Chhattisgarh Samvad

Practical Manual on Introductory Nematology

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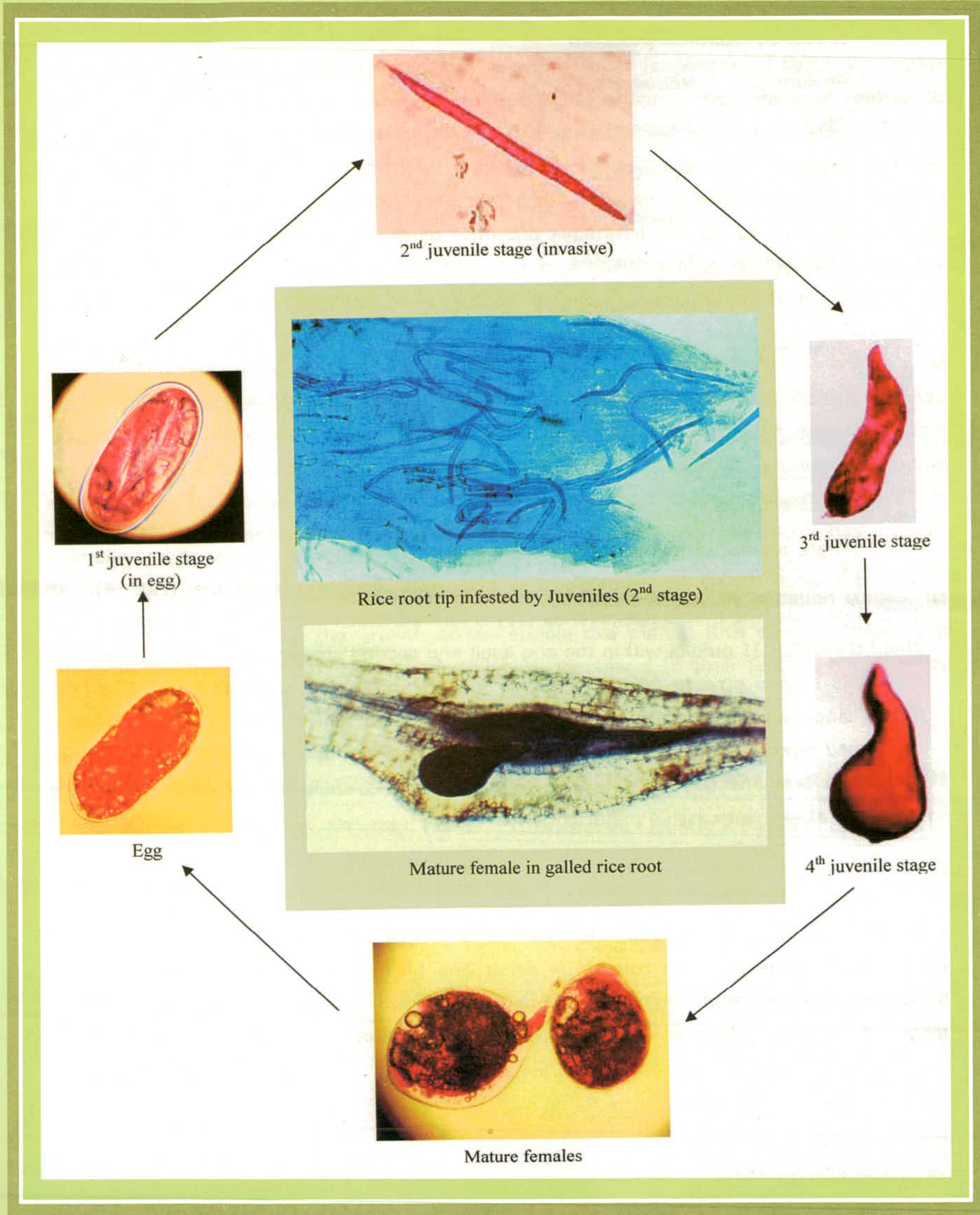
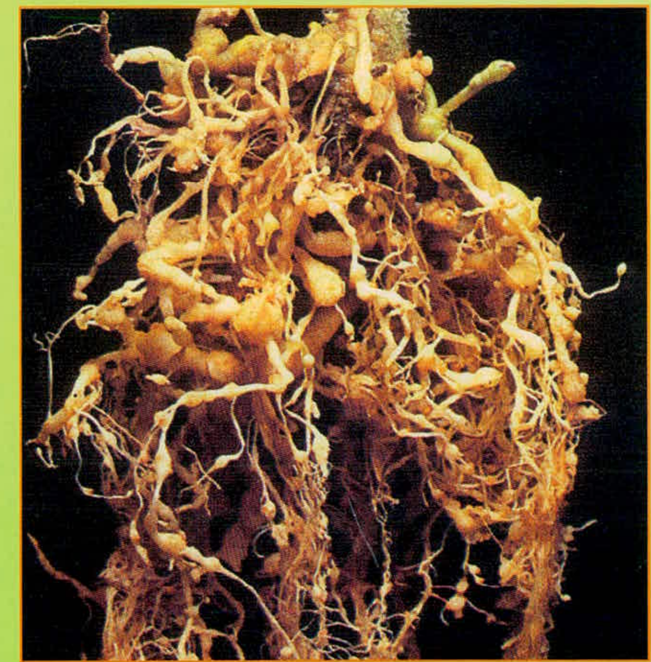


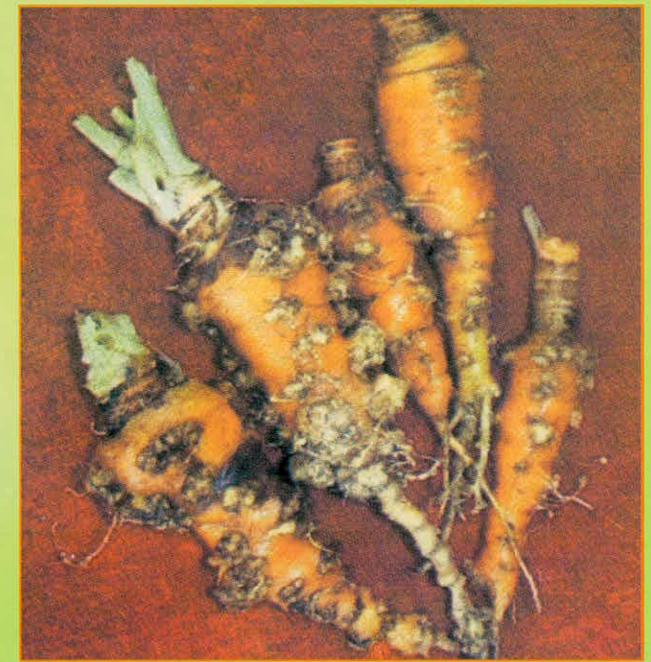
Life cycle pattern of root knot Nematode

Root knot of Papaya

Root knot of Beans



Root knot of Paddy



Root knot of Carrot

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Content

SN	Title of the Practical	Page No.
1	Collection of soil and plant sample	1-5
2	Laboratory techniques for examination of nematodes	
	1. To study the Baerman funnel method of nematode extraction.	6-8
	2. To study the Tray/dish/pie pan method of nematode extraction.	9-10
	3. To study the Sieving and gravity method of nematode extraction.	11-13
	4. To study the Centrifugal floatation method of nematode extraction.	14-15
	5. To study the Trituration or maceration method of nematode extraction.	16-17
3	Extraction of nematode eggs	18
4	Slide preparation.	19-21
5	Preparation of perineal patterns.	22-25
6	Counting of nematodes.	26
7	Symptoms of nematode damage	27-29
8	Typical life cycle pattern of nematode	30-32
9	Identification of important nematodes	
	A. Burrowing nematode (*Radopholus*)	33-34
	B. Lesion nematode (*Pratylenchus*)	35-36
	C. Rice-root nematode (*Hirschmanniella*)	37-38
	D. Root-Knot nematode (*Meloidogyne*)	39-40
	E. Cyst nematode (*Heterodera*)	41-42
	F. Citrus nematode (*Tylenchulus*)	43-45
	G. Rice white tip nematode (*Aphelencoides*)	46-47
	H. Seed and leaf gall nematode (*Anguina*)	48-49
	I. Dagger nematode (*Xiphinema*)	50-51
	Glossary	52-54
	References	55

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Exercise No.: 1

Title :- Collection of soil and plant sample.

Objective :- To collect the soil and plant sample for nematode analysis.

To diagnose nematode damage correctly, it is essential to collect samples, extract, identify and record the population of nematode associated with the injured plants or present in the rhizosphere of affected plants. To do these, following subheads will be executed necessarily step by step for exact diagnosis of nematode (s).

Sample collection

It is most essential part of diagnosis. If the samples are not collected properly, outcome of the efforts may be misleading. The nematode in a sample must truly represent the underlying population at a given time. In this context, it is necessary that investigator should know certain points like where to sample, when to sample, how to sample, care of the collected sample(s) etc. for precise and authentic result(s). All these points are elaborated as under :

(i) Where to sample

Any location where plants do not grow well (when the weather, soil fertility and other conditions are taken into consideration) could be an area of nematode damage. It is not necessary that an entire field may be affected by nematode. Soil samples should be collected from the rhizosphere of comparatively unhealthy/weak plants. Soil samples around severely affected or dead plants should not be collected because nematode populations on these plants have usually declined.

For foliar plant parasitic nematodes, plants parts (stems, leaves, blossoms etc.) should be collected showing a range of symptoms. These include plants with unusual patterns of shoot growth, galls, rots and abnormal discolourations such as browning, blackening or yellowing.

(ii) When to sample

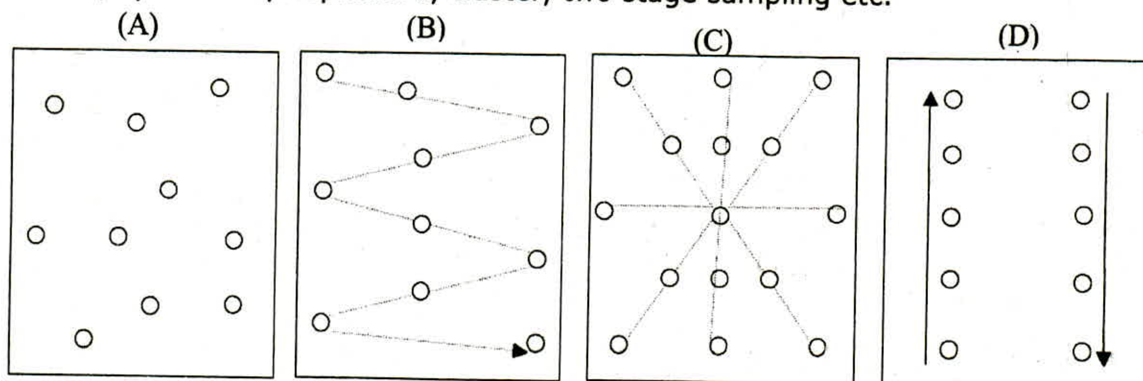
It is primarily essential to have the knowledge of life cycle of suspected/ targeted nematode(s) in soil, roots and aerial parts in determining the ideal time for

sampling in general and annual crop plants in particular, in which nematode population fluctuate periodically.

- If the identity of nematode species in sampling area is unknown, however, sampling should be done late in the growing season because nematode population is highest at that time.
- Sampling should be done when the soil moisture is slightly below the field capacity (soil neither dry nor wet). At this stage, sample collection become easier and eases in preparation for analysis.
- Sampling can be done at any time of the year, as long as sampling tools can be inserted in the soil to the proper depth. The best time is usually when damage is first noticed.
- Collected samples should be well protected from direct sunlight and store in cool (not refrigerated) and dry place until further processing.

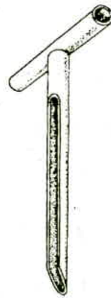
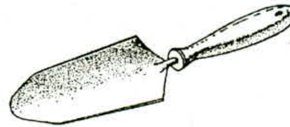
(iii) How to sample

Nematodes are never distributed uniformly in the soil. They are abundant close to plant roots and comparatively less in other parts of the field might be due to wetness, soil type and other physical and edaphic factors. Thus, sample a field systematically to obtain a sample that give precise and accurate picture of the overall population. The possible sampling patterns are simple random, stratified random, systematic, haphazard, cluster, two stage sampling etc.



(Patterns for nematode sampling (A) Random, (B-D) Systematic sampling)

Useful tools for sampling are spade, hand trowel, shovel, screw driver, soil auger {(corer) 2.5 cm in diameter}, knives, scissor, polythene sample bags (re-closable), tags, water proof marker and Veilmeyer sampling tube, if required.

