts and larval suspension of *M. javanica*, *T. semipenetrans*, or *H. cajani* at 27 ± 1 °C and *A. tritici* at 18 ± 1 °C for 48 h in incubators; record the data on mortality by observing under the stereo binocular microscope after 48 h.

11.6.5 Preparation of the Plant Leaf Extract

Procedure Wash fresh leaves of the plants in ordinary water two or three times; sterilize these leaves with 0.1 % $HgCl_2$ solution for 30 s; wash them thoroughly with sterilized water two to three times and ground with a sterilized pestle and mortar to prepare paste; prepare the extract by adding 2 g of the paste in 5 ml of sterilized distilled water; filter this through a sterilized muslin cloth till the whole liquid separates from green leaves or leaf debris; centrifuge the extract that contains more quantity of chlorophyll at 4,000 rpm for 5 min; filter the supernatant through double Whatman's filter paper No.1; prepare the extract under sterilized conditions and store in sterilized flasks in a refrigerator; for bacteria, filter the supernatant after centrifugation through microfilters and store at low temperature.

11.6.6 Efficacy of Chopped and Finely Ground Plant Leaves Mixed in Soil Under Green House Conditions

Procedure Chop and finely grind the leaves of plants at 10, 20, 40, and 80 g leaves/kg soil; mix them with sterilized soil in 15 cm pots; after a week, sow the seeds of the required crop (pigeon pea against *H. cajani*, wheat/castor/aak/bougainvillea against *H. avenae*); when the plants are 1 week old, inoculate 1,000 larvae; replicate each treatment five times; record the data on plant growth parameters and final nematode population in roots and soil after 4 months in pigeon pea and 5 months in wheat.

11.6.7 Field Evaluation of Nematicidal Plants Against Phytonematodes

Procedure Select the fields having uniform infestations of *H. avenae* and *M. javanica* to study the efficacy of chopped leaves of castor and aak at 20 and 40 g/kg soil in tomato and wheat, respectively; the desired plot size for wheat is 1 m× 1 m and for tomato 2 m× 1 m; calculate the quantity of chopped leaves to be added for each plot based on the weight of soil/plot (weight of 6′ furrow soil of 1 ha is estimated to be 2×10^6 kg); estimate the initial population for each nematode before mixing of chopped leaves for each plot separately; mix the calculated amount of each plant leaves thoroughly in each plot; arrange the treatments in randomized block design; replicate the treatments four times; water the pots individually; 15 days after adding the chopped leaves (after decomposition), take up the sowing of wheat and transplant tomato seedlings; plots receiving no plant leaves serve as control; for comparison, carbofuran 3G is applied at 2 kg a.i./ha before sowing/transplanting; record the data on final yield/plot and final nematode population both in roots and soil.

11.6.8 Technique to Isolate and Characterize the Active Principles of Nematicidal Plants

11.6.8.1 Plant Material

Dry the samples of plant materials under shade; the material may consist aerial parts of bhurt (*Xanthium stramonium), Chenopodium album, C. murale, Calotropis procera, Ricinus communis, Nerium oleander, Datura stramonium, Ipomea carnea,* palmarosa (*C. martinii* var. *motia*), lemon grass (*C. flexuosus)*, and citronella java (*C. minternianus*).

11.6.8.2 Preparation of Crude Extract

Dip the fresh, dried, and crushed parts of the plants in methanol for 48 h at room temperature and filter; remove the solvent at reduced pressure that gives extract which is stored in refrigerator for further analysis.

11.6.8.3 Isolation of Essential Oils

Obtain essential oils of bhurt, palmarosa, lemon grass, and citronella java by hydro-distillation of fresh aerial parts of the plants in a laboratory hydrodistillation unit.

11.6.8.4 Chromatographic Resolution

Resolve the crude extracts/oils by repeated column chromatography/preparative TLC over silica gel using a series of solvent system in increasing order of polarity.

11.6.9 Identification of Constituents

Routine checking of the purity of the compounds is done on silica gel G TLC plates. Record the melting points in Gensons electrical melting point apparatus; identify the compounds mainly by spectral studies; record IR (max. cm^{-1}) spectra on Perkin-Elmer infrared 157 spectrophotometer; run ^{1}H NMR spectra (in units) on Varian EM-360 instrument using TMS as internal reference and mass spectra on Jeol-JMS-D300 mass spectrometer.

11.6.9.1 GLC of Lipid Fraction

Prepare the fatty acid methyl esters from the lipid by transesterification in methanol using sodium methoxide as catalyst; separate by GLC using Aimil Nucon Gas Chromatograph series column (1/8 in. O.D.X feet) packed with 15 % polydiethylene glycol succinate on Chromosorb W, under usual operating conditions; identify the compounds by comparison of their retention times with those of authentic samples recorded under similar operating conditions.

11.6.9.2 Preparation of Acetates

The compounds amenable to acetylation are converted into acetoxy derivatives using acetic anhydride pyridine at room temperature.

11.6.9.3 Monoterpenes and Related Derivatives

Citral (XIV), geraniol (XV), methanol (XXIa), cyclohexanone (xxiv), and cyclohexanol (xxva) required in this estimation are available commercially (Aldrich Chemical Company Ltd.); prepare the N-phenyl-carbamates (xxb, xxib, and xxvb) of xxa, xxia, and xxva, respectively, by their reaction with phenyl isocyanate in refluxing benzene; prepare the esters xxc-xxa, xxib-xxid, and xxvc-xxvd by the esterification of xxa, xxib, and xxva with corresponding acid anhydride or acid chloride, 3-chloro-p-menthane (xxii), and P-3-menthane (xxiii) by treatment of menthol (xxia) with phosphorus pentachloride and p-toluene-sulfonic acid, respectively; check for the purity of all these compounds by thin-layer chromatography over silica gel G.

11.6.9.4 Bis-(3-Substituted Amino/ Aryloxy-2-Hydroxy or Phenyl-Carbamoyloxy-Propoxy) Benzenes

Prepare the compounds 1,4-bis-(3-substituted amino/aryloxy-2-hydroxy propoxy) benzene xxvii-xxix/xxx as per the standard procedure starting from the alkylation of quinol with spichlonohydrin followed by nucleophilic ring opening of the epoxide xxvii with various secondary amines or phenoxide ions; refluxing of the amines xxviii-xxix and quinol xxvi with phenyl isocyanate gives the corresponding carbamoyloxy derivatives xxxi–xxxii and xxxiii.

Phenolics: Select phenols with various substituents having electron-withdrawing or electron-donating tendency, which are commercially available.

Ethyl (Aryloxy) Acetates: Prepare these by the standard procedures, by stirring the corresponding phenol with ethyl bromoacetate in the presence of potassium carbonate at room temperature.

Azides: The esters on refluxing with hydrazine hydrate in ethyl alcohol give the corresponding azide.

11.7 Techniques of Remote Sensing

Remote sensing is a technique of measurement of acquisition of information on some property of an object or phenomenon by a recording/measurement device that is not in physical contact with the object or phenomenon under study. Remote sensing measurements make use of the visible, infrared, and microwave sensors with specific spatial and radiometric character-

istics in the acquisition of required data. The data thus acquired is stored in the form of photographs, images, or digital tapes depending on the sensor used and the mode of acquisition. The data is interpreted either manually, machine assisted, or totally automated, and the information thus obtained is used for purposes of inventory, survey, monitoring, planning, and management. Remote sensing is especially useful where speed, repetitive observation, and a synoptic view are needed. It provides an important new dimension in the detection and quantification of damage to plants, assessment of the distribution of the principal host plants or habitats of pests, and surveillance of environment factors favorable for the development, spreading, and outbreak of nematode pests.

11.7.1 Remote Sensing Techniques Used in Plant Protection

Various techniques of remote sensing applicable in plant protection against pests including plant-parasitic nematodes are as follows (Nageswara Rao et al. 1991). They include photography and videography from ground and aircraft, satellite-borne photography, multispectral scanning, thermal imaging, ground-based and airborne radar, and acoustic sounding.

The range of wavelengths employed extends from the ultraviolet end of the visible spectrum to the radars.

11.7.2 Aerial Photography

Although aerial photography cannot give as great a synoptic view as satellite imagery, it provides an accurate delineation and recording of affected area of vegetation damage (Blakeman 1990). Photographic response has been used by many workers for the detection of plant diseases, including nematode diseases. It is possible to pick up primary infection of crop plants by pathogens 2–3 days before the aboveground symptoms are visible on the ground surveys. Different levels of crop diseases can be identified using small-scale color infrared (CIR) photography (1:60,000). Changes in the appearance of the foliage and other aboveground plant parts due to the infestation by nematodes may be detected by aerial photography. The CIR infrared photography has been the most widely used technique in detecting plant stress because it portrays the combination of differential response from visible as well as near infrared spectrum (Heller 1978). Identification of host plants of pests with aerial CIR photography offers a means to plan large area pest management and eradication programs. This technique may be used to investigate the distribution of host plants of plant-parasitic nematodes. It can also be used to map areas of infestation by a specific nematode. It may be used in annual surveys to monitor the postharvest plant removal to prevent outbreaks. CIR photography can serve as a unique tool for monitoring the effectiveness of plant growth regulators/chemicals applied to manage various pests. CIR photography (1:8,000 scale) has been used to show that crop rotation from cotton to grain sorghum can significantly minimize the infestation level of the reniform nematode (*Rotylenchulus reniformis*) in cotton fields.

11.7.3 Aerial Videography

Videography has been used in various remote sensing applications for estimating freeze damage; discriminating plant species, weeds from crop plants, and infested plants from the healthy ones; etc. In a video camera, the image of remote objects is cast onto a photoconductive surface rather onto a film and forms a charge distribution that duplicates the optical image. This distribution is read by a scanning electron beam and is converted into electrical signals, which are stored on a video tape recorder.

Major advantages of videotaping include the ability to display imagery while it is actually being acquired, high sensitivity to light, suitability for digitization and analysis by computer, and low price and reusable nature of video recording tape. However, a disadvantage

Table 11.8 Satellite sensors

Type/name	Sensor type	Spectral regions (μm)	Spatial resolution (M)
IRS-1A Operational	LISS I and II	0.45–0.52	36.5 and 73
		0.52–0.59	
		0.62–0.68	
		0.70–0.86	
IRS-1B	LISS I and II	0.45–0.52	36.5 and 73
		0.52–0.59	
		0.62–0.68	
		0.70–0.86	
IRS-1C/1D	LISS I and II	0.52–0.59	23
		0.62–0.68	23
		0.77–0.86	23
		1.56–1.76	69
	Panchromatic WIFS	0.50–0.75	10
		0.62–0.68	180
		0.77–0.86	180

of commercial video systems is their low resolution (Manzer and Cooper 1982).

11.7.4 Multispectral Sensing (MSS)

This can form images over a much wider range of electromagnetic wavelength than photographic techniques. Instead of generating an instantaneous image of the whole scene, MSS uses sensors with very narrow fields of view to scan an image systematically; an image is built up as the scan progresses. Satellite sensors presently operational and those going to be available in the near future for use in pest management and plant protection are listed in the following table (Table 11.8).

Satellite-based MSS has been concentrated in two principal areas of plant protection, i.e., the detection of vegetation changes due to diseases/pests and the measurement of meteorological data. The identification of crop stress and disease from satellite data is a tough task. Timeliness of data acquisition and repetitive coverage are even more important as symptoms are often transient and closely linked to crop growth stage. Satellite sensors can cover and provide useful information on crop stress and disease when the symptoms are extensive (Epstein 1975). Satellite MSS has been used to assess disease severity on wheat. Satellite remote sensing is an ideal tool to investigate the environmental factors controlling the pest and disease development, particularly in monitoring rainfall and air temperature. Meteosat data can be used to monitor rainstorms that may affect pathogen development and for rapidly locating potential outbreaks. Several methods/programs like SPOT and Landsat TM and Advanced Very High Resolution Radiometer (AVHRR) of NOAA meteorological satellite that records vegetation index maps and rainfall estimates have been developed in detecting and studying disease aspects.

11.7.5 Radar

Radar has been extensively used as a tool in the study of long-distance migration and flight behavior of insect/disease inducing organisms. Information on its usage with respect to nematode diseases is but meager. However, in future, progress in this regard is expected, especially, aboveground feeding nematodes. These radars use a wavelength of 3–10 cm. Millimetric radar is used in case of small insect pests. Migration of a pest can be deduced from the orientation and directive properties of the radar antenna and target range from the time elapsing between transmission of the illuminating pulse and the reception of the

echo. Typical maximum detection ranges would be 1.5–2.8 km for individual pest and up to several tens of kilometers for dense swarms (flight behavior of locusts, grasshoppers, and moths of various species of *Helicoverpa*, *Spodoptera*, etc.). Radar (C-band) has been demonstrated to be an effective tool to detect blight in corn (Ulaby and Moore 1973). Microwave backscatter from crops is strongly dependent on the size of the scattering elements within a crop, crop geometry, row direction, canopy height, and the dielectrical properties of the canopies. A time series data of canopy scattering properties at different microwave frequencies (Ku, X, C, L bands) and polarizations (HH, VV, and HV) over the season could provide information about crop vigor and stress.

11.7.6 Sodar

The working of sodar is similar to that of pulse radar, except that high-frequency sound waves are transmitted rather than electromagnetic waves. Low-power, short-range sounding device has been used to count pests approaching pheromone traps (Hendricks 1980). Acoustic sounds can be used to monitor the atmospheric structure and vertical temperature profiles which favor the flight of many insect pests.

11.8 Technique of Soil Solarization

Soil solarization is a nonchemical method for managing phytonematodes and other soil microorganisms apart from weed seeds, bacteria, and fungi. Soil solarization is a unique approach to handle nematode problems. It is a potential alternative to chemical fumigation. As a nonchemical management method, it fulfills organic or low toxicity control (Ravichandra 2010). The technique is a simple and easy way to clean nursery/garden soil to grow nematode-free plants. Early summer is a great time to use solarization to clean up nematode "hot spots." Soil solarization is usually only considered as a remedy for nematode management in nursery and in the home garden. The basic procedure is to cover the soil with a clear plastic and trap the sun's heat to raise soil temperatures high enough to kill nematodes present in the soil. No plant material should be present in the area to be solarized. The extremely high soil temperatures will kill any plants left in the solarized area, and plant remains/debris will interfere with even heating.

