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Detection Survey Protocol for *Meloidogyne* spp. in Nepal

Government of Nepal
Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
Hariharbhawan, Lalitpur

March, 2025

Approved
March 12, 2025

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NPPO-Nepal, 2025

Endorsed by NPPO-Nepal on March 12, 2025

1. Background Information

With entry into the WTO, Nepal gets the opportunity to export its produce to the international markets. However, the exports from Nepal have not escalated to the same proportion as trade between developed nations. Developed countries have increased exports by using the rules of the SPS Agreement. At the moment, the Government of Nepal is obliged to use the SPS rules to exclude commodities that are posing a threat to the related industries within the country. Nepal should provide an adequate description of the health status of plant-based industries, while negotiating access to foreign trade. Prospective importers of Nepalese agriculture-related commodities assess the risk of introducing new pests based on the authentic pest information provided. Prospective importers also assess the phytosanitary measures being practiced in Nepal to reduce risk to an acceptable level. Extensive specimen-based records are the key for Nepal to negotiating with importing countries on a fair trading system. This document gives detailed guidelines for detection surveys of the nematode *Meloidogyne* spp. in the field of agriculture. Besides, it will be applicable for monitoring, surveillance, import inspection and export certification and is the basis for specimen-based records to be developed by the NPPO-Nepal.

Under the Plant Quarantine and Protection Act, 2064, article 6(2), survey and surveillance functions and responsibilities are designated to NPPO-Nepal as per the sub-clause (i) "To perform such other functions as prescribed". This technical guideline to undertaking a pest detection survey of *Meloidogyne* spp. has been prepared with a view to guiding the survey activity. This protocol is prepared for researchers, plant protectionists, teachers, and other concerned professionals. This document will be a guide to submitting specimens to a laboratory for diagnosis and preservation.

1.1 About the pest (Root-knot nematode)

Root-knot nematodes, members of the genus *Meloidogyne* (Goldi, 1892) (Tylenchida: Meloidogynidae), are one of the most economically damaging genera of plant-parasitic nematodes on agricultural crops (Eisenback & Triantaphyllou, 1991; Onkendi et al., 2014). Root-knot nematodes are polyphagous and sedentary endoparasites that attack numerous commercially significant crops (Riva, 2023) and their host range exceeds 3000 plant species (Abad et al., 2003). The genus *Meloidogyne* includes about 100 described species (Perry et al., 2009), including four of the most important species, *M. incognita*, *M. javanica*, *M. hapla* and *M. arenaria* (Moens et al., 2009; Dong et al., 2012), which are responsible for substantial losses in agriculture

around the world (Onkendi et al., 2014). *Meloidogyne* species, such as *M. javanica* and *M. incognita*, pose major threats to the production of a wide range of crops. These nematodes thrive in warm climates or regions with short winters, making them a significant threat in tropical and subtropical areas. Coyne et al. (2018) reported yield losses of up to 50% in maize caused by *Meloidogyne* spp. in sub-Saharan Africa. *Meloidogyne* species, particularly *M. incognita*, *M. javanica*, *M. arenaria* and *M. enterlobii*, are commonly found in maize (Pretorius, 2018). These nematodes cause significant damage by inducing root galls, impairing nutrient and water uptake, and ultimately reducing crop yield (Abad et al., 2003). Symptoms can be hard to diagnose, as above-ground signs such as wilting, yellowing and stunted growth may resemble nutrient deficiencies or water stress. This makes it difficult to detect root-knot nematode infections until they have caused substantial damage.

Meloidogyne spp. is a quarantine pest for China. As such, to comply with the protocol between the Ministry of Agriculture and Livestock Development of the Government of Nepal and the General Administration of Customs of the People's Republic of China on the Safety and Health Condition of Haylage Export from Nepal to China, the exported haylage must be free from *Meloidogyne* spp. However, *Meloidogyne* has not been found infecting maize crop in Nepal.

In Nepal, root-knot nematodes (*Meloidogyne* spp.) are the most commonly prevalent in vegetable crops (Baidya et al., 2017), such as tomato, broad bean, cowpea, radish, coriander, potato, pumpkin, chilly, brinjal, onion and bitter gourd in various districts, including Chitwan (PPD, 2009).

1.2 Identity and taxonomy of target pest (CABI, 2021a)

1.2.1 Identity

Preferred Scientific Name: *Meloidogyne*

Preferred Common Name: Root-knot nematodes

International Common Names

Spanish: anguilulosis de la raíz (Argentina); nematodo de nodulos

French: nématodes cécidogènes

Local Common Names

Denmark: rodgallenematoder

Finland: aekaemaeankeroiset

Germany: Älchen-Arten, Wurzelgallen

Japan: nekobu-sentyubyo

Netherlands: wortelknobbelaaltje

Norway: rotgallnematoder

Sweden: rotgallnematoder

Turkey: kok ur nematode

Nepal: Jarama ghatha banauni juka (जरामा गाँठा बनाउने जुका)

