Lesson 27

Titles of the Experiment:

1. Symptom Identification and Study of Histological Changes of Nematode Infected Plants
2. Isolation of Nematodes from Soil (Bearmann Funnel Method) and Studying Various Life Cycle Stages of Nematodes

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Introduction

This laboratory exercise is aimed to provide an opportunity for teachers to familiarize with the symptoms and the histological changes of nematode infected plants by observing above ground and below ground parts of infected plants along with various stages of the nematodes inside the plants. This also provides them to familiarize with the Baermann Funnel method used for the isolation of soil nematodes.

Theory

Nematodes are tiny, round-bodied, un-segmented, worms. Most yards typically have billions of them in the soil, feeding on organic matter, bacteria, insects and plants. Nematodes are one of the most common of all animals, but they usually can't be seen without a microscope. Approximately 10% of all nematodes feed on plants, living around or in the roots. The most well known is the root knot nematode *(Meloidogyne* spp.), because of the distinctive galls it causes on infected roots, it's wide distribution, and the wide range of plants that it attacks (including most common vegetables, ornamentals, and many other crop plants).

Morphology and Anatomy

Nematodes are simple animals, often only containing 1000 cells or less. Plant-parasitic nematodes occur in all sizes and shapes. The typical nematode shape is a long and slender worm-like body, but often the adult animals are swollen and no longer even resemble worms (Figure 1). Plant-parasitic nematodes range from 250 um to 12 mm in length, averaging 1 mm, to about 15-35 um in width. While nematodes may look dramatically different, they all share some common features. Nematodes in all or part of their life cycle are worm-shaped (vermiform), although some species become swollen and rounded in later life stages (Figure 2). The basic body plan of a nematode is a tube within a tube. They have an outer skin or cuticle that is secreted from an inner hypodermis. The muscles are attached longitudinally to the nematode’s hypodermis, allowing them to move only in the dorsal ventral direction (snake-like movement). Inside the nematode there is an inner tube, the alimentary canal, which runs inside the nematode from head to tail. Between the alimentary canal and the body wall is fluid that provides pressure against the wall to maintain body shape and allow movement. At the head of a plant-parasitic nematode is a hollow mouth spear (like a hypodermic needle) called a stylet (Figure 3). The nematode uses this stylet to puncture plant cells, to withdraw food and also to secrete protein and metabolites that aid the nematode in parasitizing the plant. The intestine ends at the rectum in the female nematode and in the middle to posterior of the nematode are the reproductive organs. Nematode species often have both males and females, but it is not uncommon for plant nematodes to reproduce asexually by parthenogenesis. In females the reproductive organs are used as traits for identification because the number of ovaries and the position of the vulva in the female nematode's body are easily seen under the light microscope (Figure 4). Male nematodes have one or two testes and they are easily identified by the presence of spicules. Spicules are copulatory structures that are used during mating to guide the sperm into the vagina of the female nematode (Figure 4).