Nursery, vegetable garden plots, and annual flower beds are the best areas for control. Soil solarization is a simple, safe, and effective alternative to the toxic, costly soil nematicides and the lengthy crop rotations now needed to manage soil nematodes. The technique utilizes the sun's heat to zap nematodes by trapping solar energy in the soil with a polythene layer. There are several procedures that must be followed closely to insure the soil temperature is raised to the level where nematode control is obtained (Killebrew 1999). Radiant heat from the sun is the lethal agent involved in soil solarization. A clear polyethylene mulch or tarp is used to trap solar heat in the soil. Over a period of several weeks to a few months, soil temperatures become high enough to kill nematodes to a depth of nearly 8 in. Nematodes may not be fully eradicated from the treated area, but their numbers in the plow layer (top 6–8 in.) will be greatly reduced, allowing successful production of a crop. In sandy or sandy loam soils, nematodes may survive at depths below the lethal temperature zone. As a result, some damage may be seen on deep-rooted crops, but those with shallow root systems should escape serious injury.

11.8.1 Points to Consider

The soil should be moist and well tilled before tarp installation. Solarization should be practiced during late spring months, or ideally at the hottest time of the year when the incidence of sunlight is greatest. Normally, 4–6 weeks is an adequate period of solarization. Six weeks is the preferred period if the technique is used in the late spring. Selection of a plastic or polyethylene cover is probably the most important step in the process. The cover must be clear and no more than 2 mm thick. It should be strong and durable enough to allow for stretching. Placement of the cover is also important.

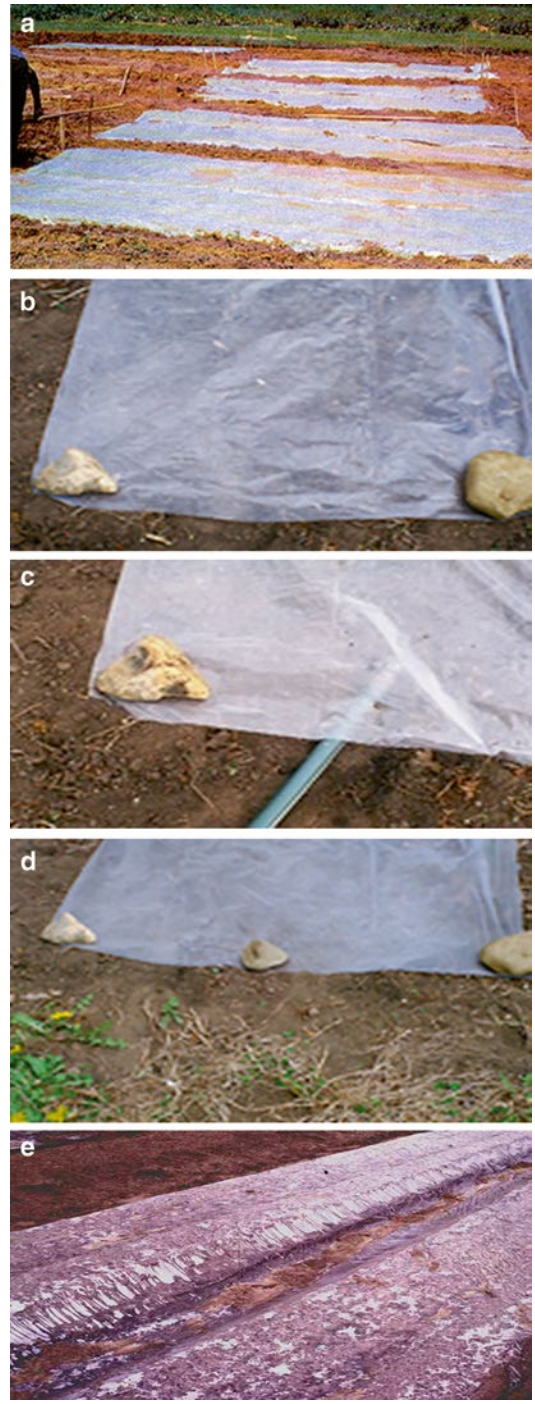
It should be stretched tight and in direct contact with the soil surface. Care must be taken to bury the edges of the tarp in the soil to a depth of at least 6 in. so as not to allow the wind to lift the tarp during the solarization period. The tarp has to remain in place for a 4- to 6-week period. When removing the tarp, avoid contamination of the treated area with untreated soil. This is also true at planting and best results are obtained when beds are prepared for planting before solarization and immediately planted after removing the tarp. Solarization also controls other soilborne organisms such as fungi, insects, and certain weeds.

Procedure

Soil Preparation: The soil to be solarized must be worked up to seedbed condition, that is, cultivated until it's loose and friable with no large clods or other debris on the soil surface (Plate 11.5a–e); a rotary hoe or rototiller will eliminate clods or other debris that create air pockets that reduce heating of the soil and keep the tarp from fitting tightly over the soil surface; a clean, flat surface will also prevent the accidental puncturing of the thin plastic mulch by debris.

Soil Moisture: Make sure moisture levels are adequate for working the soil before laying the plastic tarp; if the soil is dry, water the areas to be solarized before laying the tarp, because most soil pests are more sensitive to high temperatures in wet soil than in dry soil; when possible, lay a soaker hose or drip irrigation lines under the tarp to maintain moisture levels during soil solarization; tarped raised beds may also be watered by flood-irrigating the adjacent furrows.

Trenching: Dig a trench approximately 6–8 in. deep and wide around the perimeter of the plot. The trench will be used to bury the edges of the plastic to be placed over the plot.



Plates 11.5 (**a**)–(**e**) Steps in soil solarization (Courtesy: http://Okeechobee.ifas.ufl.edu)

11.8.2 Plastic Tarp

Use a clear, UV-stabilized plastic (polyethylene or polyvinyl chloride) tarp or sheeting 0.5–4 mils thick. The tarp material must be flexible enough to stretch across the soil surface. Using two layers of thin plastic sheeting separated by a thin insulating layer of air increases soil temperatures

and the overall effectiveness of a solarization treatment. The edges of the sheets must be buried to a depth of 5 or 6 in. in the soil to prevent blowing or tearing of the tarp by the wind. White or black plastic usually does not transmit enough solar radiation to raise soil temperatures to lethal levels for many soil pests. Thinner sheets (0.5–1 mil) are less costly, but they tear or puncture more easily.

Thicker plastic sheets (2 or more mils) should be used where damage from high winds or similar problems is likely. Patch holes or tears immediately with duct tape to prevent heat loss. Plastic mulches may be laid by hand or machine in a continuous sheet using glue or heat as a sealant and in strips over flat or raised beds. Continuous sheets are the best method for nematode control because the entire area is disinfested. Plastic strips, 2–3 ft wide, are often more convenient and economic for many bed-grown vegetable crops. Crops may be seeded or planted directly into slits or holes in UV-stabilized strip mulches after soil solarization is completed. For effective solarization, the edges of tarps laid over raised beds must be buried in the adjoining furrows. Expect some increase in pest and weed problems along the edge of the stripped mulches. Do not cultivate solarized areas, because healthy weed seed will be brought to the soil surface.

11.8.2.1 Timing

Long, hot, sunny days are needed to reach the soil temperatures required to kill nematodes. The longer the soil is heated, the better and deeper the control will be. During hot summers, a tarping period of 4–6 weeks is most favorable to control nematodes (although a slight variation is commonly seen in different regions), while a 2-week tarping period may not be very effective.

11.8.3 Other Benefits

Managing damaging nematode, soil fungi, bacteria, and pests with soil solarization can increase the growth and yield beyond expected levels. Some increases in yield may result from the control of usually minor and often unnoticed diseases. Populations of beneficial, growth-promoting, and pathogen-antagonistic bacteria and fungi quickly recolonize solarized soil, adding a biological control component to soil solarization. Plant-pathogenic fungi weakened by high soil temperatures are more susceptible to these antagonists. *Rhizobium* bacteria are also sensitive to high soil temperatures, but reduced nodulation of the roots of legumes such as peas or beans in solarized soils should be temporary. Improved soil tilth and the increased availability of essential plant nutrients such as nitrogen, calcium, and magnesium may also account for increases in plant growth following soil solarization. Soil solarization will tie up land for a period of 1–3 months. Commercial growers and gardeners must plan to pull areas out of production sometime during the 6-month period when solarization is possible. The benefits of higher crop yields with quality will reduce nematicidal use, particularly for gardeners, may far outweigh the cost and inconvenience associated with soil solarization.

11.8.4 Factors Influencing the Success

Soil characteristics such as color, structure, and depth have an effect on the effectiveness of soil solarization. Dark colored soil tends to absorb more solar radiation in comparison to light colored soils. Loose, friable soils enable heat to be transmitted throughout the soil and allow heat to go deeper. Control is much better in the top few inches of the soil and decreases with soil depth. The level of control achieved depends on a combination of the duration of the solarization process, the high temperatures reached, and the susceptibility of the weed species to be controlled. Nematodes that live in the top 2–3 in. of soil are easier to manage with solarization.

11.9 Technique of Soil Fumigation

The optional growing conditions provided for repeated cropping in intensive, irrigated horticulture favors the growth and multiplication of plant-parasitic nematodes. If not checked,

nematode pests build up to such levels that disease outbreaks progressively lower crop quality and yield, until a point is reached where crop plants are destroyed. Soil fumigation is the only answer to the problem, both as a cure for severely diseased soils and as a means of preventing soils from becoming diseased. Soil treatment with fumigant gases can reduce soil infections to safe levels and reduce the carry-over of nematodes to successive crops (Ravichandra 2010). Among various fumigants, methyl bromide is the most commonly used that gives the most efficient and economic management of plant-parasitic nematodes. It is the most effective fumigant that kills nematodes in soil and also kills nematodes within their cysts and galls and in plant residues which have not yet decomposed, viz., cyst nematodes, root knot, root lesion, pin, stylet, dagger, stem nematodes, etc. This technique is also an effective way to manage soil nematode pests present in seedbeds, nurseries, and other small plots.

11.9.1 Methyl Bromide

It is a liquid fumigant, it boils and becomes a gas at 4 °C, but this temperature is too low for soil treatment. Soil may be treated when its temperature at 15–20 cm is 8 °C, the optimal temperature, however, being about 25 °C. Methyl bromide sinks into soil at first, because it is heavy (about three and a half times heavier than air), but because it is very volatile, it is applied under plastic tarpaulins that serve to prevent its escape. It is sold in cans as a liquid under pressure. When the can is opened, all the methyl bromide flows out rapidly as a liquid or an odorless gas. Because it has not odor, it would be dangerous if not mixed with 2 % chloropicrin (trichloronitromethane) which has an odor and is highly irritating to the eyes. Methyl bromide is also obtainable mixed with larger percentages of chloropicrin and other nematicides.

Procedure Correct soil preparation is the key to successful nematode management by methyl bromide; nematodes are exposed and activated in the process of correctly preparing soils for fumigation; soils correctly prepared for methyl bromide treatment must be free of crop trash; cultivated to porous seedbed tilth to the maximum depth possible, without a plow sole; neither too wet nor too dry; about 2/3 of filed capacity is ideal; level.

11.9.2 Soil Preparation Schedule

For soil to be thoroughly ready for methyl bromide treatment, the below given order of operations is to be followed.

Clear the soil of plant trash; cultivate the soil to 40 cm depth at least. The deeper the cultivation, the better the penetration of the gas; disc at right angles to the prior operation. In heavy and medium soils, use a roller to break up any clods and obtain an even surface. For the third and last cultivation, a harrow should be attached. It will help pulverize clods and remove plant trash and will give a level surface finish. An excellent implement, especially in hothouses (green houses/poly houses), is the rotary hoe or the spade plow. It penetrates the soil to a great depth, turning and pulverizing clods without forming a plow sole, and gives a good, level surface finish. Irrigate to saturation to an 80-cm depth at least, not less than 2 weeks prior to treatment. Irrigate lightly, as required, to maintain constant moisture status (about 2/3 FC), until and especially at treatment time.

11.9.2.1 Manures

Soil should be manured before the first cultivation, but the dressing must not exceed 200 m^3/ha. If a dressing is required or if the manure is lumpy, it must be applied at least 3 months before fumigation so that it can decompose and mix with the soil. Large lumps of manure on or in the soil are to be avoided.

11.9.2.2 Chemical Fertilizers

High levels of available nitrogen are released by soil fumigation. To prevent possible excessive vegetative growth, the application of N fertilizers as a post-planting top dressing is advisable instead of a preplanting basic dressing.

Table 11.9 Dosage of methyl bromide based on crops

Crops	Dosage (in kg/ha)	Aeration (in days, by soil type and temp.)	Remarks
1. *Nurseries*: vegetables and flowers	350–500	7–14	Do not fumigate heavy soils to be used for celery nurseries
2. *Vegetables*: cucurbits, tomato, eggplant, pepper	350–500	7–14	For Beit Alpha- type cumbers, soil leaching is required
Strawberry (nursery and field)	350–500	14	–
3. *Flowers*: annual and perineal cut flowers	350–500	14–21	Even light soils must be leached before planting carnations
4. Bulbs and corms (on light soils only)	350	14	–
5. Citrus replanting	500	14	–
6. Deciduous replanting	750	14	–

11.9.3 Fumigant Application

Irrespective of the type of method chosen to apply the fumigant to soil, use of plastic tarpaulins is common, to confine this volatile gas in the soil for a 48–96 h exposure/treatment period. This variation in length of exposure is determined by the soil temperature at 15–20 cm at the time of treatment. The longer periods are required for lower temperatures. Polyethylene (polythene) tarps are recommended, either thin (0.03–0.05 mm) for one-time use or thick (0.1–0.15 mm) for reuse. Polyvinyl chloride (PVC) tarps are also used. Black tarps, made of recycled plastics, can reach a temperature of 60 °C on hot sunny days, but their use is to be avoided. Methyl bromide tends to move down slopes. On gently sloping sites, apply more gas at the upper, than at the lower end. On steep slopes, treat on the contour. To ensure that run-off water from irrigation or rain will not wash infected soil into the fumigated area, always fumigate a few meters beyond the ends of the planting rows and open a trench around the fumigated area. Crops may be planted 3–21 days after the tarps are removed. The length of this aeration period depends on the crops as shown in Table 11.9.

For light soils and/or high temperatures, the shorter aeration period is sufficient; for medium and heavy soils and all soil types at low temperatures, the longer aeration period is required; the long aeration is also desirable for direct seeded crops; if rain is expected during the aeration period, do not remove the plastic sheets but allow for aeration while protecting the soil from direct rain.

For crops sensitive to bromide residues (e.g., onion, carnation, citrus seedbeds, cotton, pepper, celery), a leaching irrigation after an initial 2–3 days aeration of 200–400 mm is essential. These crops may be planted as soon as soils have drained to suitable moisture levels. As for all nematicides, carefully read the label before starting work. Methyl bromide can be applied by various methods like in cylinders either by cold gas manual method or hot gas manual method, in cans, as capsules, through drip irrigation system, through fumigation of hothouse soils, or by machine. In most places, fumigation of hothouse soils is a commonly followed method.