1.2.2 Taxonomy

Taxonomic tree of the nematode is presented below

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Nematoda

Family: Meloidogynidae

Genus: *Meloidogyne*

Species: *Meloidogyne acronea*, *Meloidogyne ardenensis*, *Meloidogyne arenaria*, *Meloidogyne artiella*, *Meloidogyne brevicauda*, *Meloidogyne coffeicola*, *Meloidogyne enterolobii*, *Meloidogyne exigua*, *Meloidogyne graminicola*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne luci*, *Meloidogyne naasi etc.*

Note: Among the *Meloidogyne* species listed above, only *M. arenaria, M. hapla, M. incognita, M. javanica*, and *M. graminicola* are reported in Nepal. Furthermore, *M. chitwoodi* and *M. fallax* are quarantine pests of Nepal (GoN, 2071 BS). If identification is confirmed, the whole consignment and crop grown will be destroyed as per government rules and regulations.

1.3 Host range

Root-knot nematodes (*Meloidogyne* spp.) attack numerous commercially significant crops and their host range exceeds 3000 plant species (Abad et al., 2003), including vegetables, fruits, cereals, ornamental plants as well as many weeds. Many crops of botanical families such as Cucurbitaceae, Solanaceae, Poaceae, Fabaceae, Chenopodiaceae, Malvaceae, Liliaceae, Asteraceae, Brassicaceae and Apiaceae are majorly damaged by root-knot nematodes. Some of the main host plants are tomato (*Solanum lycopersicum*), brinjal (*Solanum melongena*), potato (*Solanum tuberosum*), cucumber (*Cucumis sativus*), maize (*Zea mays*), rice (*Oryza sativa),* carrot (*Daucus carota*), cotton, soybean (*Glycine max*), grapevine (*Vitis vinifera*), pea (*Pisum sativum*), cowpea (*Vigna ungiculata*), okra (*Abelmoschus esculentus*) sugarcane (*Saccharum officinarum*), tobacco (*Nicotiana tabacum*), cotton (*Gossypium hirsutum*), tea (*Camellia sinensis*) (CABI, 2021a; CABI, 2021c; Eisenback, 2020).

1.4 Nematode biology and damage symptoms

1.4.1 Life cycle and field identification

Root-knot nematodes are endoparasites that rely on the formation of a permanent feeding site within the plant's roots to complete their life cycle. The life cycle consists of six stages: eggs, four juvenile stages (J1 to J4), and the adult stage. Temperature plays a key role in the duration of the nematode's life cycle. For most thermophilic *Meloidogyne* species, the life cycle typically ranges between 20 and 30 days at temperatures of 25 to 30°C (Greco & Di Vito, 2009). Under favorable conditions, multiple generations of *Meloidogyne* can occur within a single growing season, causing significant damage to the host plant's root system.

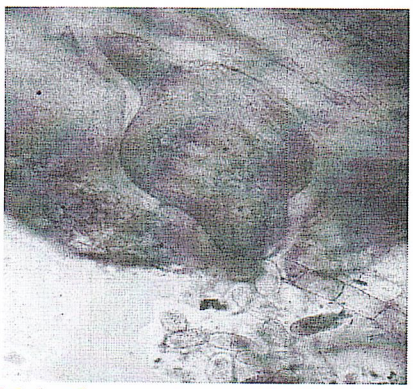


Figure 1. *Meloidogyne incognita* female egg sac in root (Eisenback, 2020)

a) **Egg Stage:** According to Moens et al. (2009), female root-knot nematodes lay eggs into gelatinous masses composed of a glycoprotein matrix secreted by the female's rectal glands, which keeps the eggs together and protects them against environmental extremes and predation. These eggs are often found within root galls or on the surface of these galls. The female root-knot nematode can produce hundreds up to 1000 eggs (Karssen et al., 2013; Subedi et al., 2020). The egg mass is initially soft, sticky and hyaline but becomes firmer and dark brown with age (Moens et al., 2009).

b) **Juvenile Stage (J1 and J2)**: First stage juvenile (J1) develops within the egg, and molts into motile and vermiform second stage juvenile (J2) which hatch out of the egg (Wale et al., 2008; Moens et al., 2009). Second stage juvenile (J2), which is infective stage, penetrates the roots of the host plant by piercing the plant cell wall with the help of stylet (Subedi et al., 2020). When conditions become favorable, particularly with the detection of a host plant, the J2 hatches and migrates toward the plant roots (Moens et al., 2009). After penetrating the host root, J2 actively moves through the root to initiate and establishes a permanent feeding site by transforming nearby root cells into giant cells, which serve as a nutrient source (Karssen et al., 2013; Moens et al., 2009).

c) **Juvenile Stages (J3 and J4)**: Under favorable conditions, the J2 stage molts into the third stage juvenile (J3) after 14 days and then progresses into the fourth stage juvenile (J4) (Moens et al., 2009). The third and fourth stage juveniles are sedentary inside the root (Karssen et al., 2013). The combined duration of the J3 and J4 stages is typically 4–6 days, much shorter than the J2 or adult stages (Moens et al., 2009). Sexual dimorphism becomes evident at the fourth stage juvenile (J4), where environmental conditions influence the nematode's sex (Karssen et al., 2013). In adverse environmental conditions such as nutrient scarcity, more males are produced, which are motile and leave the host (Subedi et al., 2020).

d) **Adult Stage**: Males are typically small and free-living in the soil, while females remain inside the plant roots, *i.e.* sedentary. Females lay eggs in a gelatinous matrix attached to their posterior end when they reach maturity (Escobar et al., 2015). After maturation, female nematodes swell and become pear-shaped (Karssen et al., 2013), often causing characteristic "root-knots" or galls (Moens et al., 2009).