Spade soil sampling tube Trowel Screw Driver Soil augar

Fig 1: Various sampling tools for soil sampling

- Sampling is best done with a coring device. In general, soil cores are taken to a depth of 15-20 cm (6-8 inches). Usually, discard the top 3-5 cm (1-2 inches) of soil layer because extremes of temperature and moisture and absence of suitable host tissues limit the nematode populations (except in turf and few other crops).
- Soil cores should be taken to a depth of 60 cm (24 inches) for tree crops and other deep rooted perineals.
- Several subsamples of soils should be collected from the fibrous feeder root zones of growing plants, around the drip line of trees and other perineals and in and between rows of annual crops (Fig.1).
- Four to 20 or more subsamples or cores should be collected depending on the size of the plant, bulk and mixed thoroughly and gently (nematodes grinds between soil particles when excessive mixing is being done and render the sample useless).
- Samples should be collected from apparently healthy to moderately affected plants.
- Samples collected should have feeder roots.

Agronomic and vegetable crops

Depending upon the size of the area, number of subsamples needed are as under :

a. For a small area (less than 500 m^2) - collect 8-10 subsamples

b. For a medium sized area (500 - 4000 m^2 or 1 acre) - collect 10-15 subsamples

c. For a large area (4000-20000 m^2 or 1 - 5 acre) - collect 20 subsamples (as many as 40, if possible)

It is important that subsamples should be from an area of a uniform soil type.

Sometimes characteristic symptoms of nematode infestation are present on the roots. If the plants are still in seedling stage or relatively small, three or four plants dig up carefully, gently remove the soil from roots and kept the plants and soil in separate bags.

Trees and woody plants

With the help of soil auger, dig 30-38 cm (12-15 inches) of soil after removing the upper 5 cm of soil around affected trees and shrubs.

Dig at the outer drip line and in the area between the outer branch tip and the stem.

Ornamentals and herbs

From the exposed soil profile around the plants where roots are visible, take small quantity and some feeder roots from various depths, mixed thoroughly and keep at least 1 Kg for processing.

In case of foliar nematodes suspected, whole crown of the leaves or 5-6 leaves and lower portion of the affected plants should be collected. Collected stems and leaves should be kept in moist conditions.

Bare soils

With the help of narrow bladed shovel and trowel, collect 20-40 subsamples to a depth of 20 cm after discarding to 5 cm soil in a zigzag manner beyond the area of subnormal production. Limit each composite sample to an area of 5 acres.

Care and labeling of collected samples

Since nematodes are living creature and they required moisture and moderate temperature to survive. In this context, it is most important to protect the collected samples from drying, cold (<4°C), heat (> 27°C), anaerobic conditions and rough handling. Collected plant and soil samples should be directly placed in the thick polythene bags (re-closable), labeled properly on the outside and kept immediately in a cool place or in insulated box. Collected samples are best examined within few hours or days after collection, if not possible then store for one

to three weeks in a refrigerator or incubator. The following information must be attached with the samples or written on the bags itself for making proper recommendations for management of nematode population.

1. Name, full address and contact number of the farmer and person collecting the samples.
2. Date of collection and date of submission for analysis.
3. Name of the plant, variety or cultivar, if known.
4. Location of the field or area.
5. Percentage/portion of the growing area represented by the sample(s).
6. Typical symptoms noticed and distribution of symptomatic plants (clustered, in spots, uniform, scattered).
7. Extent of damage.
8. Cropping history of the past three years including cultural and chemical management practices.
9. Description of weather conditions for two weeks prior to symptom development.

Exercise no. : 2

Title :- Laboratory techniques for examination of nematodes.

Objective :- 1. To study the Baerman funnel method of nematode extraction.

Stereoscopic (dissecting) or inverted compound microscope is usually used for examination of nematodes at magnification of 10-50X in incident or transmitted light. Extraction of nematodes from plant and soil sample is based on certain characteristics of nematodes like motility, size and specific gravity.

Depending on the type of nematodes and plant materials, plant parasitic nematodes can be extracted from plant and soil in many ways. Mist extraction, Baerman funnel method, soaking in water, trituration or agitation in a shaker are the methods used for extraction of nematode from roots and other plant parts like bulbs, tubers, crown, stems and leaves. Centrifugal flotation method should be used for extracting cyst forming nematodes.

Minimum four to six individuals should be examined because variation occurs in morphological characteristics of individuals in same genus or species.

- This method is generally used for extracting juveniles and active nematodes from very small samples (30-100 cm^3).
- During the incubation, eggs of nematode will hatch and subsequently second-stage juveniles will also be recovered.
- Recovery of nematode may vary according to the sample size, time of storage, nematode species, temperature etc. but majority of nematodes recovered within 24-48 h in this method.

Material required

Paper towel/ double layer muslin cheese cloth/ cotton wool (vermiform live nematode can pass through), course plastic or stainless steel screen with flat bottom 17.5 cm in diameter, glass funnel 10-15 cm in diameter with 10-15 cm long stem, rubber tubing 8 cm long and fitting around the stem of the funnel, funnel stand, stopper clamp for close off of tubing, beakers (50/100 and 250 ml).

Method

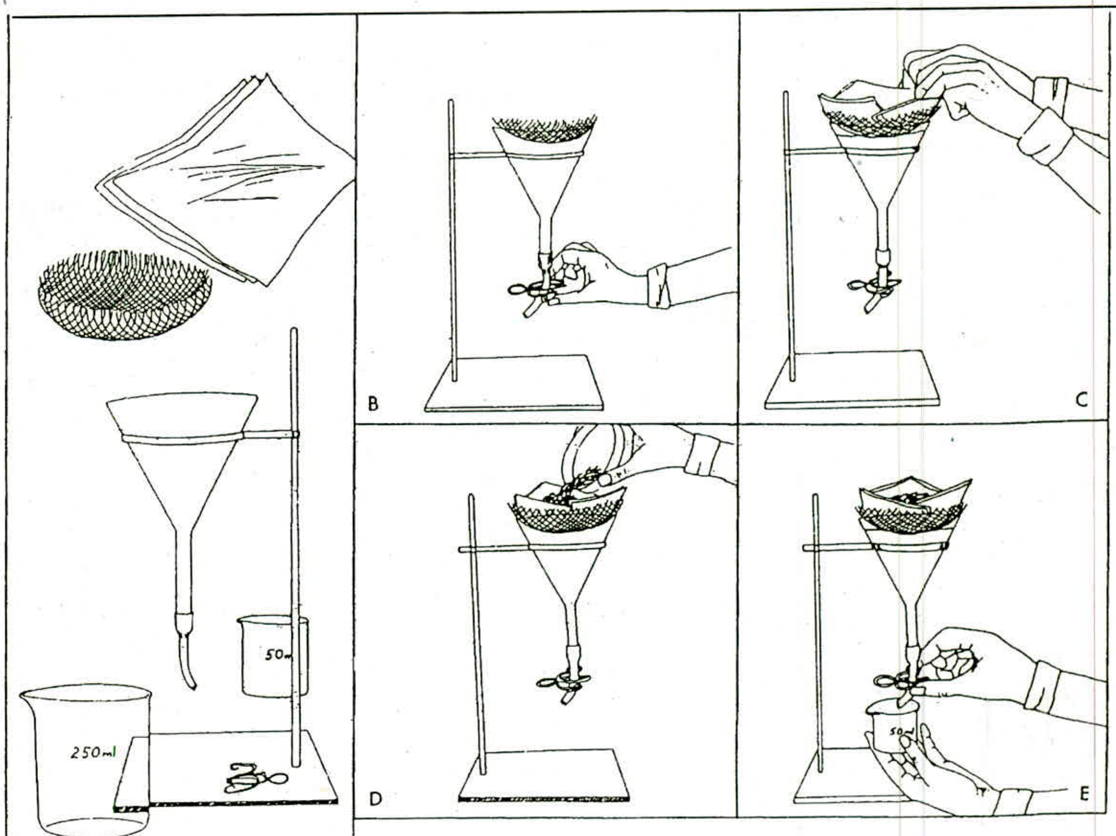
1. Arrange the funnel, tubing and screen on the funnel stand as shown in the fig. Fill the funnel with water, open the clamp and drain out sufficient water from the stem to remove air-bubbles. Again fill the water in the funnel touching the bottom of screen.

2. On upper surface of screen, put carefully any one of the filter aid listed above that retain as much debris as possible, with large pores for the vermiform nematode to wiggle through.

3. Cleaned and chopped plant tissues (5-10g) or finely crumbled soil (50 g) or composite sample (30-100 cm^3) uniformly spread over filter aid, gently pour water along the inner surface of the funnel until it just covers the plant tissues or soil kept over the filter for nematode extraction.

4. Keep this assembly at room temperature according to targeted nematode.

5. Collect 5-10 ml of water from rubber tube containing nematodes in a beaker or shallow viewing dish (Syracuse dish- grid pattern at the base for counting) at the end of extraction period.



Precautions

1. Funnel should not be overfill, just saturate the sample with water.

2. The sample will desiccate if edges of filter hang over the sides of funnel because water will wicked out.

3. Water should not be poured directly over the sample. Slowly add the water along the inner surface of funnel.

4. Funnel apparatus should be checked periodically for loss of moisture from sample due to evaporation and if so add some water into it.

5. Sometimes water containing Methylene blue (2 ppm) is used for soil samples and Streptomycin sulphate (1000 ppm) is added to plant tissues sample to avoid bacterial decay.

Advantages

1. This method is simple and the materials required are inexpensive.

2. Recovery of live nematode is fairly good even from very small samples.

Disadvantages

1. Poor oxygenation in the bottom of the funnel reduces the movement of nematode and thus recovery of live nematodes is comparatively less (90%).

2. Some nematodes are lodge on the sloping sides of the funnel.

3. Recovery of nematodes is very poor large/big samples.

Objective :- 2. To study the Tray/dish/pie pan method of nematode extraction.

This method is modified version of Baerman funnel method to overcome the disadvantages of previous method.

Materials required

Shallow evaporating dish, Petri dish or tray, set of sieves, glass and bowl, double layer of muslin or cheese cloth

Method

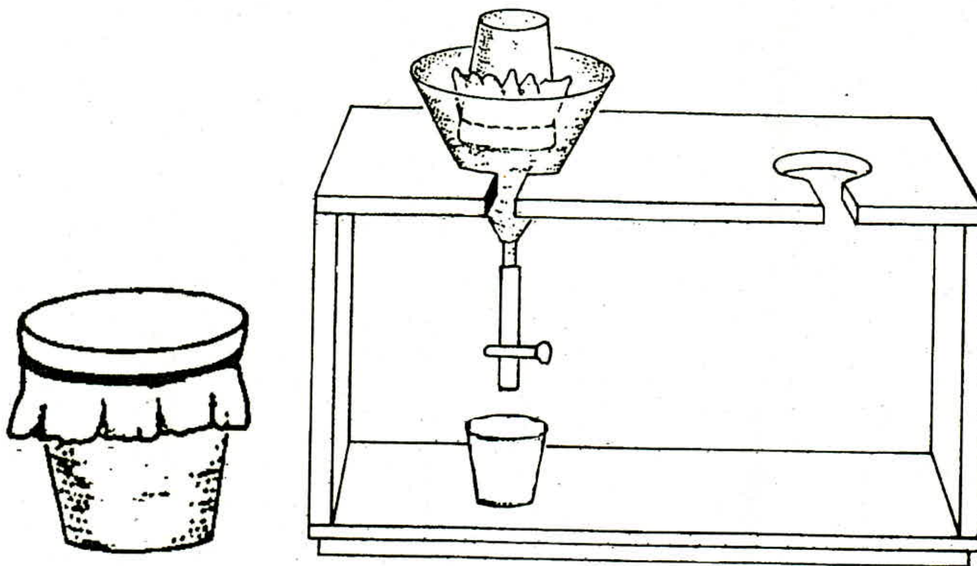
Two methods are described herewith which can be adapted according to the availability and requirement of instruments.

Method 1: *(This method is applicable for both soil and root sample)*

1. Processed soil or root samples were spread on a single ply paper towel and put on a coarse meshed screen. The whole setup then put on a plastic container (Petri dish/ saucer).
2. Water is added to the container until the soil thoroughly wet but not immersed.
3. The whole container then covered with a large Petri dish/ saucer to reduce evaporation of water.
4. The setup was then kept undisturbed for 24 hrs or more for better result.
5. Paper towel with soil are discarded and nematode suspension from the container collected in test tubes for examination.

Method 2: *(This method is best applicable for root sample)*

1. Wash the roots with tap water thoroughly and carefully.
2. Cut the roots into 2-3 cm pieces with sharp scissors.
3. Chopped root sample are placed on a coarse mesh sieve attached in a polyvinyl cylindrical tube.
4. The tube with root sample is placed inside a plastic cup.
5. The cups with root samples are then kept in a mistifier at 27°C that produces fine mist of water for 90 seconds in every 10 minutes (Seinhorst, 1962).
6. Overflow escaped through a hole in the upper side of the cup. The whole suspension in a cup is collected for the examination of nematodes.



Advantages

1. Specific equipment is not required.
2. It is easy to adopt locally available materials.
3. It extracts wide variety of mobile nematode.