11.9.4 Methyl Bromide Fumigation of Polyhouse Soils

In polyhouses and glass and plastic hothouses, physical barriers like walls and supporting posts, affect work methods and order. Uncovered

plastic houses may be regarded as being on a par with the open as far as precautions are concerned, but the generally limited ventilation of covered plastic and glass hothouses makes working to safety rules imperative. The dosage of the fumigant is normally fixed based on the crops and stage of the crop (Table 11.9). Work out the glasshouse doors as much as possible. Seal, above and below soil level, all drains and other openings that connect hothouses adjacent to the one being fumigated, so that the gas will not escape into them. Open all doors and windows to the full. Start fumigating at the bay furthest from the entry and complete it at the bay closest to it. The one-time, overall treatment should be the rule for hothouse fumigation. It has the great advantage of the gas being delivered from outside the structure. When preparing the soil, pay special attention to the usually more compacted soil close to the hothouse door. To seal the soil, trench soil away from the walls to form a channel along the wall base and a soil mound parallel to it. Brush all soil from the walls into the channel. Hold the tarp smooth against the wall, so that its margin hangs in the channel rather like a bed sheet, with the soil being pushed from the mound beneath the tarp rather like the mattress holds a bed sheet margin firmly and smoothly against a wall, the mattress (here the soil) being sealed in by the sheets under flap, in the process. The sealing around posts can be problematic. It is sometimes worth slitting the margin of the tarp and firmly taping the overlaps to the post. No soil particle must be left above soil level on wall surfaces, post bases, etc., because it could cause recontamination after fumigation. If tarps between bays are not to be glued together, allow for a 35-cm overlap at each margin.

At the end of methyl bromide delivery, close all side ventilators and hothouse doors from the outside and hang up the required warning notices on doors and in conspicuous positions around the hothouse. Roof ventilation must be left open. Unauthorized personnel (and pet animals) must be forbidden from closely approaching or entering the hothouse during and for 48 h after fumigation or until it is shown by halide detector that it is free of gas. A gas mask with a methyl bromide filter or one supplying compressed air must be worn if there is a need to enter the hothouse during treatment or after when tarp edges must be partly raised for initial aeration (in most places, the use of a compressed air breathing apparatus is compulsory). Open wide all doors and side ventilators at the end of the 48–96-h treatment time. Lift tarp edges here and there and leave the hothouse immediately. After a 2-h period, remove the lay flat tubes and tarps, and if the tarps are to be reused, aerate them in the pen before storing. Ventilate the hothouse either for 4 days or until it is shown to be free of gas by a halide detector, before allowing operations to start up again. Ensure that gas mask filters are used according to manufacturer's instructions. Begin each day with a new filter. Clearly mark those filters which are lapsed and never reuse filters. If the gas concentration is high, change the filter more often than specified.

11.9.5 Safety Precautions

11.9.5.1 Handling the Containers

Cans must be opened with special can openers; instructions for using the special openers must be rigidly followed. Empty cans should be crushed and buried away from habitations. Cylinders have a dip pipe reaching to the concave bottom; gas (and added nitrogen) pressure will make the liquid methyl bromide rise through the tube when the valve is opened and the cylinder will empty itself. Cylinders must never be handled roughly, dropped, bumped, or dragged and must never be unloaded by rope sling, hooks, tongs, etc. The heavier cylinders must be firmly cradled and secured for transport on a suitable hand or fork truck. The valve-protection cap should be removed only when methyl bromide is to be delivered from the cylinder. This cap must be opened by an adjustable or other smooth spanner only. Instruction for delivering the gas must be rigidly followed. Empty cylinders must be returned to the manufacturer with closed valves and valve-protection and cylinder caps in place.

11.9.5.2 Storing the Containers

Cans and cylinders must be stored under lock and key, away from excessive heat, and upright and

tightly capped in well ventilated, dry, cool storage areas secured from children, unauthorized persons, and animals. Cylinders should be examined periodically for leaks by means of a halide detector lamp.

11.9.5.3 While Handling Methyl Bromide

Avoid inhaling gaseous fumes and splashing liquid fumigant on the skin, eyes, etc. Clothing, shoes, bandages, rings, etc., must be removed immediately if splashed. Since splashes will cause burns, they must be washed immediately from skin or eyes with copious amounts of water. Gloves must not be worn. They impede the rapid evaporation of the fumigant from the bare hands and, by prolonging contact, will only worsen skin injury. Air contaminated shoes and clothes fully before wearing them again. Do not eat, drink, or smoke during fumigation operations and shower and change into clean clothes and shoes at the end of the operation.

11.9.5.4 Hothouse Fumigation

The general provisions noted above also hold for hothouse fumigations. There are, however, some extremely important additional safety measures required, relating to the proper use and care of approved safety equipment, which, if strictly observed, will make hothouse fumigation a safe, effective operation. The use by all operators of approved, full-face, air-purifying respirators with methyl bromide canister filters and/or compressed air breathing apparatus. The use of suitable methyl bromide detection equipment (halide leak detector, gas detection tubes, etc.) to establish that safe entry levels (i.e., atmospheric gas concentrations of less than 5 ppm) have been reached inside the hothouse. The rule that anyone not wearing an approved full-face respirator or compressed air breathing apparatus shall under no circumstances enter the hothouse throughout the fumigation operation (i.e., from the start of gas delivery until the hothouse is declared safe for entry). The rule that work must be performed by qualified, responsible personnel and that it must be done in teams (or at least in pairs), so that anyone entering the hothouse for operational reasons shall, although wearing breathing equipment, be under observation by an operator outside the hothouse. A full-face respirator must be used strictly according to the manufacturer's instructions. Always check airtight fit before use, by placing face piece over the face, kinking the hose, and trying to breathe. An airtight mask will be sucked against the face and no air will leak into it from the edges. Prevent misting of the eyepiece by using an impregnated cloth to clean it.

During gas delivery under the polyethylene tarpaulins, the methyl bromide vapor canister is to be used for a maximum of 1 h. A new vapor canister is to be fitted routinely at the start of each day's operations. This vapor canister is suited to a safe upper working limit of 20 ppm – if it becomes necessary to come in contact with higher concentrations (e.g., when a lay flat or manifold must be reattached or a tear must be repaired), wear a compressed air breathing apparatus. Never reuse partially used canisters, even if not time-expired, and always keep them apart from new ones. Crushing the inlet of used canisters will insure that they are not used again. When using compressed air breathing apparatus, move to an area free of gas immediately if the warning whistle indicates the level of air in the cylinder is low. When wearing a respirator, a person with punctured eardrums may draw fumigant vapors in through his ears as a result of creating a slight negative pressure during inhalation. Any fumigant drawn this way will be exhaled into the inside of the respirator face piece and a poisonous concentration may build up inside the respirator. It is usually possible for persons with this defect to obtain complete protection by using cotton earplugs covered with oil.

11.10 Microplot Techniques

Microplots refer to the small field plots with barriers to retain the nematodes, which are useful to differentiate damage caused by single specie/genus of nematode and related population dynamics (Barker et al. 1985). They are specifically designed to overcome many of the difficulties associated with field plots. Microplots that give more control of the experimental variable than do

typical field experiments have been used by many scientists. Using microplots, types of barriers that may be constructed, methods of installations with various nematodes can be determined.

Microplot experiments yield useful quantitative data for most crops. They are useful to develop estimates of the effects of several nematode species on growth and yield potential of a given crop; to determine the relative importance for various physical and climatic parameters like soil type, temperature, and soil moisture; to study the differential responses of several cultivars to specific species; to characterize the population dynamics of several nematodes; to determine reproductive factors, equilibrium densities, and rates of decline of a single nematode species; to characterize nematode–host interactions; to identify and differentiate the roles of some soil microflora and microfauna associated with various disease complexes; to develop and evaluate nematode management tactics; to determine the differential efficacy of several nematicides on single and multiple nematode species; to manage nematodes in the study of biocontrol agents; and to determine the effect of rotation regimes and other management practices on nematode populations.

11.10.1 Types of Microplots

Most microplots used for nematological research vary in size from 25 × 25 cm to 1 m^2 soil surface area (Plates 11.6 and 11.7). Since available soil moisture becomes limited quickly, the smaller plots may pose problems for moderate- to large-sized plots, which can be minimized when automated irrigation facilities are adopted. Perennial plants may need a plot size of 5 × 5 m, while plots of approximately one-half m^2 are enough for most annual plants and also for small perennial plants.

11.10.2 Preparation and Infestation of Microplots

Preparation of microplots for infestation by the specific numbers and types of nematodes needs considerable effort and expense. Treat the soil with a fumigant like methyl bromide at a rate of 50–150 g/m^2 to destroy nematodes and other soil pests. Till the soil of each plot to a depth of 20–30 cm and 3 or more holes at least 40 cm deep should be made in each plot to facilitate penetration by the fumigant. Cover the entire plot area with plastic prior to treatment with the fumigant. It is advised to avoid introducing nematodes or plants into the plots for 1–2 months after treatment. Tillage of the soil two to three times after removal of the plastic cover will enhance the dissipation of the toxic gas. Nematode inocula to be used to infest microplots to the desired levels may be collected from greenhouse or monoxenically grown culture (Townshend and Potter 1980). Before the harvesting of nematode inocula, allow

Plate 11.6 Microplots with different treatments

Plate 11.7 Microplots-General view

plants to grow for 10–12 weeks. Standardized, chopped, infected roots or NaOCl-extracted eggs may be utilized to infest the plots to desired levels. It is better to add mycorrhizal fungi like *Glomus* spp. and other beneficial organisms like *Rhizobium* spp. for legumes into each plot at the time of nematode infestation. Add these organisms at the same rates to all plots including the control plots.

If infected root material is used to establish the required level of nematode level, a suitable quantity of healthy root material should be added into the control plots and lower density plots to give an equal amount of root material for reach plot. Several factors influence the quality of data obtained from microplots like preparation, precise infestation, and thorough mixing of the inoculum into each plot. Due to minimal competition, an abnormal result may be encountered sometimes like an annual crop yield two- to three-fold more than that which occurs with normally spaced plant populations that can be overcome with continuous normal spacing of plants within and between the microplots.

11.10.3 Major Limitations of Microplots

As with greenhouse and phytotron experiments, results obtained in microplots should be validated in parallel full-scale tests. Data thus obtained may be applicable to limited geographical and climatic regions. Contamination with multiple species is a major problem in microplots, particularly with nematodes like *Paratrichodorus* spp., which are difficult to eradicate with fumigants. Special efforts are needed to eradicate nematodes on deep-rooted plants prior to initiating experiments. There is a need for much hand labor and frequent use of expensive nematicide treatments. Data interpretation and inferences normally cannot be extrapolated directly to the field situation as in the studies involving the relationship of initial nematode densities to growth and yield of the plant.

11.11 Techniques of Evaluating Nematicides

To know the nematicidal activity of a wide range of agricultural chemicals, evaluation techniques are necessary. They are helpful to understand the influence of systemic nematicides on nematodes by continued and discontinued exposure and to know the persistence of systemic nematicides in the soil under various conditions. Experiments with nematicides provide convincing evidence that yields can be increased by nematode control. If use of nematicides is economically feasible, experiments

provide useful information on methods, results, and profits. When the use of nematicides is not profitable, demonstration of yield increases provides a strong incentive to find other nematode control methods. Experiments demonstrating yield increase in farm fields are evidence that nematologists are working on a subject which will be of benefit to farmers and will increase food supplies. Standardized nematicide experiments by cooperators of international projects may provide comparison of data from different parts of the world (Taylor and Sasser 1978).

11.11.1 Procedure for Measuring the Root-Knot Infestation by Using the "Indicator Plants"

Collect soil samples at 5-m intervals. Each sample should be taken from an area about 1 m^2 and should be a composite of four samples of about 500 cm^3 each. Samples taken from the soil layers about 10–20 cm deep are best. Number the samples and make a drawing showing the location of each. Place the composite samples in pots (tin cans/plastic bags) and plant two or three tomato seedlings grown in nematode-free soil or ten tomato seeds in each pot. Fertilize lightly and water every day. If seeds are planted, remove all but two or three seedlings from each pot. Allow to grow for 4 or 5 weeks and examine the roots. These are indicator plants to be used for estimating infestation of the soil. Remove the tomato plants from the pots and wash off the soil. Examine the roots for *Meloidogyne* knots. Rate each plant by using the scale (0 = no galls, 1 = 1–10 galls, 2 = 11–20 galls, 3 = 21–50 galls, 4 = 51–100 galls, 5 = 101 or more galls).

The results will give an indication of the nematode infestation of the plots. Make a diagram of the plots, locating the samples and indicating gall rating. Fields can also be selected at the end of a crop season by examination of living roots remaining in the soil. If the crop has a moderate infection and roots are not excessively decayed, there will probably be a good infestation for experimental purposes the next season.

11.11.2 General Methods

11.11.2.1 Preparation of Plots

Plow the soil and prepare it for planting (Taylor and Sasser 1978). Lay out plots. For example, if sixteen plots are needed, plots may be in four blocks of four plots each. Plots may be square or rectangular according to the shape of the land. Mark each plot with a stake showing the treatments. These stakes should be 4 or 5 cm wide and 50–60 cm long. They should be strong enough to last for the duration of the experiment.

11.11.2.2 Treatments

The four treatments are as follows:

1. Control, no nematicide
2. One-half the amount recommended by the manufacturer of the nematicide
3. The amount recommended by the manufacturer
4. Two times the amount recommended

Each treatment is applied to four plots randomized so that no two plots receiving the same treatment are adjacent.

11.11.2.3 Preparation for the Treatment

Calculate the number of grams of granular nematicide or the number of milliliters of liquid needed for each plot (T3), that is, the amount recommended by the manufacturer of the nematicide. The information is obtained from the label of the container or from the manufacturer's circulars. The amount may be specified as application rate per acre or per hectare. The following table (Table 11.10) gives amounts per square meter and amounts per 100 m of row for various application rates (Taylor and Sasser 1978).