Figure 1: Swollen worm like nematode body

Figure 2: Root knot female nematode with a swollen rounded body

Figure 3: Plant parasitic nematode with a stylet

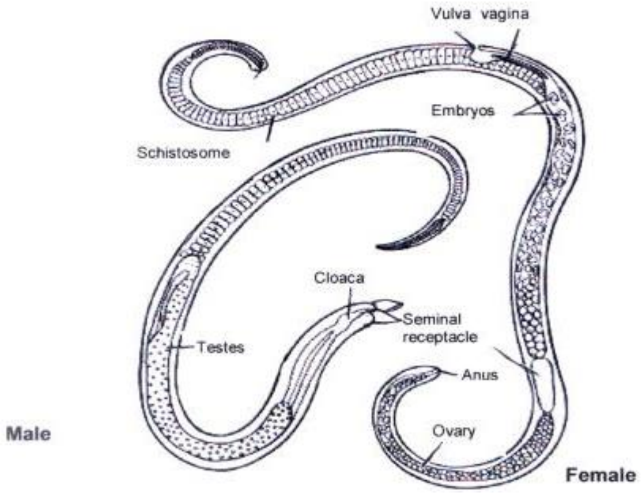

Figure 4: Male and female nematodes

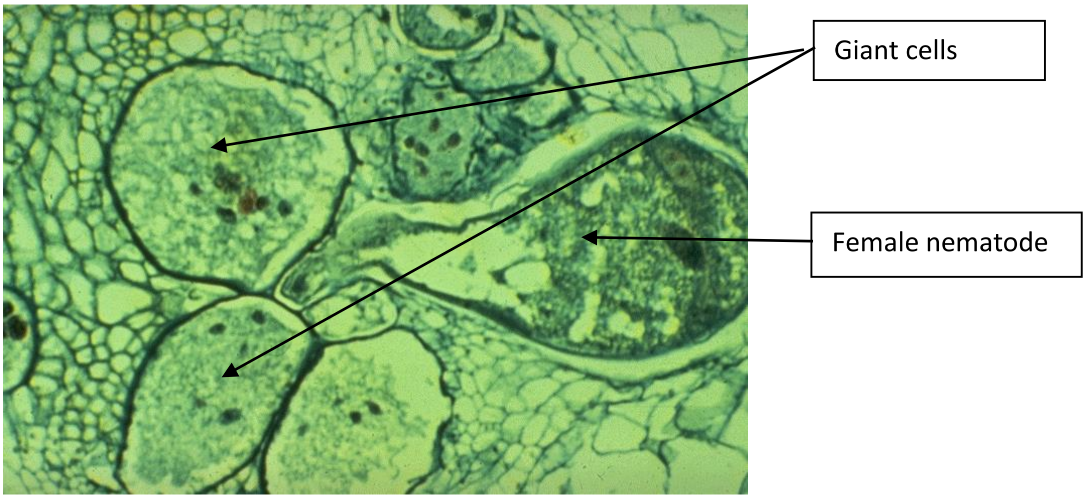

Figure 5: Giant cells produced in a plant root tissue

The common plant-parasitic nematode genera are fairly easy to identify to that level using a standard compound microscope. Identification of nematodes to the species level often requires detailed morphological analysis, growth of the nematode on different host plants, or DNA or isozyme analysis. Common morphological features used in nematode identification include the mouth cavity (presence or absence and shape of a stylet), the shape and overlap of the pharyngeal glands with the intestine, size and shape of the nematode body at the adult stage, size of the head, tail, and number and position of ovaries in the female. One of the guide to nematode identification is "Plant-Parasitic Nematodes: a Pictorial Key to Genera" by William F. Mai *et al.,* 1996.

Classification

All plant nematodes belong to the Kingdom Animalia and Phylum Nematoda. Most of the important parasitic genera belong to the Order Tylenchida and few belong to the Order Dorylaimida. Classification of a root knot nematode is as follow.

Kingdom: Animalia
Phylum: Nematoda
Class: Secernentea
Order: Tylenchida
Family: Heteroderidae
Genus: *Meloidogyne*
Species: *Meloidogyne incognita*

Plant parasitic nematodes are either ectoparasites or endoparasites. Ectoparasites normally do not enter root tissue but feed only from the outside on the cell near the root surface. Endoparasites enter the host and feed from within. Both of these can be either migratory (live freely in soil and feed on plants without becoming attached) or sedentary (feed the plant being inside or becoming attached).

Nematode Dissemination

While nematodes are motile animals, most are able to move no more than a meter through the soil within their lifetime. However, this lack of long distance crawling does not mean nematodes cannot rapidly spread from field to field. Farm equipment and even muddy shoes contaminated with nematode-infested soil can rapidly disperse nematodes. The movement of water during floods and irrigation can disperse nematodes over long distances. Likewise the movement of nematode infected plants, seeds, and bulbs can give nematodes international tickets to travel the world if plant quarantine officials are not careful. The ability of nematodes to form environmentally resistant stages makes their dissemination even easier, since dried nematodes can be blown with the wind or plant debris over large geographical regions. Even migrating birds are suspected to be able to carry nematodes along their flight paths, assisting the nematodes in their quest for new homes. Essentially any process that moves soil or plant tissue has the ability to disperse plant nematodes, making them difficult plant pathogens to quarantine.

Plant-Nematode Interactions

Nematodes feed on all parts of the plant, including roots, stems, leaves, flowers and seeds. Nematodes feed from plants in a variety of ways, but all use a specialized spear called a stylet.

Often nematodes withdraw the contents of plant cells, killing them. When this type of feeding occurs, large lesions are formed in the plant tissue). Some nematodes do not kill the plant cells they feed upon but "trick" the plant cells to enlarge and grow, thus producing one or more nutrient-rich feeding cells for the nematode. These feeding cells enable long term feeding associations, and form by repeated nuclear division in the absence of cell division (giant cells) or by the incorporation of adjacent cells into a syncytium formed by the breakdown of neighboring cell walls (Figure 5). Collectively, nematodes can feed on almost any plant cell type, and form a variety of feeding cell types. The number of feeding cells can vary from one to a half dozen depending on the nematode species.