Disadvantages

1. Large and slow moving nematodes are not extracted very well.
2. The extractions sometimes quite dirty and therefore difficult to count the nematodes.
3. The proportion of nematodes extracted can vary with temperature.
4. Maximum recovery takes 3-4 days.

Objective :- 3. To study the Sieving and gravity method of nematode extraction.

This method is given by NA Cobb. In this method, active, dead, sluggish, cysts and eggs of nematodes are better recovered in fairly less time (30 min) even from large samples (1.5 kg).

- Sieve is the main component of this method. The sieve is designated by a mesh number which indicate the number of openings per inch of surface (60 mesh means 3600 openings per square inch).
- Sieves are selected according to size of the nematode expected to be extracted and soil type. For fine textured soil, 20, 100, 200, 325, 500 and 625 mesh sieve may be used. Use 60 or 80 mesh sieve for extraction of cysts and eggs last in the series and backwash it into a pan.
- Generally sieves are used singly not used after staking them. It reduces the efficiency of recovery.
- Wash the sieves in hot water, never use detergent or soap which plug the openings of fine sieves.

Material required

Series of at least 8 sieves, Syracuse dishes or Petri plate, wash bottle, beakers and flasks of various capacities, big pans.

Method

1. Collected soil samples thoroughly mix and about 100-500 g representative sample pour into 3.5 L of water in a pan or bucket (A), stir well up to homogenization so that nematodes become free from sample and suspended into water.
2. Decant muddy water into another pan or bucket (B) through 20 mesh sieve after setting the above mixture for 1 min to remove heavy soil particles and other material.
3. Resuspend the sediment in another one L of water to recover maximum number of nematodes. Repeat the step 2 and discard sediment.
4. The contents of bucket (B) stirr well, allow to settle for 1 min, pour it in clean bucket (C) through 150 or 200 mesh sieve. Discard sediment.

5. Gentle stream of water apply on the angled sieve (step 4) and collect washing in clean pan (small nematodes and eggs) and collect large nematodes on the upper surface of the sieve by backwashing with gentle water streaming onto lower surface in the beaker.

6. Repeat step 5 using 325 mesh followed by 500 mesh sieve for more nematodes.

7. Resuspend the small nematodes and eggs collected in the clean pan in step 5 and concentrate them through backwashing of 325 mesh sieve in to beaker.

8. Allow the collected contents of beaker settle down for 20 min and decant carefully upper 30 ml and remaining contents examined for presence of eggs and nematodes.

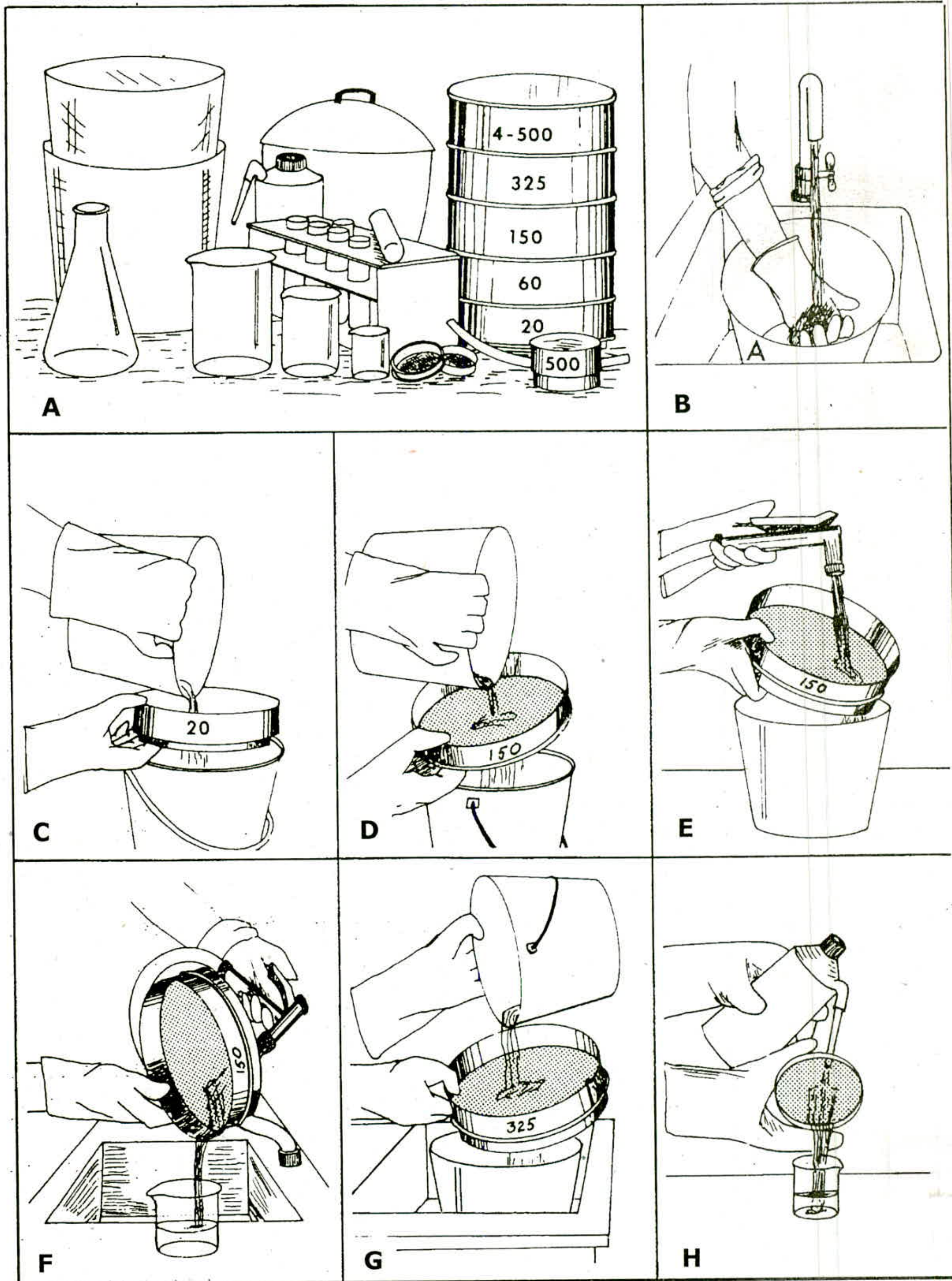
The contents collected from washing of sieves may be processed further through Baermen funnel method for additional separation of nematodes from soil particles. majority of nematodes pass through filter within 24 h and collected almost free of debris.

Advantages

1. For examination, nematodes are available in very less time.
2. Since this method is not dependent on nematode movement, active, dead, sluggish, cysts and eggs of nematodes are better recovered.
3. Recover nematodes from even large samples.
4. Suitable for extracting nematodes from wet soil.
5. Useful for cyst extraction from soil.

Disadvantages

1. Sieves are expensive and method needs experienced worker.
2. Due to the presence of fine particles, nematodes are difficult to see.
3. Nematodes can be easily damaged.


4-500
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Objective :- 4. To study the Centrifugal floatation method of nematode extraction.

This method is more efficient than the Baerman funnel method and Cobb's sieving and gravity method for extracting nematodes. This method is often used to clean extracts obtained in sieving or elutriation.

Materials required

Series of at least 8 sieves, Syracuse dishes or Petri plate, wash bottle, beakers and flasks of various capacities, big pans, centrifuge, centrifuge tube and rack, sucrose solution.

Method

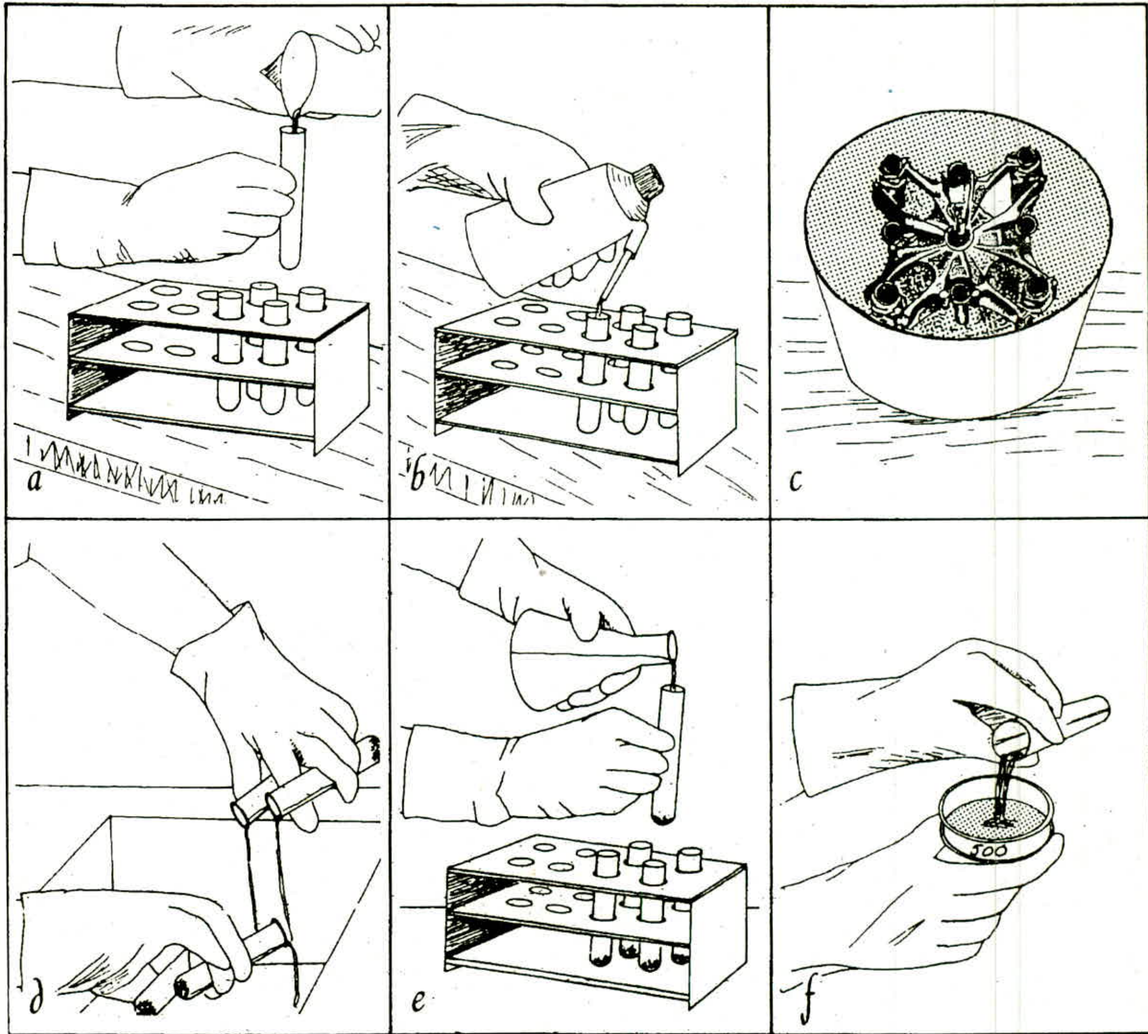
1. Extract the nematodes by following Cobb's sieving and gravity method or by elutriation.
2. Nematodes are concentrated in 20-30 ml of water and equally distributed in 50 ml round bottom centrifuge tubes.
3. Centrifuge the tubes at 2900 x g for 4-5 min to extract vermiform nematodes, 1150 x g for cyst nematodes. At the end of centrifugation, nematodes and eggs will be concentrated at the bottom of tubes in the form of pellet.
4. Drain off the supernatant carefully without re-suspending the pellet.
5. Refill the tubes with sucrose solution halfway to the top and thoroughly mix with stirrer (a. Dissolve 673 g sucrose in 1 L of water to suspend vermiform nematodes, b. Dissolve 1210 g sucrose in 1 L of water to suspend cyst nematodes).
6. Centrifuge above at 2900 x g for 60 sec to extract vermiform nematodes and 1150 x g to extract cyst nematodes. The nematodes will be recovered on top of the tubes.
7. Pour the supernatant on 300-625 mesh sieve and backwash the nematodes on the sieve in to a Syracuse or viewing dish (Extracted nematodes should not be remain more than 2 min in sucrose solution to avoid distortion of nematodes by osmotic pressure of solution).

Advantages

1. This method can be used to process large soil samples in few minutes.
2. Recovery of live, dead, sluggish, sedentary nematodes and eggs is excellent.

Disadvantages

1. This method is applicable for soil samples only.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Objective :- 5. To study the Trituration or maceration method of nematode extraction.