Table 11.10 Calculating application rates for nematicides

Application rates for liquid nematicides				Application rates for granular nematicides			
US gal/acre	L/ha	ml/m^2	ml/100 m of row 30 cm wide	lb/acre	kg/ha	g/m^2	g/100 m of row 30 cm wide
1.0	9.353	0.94	28.06	1.0	1.121	0.11	3.36
2.0	18.706	1.87	56.10	2.0	2.242	0.22	6.73
3.0	28.059	2.81	84.18	3.0	3.363	0.34	10.09
4.0	37.412	3.74	112.24	4.0	4.484	0.45	13.45
5.0	46.765	4.68	140.30	5.0	5.605	0.56	16.81
6.0	56.118	5.61	168.35	6.0	6.726	0.67	20.17
7.0	65.471	6.55	196.41	7.0	7.847	0.78	23.54
8.0	74.824	7.48	224.47	8.0	8.968	0.90	26.90
9.0	84.177	8.42	252.53	9.0	10.089	1.01	30.27
10.0	93.530	9.35	280.59	10.0	11.210	1.12	33.63

Notes
1 US gal = 3785.3 ml = 0.832 imperial (British) gal
1 m = 3.281 ft = 1.094 yd = 39.37 in.
1 ha = 10,000 m^2 = 2.471 acres = 11,959.64 yd^2
1 acre = 43,560 ft^2 = 4,840 yd^2 = 4,046.9 m^2
1 US gal/acre = 9.353 L/ha
1 kg = 2.205 lb
1 lb/acre = 1.121 kg/ha

Check the calculations for T3 carefully. Then calculate one-half the amount for T3 plots and two times the amount for T4. Assemble all materials and equipment required and make a trial application on a piece of ground not to be used for the experiment. If this goes well, proceed with the application. Make a diagram of the plots and the field. This will help in locating the plots even if the stakes are lost and also in finding their location again in the future.

11.11.2.4 Planting and Care of the Plots

After the waiting period recommended by the manufacturer of the nematicide, plant the plots, following the best procedure being followed in the region. It is suggested that the experimental plots be weeded and cultivated. Plots should be visited at frequent intervals to be sure that nothing is going wrong, like infestation by insect/disease or accidental interference.

11.11.3 Publicity to the Farmers

Before harvest when plots show large differences, it is better to invite farmers to visit the plots, explain them about the procedure followed there, and advise them how to improve the yields by controlling nematodes. If possible, prepare and distribute the leaflets/handouts/literature on the importance of the particular nematode problem of that area with all details including the symptoms, spread, life cycle, survival, and management with more stress on the integrated management including the major insect pests, diseases, weeds, and nutrient deficiency, with good quality photographs. Arrange radio talks and television programs on the specific problem in detail rather than a general talk/show on nematode problems of several crops. Field days/farmers' meetings may be arranged during the completion of the experiment/demonstration in collaboration with the line department officials of agriculture, horticulture, sericulture, etc., and

also scientists of the nearby research stations/ institutes. The program may be given a wider publicity through local newspapers to reach large number of farmers.

11.11.4 Collection of Data

During the growing season, make notes of any differences in growth, death of plants, or wilting. If possible, take numerical data, plant height, percentages of dead or wilted, etc. Four to six weeks after planting, dig 5–10 plants from each plots and rate for galling as with the indicator plants. At harvest time, bags, crates, or other containers will be required to hold the produce, preferably one for each plot. Scales to obtain weights are needed. Data include yield of each plot, and if the produce is usually graded, the weight of each grade to be taken. After harvest, dig up the plant roots and rate them for root-knot galls, using the same method as for indicator plants.

Calculations: Calculate weights and value of produce from each plot and averages of the four plots of each treatment. This data can be analyzed by standard methods of analysis of variance.

Reporting: Prepare a report of the experiment in good form for publication and send to the appropriate journal.

General: Photographs of difference in growth in plots are useful. These may be color slides for use at scientific meetings and also black and white for publication, if needed. It may be possible to photograph the harvest in a way which will show differences between treatments and control. Be sure that administrators and supervisors have a full report of results.

11.11.5 Types of Specific Techniques

Three major types are common.

1. The penetration inhibition test (PI TEST)
2. The therapeutic test (T TEST)
3. The modified gall index test (GI TEST) (Bunt 1975)

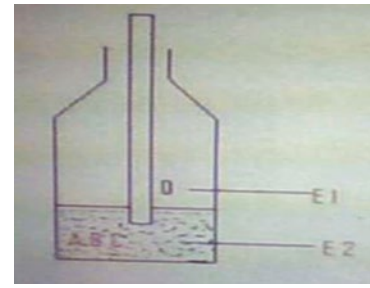


Fig. 11.5 Penetration inhibition test

11.11.5.1 The Penetration Inhibition Test

This test rates the property of all known nematicides to inhibit invasion by repellence, narcosis, or antifeeding.

Procedure Pipette a nematode suspension (*Ditylenchus dipsaci*) of 1 ml comprising about 400 specimens (B) into a 10-ml glass vial containing 5 ml of dry silver sand (A) (Fig. 11.5). Another 1 ml with the substance to be tested in the desired concentration (C) is also pipetted into vial. The sand is then just saturated. After 24 h incubation at room temperature, a 4-cm stem section of field bean, *Vicia faba* L., is placed a few mm into the moist sand (D). After another 24 h, the infective fourth larvae invade particularly the lowest 1 cm of the stem, which is washed free from adhering sand and cut off. After cutting the stem section once longitudinally, the pieces are placed in a counting tray in 5 ml water for 24 h to extract the number of penetrated but still viable nematodes (E_1), which are examined and counted after extraction. Untreated stem pieces in water instead of chemical solution usually have about 100 nematodes. The silver sand can also be analyzed for surviving *D. dipsaci* by placing the sand sample on a nematode extraction filter in a counting tray (E_2). Before field bean stem sections were found suitable as bait for *D. dipsaci*, pieces of potato tubers and carrots and also stem sections of potato and other plants should be tested.

11.11.5.2 The Therapeutic Test

This test is used to investigate nematicides which might affect nematodes already in

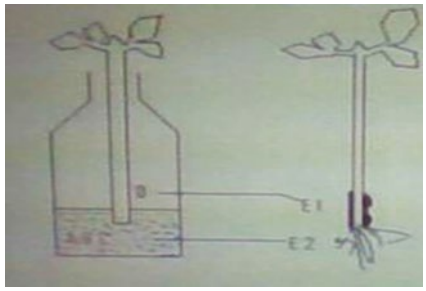


Fig. 11.6 Therapeutic test

infested plants. It is useful to study the effect of delayed treatment when nematicides have to be converted by the plant into nematicidal active derivatives; such nematicides may be missed in the PI test.

Procedure The test follows the procedure of the PI test. A 1-ml suspension with 400 *D. dipsaci* (B) and 1 ml solution of the test chemical (C) are pipetted into a vial containing 5 ml of dry sand (A), just saturating the sand (Fig. 11.6). After incubation at room temperature for 24 h, a tomato cutting is placed a few mm in the moist sand (D). At 15–20 °C it takes 6–8 days for *D. dipsaci* to cause swellings and discolor the tomato stem just below to a few cm above the soil (E_1). The silver sand is analyzed for surviving nematodes by placing the sand sample on a nematode extraction filter in a counting tray (E_2). An effective nematicide prevents nematode attack and no symptoms are seen. One week after placing the tomato cutting, the attack may be evaluated with the naked eye. Two to four replicates suffice for reliable results over a range of concentrations of a candidate nematicide. Thus, some hundreds of compounds can be tested by one person per week. The influence of the chemicals upon egg laying and numbers of larvae produced may also be determined. If further observation is needed, fertilizer is added and the plants are allowed to grow for 2–3 weeks. The first new larvae have then appeared if an inoculum of L_4 stage larvae is used. If such a longer growth period is desired, vials of a greater content, e.g.,100 ml instead of 10 ml, can be used to keep the sand moist. The cuttings can be inoculated in a small vial and the rooted cuttings infested with *D. dipsaci* transplanted to a larger container later on. For a complete therapeutic test, the cuttings can be allowed to root in the sand and *D. dipsaci* allowed invading the stems for 3–4 days before the chemical is introduced. A therapeutic systemic will prevent multiplication or kill the nematodes in the plant tissue. If more than 200 *D. dipsaci* specimens are used as inoculum, the first symptoms are visible after 3–4 days, but they do not become severe if effective systemics are added.

The test may also be used to study adsorption rate of a nematicide on organic matter and on other soil components. Inoculated, rooted cuttings transplanted to various soils are treated with a range of concentrations of the test chemical; the influence of adsorption can be estimated from the test plants by evaluation of symptoms, egg laying, number of larvae and adults, and sex ratio of the adult nematodes. The best time for evaluation depends on the information wanted. The techniques for nematode extraction are the same as for the PI test.

11.11.5.3 The Modified Gall Index Test

This technique is used to study the nematicide effect against *Meloidogyne* spp. in pots/greenhouse.

Procedure The gall index or GI test resembles the T test, but *M. incognita* is used instead of *D. dipsaci* and root galling is recorded instead of stem swellings (Fig. 11.7). A 1-ml suspension with about 500 *M. incognita* larvae (B), obtained by placing egg masses upon a nematode extraction filter, and a 1 ml solution of the chemical to be tested (C) are pipetted into a vial containing 5 ml silver sand (A). After incubation at 20 °C for 24 h a tomato seedling, from which the roots are taken off, is placed a few mm into the moist sand (D). Some days later new roots are formed and active *M. incognita* larvae in the sand will penetrate and initiate galling of the roots. After 7 days the roots are washed free from sand and are rated for galling under the dissecting microscope. With the naked

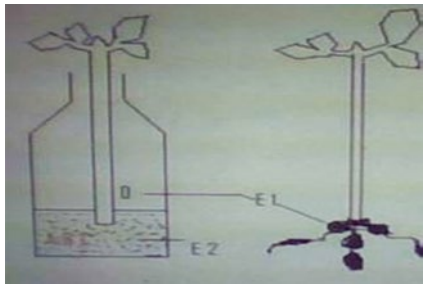


Fig. 11.7 Modified gall index test

eye the degree of galling can be estimated after about 10 instead of 7 days. The results may be given as estimates, but counted num ber of galls on the root is more exact (E_1). Surviving larvae can be extracted by placing the sand on a nematode extraction filter for 24 h (E_2).

The modifications compared to the original gall index test are as follows: tomato seedlings without roots are used, the bioassay takes place in vials of 10 ml comprising 5 ml pure sand, less chemical is required, the whole test is shortened from 6 weeks to 10 days, and less labor and greenhouse space is needed.

11.11.6 Points to Remember

The PI and T test appear to be adequate for screening large numbers of chemicals to detect nematicidal activity independent of the mode of action. Each method has advantages and disadvantages, but both tests cover a wider field of nematicidal effects and are easier to handle than methods in current use. In special cases, the GI test may be preferable, e.g., for tropical regions and also if microscopes are not available for evaluation; the last reason also holds for the T test. The advantages of all these tests is that they are bioassays; the test plants or plant parts are also used to indicate phytotoxicity of candidate nematicides, namely, by blackening of stem parts in the PI test and by various other symptoms in the GI and *T* tests. A phytotoxic systemic may damage the top leaves of the young seedlings and phytotoxic fumigants more often damage roots or lower parts of the stem. In a large-scale primary screening program, it is possible with each of the three techniques to test about 250 substances per person per week.

11.11.7 Preliminary Screening of Nematicides

11.11.7.1 Water Screening

Add 1 or 2 ml of nematode suspension containing approximately 50 juveniles into a vial. Add equal amount of stock solution of nematicide to give half the concentration prepared. Keep four replications of each treatment and check. Examine under stereo binocular microscope after fixed time.

11.11.7.2 Sand Screening

Add 1 or 2 ml of nematode suspension containing approximately 50 juveniles into a vial. Fill 2/3 of vial with thoroughly washed sand. Add 1/10th of the volume of the vial the prepared concentrations of the nematicide. Shake to mix sand, nematodes, and the chemical. Fill the vial with dry sand. Keep screw for 12 h. Wash the contents of the vial into a beaker and decant. Examine under a stereo binocular microscope.

11.11.7.3 Test Systems

Several test systems are available for the evaluation of nematicidal effects of fungal extracts or the ability of fungi to act as biocontrol organisms. Important ones are as follows.

11.11.7.4 The Microtiter Plate Assay

It is a fast assay for testing the nematicidal activity of crude extract or pure compound. Large numbers of samples can be handled while using relatively small sample amounts.

11.11.7.5 Water Agar Assay

In the water agar assay, the antagonistic properties of fungi for a potential use in biocontrol can be evaluated. These fungi can be divided into endoparasitic species which grow only within their host and precious species which grow in the soil and form special mycelial structures

functioning as nematode traps. Nematode trapping is restricted to mobile host stages whereas parasitism can be performed on mobile or immobile stages. A migration assay with lettuce seedlings in a Sephadex resin can be used for detecting toxic effects of fungal extracts or isolated compounds and their interference with the migration of larvae to the root tips. Phytotoxic effects of the compounds may also be seen. The ability of a compound to interfere with the invasion of second-stage larvae into the root tips and the gall formation can be evaluated in a test system for protective activity on agar plates. This assay also gives information about the stability of the compound and the duration of the effectiveness of the compound under favorable nematode growing conditions.

11.11.8 Techniques to Test the Efficacy of Nematicides

These techniques may be used for various purposes, viz., to evaluate the effects of chemical/nematicide treatments on hatching and emergence of juveniles and to obtain juveniles for in vitro evaluation of chemicals and for inoculations to evaluate chemical soil treatments (Steele 1978).

11.11.8.1 Procedure to Evaluate Nematicidal Effects on Hatching and Emergence of Juveniles from Cysts

Separate newly hatched cysts from sugar beet roots and soil by washing, floating, and decanting suspended debris into screens. Select the newly formed cysts with eggs and juveniles, manually separate from washed debris, and store until required. Transfer 2–5 ml of test solutions to the portion cups using a precalibrated pipette. Transfer groups of 20 nematode cysts with a fine bristle brush to a small wedge-shaped piece of filter paper before going to the collection cups (sieves). This prevents dilution of the test solution during transfer of the cysts. Incubate the hatching vessels and their contents at 24 °C during the entire test period. Treat the cysts with the nematicidal solutions for 1–7 days. Transfer to tap water, which is changed daily, for 4 days to remove the test materials. Place the cysts in a hatching agent for 2–4 weeks. Inoculate the host plant (e.g., sugar beet) with the cysts to evaluate nematode viability.

11.11.8.2 Procedure for Hatching Juveniles

Add washed and screened root debris containing cysts to a large screen (about 10 cm in diameter), which is placed in a funnel containing a hatching solution. Adjust the level of the solution so that the debris is wet but not completely covered. Insert the stem of the funnel through the top of the refrigerator cabinet. Maintain the cabinet interior around 8 °C and that of the solution bathing the cysts at 24 °C using an electronic thermoregulator equipped with a thermistor and heating tape. If ambient temperatures are above 24 °C, supplementary heating of the solution may not be needed.