Symptoms caused by nematodes in plants:

Nematode infection of plants result in the appearance of symptoms on roots as well as on the above ground parts of the platns. The aboveground symptoms of nematode damage to roots are relatively nondescript, including nutrient deficiency, yellowing of foliage, necrotic lesions, twisting or distortion of leaves and stems, incipient wilt, stunting, poor yield and sometimes plant death. Root symptoms may appear as root lesions, root knots or root galls, excessive root branching, injured root tips and root rot when accompanied by plant pathogenic bacteria.

General life cycle (disease cycle) of a plant pathogenic nematodes

The disease cycle occurs during the growing season of the host plant. An appropriate host as well as appropriate environmental conditions must be present for disease to occur. Nematodes are spread to an appropriate host through various mechanisms including: water (rain and irrigation) and humans. Signals from the host will trigger hatching of nematode eggs in soil. Nematodes go through various stages during their life cycle. Only the second juvenile stage can infect plants. Most nematodes mechanically penetrate a host using the stylet. Nematodes will actively move from one cell to the next by their own power. Most infections by nematodes are localized (contained within a small area of the plant). A single nematode can do little damage to a plant root. However, when large numbers build up in the soil over several growing seasons, many root areas can be attacked at once and significant damage can result. Nematodes will survive the winter and unfavorable conditions in roots of dormant plants, in infected plant debris, in seeds and in bulbs or as eggs in the soil. Once penetration occurs, a nematode must come in contact with susceptible cells for infection to take place. Once infection occurs, symptoms may be visible immediately or after some time (life cycle is described in detail under *Meloidogyne* spp., root knot nematodes).

Stem and bulb nematodes:

Stem and bulb nematodes (*Ditylenchus* spp.) are, as their name suggests, nematodes that attack the upper and lower parts of plants (Figure 6). They use water films to migrate up the stem of the plant and therefore are more damaging under wet conditions. The infectious stage of the stem and bulb nematodes is the fourth stage juvenile. This stage often enters emerging plant tissues below ground, but can crawl up stems in a film of water and enter shoots via buds, petioles, or stomata. Once in the host plant, they destructively feed as migratory endoparasites, molt into adults and reproduce. The nematodes hatch from the egg as J2 and continue to feed, molt and reproduce, extensively macerating and distorting the plant tissue. Once the plant is destroyed or winter arrives, the stem and bulb nematode juveniles arrest their development at the environmentally resistant J4 stage and overwinter. Fluffy masses of dried (cryptobiotic) Ditylenchus can be seen on the surface of bulbs and are known as "nematode wool." Once environmental conditions are favorable, the cryptobiotic J4 become active and their life cycle resumes.

Seed gall nematodes:

Seed gall nematodes (*Anguina* spp.) were the first plant-parasitic nematodes to be described in the scientific literature in 1743 (Figure 7). These nematodes migrate as J2s in water films to the leaves of plants where they feed as ectoparasites at the tips, causing distortion of the leaves. Once the plant starts to flower the J2 penetrates the floral primordia and starts to feed on the developing seed. Once in the seed, the nematode undergoes its molts, continues to feed, and eventually kills the seed to form a blackened "cockle" (seed gall). The adults sexually reproduce, the eggs hatch as J1 and then quickly molt into a J2 survival stage. The environmentally resistant J2 desiccates with the seed gall and overwinters. The nematodes in the seed gall can survive for 30 years if kept in a dry location. When proper moisture and temperature conditions arise, the cryptobiotic J2 becomes active and starts the life cycle over again.

Figure 6: *Ditylenchus* spp. in onion bulb tissues

Figure 7: Life cycle of *Anguina*, the seed gall nematode

Figure 8: *Aphelenchoides* spp., the foliar nematode

Figure 9*: Meloidogyne spp.,* the root knot nematodes

Foliar nematodes:
Foliar nematodes are in the genus *Aphelenchoides.* The adult nematodes migrate in water films on the stems to the leaves of their host plant and penetrate the leaves through natural openings (stomata) (Figure 8). Once in the leaves the nematodes migrate, destructively feed, molt and lay eggs. The feeding activity of the nematodes causes characteristic interveinal chlorosis and necrosis of the leaf, ultimately killing it. The nematodes are able to move from leaf to leaf if the proper (moist) environmental conditions exist and can severely damage a plant. In the winter the adult nematodes persist in the dead leaves until favorable conditions arise in the spring. If the dead, nematode-infested leaves are moved or blown around this will help disperse the nematode near new host plants.