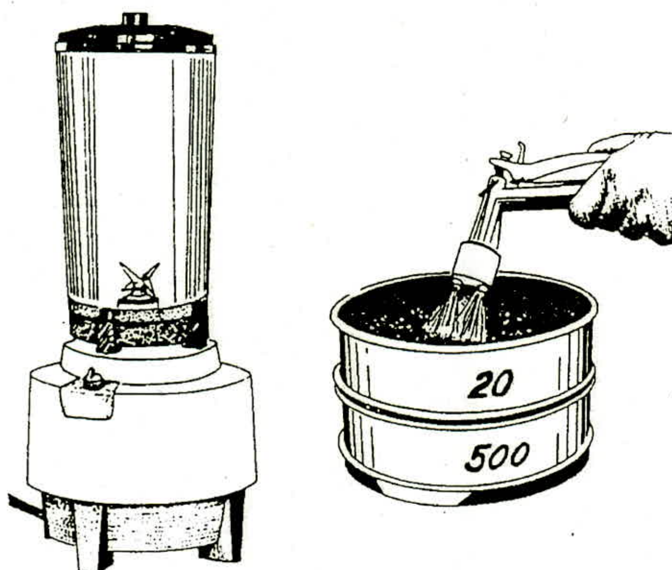
This method is very good for extracting migratory and sedentary nematodes from bulbs, corms, cloves, crowns, storage roots, leaves and small plants.

Materials required

Electric food blender, 20 and 500 mesh sieve, fogger nozzle and beaker or Syracuse dish.

Method

1. Gently wash plant part(s) with water to remove soil particles.
2. Plant part cut in to pieces and 10 g of it put into electric food blender with 200 ml water.
2. Run the blender at medium speed for 20-50 sec without undue damage to the nematodes.
3. Blended or slurried mixture of plant parts poured on the 20 mesh sieve which is nested on top of a 500 mesh sieve. With the fogger nozzle, wash the blended material.
4. Discard the material present on the 20 mesh sieve. Nematodes pass through it and collected on the 500 mesh sieve.
5. Backwash the 500 mesh material into beaker of Syracuse dish for examination or material processed in Baerman funnel method for recovery of nematodes.



Advantages

1. This method is quick and useful for extracting nematodes especially from below ground parts.
2. This method is commonly used for extracting *Meloidogyne* eggs in roots to be used for inoculation.

Disadvantage

1. Blending action may release toxic substances from the plant parts that can·kill or immobilized nematodes.
2. Movement of nematodes sometimes inhibited by slurried or thick mass of blended plant parts resulting in less recovery of targeted nematodes.

Exercise No.: 3

Title :- Extraction of eggs.

Objective :- To study of extraction of eggs from cysts.

Eggs of root knot and other related nematodes can be collected egg matrices by dissolution of gelatinous matrix surrounding the eggs.

Materials required

1. Infected roots should be washed gently to remove soil particles.
2. Roots are cut into pieces, dip into 5.25% solution of NaOCl, agitate rigorously and diluted four times to make 1% solution.
3. Pour the solution over sieve, wash the collected eggs with tap water to remove NaOCl solutions.
4. Suspend the eggs in water for examination.

With the combination of wet sieving and decanting, cysts can be collected.

1. Dissolve the well mixed 1/2 cup (100 cm^2) soil sample into two quarter of water.
2. Pour the soil suspension on a 60-mesh sieve and backwash the deposits with water in separate pan.
3. Re-pour the suspension on a 60-mesh sieve, hold the screen at an angle to concentrate the debris and cysts.

5. With the help of rubber stopper gently rub the cysts to release the eggs. Wash the sieve over large beaker with gentle stream of water.
6. In round bottomed centrifuged tube, concentrate the eggs by centrifugation (800 rpm).
7. Decant the supernatant carefully by leaving about 2 ml of it above the pellet.
8. In 50 ml conical centrifuge tube, stir the pellet and a layer of slurry over sucrose gradient (consist of 10 ml of 50% sucrose overlaid with 10 ml of 40% sucrose and then 10 ml of 20% sucrose).
9. Centrifuge for 5 min at 800 rpm.
10. In a band in 40% sucrose layer, eggs are concentrated also contained hatched juveniles.
11. Siphon off the band, pass through 250-mesh sieve which removes the juveniles and collect the eggs on 450-mesh sieve.

Exercise No.: 4

Title :- Slide preparation.

Objective :- To prepare microscopic slides for examination of nematode.

I. Temporary slide

For quick identification or study of features best seen in unfixed specimens. For this, temporary slide is prepared as under :

1. Transfer live specimens to small drop of water on a glass slide.
2. Heat slide over the flame of spirit lamp for a while (Check the nematode movement till stationery)
3. Apply cover slip and seal around edge with nail varnish, allow to air dry and examine under microscope.

II. Permanent slide

Preparation of *M. graminicola* for identification using light microscope

It is essential that the nematode populations used in the screening are identified accurately at the species level. This protocol describes a routine method by which whole nematodes are prepared for light microscopic observation. Good results may be obtained when the nematodes are killed quickly and fixed in one process with hot formaldehyde (Seinhorst, 1966), transferred to glycerol by the ethanol-glycerol method (Seinhorst, 1962) and mounted on glass slides with the wax-ring method (de Maeseneer and d'Herde, 1963). These glass slides can be stored permanently and the preserved nematodes can be used as reference specimens. This routine method is good for the preparation of second stage juveniles of *M. graminicola* but is not suitable for the preparation of female root-knot nematodes.

A. Killing and fixing the nematodes

- Concentrate the nematodes as much as possible in a very small drop of water in a glass cavity vessel (for instance a glass staining block of 4 x 4 x 1.5 cm),
- Boil the same volume of 8 % formaldehyde for 20-30 sec. in water bath at 70-90°C,
- Add the hot 8 % formaldehyde as fast as possible to the drop of water containing the nematodes (resulting formaldehyde concentration: 4%).

B. Transfer of the nematodes from formaldehyde to ethanol

- Prepare solution I (10 ml of 4 % formaldehyde + 1 drop glycerol/100 ml ethanol),
- Fill a glass staining block with solution I,
- Transfer the nematodes with a needle from the 4 % formaldehyde to solution I in the glass staining block, leave for a day to allow the fixative to penetrate and act on all the tissues,
- Fill a closed glass vessel (for instance a desiccator) to approximately 1 cm depth with 95 % ethanol,
- Place the glass staining block on a support in the desiccator so that it stands above the layer of ethanol (wick pot),
- Close the desiccator tightly,
- Place the desiccator for 1 night in an incubator at 35°C. This will allow all the water in the suspension with the nematodes to be replaced with ethanol.

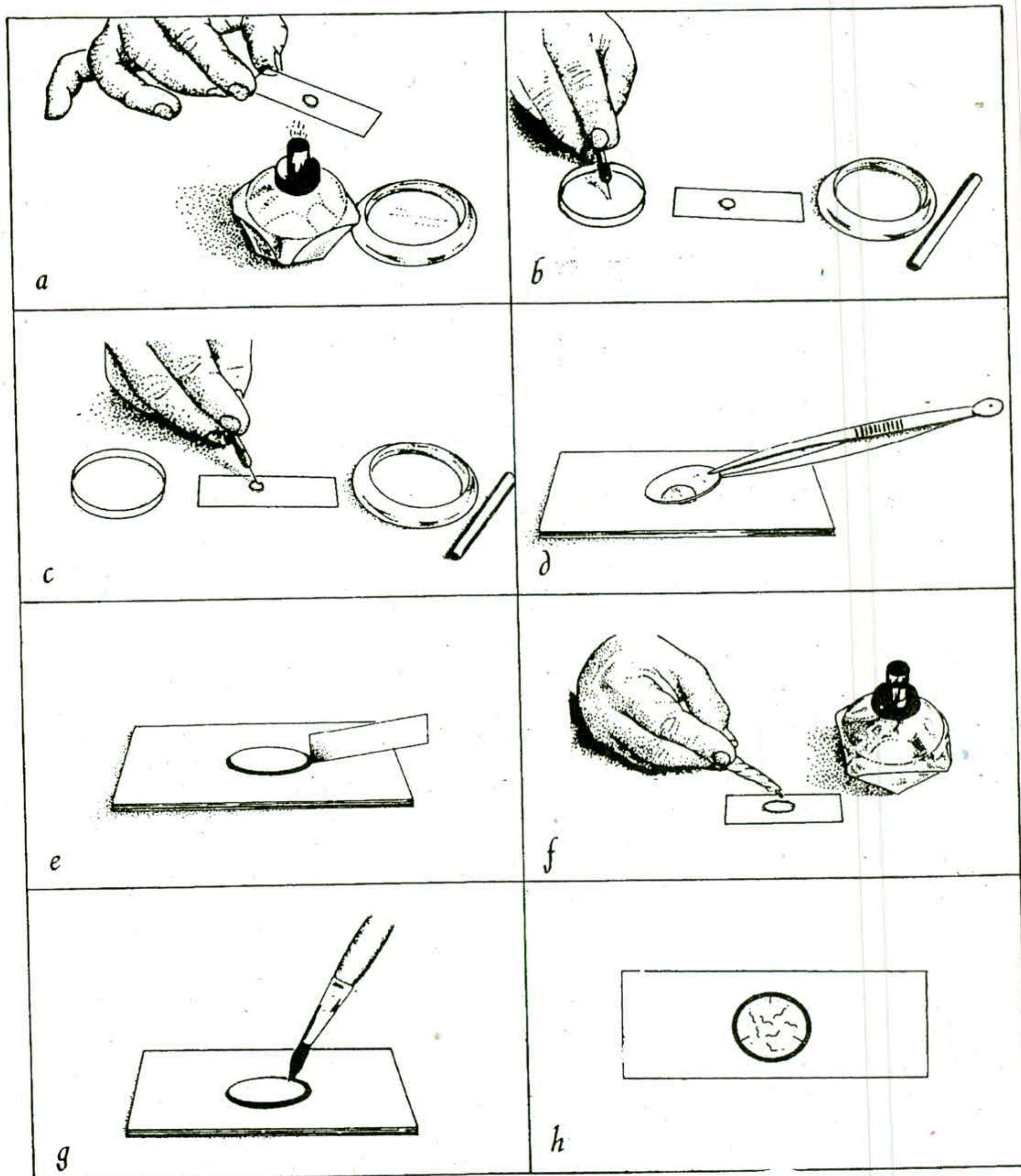
C. Transfer of the nematodes from ethanol to glycerol

- Take the glass staining block out of the desiccator (ethanol will have replaced the 4 % formaldehyde),
- Cover the glass staining block partially with a cover glass,
- Place the glass staining block in an incubator at 35°C,
- Check after 15-20 min., if the ethanol has evaporated. When it has, add a few drops of solution II (20 ml of 95 % ethanol + 2 drops glycerol/100 ml distilled water),
- Repeat this process several times until the ethanol has evaporated,
- Add a few drops of glycerol (just enough to immerse the nematodes).

D. Preparation of glass slides

- Heat the 1.5 cm diameter tip of a copper tube in a flame,
- Dip the hot tip in paraffin wax,
- When the paraffin wax has melted, press the tip on a glass slide making a thin wax ring which will soon solidify,
- Put a small drop of glycerol in the middle of the wax ring,

- Transfer the nematodes with a needle and place them in the centre of the glycerol drop (10 nematodes/glycerol drop),
- Cover with a cover glass,
- Place the glass slide on a hot plate for a few seconds (the wax ring will melt allowing the cover glass to settle down thus confining the glycerol to the centre of the ring),
- Place the glass slide on a cool surface (the wax ring will soon solidify),
- Seal the cover glass (for instance with nail varnish).



Exercise No.: 5

Title :- Preparation of perineal patterns.

Objective :- To prepare perineal patterns for species identification.

The perineal patterns of meloidogyne species are fingerprint-like patterns of grooves and ridges around the tail terminus of mature females. Perineal patterns are useful in species identification (Hartman and Sasser, 1985).

- Perineal patterns of mature females were prepared by standard procedures. Root tissues were teased apart with forceps and half spear to remove adult females.
- The lip and neck regions of the nematode were excised, and the posterior end was cleared in a solution of 45% lactic acid to remove remaining body tissues.
- With the help of a dissecting needle and scalpel, the cuticle around the perineal pattern is trimmed away.
- The cuticle piece with the perineal pattern is transferred to glycerol onto a slide surface with the outer surface uppermost.
- Cover slip is applied and sealed with nail varnish.