11.11.8.3 Procedure for Evaluating Chemical Effects on Hatched Second-Stage Juveniles

Treat newly hatched second-stage juveniles for 24 h at 24 °C with aqueous solution of 1, 5, 10, 25, 50, and 100 µg/ml of test chemical. Estimate the effects of chemical treatments on mobility by placing the juveniles on tissue paper supported by collection cups (sieves) that are in turn placed in the chemical solution. Juveniles remaining on the tissue paper are assumed to be either immobilized or incapable of purposeful movement (disoriented). After the initial treatment, wash the juveniles with several liters of tap water. The nematodes are easily concentrated in a small volume of water using a Buchner-type funnel with fritted disc. Inoculate the roots of the host plant (sugar beet) grown in a steam-sterilized sand–soil mixture with treated and nontreated juveniles. At 18 and 30–35 days post inoculation, harvest the plants. Examine the roots and soil for adult nematodes. Specify the numbers of juveniles added per plant or unit weight of soil. Not less than 2,000 juveniles or eggs and juveniles from 20

selected viable cysts should be added per plant. If cysts are used, estimate the numbers of eggs and juveniles by counting them or by hatching the eggs in a solution containing a hatching agent.

11.11.8.4 Procedure to Assess the Efficacy of Nematicides

Evaluate the nematicides' efficacy one or more times during the growth period of the host plant. Obtain at least one count before production of the second nematode generation. Take up the counts on nematodes extracted from soil or plant tissues and may include any or all stages. To get the counts of adult males, harvest plants 18 days after inoculation and place roots on funnels in a moist chamber for 5–10 days. For counts of adult females, harvest the plants 30–35 days after inoculation.

11.11.8.5 Techniques to Evaluate Nematicides for Systemic Eradication of Root-Knot Nematodes

The objectives may include the evaluation of a nematicide for systemic eradication properties against root-knot nematodes (*Meloidogyne* spp.) present inside the root tissue prior to chemical treatment. This technique helps in evaluation of nematicides and their potential for post-planting use (Thirugnanam 1978).

Inoculum Procedure (Modified Baermann's Technique)

Wash the root-knot infected host (e.g., tomato) roots containing egg masses free of soil in tap water. Cut into small pieces and macerate in a blender for about 30 s. Pour the macerate evenly onto a screen (15–25 mesh) that is already layered with wet strength tissue and supported by two Petri dishes at one end in an inclined shallow tray. Add water to the tray and maintain at a level sufficient to keep the tissue layer moist during the incubation periods. During this period, the juveniles hatch on the screen and migrate toward water. Aeration of water keeps them alive. After 5–7 days of incubation, the aqueous larval suspension is stirred well to get uniform distribution of nematodes for microscopic counting. Dilute the aliquot further with water to facilitate counting.

Inoculation Procedure Wash 3–4-week-old seedlings grown (4–6 leaf stage) in steam-sterilized soil in running water to free the roots from soil particles. Place the root system of each seedling in a Petri dish. Pipette out 2 ml of aqueous nematode suspension containing about 500 second-stage juveniles directly onto the roots. Sprinkle the steam-sterilized soil over the entire rot system. Maintain this soil moist throughout the inoculation period. After 24 h, remove the seedlings and wash their roots thoroughly in running water prior to chemical treatment.

Nematicidal Treatment

Soil Incorporation: Treat the steam-sterilized soil with test compounds to obtain desired dosages. Apply the chemicals by thorough incorporation, drench, or surface treatment or by fumigation in the case of volatile compounds. Plant the seedlings exposed to nematode infection for 24 h in treated soils. Appropriate checks/standard chemical treatments/replications are to be maintained.

Foliar Application: Hold the seedlings horizontally and spray the foliage to run off with test compounds. Cover the root system, during spraying, to prevent direct contact with the chemical. When leaves are dry, pot the plants in steam-sterilized soil. Appropriate checks/standard chemical treatments/replications are to be maintained.

11.11.8.6 Determination of Eradicant Activity

Extraction of Eggs

After 4–6 weeks of treatment, remove the plants carefully from the soil. Wash the root system in water. Determine the number of eggs on each plant by extraction with 0.5 % NaOCl for 10 min and microscope counting. Express the eradicant activity of the test compound as the percent control of egg production using the following formula.

$$\text{Percent control of egg production} = \frac{\text{Number of eggs in check plant} - \text{Number of eggs in treated plant} \times 100}{\text{Number of eggs in check plant}}$$

11.11.8.7 Technique to Test the Phytotoxicity of a Nematicide

After having disinfested the soil with a nematicide, particularly in case of volatile chemicals, the grower may need to start planting or sowing as soon as possible, however, running the risk of phytotoxicity. The "watercress germination" test is a useful method to establish whether traces of the nematicides are still present in the soil.

Procedure Mix the soil thoroughly. Take a sample of 300 ml and pour this in the jars. Treat the soil with one of the nematicides. Close the jars with the lid or with plastic film. Ten days after treatment a small pad or moist cotton wool with watercress on it is suspended in the jar. The germination is indicative of the disappearance of the nematicide.

11.11.8.8 Points to Be Considered During Field Evaluation of Nematicides

Various test materials and environmental and cultural conditions influence the results of evaluation of nematicides under field conditions. The information that should be recorded during field testing of experimental nematicides for an effective evaluation is given hereunder (Johnson 1978).

Test Materials

Compare all the test materials with an untreated control and with a known standard, normally one of the materials currently recommended. The knowledge on the biological activity and chemical and physical properties of the test material is necessary. Review pertinent literature or technical reports before designing field trials.

Formulation

Record the following: formulation type, viz., emulsifiable concentrate, wettable powder, flowable, water soluble, and granular (mesh size); the names and percentage of every ingredient in the formulation; lot number on the package label; and dates sent and received. If a nematicide is diluted before application, specify the amount of diluent used by common and chemical names.

Rate or Rates of Application

Mention clearly and precisely the rates as formulation and active ingredient in one or more of the following terms: the quantity per unit of area if treated overall (broadcast), the quantity per linear distance and row spacing if row treated, and width of band and row spacing if band treated.

Number and Timing of Applications

Record the dates including the month, day, and year of preplant or postplant applications or both. Proper timing of application is very important. Specify the time of application in terms of crop planting date, emergence date, growth stage, preharvest interval, and intervals between applications. Specify the levels of the target nematode population and incidence. Record the plant size, stage of growth, or number of days since emergence for postplant applications or a combination of these.

Method of Application

Specify the method of application including specialized equipment. Specify the appropriate terms like spraying, injecting, soaking, spacing, rinsing, and flooding. Specify details in case of soil application like band width, row spacing, chisel spacing, depth of application, and time interval between application and incorporation. If applied on the surface, mention the method and depth of incorporation, if any. Row application details should include furrow row, band over row, or side dressed (preplanting, at planting, postplant, post emergence). For side-dressed applications, mention the placement in relation to seed or plant. Treatment may be broadcast, strip, row, site, root dip, or foliar spray and may be injected into the soil or applied to the soil surface as a drench, spray, granule, or solution in irrigation

water. Incorporate the granules into the top few centimeters of soil, or the active ingredient may be washed from the granules by irrigation or rainfall. Take care to prevent recontamination of treated areas by cultivation of other means, whereby soil from nontreated areas is blended with soil from treated areas. Application methods in irrigation water should specify overhead sprinkler, row or furrow, and basin.

Environmental and Cultural Conditions

Record the information on pretreatment, attreatment, and posttreatment environmental and cultural factors that might affect the efficacy of the nematicides. Sometimes, inconsistent/erratic results of incomplete experiments may occur due to some effects of these factors as relative humidity, wind, rainfall, and air temperature during the test period. Consider and explain/correlate the relationship among environmental and cultural factors to host, nematode, and nematicides.

Soil factors should include the identity of target and nontarget nematodes and their relative density before, during, and after testing; temperature; soil types including textural variations with depth; pH; field capacity; nutrient levels; organic matter percentage; presence or absence of crop trash/refuse; percentage of soil moisture; an estimate of drainage; amounts and frequency of rainfall or irrigation or both and type of irrigation (flood, sprinkle, row, basin etc.); other information that may affect the application and performance of the nematicide being tested; the occurrence and quantity of other organisms that affect crop growth and nematode populations; the application of fertilizers, lime, or other soil amendments like herbicides, fungicides, bactericides, or insecticides; and the cropping history and pesticide usage.

11.12 Crop Loss Assessment Due to Nematodes

Yield losses in crops due to phytonematodes are influenced by several criteria including the pathogenicity of the species of nematode involved, the nematode population density at planting, and the susceptibility and tolerance of the host and by a range of environmental factors. Because of this, available models only estimate yield losses as proportions of the nematode-free yield. Estimating threshold levels further involves various economic calculations. Consequently, predicting yield losses and calculating economic thresholds for most nematode/crop problems is not yet possible. What is needed is more field-based information on the relationship between nematode population densities and crop performance, and various approaches to obtaining such data are described. Measuring the population density, especially of *Meloidogyne* species, is a major problem (Trudgill 1992).

Major objective of crop damage assessment and prediction is to form a basis for nematode management decisions. A fundamental principle of nematode management is that disruptive management techniques should only be used if and where necessary (Ferris 1981). The decision basis may be economic but constrained by environmental considerations; it requires knowledge of the relationship between the organisms present and the expected crop loss. Environmental monitoring may be involved to determine the occurrence of conditions dictating the emergence or arrival of nematodes in the field, rather than by direct analysis and quantification of population levels. Management decisions based on knowledge of the relationships involved may be made at the plant, field, farm, crop, regional, national, international, or global levels. There should be some measure of the confidence which can be placed in the predictive capabilities of models used at each level of management decision. Users of crop loss models include growers for crop management decisions, nematode management advisors for developing and justifying appropriate management systems, and extension and advisory services. Another objective for crop damage assessments is to allow quantification of damage caused by phytonematodes at various levels of structural organization, so that research objectives and priorities can be planned, funded, and administered. The information is also used by horticultural economists and resource planners for nematicide development and benefit

assessment and by national and international policy-makers in determining the allocation and availability of food and fiber throughout the world.

Reliable sampling of nematode communities in crop loss assessment provides basic characterization of the population dynamics and damage potential of given species in a particular host–cultivar environment (Barker et al. 1985). Loss assessments for annual plants may be based on samples taken at time of planting, mid-season (where nematicide or resistant-cultivar treatments are used), and at harvest. Population levels at, or shortly after, harvest are most useful in projecting nematode hazards for a subsequent crop, especially where survival rates are known. Root symptoms, root-gall, or root-necrosis indices, taken at mid-season or at harvest, also can be correlated with yield losses caused by *Meloidogyne* spp. and associated fungi. A composite sample of 20–50 soil cores (2.5 cm diameter × 20 cm deep) collected in a stratified or systematic pattern over a 1- to 2-ha area provides nematode population estimates within about 30–50 % of the mean. The major problems encountered in relating these estimates to potential hazards and eventual crop losses are related to the striking variation in crop–nematode responses as influenced by environment, cultivar, and general crop management practices. Relative precision in crop loss assessment should be improved as more research on crop–nematode responses and sampling methodology is completed.

Disease assessment is the process of determining disease intensity in a population of plants using an accepted method. Disease incidence is the proportion of plants infected in a population, commonly expressed as a percentage, while disease severity is the proportion of plant tissue infected. The FAO has used disease intensity to mean either disease incidence or disease severity (Chiarappa 1981). Nematode-induced crop losses are a feature of both primitive and modern agricultural systems involved in food and fiber production. Because crop losses continue to be encountered while agriculture generally has become more input and energy intensive, much research has been directed toward identifying the causes of loss, quantifying losses, and forecasting losses for disease management.

11.12.1 Strategies for Loss Assessment

Using various approaches, crop loss data may be obtained dependent on the resolution of the data required for decision making and the definition of loss used. Crop loss actually refers to the difference between attainable yield and actual yield, according to the FAO (Chiarappa 1981). Loss is the reduction in quantity and/or quality of the economic yield of a crop. Some workers suggest that it is necessary to obtain loss data in quantities like kg/ha, while others feel that knowing the magnitude of loss (e.g., 1 = none, 5 = very severe) may be acceptable for setting policy on crop protection.

The major approaches employed to collect regional crop loss data are mentioned below (Teng 1981).

1. *Expert Testimony*: In this approach, knowledgeable scientists are asked to make a "statement of authority" on the extent of loss based on their experience with the crop and diseases in an area.
2. *Enquiries*: Estimates are solicited from a broad range of people concerned with the production of a crop in an area and a consensus is developed on the extent of loss. This approach resembles the "delphi" procedure used in research management.
3. *Literature Reviews*: Evaluation of published work not specifically designed for loss assessments for its value in giving estimates, for example, multiplication chemical and cultivar evaluation trials.
4. *Remote Sensing*: Satellite imagery is used in estimating crop area, crop yield, and crop loss. This is best suited for diseases that result in total plant loss, as in nematode caused diseases.
5. *Experiment/Survey Approach*: Yield and crop loss models are developed through field experiments and the models coupled with survey data to provide regional loss estimates. This is supposed to be the most objective method of deriving crop loss data for decision making at all levels (James and Teng 1979).

There is no single perfect approach for collecting regional crop loss data that has been generally accepted. While other approaches with the exception of remote sensing are mostly indirect that can be used to improve the reliability of direct approaches like experiment and remote sensing, the experiment/survey approach is the most direct and empirical approach, which requires several strategies, viz., quantification of nematode/disease, collection of data to measure the disease–loss relationship, modeling of the disease–loss relationship, and development and use of regional crop loss databases.

11.12.2 Quantification of Nematode/Disease

Nematodes have generally been quantified in terms of their numbers per unit of soil or plant part. Number of nematodes may be related to the intensity of plant symptoms, which in turn is a visual indication of the stress imposed on the plant, resulting in measurable loss. With nematodes, preplant nematode density is the most common descriptor used in quantitative relationships of loss. Field disease assessment is done normally, by using disease keys, standard area diagrams, remote sensing, and population counts.

Disease keys and standard area diagrams rely on the determination of severity in comparison with a predefined key or series of diagrams depicting different degrees of severity. The severity assesses for a plant part like a leaf includes the infected area as well as any accompanying chlorosis or necrosis. Remote sensing has been successfully used for assessment of losses due to nematode pests that cause total plant loss but has been only marginally successful with pests that affect only plant parts (James and Teng 1979). Population counts are widely used in quantifying diseases caused by nematodes. A problem in loss assessment is the determination of a representative mean value of the nematode/disease in a cropping unit using the designated method of assessment. Sampling for diseases populations is a relatively under-researched area in comparison with insect sampling. The distribution of a nematode/disease in any spatial unit may be mathematically described as a frequency distribution with estimated parameters, for example, normal or negative binomial.