Root knot nematodes:
Root knot nematodes are in the genus of *Meloidogyne*. They occur the world especially in the areas with worm or hot climates and short or mild winters. Sri Lanka being a tropical country most of our crops including ornamental plants and vegetables are severely attacked by some species *Meloidogyne (M. incognita*). They attack more than 2000 species of plants, including almost all cultivated plants, and reduce world crop production by about 5%. Losses in individual fields however may be much higher. Infection in plants shows above ground as well as below ground symptoms. Above ground symptoms are reduced growth and fewer small, pale green or yellowish leaves that tend to wilt in warm weather. Flowers and fruits are few and of poor quality. Infected roots develop the typical root knot galls that are several times large in diameter as the healthy roots. Several infections along the roots give the root a clubbed appearance and sometimes a bushy root system (figure 9).

Life Cycle of *Meloidogyne spp.*
All nematodes pass through an embryonic stage, four juvenile stages (J1–J4) and an adult stage. Nematode Juveniles hatch from eggs as vermiform, second-stage juveniles (J2), the first molt having occurred within the egg. Newly hatched juveniles have a short free-living stage in the soil, in the rhizosphere of the host plants. They may reinvade the host plants of their parent or migrate through the soil to find a new host root. J2 larvae do not feed during the free-living stage. Briefly, second stage juveniles invade in the root elongation region and migrate in the root until they became sedentary. Signals from the J2 promote parenchyma cells near the head of the J2 to become multinucleate to form feeding cells, generally known as giant cells, from which the J2 and later the adults feed. Concomitant with giant cell formation, the surrounding root tissue gives rise to a gall in which the developing juvenile is embedded. Juveniles first feed from the giant cells about 24 hours after becoming sedentary.

After further feeding, the J2s undergo morphological changes and become sausage like. Without further feeding, they molt three times and eventually become adults. In females, which are close to spherical, feeding resumes and the reproductive system develops. The life span of an adult female may extend to three months, and many hundreds of eggs can be produced. Females can continue egg laying after harvest of aerial parts of the plant and the survival stage between crops is generally within the egg.

Learning Outcomes:
After completion of this laboratory session, you should be able to :

(i) demonstrate skills in identifying nematode infected plants by studying the macro symptoms and histological changes.
(ii) demonstrate skills in isolating nematodes from soil using Baermann funnel method
(iii) demonstrate skills in identifying different stages of the life cycle of nematodes.

Materials/Equipment:

Nematode infected plants
Glass slides
A light microscope
Glass funnel (12 -15 cm in diameter)
A stand

Methodology/Procedure:

Symptom identification and study of histological changes of nematode infected plants:

(a) Observe the symptoms present in the disease specimens provided
(b) Make line drawing of all specimens.
(c) Obtain cross section of root knots and observe under the light microscope. (Note the giant cell formation in the cortex and observe clusters of giant cells within the vascular region. Also note female nematode embedded near the vascular bundles or in the cortex).

Studying stages of the life cycle of *Meloidogyne* spp.

(a) Isolation of eggs and larvae of *Meloidogyne* spp.

i. Observe the root knots of given specimens under the dissecting microscope and identify the eggs sacs of *Meloidogyne* spp.
ii. Remove the egg sacs (white/ brown in colour) clinging to the outsides of the root using a dissecting needle and crush it on a drop of water placed on a glass slide.
iii. Observe eggs in all stages of development, larvae and sometimes male.
iv. Identify them and make suitable drawings.

(b) Isolation of female *Meloidogyne* spp. from root knots

i. Place a small piece of root tissues with root knots of given specimens in a shallow dish with a little water.
ii. Then place the dish under the dissecting microscope and pick the tissue apart using two dissecting needles and check the presence of pale white collared and pear shaped female.
iii. Make suitable drawings.

Baermann funnel method:

A baermann funnel consists of a fairly large glass funnel (12 - 15 cm in diameter) to which a piece of rubber tubing is attached, with a clamp placed on the tubing.

(a) Bring soil samples from the field.
(b) Place the funnel on a stand and fill with water.
(c) Place the soil sample in the funnel on porous wet- strength paper
(d) Pour water into the funnel till all the soil being below the surface of water
(e) Allow the soil in the water to stand overnight or several hours (24 hours).
(f) Take the water in the rubber tube in to a Shallow dish
(g) Place drops of water from the dish on glass slides and observe the nematodes in various stages of their life cycle through a light microscope.
(h) Make suitable drawings.

Recommended Reading:

Agrios G N (2005). Plant Pathology. Fifth edition. Elsevier Academic Press

Mai W F, Mullin P G (1996). Plant parasitic nematodes: A pictorial key to Genera. Second edition. U S A; Comstock Pub Assoc