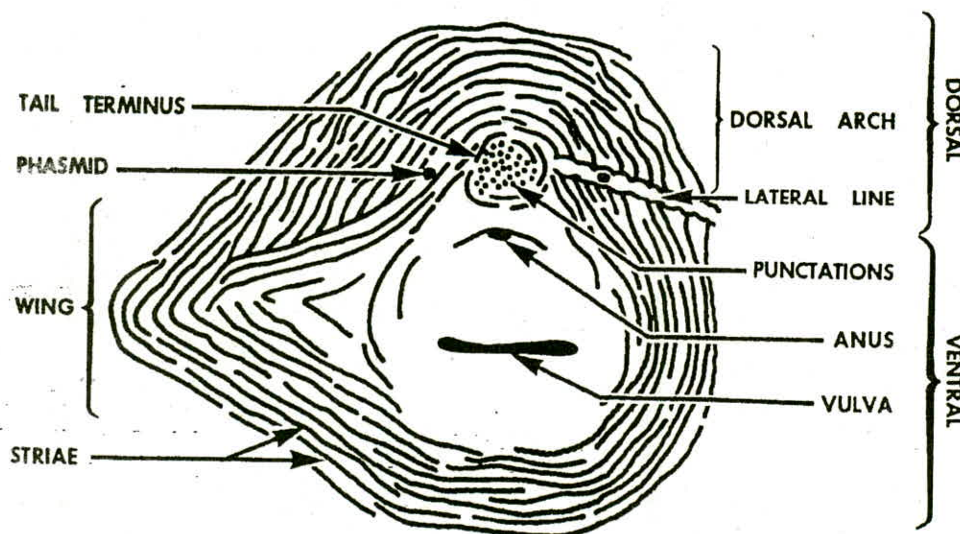


Fig.:- General morphology of a perineal pattern in *Meloidogyne*

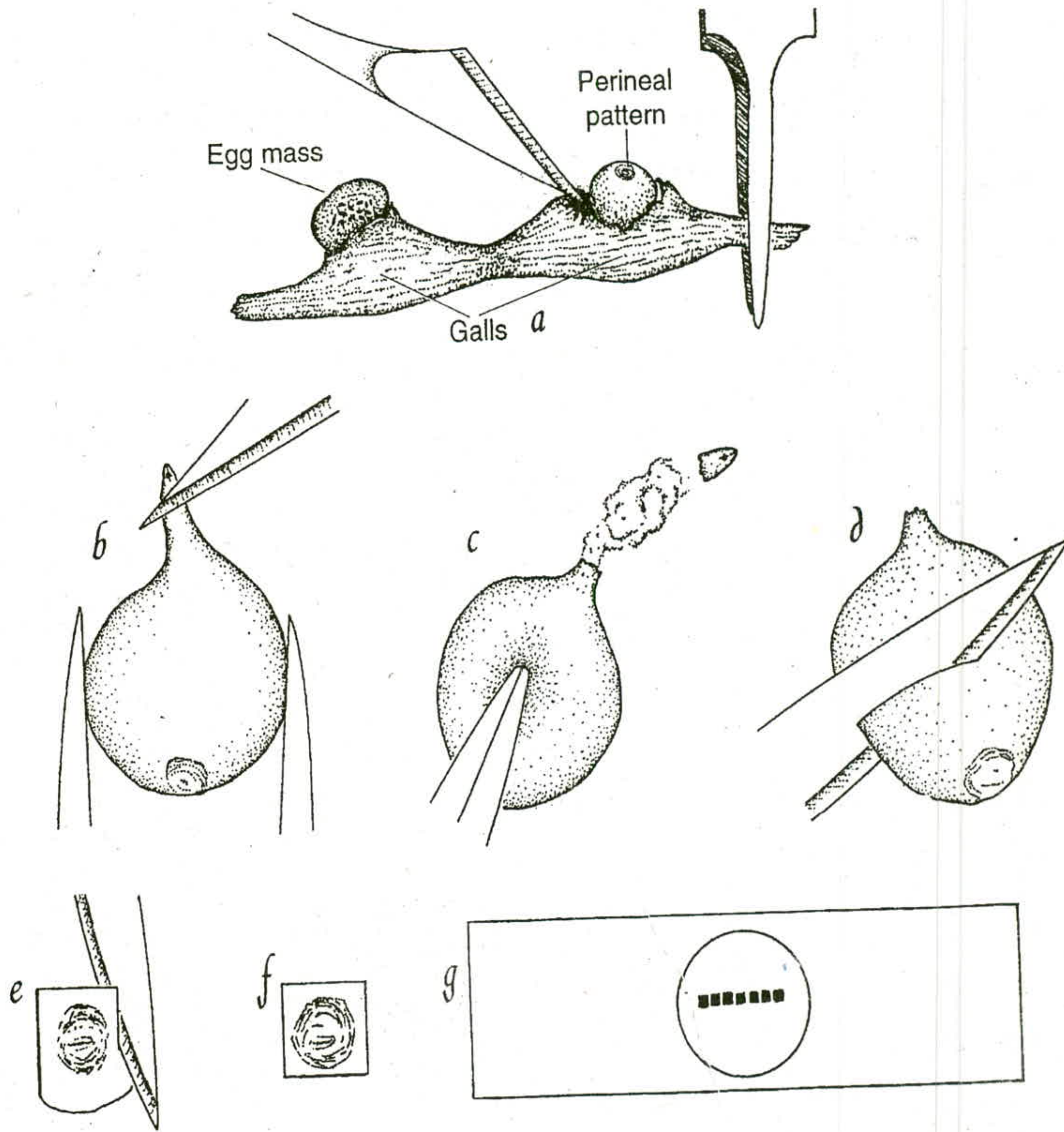
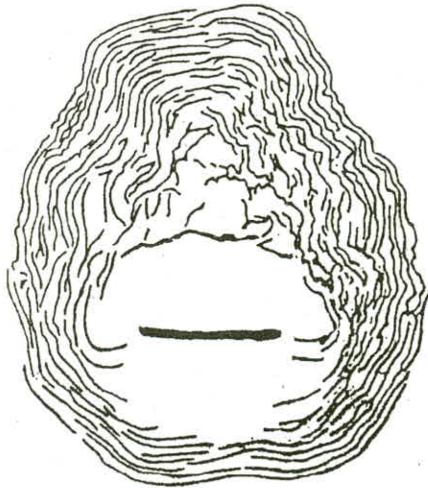
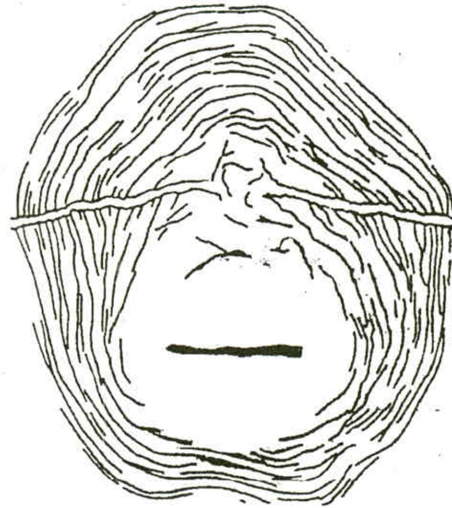


Fig.: Preparation of cuticle for examination of perineal patterns in *Meloidogyne*.



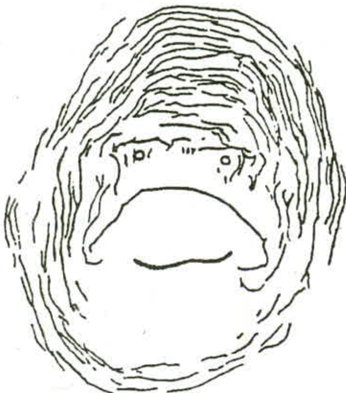
M. incognita



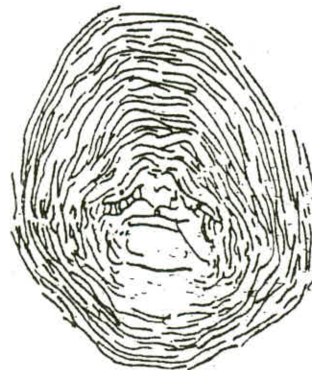
M. javanica

M. arenaria

M. hapla



M. naasi



M. ardenensis

Fig.: Perineal patterns of six species of *Meloidogyne*

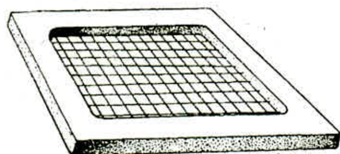
Exercise :- Prepare perineal patterns and draw the diagrammes.

Exercise No.: 6

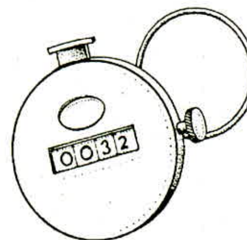
Title :- Counting of nematodes.

Objective :- To study of technique for Counting of extracted nematodes.

- Extract nematodes from a known weight of plant tissue or volume of soil using one of the previously described methods.
- Concentrate the extracted suspension to a precise known volume in a measuring cylinder or graduated tube (e.g. 10 ml).
- Shake or stir the suspension immediately before removing aliquots.
- Use a wide mouth pipette to remove aliquots, to prevent blockage by debris. Pipette tips can be cut if they are too narrow.
- Carefully pipette aliquots into the counting dish, avoiding splashing.
- If only a few nematodes are present, count them in the total suspension volume.
- If nematode density is high, count the nematodes from an aliquot (e.g. 1 or 2ml). Dilution of the suspension may be necessary to aid counting, for example doubling the volume.
- Count all the nematodes in the counting dish in a systematic way following the gridlines on the dish (De Grisse counting dish). Sometimes nematodes may float on the surface, but adding a tiny spot of liquid soap overcomes this.
- Use a tally counter to count the various different nematodes present.
- Return the counted aliquot to the suspension after counting.
- Repeat using 2-3 aliquots per sample and then calculate the mean for the combined aliquot score before calculating the total nematode number per sample.
- The mean number of nematodes calculated from the aliquots should be multiplied by the total volume of the suspension to calculate the total number in the plant tissue or soil that they were extracted from (e.g. 100 ml soil or 5 g root).



De Grisse counting dish



Hand Tally counter

Exercise No.

Title :- Symptoms of nematode damage.

Objective :- To study the symptoms of damage caus[illegible] in plants.

A. Above ground symptoms

1. Stunting, reduced foliage and dieback
2. Poor yield
3. Yellowing (chlorosis) of leaves
4. Wilting
5. Early senescence
6. Fruit drop and poor or malformed fruits
7. Interveinal discoloration and necrosis of leaves (e.g. *Aphelenchoides ritzemabosi* on chrysanthemum leaves.
8. Stem, leaf and seed galls (e.g. *Anguina* spp. on leaves and seeds of grasses and cereals).
9. Twisting of leaves and white tips (e.g. *Aphelenchoides besseyi* on rice).
10. Twisting of leaves and raised yellow lesion on stems and leaves (e.g. *Ditylenchus dipsaci* on daffodils and onions).
11. Twisted panicles and empty grains (e.g. *Ditylenchus angustus* on rice).
12. Yellowing and rapid death of pine trees (*Bursaphelenchus xylophilus*).
13. Yellowing and collapse of palm leaves followed by rapid death. Red necrosis of internal stem tissues usually in a ring (*Rhadinaphelenchus cocophilus* on coconut and oil palm).
14. Distorted apical growth and crimping of leaves and inflorescence (*Aphelenchoides besseryi* and *A. fragariae* on strawberry).

elow ground symptoms

1. Reduced root systems, particularly reduction secondary feeder roots.
2. Abnormal roots-
    a. Overall galling of roots, often severe. Large uneven galls (most *Meloidogyne* spp.)
    b. Overall galling of roots, often severe. Small rounded galls (*Nacobbus*, some *Meloidogyne* spp.)
    c. Root-tip galling rounded (*Longidorus, Hemicycliophora*, coffee *Meloidogyne* spp.)
    d. Swollen, hooked root tips (*Subanguina* spp., *Xiphinema* spp., *Meloidogyne graminicola*).
    e. Clumping of lateral roots in a ball (some Meloidogyne spp. *Heterodera* spp.).
    f. Necrotic root lesions or overall necrosis of roots (*Pratylenchus* spp., *Radopholus* spp. *Hirschmanniella* spp., some *Helicotylenchus*).
    g. Accumulation of soil particles and debris on roots (*Tylenchulus semipenetrans*).
    h. Stubby roots (*Paratrichodorus, Trichodorus*)
3. Cysts (white, yellow or dark-brown specks root surface (*Heterodera, Globodera*).
4. Internal rotting of tubers, corms and bulbs (*Ditylenchus, Pratylenchus, Scutellonema*).
5. Galled or warty tubers (potato, yam) *Meloidogyne* spp.).
6. Surface cracking of tubers (potato, sweet potato, yam) (*Ditylenchus destructor, Scutellonema bradys, Pratylenchus coffeae Meloidogyne* spp.).
7. Lesions on groundnut pods (*Pratylenchus, Criconemella*).
8. Brown and shriveled groundnut seeds (*Aphelenchoides arachidis, Ditylenchus africanus*).

Exercise :- Draw the diagrammes of damage symptoms caused by nematodes in plants

Exercise No.: 8

Title :- Typical life cycle pattern of nematode.

Objective :- To study the typical stages of life cycle of nematode (eg. *Meloidogyne* spp.).

There are six stages in the life cycle of *Meloidogyne* spp.- the egg, four larval or juvenile stages and the adult (male or female). The life cycle of *Meloidogyne* is simple (Fig.). After embryogenesis, the first stage juvenile (J1) is formed which is still enclosed within the egg-shell, thereafter J1 moults within the egg itself and second stage juveniles (J2) hatches out. It moults second time to form third stage juveniles (J3), and another moult give rise to fourth stage juvenile (J4) which later on develops into fully mature male/female having developed reproductive system. Usually the juveniles are similar to adults in general shape, except for size and the development of reproductive system. The size of the nematode increases at each successive moult.