Preliminary indications of the type of distribution are obtained by examining the mean: variance ration of the sample mean of disease intensity. Knowledge of the type of distribution in a field enables sampling protocol to be designed to obtain a representative mean in an economic manner (Lin et al. 1979). Nematode populations commonly occur as clusters, suggesting that the pattern of taking samples from a field is important (Barker and Olthof 1976). Various microprocessor techniques have been designed for use in fields. Portable, low-cost data acquisition system for measuring canopy reflectances, which may be used for determining the mean effect of a pest on a crop in terms of reduced crop vigor; laboratory-based video image analysis unit to measure the area of infected leaf tissue and proportion of infection; testing the taping of images of diseased leaves in the field with portable video cameras and analyzing the images in the laboratory; image analysis is routinely used for measuring the root area of plants and it is conceivable that there will be developments allowing its use for measuring nematode number in a sample (Lindow and Webb 1983).

11.12.3 Collection of Data to Measure the Disease–Loss Relationship

It represents the experimental phase of a disease–loss program, in which field data are collected either from fields with natural epidemics or from experimental treatment plots with different disease intensities. Several major methods have been briefed hereunder.

11.12.3.1 The Single Tiller/Plant Method

This method is used for collecting data to model the disease–loss relationship in fields in any cropping area, with varying disease in one season, and it includes the steps like tagging of hundreds

of tillers/shoots with care being taken to select tillers reflecting a wide range of severities, including zero and maximum disease; visit the fields using a predefined survey procedure, and in each field, disease intensity is assessed and tillers are harvested; each tiller becomes a single datum point for regression analysis; this method is a derivation of the paired-plant method in which pairs of healthy and diseased plants are tagged and observation is made on them through the growing season; natural epidemics are used in this method and there is economy of labor, space, and time (James and Teng 1979); it has mathematical limitations and models developed have only been able to explain a small proportion of the variation in yield due to disease; interplant differences in yield are a major source of variation in single tiller/plant studies. It may be improved by using measurements of plant parts not affected by disease, but related to potential yield, to correct for differences in observed yield and are able to reduce some of the variation.

11.12.3.2 The Synoptic Method

Initial procedures are to determine which of the parameters measured are appropriate in the yield-loss analysis. An advantage of the approach is that it requires and promotes consideration of all the factors likely to influence crop yield or crop loss (Ferris 1981). Predictive regression models are based on those variables deemed relevant by the initial analyses. As mentioned earlier, a real danger in the study of crop damage from a disciplinary standpoint is that factors outside of the discipline are not considered. This results in inflated estimates of crop losses by additive consideration of losses from individual pests. Consequently, interaction and discounting effects of damage overlap are ignored.

The synoptic approach represents a classical mutivanate statistical version of the systems analysis approach to an agroecosystem. All significant components of the system are considered. The complexity of the system is reduced through principal components analysis and determination of those parameters which provide the greatest explanation of the observations. In a systems analysis approach, the parameters and state variables considered important in determination of the output variables to be monitored would be selected intuitively. Generally, they would be in greater number and in greater detail than necessary and would be reduced by trial simulations of the system. Sensitivity analysis determines which state and input levels affect the system when they change. The multivariate statistical approach (Stynes et al. 1979) would be a rational and logical way of choosing the components for an agroecosystem model for a systems analysis and simulation approach to the assessment of crop damage.

11.12.3.3 Field Plot Techniques

Plots arranged using an experimental design like a randomized complete block are common in crop loss studies. The plots are either paired treatment or multiple treatment, where treatments are desired levels of disease or pathogen population. In crop loss assessment, the aim of treatments is to ensure that epidemics with different characteristics are generated using methods that may not necessarily be economic (James 1974). In the paired plot approach, healthy (protected) and infected (unprotected or inoculated) plots are situated near each other to constitute a replicate and the pairs repeated over many locations. With multiple treatment experiments, treatment extremes range from healthy (no disease) to maximum disease, with intervening levels of disease as the other treatments. Levels of disease have been generated on cultivars with different susceptibilities but comparable potential yield, by variation in planting date, by use of chemicals, and by use of differential inoculation.

Nematode population can be varied by growing hosts or nonhosts prior to experimentation, by inoculation, or by mixing infested with noninfested soil (Seinhorst 1981). Plot size is a major consideration in this technique. Use of microplots is a common practice by many nematologists. In practice, there is a trade-off between reducing inherent yield variation by increased plot size and increasing the variation due to soil factors when plot size is increased. In general, small plot size, as opposed to large plot size, results in higher variation between plots and

requires a larger number of replicates for the same difference between two treatments to be detected. The relationship between yield loss and disease at different growth stages has been conceptualized as a three-dimension response surface, where at each growth stage, disease–loss may be represented by curves. Experiments to model nematode population–loss relationships commonly focus on the initial nematode population. The precision of nematode models could be greatly improved if data is also collected on population levels during the cropping season.

11.12.4 Mathematical Models for Crop Damage Assessment

Models predicting final nematode densities from initial preplant nematode densities are generally based on modifications of the Verhulst logistic model (Seinhorst 1970). Models proposed for describing the relationship between numbers of nematodes and plant growth have been developed by Seinhorst (1965). They are elegant in concept, recognizing the decreasing influence per nematode as population densities increase. In general, the models pertain to annual crops and predict proportional yield loss in relation to preplant nematode populations. All these models are well described (Ferris 1981). One rationale for developing mathematical models is the simplification of the real world. Attention is focused on the components of the system that are critical as predictors of its output and, further, on the environmental parameters which affect the interactions among the components. Therefore, the model acts as a conceptual framework within which literature can be searched and current information assessed. It provides a summary of the state of knowledge of the system, and it allows the organization of available data and the determination of research needs by focusing on information gaps. A series of steps are prescribed in the development of the mathematical model. The regimentation involved in the procedure forces simplification and understanding of the system.

Step 1: The limits of the universe to be considered in the model are defined. It may be a horticultural field and the nematode communities limiting the production. Such a model would be extremely complex and involve a series of interacting subsystems. A simpler model would be a single plant and the phytonematode community parasitizing its root system. A danger of this simplification is that the resultant model may be misrepresentative in isolating one set of stresses on the growth of the plant and considering them apart from other interacting stresses in determining crop loss. Another simpler and less realistic situation is to consider the effect of parasitism of one nematode species on plant growth.

Step 2: The components of the system are defined. In the simplest case described, the components might consist of the nematode population system, the photosynthetic system of the plant, and the harvestable yield of the crop; even simpler components might be the numbers of nematodes and the amount of harvestable yield.

Step 3: The relationship between these components should be quantified. With nematodes on annual crops it is well documented that predictable relationships can be developed between harvestable yield and preplant nematode densities.

Step 4: The effect of environmental parameters on the defined relationship should be determined. This allows use of the model under a range of environmental conditions.

Step 5: Validation of the model is necessary before any quantification of crop loss due to nematodes is possible. One result of validation might be a realization that the original definition of the system was too narrow and that interaction with other organisms and other environmental conditions should be considered.

11.12.5 Types of Models

The empirical disease–loss models available in literature may be classified into various types, viz., single-point models, multiple-point models, integral models, response-surface models, nonlinear models, synoptic models, critical-point models, and simulation models.

11.12.5.1 Single-Point Models

These models relate to disease intensity at a specific time in the life of a crop, either a critical growth stage or a predetermined number of days into the growing season. Several models have been explained in case of fungal diseases and not much for nematode diseases, although similar concept holds good for nematode also. For example, in wheat stem rust, loss may be estimated from the percent stem rust severity at the ¾-berry stage (X) using this model (Romig and Calpouroz 1970).

$$\%\text{Loss} = -25.53 + 27.17 \ln X$$

In this example, the ¾-berry stage is identified as a time when the crop is most sensitive to rust. Fitting a single-point model to a data set does not imply that no other growth stages respond to diseases but rather that a particular stage only shows good statistical correlation. It is necessary to incorporate some physiological knowledge into regression models to ensure that the models are biologically meaningful. Another form of single-point model is that for estimating losses in potato due to late blight from the number of blight-free days (X), using the model as proposed by Teng and Gaunt (1985).

$$\text{Yield, t / ha} = 234.0 - 1.706\,X$$

Single-point models are the most commonly used disease–loss models mainly because they require relatively less data to develop. However, their application appears restricted to short-duration, late epidemics with stable infection rates. This model type assumes that disease dynamics before and after the single-point in fields resembles that encountered in the original experiments.

11.12.5.2 Multiple-Point Models

These models relate yield loss to several disease assessments during the life of a crop. The disease descriptors used in models are either disease increments during a defined period or disease intensities at identified growth stages. For example, use of model for estimating wheat yield loss due to leaf rust from three growth stages, X_2 (% rust/tiller at boot stage), X_5(% rust on flag leaf at early berry stage) and X_7 (% rust on flag leaf at early dough stage), using the model (Burleigh et al. 1972).

$$\%\text{Loss} = 5.3788 + 5.5260X_2 - 0.3308X_5 + 0.5019X_7$$

This type involves repeated measurement of the disease progress to predict losses. Such models have not been explored thoroughly in nematology (Ferris 1981). They may have potential for use with perennial crops but are unlikely to be useful with annual crops. In perennial crops, yield during a current year is frequently influenced by the amount of stored products accumulated during a previous year, which would be affected by previous levels of nematode stress. Consideration of nematode populations at a point in time in successive years might allow prediction of yield losses. The cost involved in sampling to determine population development at various points during the growing season would probably become limiting in the use of these models in annual crops. Further, the lack of available management techniques during the growing season would negate their usefulness. The workers who developed this model observed that though they could determine several single-point models from the same data, the multiple-point model explained the most variation in yield loss due to rust. Multiple-point models are particularly suited for epidemics that are long in duration, have unstable infection rates, and affect more than one yield component.

11.12.5.3 Integral Models

These models relate loss to a disease descriptor derived from summing disease intensities over a specified period of crop growth (Vander Plank 1963). Vander Plank proposed the area under the disease progress curve (AUDPC) as a method for analysis of wheat stem rust data. The model for estimating loss in cowpea due to leaf spot caused by *Cercospora* is an example (Schneider et al. 1976).

$$\%\text{Loss} = 0.43\,\text{AUDPC} - 14.95$$

AUDPC, in general, cannot distinguish between late or early epidemics since two progress curves with very different onset times and

infection rates could give the same area under the curve. AUDPC models can be applied for short-duration, late epidemics. The predictive ability of AUDPC models can be improved by assigning weighting factors to the disease assessments made at different growth stages which are used to calculate the AUDPC (Hills et al. 1980).

11.12.5.4 Critical-Point Models

The classical models of Seinhorst relating expected plant growth to preplant nematode population levels fall into this category. These involve measurement of a nematode population at one point in time, usually before planting, and they predict losses based on knowledge of some damage function. Critical-point models are frequently used in plant pathology. They allow prediction with a minimum of measurement and monitoring. The development of critical-point models has certain advantages for nematodes over other pest groups. Nematodes are relatively slow-reproducing organisms compared to fungi or bacteria. They do not have winged stages which may result in crop invasion at unpredictable times. For annual crops, the critical population density is that which is present at the time of planting. This allows the development of predictive relationships between preplant population densities and crop growth (Ferris 1981). The existence of such relationships is fortunate, since most management alternatives, including varietal selection and soil fumigation, are preplant decisions. Since the nematode population is present at the time of planting, it is not necessary to consider the phenological state of the crop at the time of pest invasion, as might be the case with a foliar pathogen or an insect pest.

The basic damage function model of Seinhorst (1965) $[y = m + (1 - m)zW\text{-}]I$ has a strong foundation in biological theory. Critical-point relationships have been used in determination of economic threshold levels of nematode populations. The nature of both the empirical and theoretical critical-point models is governed by three parameters: the tolerance level, the slope of the lines, and the minimum yield. These parameters are influenced by environmental and physiographic conditions. There is a need for researching the relationship between environmental conditions and the magnitude of the determinant parameters to allow interpolation and generalization of the critical-point models to a range of conditions. Critical-point models are usually developed for single nematode species, often in microplots. Another approach to generalizing critical-point models for multispecific communities is to make use of available information on nematode feeding, habits, biology, pathogenicity, and ecology. A "critical species" model is developed for crop damage relative to a nematode parasite of significant importance, and the pathogenicity of other nematode species is weighted relative to the critical species.

11.12.5.5 Simulation Models

Simulation models mimic the biology of the interaction of nematode, plant, and environment at their prevailing levels at any point in time. Rather than predicting crop damage based on a population density at a single point in time or at a series of points in time, such models are real-time explanatory models that are descriptive of both pest and plant biology. They deal with the actual conditions influencing the interaction rather than assuming average seasonal conditions affecting the relationship. Simulation models, when initially implemented, require frequent biological monitoring for confirmation. As confidence is gained in the model, monitoring can become less frequent (Ferris 1981). Simulation models deal with relative rates of growth of the nematode pest and plant populations. In poikilothermic systems, the metabolic rate is proportional to the temperature to which the system is exposed. Between definable upper and lower limits, the cumulative development is proportional to the cumulative heat to which the system is exposed. In damage assessment modeling, it is useful to consider the plant as a supply/demand system. The energy supply of the plant is a function of the rate of photosynthesis and the amount of photosynthetic surface at any point in time. The energy fixed is transformed into various plant parts according to a genetically determined sequence of growth priorities. More data are necessary to construct and use simulation models than are needed for critical- and multiple-point models. Plant and pest biology must be known to develop the

explanatory framework of the system. Much required information on growth and development of the organism and plant relative to environmental conditions can be extracted from the literature once this framework is established.

A major problem in real-time simulation with available weather data is that ambient conditions of temperature and moisture may not be the microclimate perceived by the plant or the pest. The microenvironment for a pest shaded under a plant leaf is very different from ambient conditions (Ferris 1981). Similarly, conditions for a nematode 6 in. below the soil surface differ from ambient atmospheric conditions. Soil texture, irrigation status, phenological growth stage, and plant shading effects are all important. There is a real need for on-site measurements of microclimate and for models which predict microclimate from ambient conditions relative to growth status of the plant and physical environmental parameters. A critical portion of simulation models is the coupling between subsystems to allow determination of crop damage relative to varying pest densities attacking the plant over time and to varying rates of plant growth. Both plant and pest subsystems are dynamic, and the state and nature of the interaction varies constantly. The modeling rationale is that the rate of photosynthesis is proportional to the efficiency of the root system, which is related to the proportion of the root system not damaged by vascular disruption. Simulation models are useful for research and analysis of crop damage through the partitioning of energy flow. They are also useful for prediction in the pest management decision process. A simulation model may be the most logical way of abstracting and conceptualizing the system in an analysis of the crop damage due to nematodes and other biological and environmental stresses in perennial crops. The numbers of computations involved in real-time simulation models require the use of a computer. The advent of high-speed, low-cost computers has made the use of simulation models a more practical reality. The flexibility of computer and simulation languages allows the modeler certain advantages not available in classical, analytical approaches, such as critical-point models.