Description of developmental stages of life

Eggs : The eggs are laid in a gelatinous matrix. The eggs are oval shaped and very much smaller, about 50-100 x 25-50 µm in size.

Juveniles : Juvenile 1 is remain inside egg-shell and after hatching J2 stage formed. J2 stage is vermiform; about 280-500 µm long, stylet slender, about 10 µm long with rounded basal knobs, tail elongate conoid with pointed tip (spike tailed stage). In J2 stage, sex differentiation occurs. The juvenile destined to become females acquire V shaped genital primordium while in males, it is I shaped. J3 and J4 stages grow in width, genital primordial develop further but they are non-feeding stages due to lack of stylet.

Mature female : Swollen, melon like with elongated neck at anterior end; body wall soft, white and non forming cyst, stylet short with well developed basal knob, oesophagus well developed, median bulb large, isthmus short, gonoducts coiled occupying most of the body, tail absent, anus and vulva terminal.

Male : Vermiform, 1.5-2.0 mm long, stylet and oesophagus well developed, spicules slender, gubernaculum simple, bursa absent.

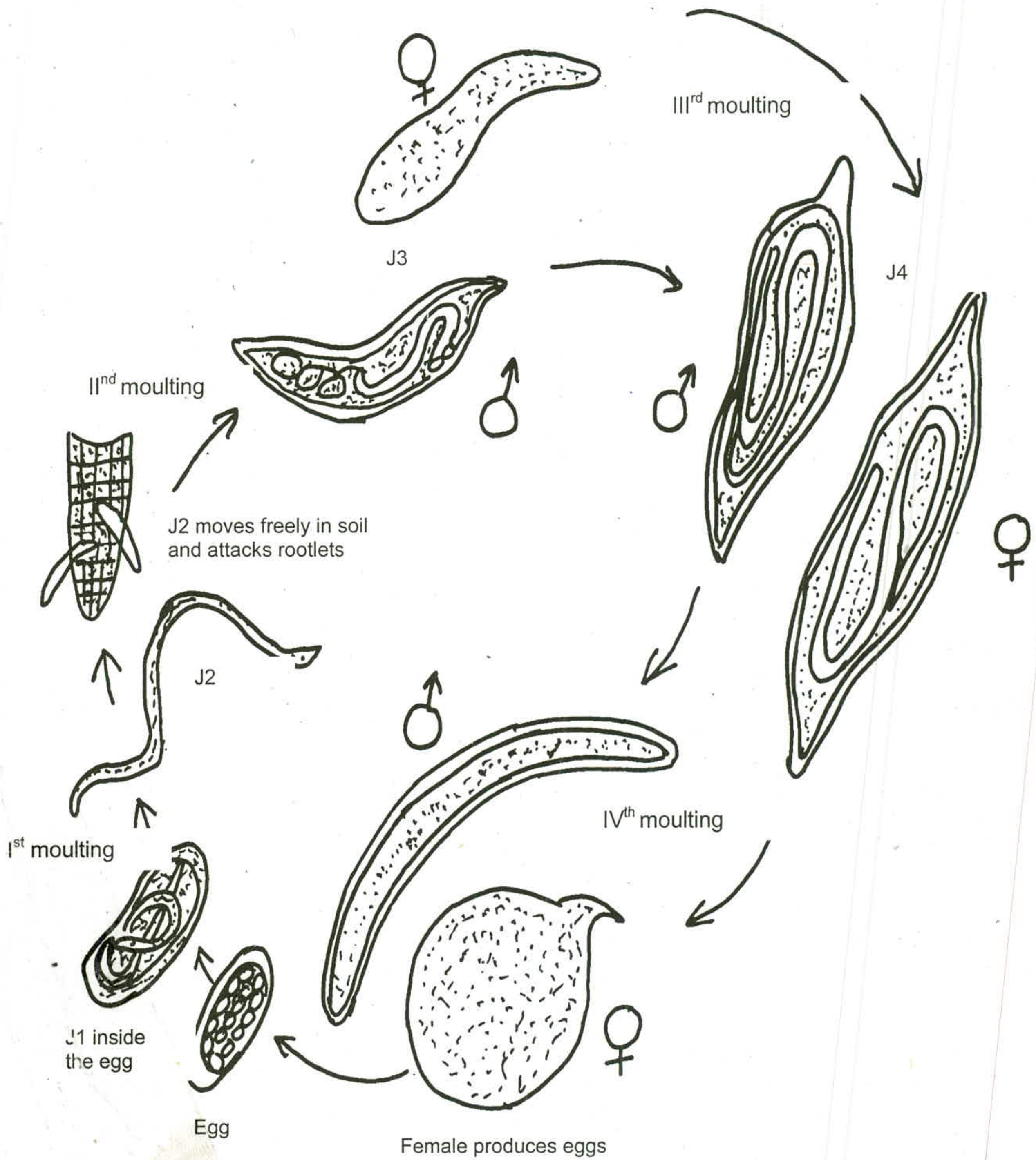


Fig.:- Typical life cycle pattern of *Meloidogyne* spp.

Exercise :- Draw the life cycle pattern of a nematode.

Exercise No.: 9

Title :- Identification of important nematodes.

Objective :- To study of features of important nematodes for identification.

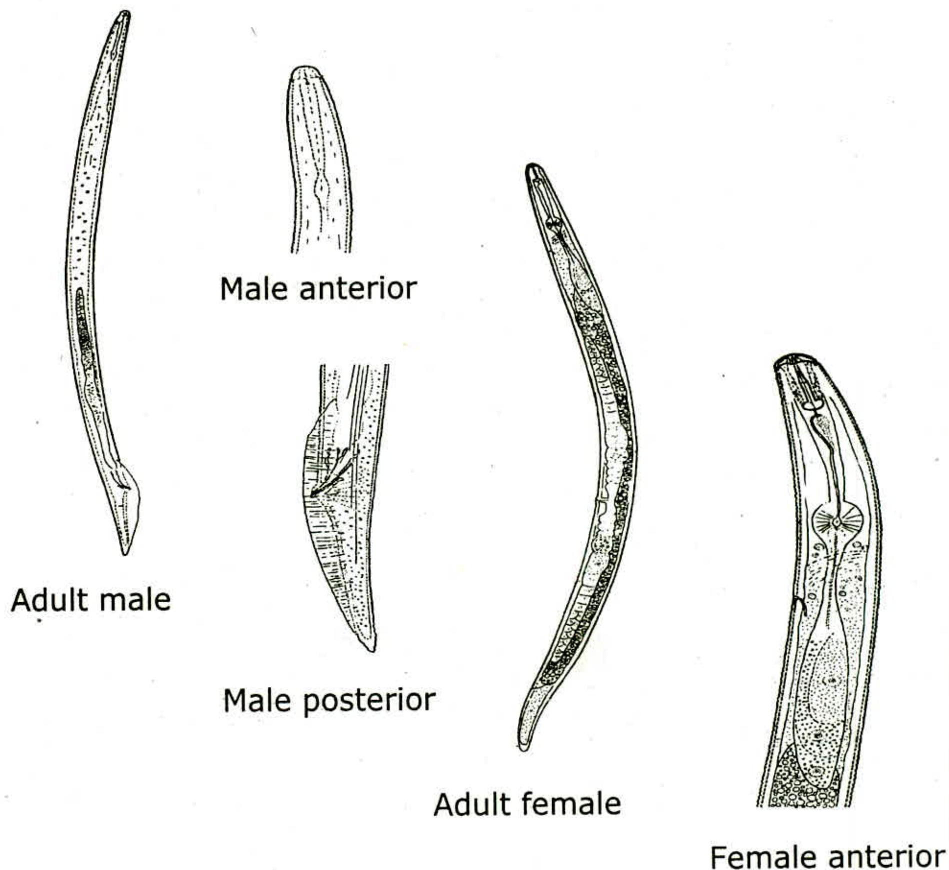
A. Burrowing nematode (*Radopholus*)

Parasitism and habitat

Endoparasitic on few plants particularly on banana and citrus. All stages are found in root tissues and in the rhizosphere.

Main Morphological Characters

Body	:	4.4 - 0.9 mm
Lip	:	Typically round in female and in males set off and knob like.
Stylet	:	Short and stout in female and it is very slender and rudimentary.
Oesophagus	:	Forming a lobe, dorsally overlap the intestine.
Ovaries	:	Two
Vulva	:	Located at the middle of the body
Tail	:	Tapers to a blunt end in female and in male the tail is long with bursa.
Resembling genus	:	*Hirschmanniella* (This genus is slender and long compared to R. *similis.*)



Exercise :- Draw the observed labeled diagramme.

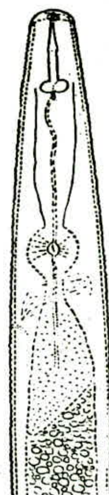
B. Lesion nematode (*Pratylenchus*)

Parasitism and habitat : Migratory endoparasite, feeding in root cortex of many plants. All stages are found in root or soil. Males common in many species, unknown or less common in others.

Main Morphological Characters

Body	:	4.4 - 0.8 mm
Lip region	:	Slightly set-off from body
Stylet	:	Typically short, strong with massive knobs
Ovary	:	Typically on and posterior ovary rudimentary to form a post uterine sac
Vulva	:	Typically on the posterior fourth of the body (75-80%)
Tail	:	Nearly round to pointed and in the case of male, tail has bursa
Resembling genus	:	*Radopholus* (Two ovaries present instead of one and great morphological difference between male and females observed in *Radopholus.*)

Adult female


Male anterior

Male posterior

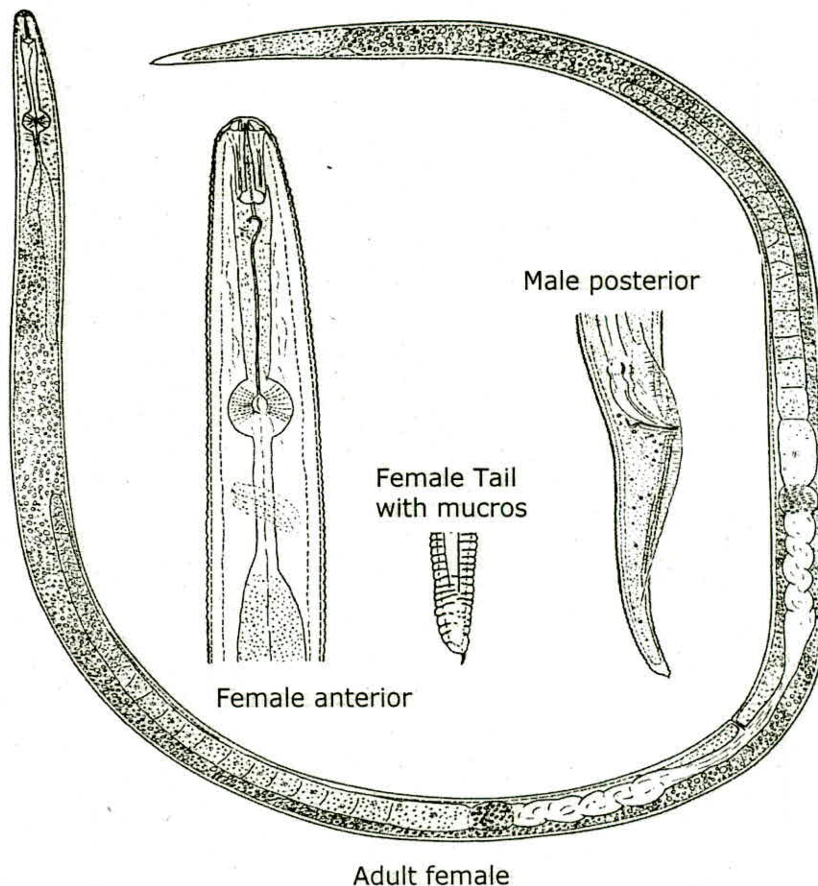
Female posterior

C. Rice-root nematode (*Hirschmanniella*)

Parasitism and habitat : Endoparasitic on rice, grasses sedges etc.

Main Morphological Characters

Body	:	Typically long, slender (1.2 to 3.0 mm or more) and annulated
Stylet	:	Typically short with rounded knobs
Vulva		Typically near the middle of the body
Ovary	:	Median, ovaries two and amphidelphic
Oesophagus	:	With conspicuous median bulb; oesophageal glands in a lobe, overlapping anterior end of the intestine ventrally.
Tail	:	Bluntly pointed with short sharp projections (mucros); male tail long with bursa
Resembling genus	:	*Radopholus* (body much shorter, always less than 1.0 mm and sharp projection on the tail is absent.)



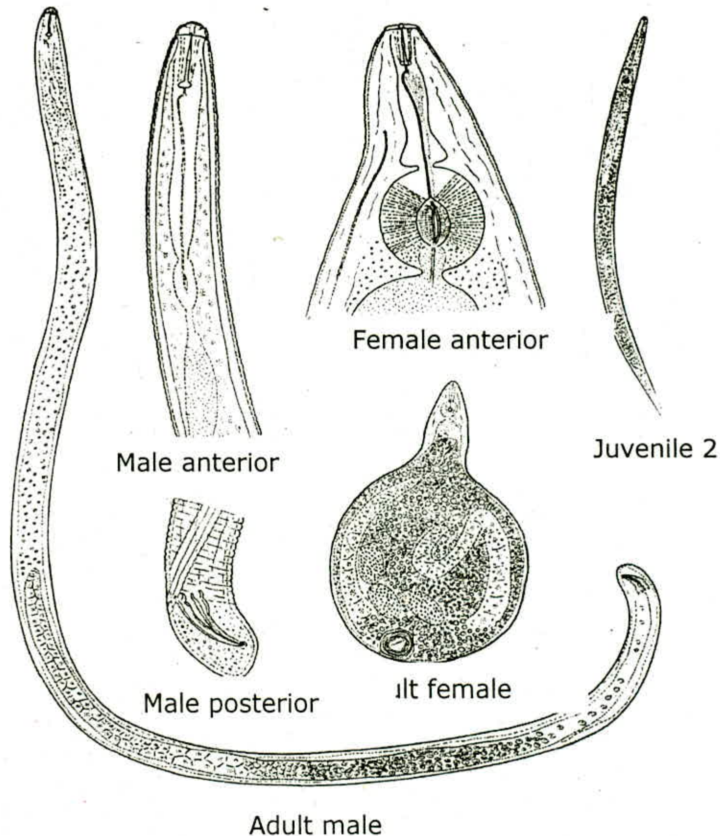
Adult female

D. Root-Knot nematode (*Meloidogyne*)

Parasitism and habitat : Females as well as third and fourth stage juvenile are sedentary endoparasites on many plants. Males and second stage juvenile are migratory and can be located in soil also.

Main Morphological Characters

Body	:	Elongate juvenile (0.5 mm) and males (1.0-2.0 mm); typically saccate, spheroid with a distinct neck in females (0.8 mm long and 0.5 mm wide)
Stylet	:	Strong with rounded knobs in males; in females more slender than in males or juveniles with strong basal knobs
Oesophagus	:	With very large median bulb followed by a short isthmus
Excretory pore	:	Often seen with part excretory tube in the area between posterior part of stylet knobs and opposite to median bulb
Vulva and anus	:	In females, typically opposite to neck and surrounded by a pattern of fine lines resembling human fingerprints. (these are used for identification of species in this genus)
Spicules	:	Very near the terminus of males; bursa absent
Resembling genus	:	*Heterodera*

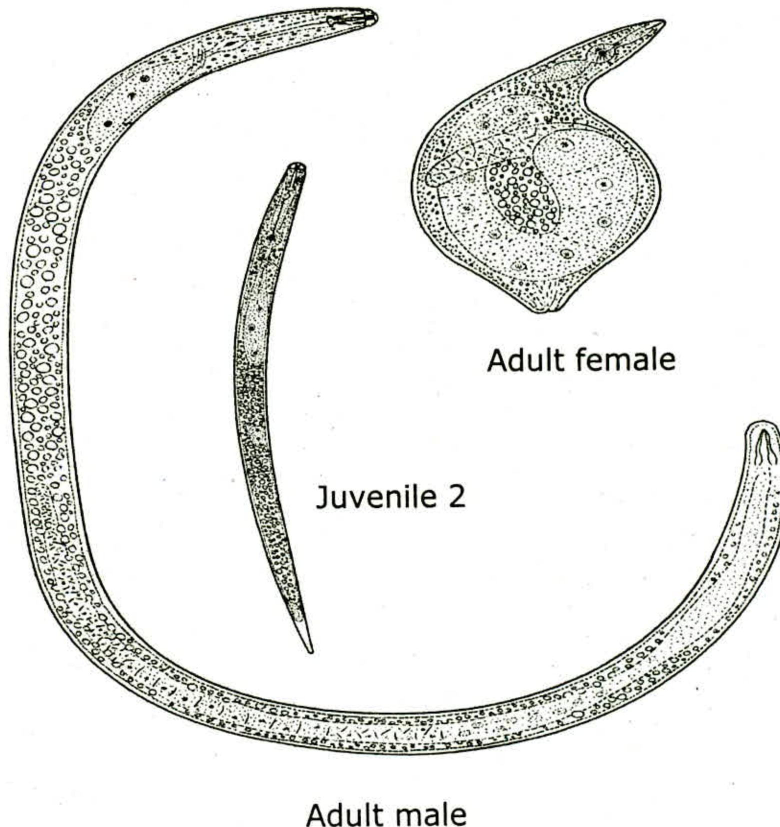


E. Cyst nematode (*Heterodera*)

Parasitism and habitat : Parasitic on many plants mostly in temperate zone (notably potato, sugar beet, oat and other grains, clover, soybean and various crucifers). Adult females with neck embedded in plant roots and the body exposed. Juveniles, males and cysts found in soil.

Main Morphological Characters

Body	:	Slender in males (1.0 to 2.0 mm) and juveniles (0.3 to 0.6 mm); in females, typically swollen lemon-shaped (0.5-0.8 mm in length), white or yellow in colour; Cysts dark brown, lemon shaped (0.8 mm long and 0.5 mm wide) or nearly the same shape as that of *Meloidogyne* female.
Stylet	:	Short in males with rounded basal knobs and in juveniles, more than 0.02 mm long
Oesophagus	:	With well developed median bulb and lobe extending back and overlapping the intestine.
Spicules	:	Near the posterior end of females
Resembling genus	:	*Meloidogyne* (stylet of juveniles only 0.01-0.014 mm long; adult females fully embedded in roots in case of *Meloidogyne*)
Potato cyst nematode	:	(*Globodera*): The adult females are globular in shape and hence, the genus is named as *Globodera*



Adult male

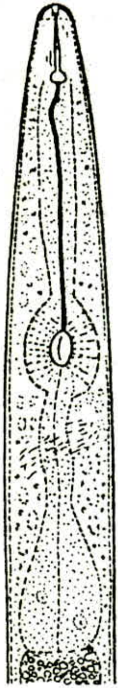
F. Citrus nematode (*Tylenchulus*)

Parasitism and habitat : Endo-parasitic on roots of citrus and other plants. Slender young females, males and juveniles are found in soil (where they can be easily overlooked or mistaken for juveniles of other species). In case of mature females they protrude from roots, often in clusters as semi-endoparasites.

(Note : Mature females are usually masked by egg masses to which soil particles adhere. For best observation, remove egg mass)

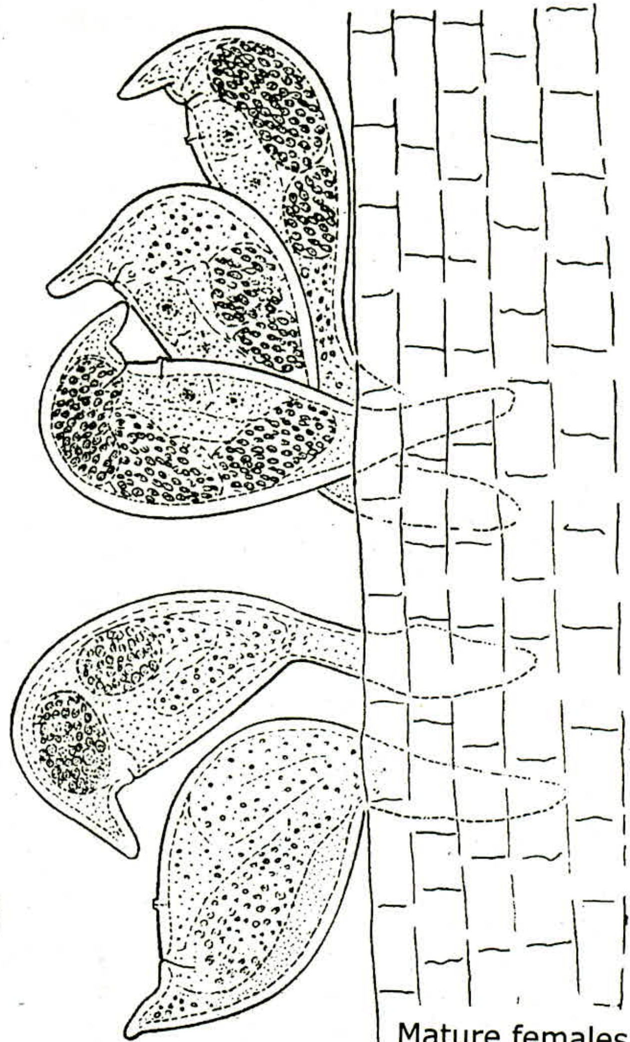
Main Morphological Characters

Body length	:	Small in all stages (0.20- 0.50 mm); in mature females, typically swollen
Stylet	:	Small in juveniles and males and well developed in young females
Oesophagus	:	With distinct posterior bulb in juveniles; young male and immature females.
Vulva	:	Prominent in the posterior end of young and adult females.
Excretory pore	:	Typically situated posteriorly in a protuberance just anterior to the vulva.
Anus	:	Absent or difficult to see in all immature stages
Bursa	:	Absent



Female anterior

Young female

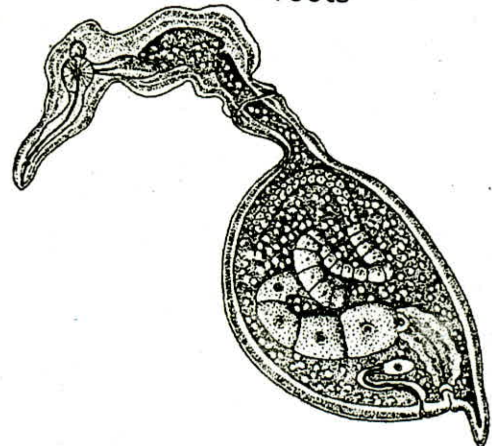


Mature females on roots

Male posterior

Adult male

Juvenile



Mature female

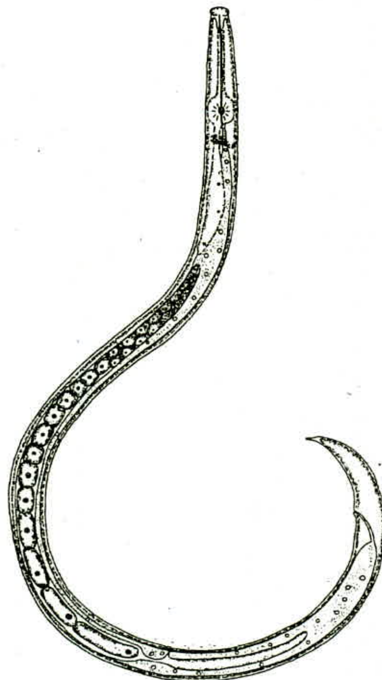
Exercise :- Draw the observed labeled diagramme.

G. Rice white tip nematode (*Aphelencoides*)

Parasitism and habitat : Migratory ecto and endoparasitic on rice leaves, buds, stem and grasses, sedges etc.

Main Morphological Characters

Body	:	Females are elongate (0.5-1.2 mm long) and very slender, cuticle is marked by fine transverse striations, male almost similar to female
Stylet	:	Slender, very short (9-17 µm), with needle like anterior portion, small to minute but distinct basal knobs.
Vulva		Transverse slit located in the posterior third of the body. The vagina usually slopes forwa5rd from the vulva.
Excretory pore	:	Opens at the level of the nerve ring or close behind it.
Ovary	:	Single, outstretched anteriorly, long post uterine sac.
Testis	:	Single, outstretched
Oesophagus	:	Procarpus long, slender and cylindrical, median bulb is large, esophageal gland overlaps the intestine,
Tail	:	Conoid, long, uniformly tapered to a blunt or acute tip. One to four mucros present on the female tail, mucros may be absent in male tail, mail tail frequently curved ventrally, spiculed paired, curved ventrally (rose thorn).
Resembling genus	:	*Aphelenchus and Rhadinaphelenchus.*



Adult female

Female tail with mucros

Male tail

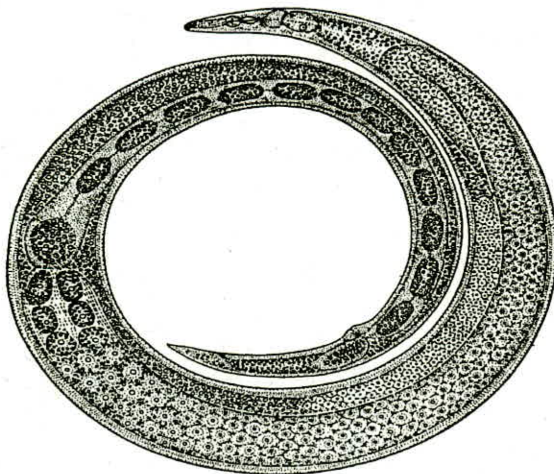
Exercise :- Draw the observed labeled diagramme.