11.12.5.6 Other Models

Teng and Gaunt (1981) conceptualized the relationship among disease, stage of the crop growth, and loss as a three-dimension response surface, which has been generalized as % Loss = f (disease, crop stage), thereby enabling loss estimation if the disease intensity and growth stage are known. This response-surface model may also be considered as integrated series of single-point models and various models have been developed fitting this concept. Response models require substantially more data to develop than the other models discussed previously and have led to research on alternative ways of experimentation to collect data, as explained in the previous section. Most disease–loss models assume a linear relationship, while it is generally recognized that biological relationships may be nonlinear (Madden et al. 1981). More than one model can commonly be found to fit a set of experimental data on any disease–loss system.

Although it is generally advisable to collect more data than is required for modeling because of the lack of prior knowledge on the form of the model, with some diseases and crops, however, enough is known of yield physiology to enable postulation of potential relationships (Teng and Gaunt 1981). This approach helps in guiding the design of experiments and pinpoint growth stages where it may be useful to have more treatments. The intended use of a model is another consideration in determining the form of the model, whether it is single point or multiple point. Several single-point models may be required in surveys, where fields may be visited only once. A multiple-point or integral model may be required to forecast potential yield loss that can account for fluctuating rates of disease progress in response to factors like nematicide application.

11.12.6 Modeling of the Disease–Loss Relationship

A mathematical model is a concise way of representing any system. In crop loss experiments, the usefulness of the data generated would be limited if the data is not reduced into a simple form.

Because of the many forms of the disease–loss relationship, there is no universal mathematical model to fit all these forms. The forms of relationships range from linear to sigmoid, and there are nine possible shapes of the disease–loss curve, according to Teng and Gaunt (1985). The mathematical description of the relationship depends on the disease descriptor (independent variable) used, like disease severity at one growth stage or area under the disease progress curve. In case of plant-parasitic nematodes, the log of nematode density is commonly used as the independent variable (Barker and Olthof 1976). The majority of mathematical models describing the disease–loss relationship have been derived using least squared regression techniques, although, recently, simulation modeling has been also tried (Ferris 1978). With regression models, several statistical criteria can be used to evaluate each model: F, r, s, and t (James and Teng 1979). Assumptions in the data collected for modeling need to be recognized and tested. For example, regression assumes that the variables show a normal distribution, yet this assumption is often violated in taking samples of disease data.

Among specific needs are the development of crop loss assessment methodology, the development of models and survey systems for collating the crop loss information and measurements, and estimates of confidence intervals and reliability which can be placed on the data developed.

11.12.7 Model Validation and Prediction

A problem with validating any model used to predict plant growth relative to a nematode population is that crop yield in a field without nematode stress will vary, even in the same region, due to microclimate differences and grower expertise (Ferris 1981). Seinhorst (1965) approached this problem using a relative yield measurement with yield loss represented as proportional decrease from the maximum yield for that field. A further generalization is the concept of minimum yield below which no further reduction was seen even at high nematode densities. Measurements of actual crop yield relative to nematode communities assessed prior to planting and weighted relative to edaphic and physiographic conditions can be used for validations of the conceptual models. This validation may result in a perception that the simplistic approaches suggested are inadequate and reveal a need for further consideration of the interaction among the determinant factors. A convenient tool for handling the problem of multiple-species nematode communities, differential host ranges, and varying environmental and edaphic requirements is to use an interactive scenario on a minicomputer. Information would be input for the actual conditions of a field for which the prediction is being made. Hence, the damage prediction is customized according to the nematode community, environmental conditions, crop type, and grower expertise. Management decisions are based upon the best current information of nematode biology, ecology, and expected damage (Ferris 1980).

Howard Ferris (1981) opined that the implementation of quantitative approaches involves real problems. The cost-effectiveness and reliability of nematode population assessment are poor. Since any use of predictive models in a management mode is based on population assessment, reliability becomes critical. There is a tremendous need for development of basic damage functions for key nematode species and crops as a basis for predicting yield losses from preplant densities. Simulation models require systems approaches to nematode biological problems and the availability of suitable plant models with which pest models can be interfaced.

Parts of farmers' fields are intensively sampled throughout the season (Stynes 1975). Variables measured include disease, nematodes, insects, soil, and water properties. Models are developed which explains a significant proportion of the yield variation caused by several factors. This method allows crop loss profiles to be developed, showing the contribution of each constraint in reducing attainable yield to actual yield. A limitation both for this and the single tiller method is that the range of disease severities for each disease may not be wide enough in any season, leading to an underestimation of its importance as a yield constraint.

11.12.8 Parameter Measurement and Estimation

Predictions of yield loss based on the assessment of nematode populations are limited by the reliability of the soil samples representing the field population. Problems in reliably estimating nematode population densities are well documented. They relate to variation in the macro- and microdistribution of nematodes as a function of their edaphic and environmental requirements and of their biological requirements in terms of feeding habits and reproduction pattern. Phytonematodes are primarily distributed according to the root system of their host, but their distribution and density varies with soil texture, previous cropping history, soil moisture-holding capacity, drainage and cultural patterns, and plant spacing (Ferris 1981). The vertical distribution of nematodes varies with food availability, root distribution pattern, and cultural and tillage procedures. Nematodes aggregate around specific feeding sites, such as root tips, and around areas of egg deposition, particularly in those species which are sedentary and deposit eggs in masses. The most reliable method of determining potential nematode stress on a crop would be to extract and count the nematodes from all of the soil in a field. A more practical reduction is to represent the field by a series of soil samples.

It is often desirable to assess or predict crop losses for areas larger than a single field. In such cases it is necessary to have data on nematode densities and distribution on a regional basis. In areas where nematode advisory programs have been conducted by public institutions for many years, a wealth of quantitative information is available on nematodes. Such information includes sampling sites, cropping histories, soil texture, and nematode population densities. If yield-loss prediction models were available, such data would allow assessment of losses, either predicted or historical, on a regional basis, according to the actual distribution of the nematode parasites of each crop. It is a practical impossibility to measure the response of each variety of a crop to each of its nematode parasites under every set of environmental conditions. Levels of horizontal resistance and tolerance will, however, dictate the expected quantitative response of individual varieties to the same nematode density. An approach to the problem is to determine the damage function relative to a range of nematode population densities for one common variety of the crop and to rank all other useful varieties in terms of their relative intolerance by greenhouse and field measurements of growth under nematode stress (Ferris 1980). The intolerance can be expressed on a zero to one basis relative to the least tolerant variety and used in weighting the damage function for specific cultivars.

It may be possible to evaluate the environmental suitability of the physiographic region and edaphic conditions based on literature currently available on nematode biology and ecology. The use of textural preference indices (Ferris and Duncan 1980) involves transforming the effect of the soil texture on nematode multiplication to a zero to one scale, in which one represents the maximum multiplication detected in a set of experimental data. Soil texture is arbitrarily quantified and a tripartite model fitted to the data with an optimal range of soil texture and a region of declining favorability on either side. For any nematode species, any portion of this tripartite model may be inoperative. Nematode counts from soil samples can be weighted for use in predictive models. Similar information can be gathered on temperature requirements of nematodes and used to express a regional preference or physiographic preference index for each nematode species. Depending on the location of the nematode community and the crop to be grown, it may be possible to weight the populations in that community according to their physiographic and textural preference indices, pathogenicity relative to a major pathogen of the region, and the relative intolerance of the host variety to be grown. This allows development of a weighted nematode stress total which is on the same relative basis as the population series for which the nematode damage function was determined (Ferris 1980). It allows prediction of potential yield losses for the proposed crop which can be used for management purposes and which are customized to the physiographic region, nature of the nematode community, the edaphic conditions, and crop variety.

11.12.9 Development and Use of Regional Crop Loss Databases

Regional crop loss databases have been developed for many countries through the use of sample surveys and the indirect methods previously dealt. This endeavor represents the next phase in most crop loss programs (James 1974). A regional database is considered to be a set of crop loss data collected over several definable spatial units like counties. Although the method of procuring the crop loss data may be different, it is common that such databases provide information on the prevalence/distribution, incidence and severity of diseases, and/or the related losses in percentage or tonnage (Teng and Gaunt 1981). Sound statistical sampling methods are to be used to determine field averages and to select representative fields. Field selection that represents a region has been based on simple random sampling, stratified sampling, or multistage sampling.

Stratification based on county or similar lines, crop reporting districts, or state boundaries appears to be most common in the literature, although stratification according to soil types, agroecological zones, or production systems has more interpretative value to scientists. The literature on ad hoc disease surveys is plentiful, and although not allowing historical trend analysis, these surveys have been useful in identifying diseases for further research and extension. With development in computer technology, isoloss lines have been plotted for Western Europe showing the mean annual loss in cereals due to each key pest. A combination of expert opinion, mail surveys, and literature reviews has been used to provide the data. Risk maps have also been plotted to show parts of the region where a certain disease has historically caused significant loss. A national pest survey database has also been developed in several countries for use in expert certification. Such and the Cooperative National Plant Pest Survey and Detection programs rely on computers and telecommunications for exchange of weekly pest data among states and central computer and will be capable of providing real-time pest and crop data during the growing season.

Individual state systems for computerized, online pest management are also in place. In Europe, the Dutch "EPIPRE" system of national pest management has been extended to other countries (Zadoks 1983). This system projects increases in pest populations and the potential economic loss, thereby providing a basis for control. In several countries like the USA, research has been directed at linking regional crop loss databases to decision making at the farm level. The "RUSTMAN" model for timing fungicide sprays on sweet corn makes recommendations based on rust severity in the field, estimated field loss, and rust severity in surrounding fields (Teng and Montgomery 1982). With increasing miniaturization of electronic equipment, matched by increasing computer power, it has been possible to deploy highly sophisticated models of nematode-loss systems in farmers' fields for nematode monitoring and management.

11.12.10 Nematode Damage Models

The nematode, the host, and the environment are the three interacting variables influencing the extent of yield loss in infested soils. An understanding of the mechanisms and principles involved in these interacting relationships is basic to being able to predict yield reductions from estimates of preplanting nematode population densities (Trudgill 1992). When modeling the damage caused to plants by root-feeding nematodes, several basic principles are to be considered like damage is proportional to the nematode population density; the degree of damage is influenced by environmental factors; and the yield harvested is determined by the amount of light intercepted by the crop, by how efficiently the intercepted light is converted into dry matter, and finally by how that dry matter is partitioned into non-harvested and harvested yield. For some crops significant variations in moisture content will also affect final yield; the above principles are more complex in practice.

Damage may be proportional to the nematode population density, but there are several qualifications of this statement, viz., the relationship is usually curvilinear, increasing numbers of nematodes having proportionally diminishing effects; there is some evidence that at low densities the host plant can repair the damage and that growth may even be slightly stimulated; Seinhorst (1965) termed the population density (Pi) at which damage first became apparent as the tolerance limit (*T*); equally, at very high values of Pi, increasing numbers of nematodes may not further reduce dry matter productivity. This minimum yield is termed "*m*." There are various reasons for the occurrence of "*m*"; there may be some growth before attack starts or after it finishes, and a significant biomass may be planted (e.g., potato tubers). However, "*m*" applies to total dry matter and because of effects on partitioning, the harvest value of *m* may be greater or less than that for total dry matter; the third parameter in the Seinhorst equation is *z*, a constant slightly less than one. The equation is as follows:

$$Y = m + (1 - m)z^{(\mathrm{Pi} - T)}$$

For $\mathrm{Pi} > T$

$y = 1$ where Pi is less than / equal to T

where y is the yield.

An important qualification is that y is expressed as a proportion of the nematode-free yield. Hence, according to Seinhorst, the greater the yield potential, the greater the loss in tons per hectare for any value of Pi. The Seinhorst equation is usually plotted with Pi on a logarithmic scale, producing a sigmoidal curve. In practice T is usually small and the Pi value at which m is reached is so large that it is only the central part of the curve that is of practical use. This approximated to a straight line (Oostenbrink 1966). The equation for such a line is $y = y\,(\max) - \text{slope constant} \times \log \mathrm{Pi}$.

The slope of the regression varies for several reasons. These include differences in pathogenicity (capacity to cause damage) between species, e.g., *Meloidogyne* spp. may be inherently more damaging than *Tylenchus* but there is no measure of their relative pathogenicities. Different plant species and varieties within species differ in their tolerance (capacity to withstand nematode damage). Also, there are large environmental influences on the damage suffered and particularly how that damage is translated into effects on final yield. An important consideration, often overlooked, is the basis of measuring Pi. Usually it is given as numbers per gram of soil. A more appropriate measure is per unit volume of soil as this allows for bulk density differences. Numbers per gram of root is probably the most appropriate, but is difficult to measure because it is always changing. This latter aspect becomes important when trying to relate results from experiments where root densities are very different, e.g., pot and field trials.

A further problem is encountered when considering damage by nematodes that have two or more generations in the lifetime of a crop. Usually the Pi is measured at planting, but on a good host population of, for example, *Meloidogyne* spp., they can increase from below the value of T to a level in mid-season where they cause significant damage. Even so, it is a race between increasing Pi and increasing plant size that brings with it increasing tolerance (in Seinhorst terms, increasing *m*). In such situations suitability as a host (susceptibility) and tolerance can have a marked effect on the degree of damage. To conclude, both the Seinhorst and Oostenbrink equations are, without the addition of a substantial amount of additional information, purely descriptive and cannot be used to predict actual yield losses.

11.12.11 Approaches of Estimating Yield Losses

Pot studies can be used to determine some of the basic information on yield-loss relationships, but because of environmental differences and interactions, field studies are also needed. There are two major approaches, viz., to use nematicides at relatively uniformly infested sites and to work at sites with a range of population densities but which are uniform in other respects (Trudgill and Phillips

2006). A combination of both approaches is often a happy compromise. The former gives practical information on the effectiveness and potential value of a particular treatment but tells little about the nature of the relationship. It also suffers from the criticism that nematicides have a range of side effects. The latter has the benefit of producing information on the relationship between Pi and yield, but it requires experimental errors to be minimized. Because Pi estimates have large errors, accuracy is improved by reducing plot size and by taking and processing multiple samples from each plot.

However, plot size must be large enough to obtain a realistic yield and adequate guard plants are essential. Another option is to establish many small plots in large but otherwise uniform fields. These can be at random, in a grid pattern or along known trends in Pi. The plots can be split and a nematicide applied to one-half. For each plot the Pi and yield are determined. The results will produce a scatter of points, hopefully with yield decreasing as Pi increases. Much of the scatter is due to errors in estimating Pi and yield, and it can be minimized by taking the average of all the results within each error band. Such an approach requires a wide range of initial populations, a uniform field, a large number of plots (100 or more), and the plots to be part of an otherwise uniform crop.