H. Seed and leaf gall nematode (*Anguina*)

Parasitism and habitat : Migratory endoparasitic inducing galls on leaves, stem and seeds.

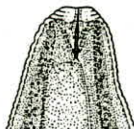
Main Morphological Characters

Body	:	Large, robust and stout, male smaller, slender than female and have a swollen midsection, female obese, tapered towards both ends, usually strongly curved (coiled), male slightly curved, lips low, flattened and distinctly set off.
Stylet	:	Short (8-10 µm), weak with small, well developed, rounded knobs.
Vulva		Near the posterior end of the body and has prominent lips.
Ovary	:	Single, ends in cap cell, small rudimentary post uterine sac.
Testis	:	Single, well developed
Oesophagus	:	median bulb has a distinct valve, esophageal gland overlaps the intestine in a basal bulb, isthmus is crossed by nerve ring.
Tail	:	Conoid, uniformly tapered to an obtuse or rounded tip.
Resembling genus	:	*Aphelenchoides, Cynipanguina, Subanguina*

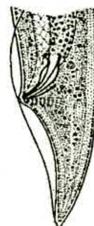


Adult female

Adult female head



Head



Male tail

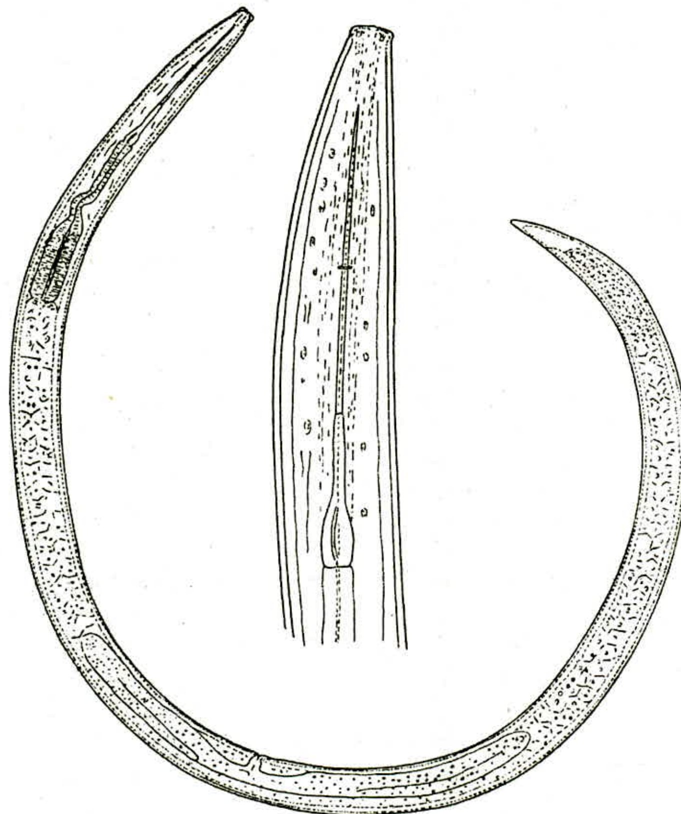
Exercise :- Draw the observed labeled diagramme.

I. Dagger nematode (*Xiphinema*)

Parasitism and habitat : Ecto-parasitic on many plants and often associated with perennial plants. All stages are found in the rhizosphere.

Main Morphological Characters

Body	:	Typically long (1.5 to 5.0 mm), slender without annulations
Stylet	:	Typically long, very slender having flanged knobs and "guiding ring" located near the base of the stylet
Oesophagus	:	Anterior tube slender and the posterior part wide
Ovaries	:	One or Two
Vulva	:	Located near the middle of the body or near end of oesophagus when only one ovary is present
Tail	:	Bluntly rounded or with projection on the ventral side
Resembling genus	:	*Longidorus* and *Paralongidorus* (in these two genera flanged stylet knobs absent and guiding ring located near anterior end of the stylet.)



Mature female

Exercise :- Draw the observed labeled diagramme.

Glossary

Basal knobs- Protuberance (usually three) at the base of the stylet, functioning as posterior points of attachment of the stylet muscles; also called stylet knobs.

Bilateral symmetry - Body devisable into two equal counterparts or mirror image halves through dorso-ventral plane.

Buccal/mouth cavity- The cylindrical or sub-globular structure connecting the oral opening with the interior portion of oesophagus and armed with a protrusible spear or stylet; also called buccal capsule.

Bursa – Lateral cuticular extension present at the posterior end of the male nematode, clasps the female during copulation.

Chlorosis – The plant physiological disorder in which the chlorophyll is deficient and photosynthesis is limited.

Cloaca- Common part of male digestive and reproductive systems that opens outside through a common cloacal aperture, emptying through the anus.

Cuticle- Non cellular, external covering of body wall, secreted by hyphodermis.

Cyst- The dark, hardened body or oxidized cuticle of dead adult female of *Globodera* and *Heterodera* species that provide protection to eggs contained within it.

Disease- Harmful physiological processes due to continuous irritation by a primary causal agent.

Ectoparasite- A parasite living and feeding on a host from outside.

Egg shell- Outermost covering of egg containing chitin inside where the development of embryo takes place.

Egg mass- Gelatinous matrix secreted by female nematode in which eggs are laid.

Embryogenesis- Sequential development of embryo from single celled egg.

Endoparasite – A parasite that enters the host tissue and feeds from within.

Gall- An abnormal swelling or localized outgrowth (tumor) on roots, often more or less spherical or fusiform, composed of unorganized cells produce by the plant as a result of attack by nematode (eg. *Meloidogyne* sp.)

Genital primordium- The group of initial cells in juveniles which give rise to reproductive system of adults.

Giant cells- A multinucleate mass of protoplasm formed by the division of nucleus without cell division or cell wall formation (eg. in *Meloidogyne* infested tissue).

Gonads- A sex organ; part of reproductive system responsible for production of germ cells or gametes (ova/sperm). Ovary in female and testis in male.

Gonoduct- Part of reproductive system responsible for carrying germ cells from gonads to the exterior.

Gubernaculum- The grooved, sclerotized plate like hard structure located to the posterior to the spicules of male nematode; guide the movement of spicules.

Hatching- The process involving the emergence of juvenile from egg shell, eclosion.

Hyperplasia- An abnormal increase in the number of cells of a tissue.

Hypertrophy- An abnormal increase in the size of cells of a tissue.

Hypodermal chords – Dorsal, ventral and lateral protrusions of hypodermis into the pseudocoelom.

Hypodermis- A thin, cellular or syncytial layer of body wall lying beneath the cuticle, responsible for the production and maintenance of cuticle.

Infective stage- The stage in the life cycle of a parasite that invades the host tissue and establishes the infection.

Isthmus- The narrow portion of the oesophagus between the metacarpus and the basal region or posterior bulb.

Juvenile or larva- Any of four life stages of nematode between embryo and adult; an immature nematode which does not yet have functional gonads.

Median bulb- The enlarged, usually ovate, posterior portion of carpus (in the middle portion of oesophagus), frequently containing a valve.

Knots- A localized abnormal swelling of plant tissue due to infestation of nematode.

Migratory- Capable of moving about freely, within or outside the host; vagrant.

Morphology- Study of forms, structure and development of organisms.

Moulting- To shed or cast off cuticle or body encasement in the transition from one developmental stage to the next to permit growth; ecdysis.

Nematode- Worm-like organism defined as triploblastic, bilaterally symmetrical, un-segmented, pseudocoelomate, invertebrates, with 4 hypodermal chords, a tri-radiate oesophagus, circum-oesophageal nerve ring and lacking specialized organs for respiration and circulation.

Nerve ring- The centre of nervous system or brain in nematode, in the form of a ring encircle the oesophagus, composed of nerve fibers, cell bodies of neurons and associated ganglia; also called the circum-oesophageal commissure.

Oesophagous- Anterior portion of alimentary canal between stoma and intestine, lined internally with cuticle.

Parthenogenesis- Reproduction of egg without fertilization.

Perineal pattern- A fine, wavy, fingerprint-like characteristic arrangements of cuticular striae near the perineal (area around anus, vulva, phasmid and tail) region in *Meloidogyne* females. This pattern is more or less an identifying characteristics of the species.

Pseudocoelom- The space between wider outer tube (body wall) and shorter inner body tube (alimentary canal) of nematode body; also called as body cavity. It is devoid of mesodermal epithelial lining.

Pseudocoelomic fluid – The watery lymph like nutritive fluid in the pseudocoelom in which internal organs are suspended.

Rhizosphere- The area in soil immediately surrounding plant roots and influenced by them.

Sedentary- Staying at a place, stationary, sessile.

Spicule- A pair of heavily cuticularized male copulatory organ.

Stylet or spear- A scletorized, slender, axially located, hollow feeding structure located in the buccal cavity or mouth cavity.

Syncytium-A multinucleate mass of protoplasm form by the dissolution of walls of adjacent cells.

Triploblastic- Possessing three germ layers i.e. ecto, meso and endoderm.

Triradiate- Having three radiating sides (used for the lumen of oesophagus).

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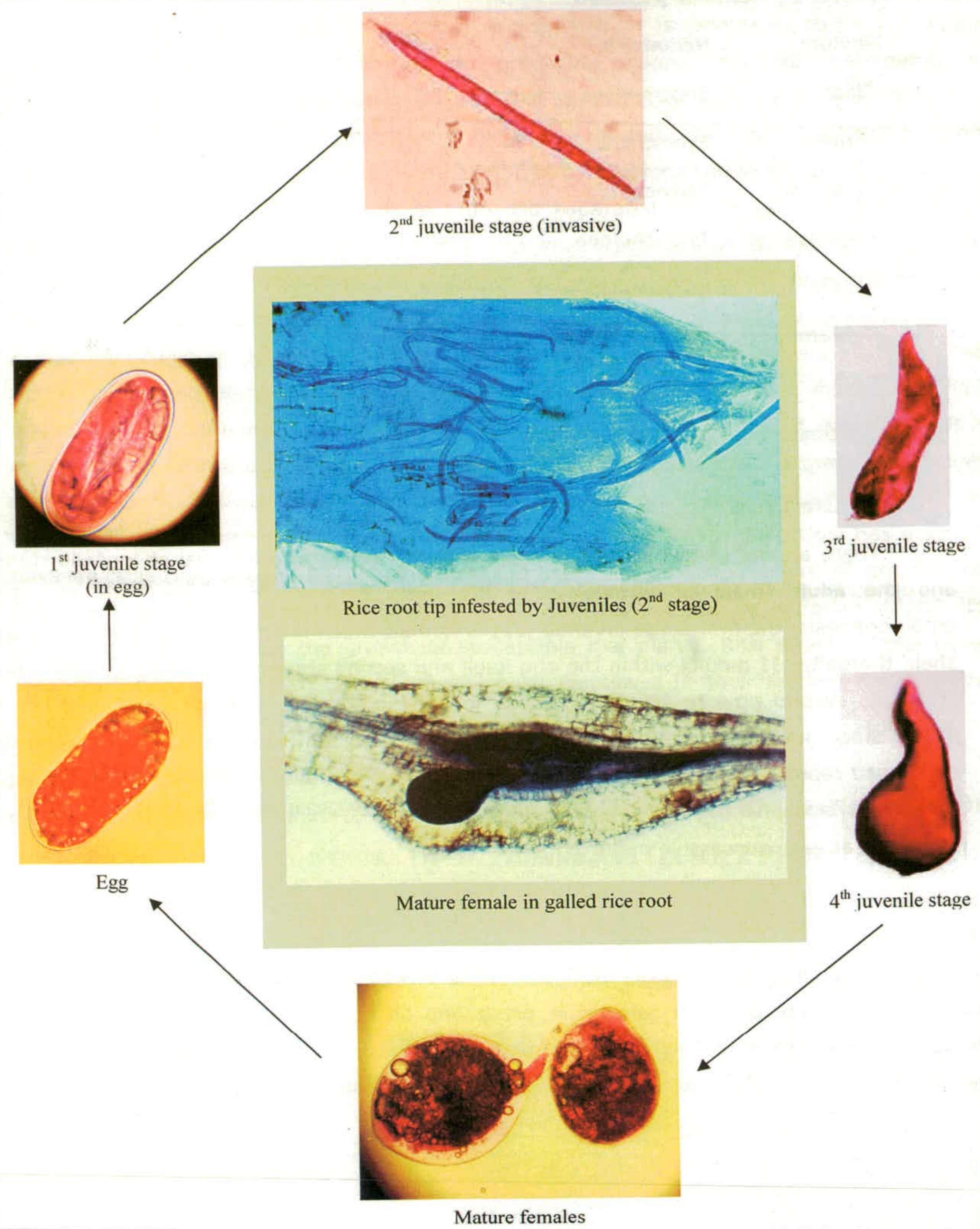

Life cycle pattern of root knot Nematode

Root knot of Papaya

Root knot of Beans

Root knot of Paddy

Root knot of Carrot