Chemical management of *Tylenchulus semipenetrans* consistently increased yield of grapefruit on sour orange rootstock in Texas (Timmer and Davis 1982). In this study, data from chemical control tests conducted from 1973 to 1980 were analyzed to determine the relationship between nematode counts and grapefruit yield and fruit size. The correlation between yield and nematode counts was negative ($r = -0.47$) and highly significant ($P < 0.01$). The data best fit the exponential decay curve: $y = 160.3x\,(-0.0000429)$ where y = yield in kg/tree and x = nematodes/100 cm of soil. The correlation between fruit size and nematode counts was not significant because yield and fruit size were inversely related. Yield loss in an average untreated orchard was estimated to be 12.4 tons/ha. Economic loss to citrus nematode in Texas grapefruit, assuming no treatment and an average on-tree price of S60/ton, was estimated to be S13.2 million annually.

References

Barker, K. R. (1985). The application of microplot techniques in nematological research. In K. R. Barker & C. C. Carter (Eds.), *An advanced treatise on meloidogyne, vol. I – Methodology* (pp. 127–134, 223pp). Raleigh: North Carolina State University Graphics.

Barker, K. R., & Olthof, T. H. A. (1976). Relationships between nematode population densities and crop responses. *Annual Review of Phytopathology, 14*, 327–353.

Barker, K. R., Townshend, J. L., & Michell, R. E. (1978). Determining nematode population responses to control agents. In *Methods for evaluation plant fungicides, nematicides and bactericides* (pp. 114–125, 141pp.). St Paul: The American Phytopathological Society.

Barker, K. R., Schmidtt, D. P., & Imbriani, J. L. (1985). Nematode population dynamics with emphasis on determining damage potential to crops. In K. R. Barker, C. C. Carter, & J. N. Sasser (Eds.), *An advanced treatise on meloidogyne: Vol. 2. Methodology* (pp. 135–148). Raleigh: North Carolina State University Graphics.

Barker, K. R., Gooding, G. V., Elder, A. S., & Eplee, R. E. (1972). Killing and preserving nematodes in soil samples with chemicals and microwave energy. *Journal of Nematology, 4*, 75–79.

Bhatti, D. S. (1988). *Utilization of toxic plants for the control of nematode pests of economic crops*. Final technical report (231pp). Hisar: Haryana Agricultural University.

Blakeman, R. H. (1990). The identification of crop disease and stress by aerial photography. In M. D. Steven & J. A. Clark (Eds.), *Applications of remote sensing in agriculture*. London: Butterworths.

Bridge, J. (1985). A technique for cryo-preservation of viable juveniles of *M. graminicola. Nematologica, 31*, 185–187.

Bunt, J. A. (1975). Effect and mode of action of some systemic nematicides (Technical Bulletin, 127pp.). Wageningen: Department of Nematology, Agricultural University.

Burleigh, J. R., Roelfs, A. P., & Eversmeyer, M. G. (1972). Estimating damage to wheat caused by *Puccinia recondite tritici. Phytopathology, 62*, 944–946.

Byrd, D. W. Jr., Kirkpatrick, T., & Barker, K. R. (1983). An improved technique for clearing and staining plant tissue for detection of nematodes. *Journal of Nematology, 14*, 142–143.

Chiarappa, L. (1981). *Crop loss assessment methods*. Slough: FAO/CAB.

Cobb, N. A. (1918). One hundred new nemas (Type species of 100 new genera). *Contribution to a Science of Nematology*, (9), 217–343.

Daykin, M. E., & Hussey, R. S. (1985). Staining and histopathological techniques in Nematology. In K. R Barker & C. C. Carter (Eds.), *An advanced treatise on meloidogyne, vol. I – Methodology*

(pp. 39–48, 223pp.). Raleigh: North Carolina State University Graphics.

Doncaster, C. C. (1962). Sealing microscopical water mounts with soft wax. *Nematologica, 7*, 2588.

Epstein, A. H. (1975, August 19–21). The role of remote sensing in preventing world hunger. In *Proceedings of the fifth biennial workshop on color aerial photography in the plant sciences and related fields EROS Data Center, Sioux Falls, South Dakota* (pp. 61–65). Sioux Falls: American Society of Photogrammetry.

Feder, N., & O'Brien, T. P. (1968). Plant microtechnique: Some principles and new methods. *American Journal of Botany, 55*, 123–142.

Fenwick, D. W. (1940). Methods for the recovery and counting of cysts of *Heterodera schactii* from soil. *Journal of Helminthology, 1*8, 155–172.

Ferris, H. (1978). Nematode economic thresholds: Derivation, requirements, and theoretical considerations. *Journal of Nematology, 10*, 341–350.

Ferris, H. (1980). Plant-parasitic nematode distributions in an alfalfa field. *Journal of Nematology, 12*, 164–170.

Ferris, H. (1981). *Mathematical approaches to the assessment of crop damage* (Plant parasitic nematodes, Vol. 111). London: Academic.

Ferris, H., & Duncan, L. W. (1980). Consideration of edaphic factors in quantifying nematode stress on plant growth. *Journal of Nematology, 12*, 220.

Franklin, M. T., & Goody, J. B. (1949). A cotton blue-lactophenol technique for mounting plant parasitic nematodes. *Journal of Helminthology, 22*, 175–178.

Godfrey, G. H. (1929). A destructive root disease of pine-apples and other plants due to *Tylenchus brachyurus n.sp. Phytopathology, 19*, 611–629.

Godfrey, G. H. (1935). A method of estimating the degree of nematode infestation. *Soil Science, 38*, 3–27.

Hafez, S. L., & Pudasaini, M. P. (2012, November 8). Sample the soil: Managing nematode populations. *Potato Grower*.

Heller, R. C. (1978). Case applications of remote sensing for vegetation damage assessment. *Photogrammetric Engineering and Remote Sensing, 44*, 1159–1166.

Hendricks, D. E. (1980). Low frequency sodar device that counts flying insects attracted to sex pheromone dispensers. *Environmental Entomology, 9*, 452–457.

Hills, F. J., Chiarappa, L., & Geng, S. (1980). Powdery mildew of sugarbeet: Disease and crop loss assessment. *Phytopathology, 70*, 680–682.

Hooper, D. J. (1990). Extraction and processing of plant and soil nematodes. In M. Luc, R. A. Sikora, & J. Bridge (Eds.), *Plant parasitic nematodes in Subtropical and tropical Agriculture* (pp. 45–68, 629pp.). Wallingford: CAB International Institute of Parasitology.

Hooper, D. J. (1970). Drawing and measuring nematodes. In J. F. Southey (Ed.), *Methods for work with plant and soil nematodes* (Technical bulletin 2, pp. 59–65, 148pp.). London: Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationary Office.

Hoper, D. J. (1970). Culturing nematodes. In J. F. Southey (Ed.), *Methods for work with plant and soil nematodes* (Technical bulletin 2, pp. 96–114, 148 pp.). London: Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationary Office.

James, W. C. (1974). Assessment of plant disease and losses. *Annual Review Phytopathology, 12*, 27–48.

James, W. C., & Teng, P. S. (1979). The quantification of production constraints associated with plant diseases. *Applied Biology, 4*, 201–267.

Johnson, A. W. (1978). Test materials and environmental conditions for field evaluation of nematode control agents. In *Methods for evaluation plant fungicides, nematicides and bactericides* (pp. 106–108, 141pp.). The American Phytopathological Society.

Killebrew, F. (1999). *Nematode control in the garden through solarization, Bull* (7pp). Mississippi: Mississippi State University Extension Service.

Lin, C. S., Poushinsky, G., & Mauer, M. (1979). An examination of five sampling methods under random and clustered disease distribution using simulation. *Canadian Journal of Plant Science, 59*, 121–130.

Lindow, S. E., & Webb, R. R. (1983). Quantification of foliar plant disease symptoms by microcomputer designed video image analysis. *Phytopathology, 73*, 520–524.

Madden, L. V., Pennyspacker, S. P., Antle, C. E., & Kingsolver, C. H. (1981). A loss model for crops. *Phytopathology, 71*, 685–689.

Manzer, F. E., & Cooper, G. R. (1982). Use of a portable videotaping for aerial infrared detection of potato diseases. *Plant Disease, 66*, 665–667.

Mc Beth, C. W., Taylor, A. L., & Smith, A. L. (1941). Note on staining nematodes in root tissue. *Proceedings of the Helminthological Society of Washington, 8*, 26.

Minderman, G. (1956). Aims and methods in population researches on soil-inhabiting nematodes. *Nematologica, 1*, 47–50.

Nageswara Rao, P. P., Jayaraman, V., & Chandrasekhar, M. G. (1991). Applications of remote sensing in plant protection. *Technical Bulletin National Natural Resources Management System*, Indian Space Research Organization, 1–2pp.

Oostenbrink, M. (1960). Estimating nematode populations by some selected methods. In J. N. Sasser & W. R. Jenkins (Eds.), *Nematology* (pp. 85–102). Chapel Hill: University of North Carolina Press.

Oostenbrink, M. (1966). Major characteristics of the relation between nematodes and plants. *Meded Landbouwhogeschool Wageningen, 66*, 1–46.

Ravichandra, N. G. (2010). *Methods and techniques in plant nematology* (595 pp). New Delhi: Prentice Hall of India Publications.

Reid, E. (1955). A rolling method for opening cysts of potato eel worm. *Plant Pathology, 4*, 28–29.

Romig, R. W., & Calpouroz, L. (1970). The relationship between stem rust and loss in yield of spring wheat. *Phytopathology, 60*, 1801–1805.

Schneider, R. W., Williams, R. J., & Sinclair, J. B. (1976). *Cercospora* leaf spot of cowpea: models for estimating yield loss. *Phytopathology, 66*, 384–388.

Seinhorst, J. W. (1950). De betekensis van de toestand van de grond voor het optreden van aantasting door het stengelaaltje (*Ditylenchus dipsaci* (Kuhn) Filipjev). *Tijdschr PlZiekt, 56*, 289–348.

Seinhorst, J. W. (1962). Modifications of the elutriation methods for extracting nematodes from soil. *Nematologica, 8*, 117–128.

Seinhorst, J. W. (1965). The relation between nematode density and damage to plants. *Nematologica, 11*, 137–154.

Seinhorst, J. W. (1966). The relationships between population increase and population density in plant-parasitic nematodes. I. Introduction and migratory nematodes. *Nematologica, 12*, 157–169.

Seinhorst, J. W. (1970). Dynamics of populations of plant parasitic nematodes. *Annual Review of Phytopathology, 8*, 131–156.

Seinhorst, J. W. (1981). Growth and yield of oats at a range of *Heterodera avenae* densities and under different watering regimes. *Nematologia, 27*, 52–71.

Southards, C. J. (1965). Host parasite relations of the lesion nematode, *Pratylenchus brachyurus, P.zeae* and *P.scribneri* and flue-cured tobacco, Ph.D. thesis. Raleigh: North Carolina State University, 101pp.

Southey, J. F. (1970). *Laboratory methods for work with plant and soil nematodes* (Technical bulletin 2, 148pp). London: Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationary Office.

Southey, J. F. (1986). *Laboratory methods for work with plant and soil nematodes* (Ministry of Agriculture, Fisheries and Food Reference Book No. 402, 202 pp). London: HMSO.

Steele, A. E. (1978). Tests for nematicidal efficacy using larvae of *Heterodera schachtii*. In *Methods for evaluation plant fungicides, nematicides and bactericides* (pp. 105–108, 141pp.). Minnesota: The American Phytopathological Society.

Stynes, B. A. (1975). *A synoptic study of wheat*. Ph.D. thesis, University of Adelaide, South Australia, 214pp.

Stynes, B. A., Wallace, H. R., & Veitch, L. G. (1979). A synoptic approach for crop loss assessment used to study wheat. I. An approach of the physical and chemical soil properties in the study area. *Australian Journal of Soil Research, 17*, 217–225.

Taylor, A. L., & Sasser, J. N. (1978). *Experimental and agronomic use of nematicides* (International Meloidogyne Project). Raleigh: North Carolina State University.

Teng, P. S. (1981). Data recording and processing for crop loss models. In L. Chiarappa (Ed.), *Crop loss assessment methods-supplement 3* (pp. 105–109). FAO/CAB, Slough.

Teng, P. S., & Gaunt, R. E. (1981). Modeling systems of disease and yield of loss in cereals. *Agricultural Systems, 6*, 131–154.

Teng, P. S., & Gaunt, R. E. (1985). Modeling systems of disease and yield loss in cereals. *Agricultural Systems, 6*, 131–1541.

Teng, P. S., & Montgomery, P. R. (1982). RUSTMAN: A portable, microcomputer based economic decision model for sweet corn common rust control (Abstr.). *Phytopathology, 7*2, 1140.

Thirugnanam, M. (1978). Evaluation of nematicides for systemic eradication of root-knot nematodes. In *Methods for evaluation plant fungicides, nematicides and bactericides* (pp. 103–104, 141pp.). The American Phytopathological Society.

Timmer, L. W., & Davis, R. M. (1982). Estimate of yield loss from the citrus nematode in Texas grapefruit. *Journal of Nematology, 14*, 582–585.

Townshend, J. L., & Potter, J. W. (1980). Population behavior of *Meloidogyne hapla* under four forage legumes in microplots. *Canadian Journal of Plant Science, 60*, 293–294.

Trudgill, D. L. (1992). Resistance to and tolerance of plant-parasitic nematodes in plants. *Annual Review of Phytopathology, 29*, 167–192.

Trudgill, D. L., & Phillips, M. S. (2006). Resistance to and tolerance of plant-parasitic nematodes in plants. *Annual Review of Phytopathology, 29*, 167–192.

Ulaby, F. T., & Moore, R. K. (1973). *Radar spectral measurements of vegetation* (RSL Technical Report, pp. 177–240). Lawrence: Univ. Kansas Centre for Research Inc.

Vander Plank, J. E. (1963). *Plant diseases: Epidemics and control* (349pp). London: Academic Press.

Wehunt, E. J. (1973). Sodium-containing detergents enhance the extraction of nematodes. *Journal of Nematology, 5*, 79–80.

Zacheo, B. (1987). Stimulation of respiratory pathways in tomato roots infested by *Meloidogyne incognit*a. *Nematologica Mediterranean, 15*, 235–251.

Zadoks, J. C. (1983). An integrated disease and pest management scheme, EPIPRE, for wheat. In *Better crops for food* (Ciba Foundation Symposium 97, pp. 116–129). London: Pitman Books.