Root-knot nematodes have two reproductive strategies: sexual (amphimixtic) and asexual (parthenogenetic) (Chitwood & Perry, 2009). In amphimixtic reproduction, males are obligatory for copulation (Eisenback &

Triantaphyllou, 1991), with sperm being stored in the spermatheca (Pretorius, 2018). In parthenogenetic reproduction, females reproduce without males, with eggs dividing by meiosis to develop further (Eisenback & Triantaphyllou, 1991). This asexual reproduction is key to the pathogenic success of some species (Chitwood & Perry, 2009).

1.4.2 Damage symptoms

Above-ground symptoms of root-knot nematode infestation in maize are typically seen as uneven patches of stunted or poorly growing plants (Pretorius, 2018). Infected plants often show signs like yellowing (chlorosis), wilting, and poor vigor (CABI, 2021b), which can be mistaken for nutrient deficiencies or water stress. This is due to the nematodes feeding on the roots, impairing the plant's ability to absorb water and nutrients (Karssen et al., 2013). Below-ground, symptoms can vary depending on the nematode population density. For instance, maize seedlings in areas heavily infected with root-knot nematodes may develop root galls (knots), while older plants in these areas might not show visible galls (Pretorius, 2018).

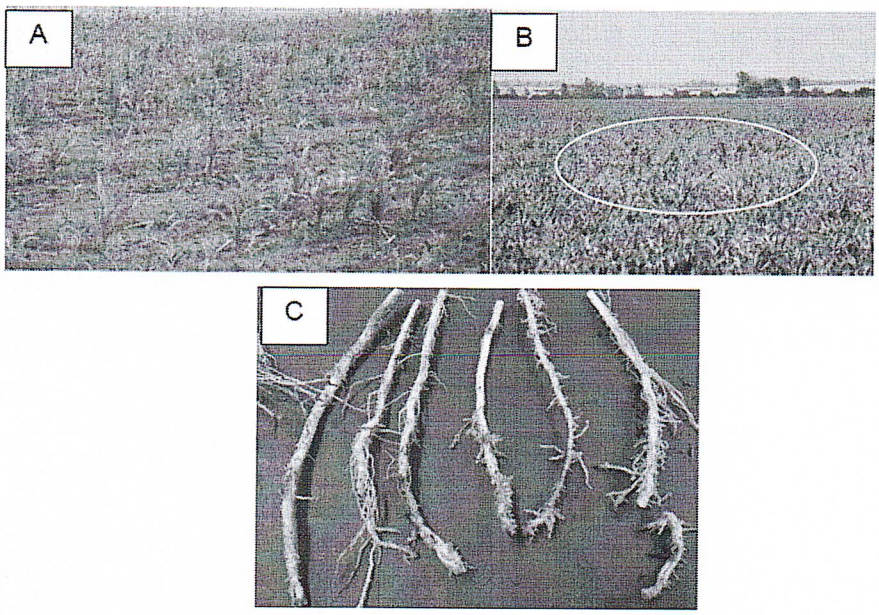


Figure 2. Above ground damage symptoms of root-knot nematode (*Meloidogyne* spp.) in maize (**A** and **B**); and galled and stunted maize root due to root-knot nematode (**C**) in South Africa (Photo Source: **A**; Chris Schmidt, North-West University; **B** Driekie Fourie, North-West University and **C** Suria Bekker, North-West University, Potchefstroom, South Africa).

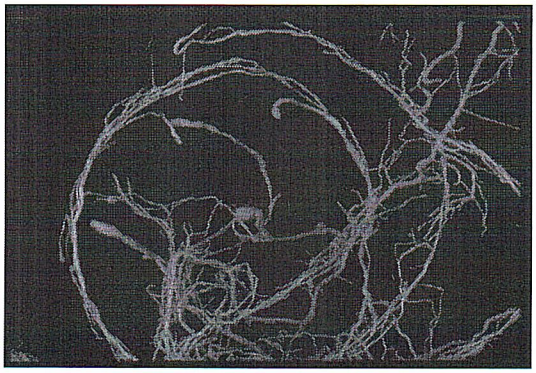


Figure 3. Maize root infested with *Meloidogyne incognita* (Eisenback, 2020)

1.5 Mode of dispersal

a) **Natural Dispersal:** Natural dispersal in the field is very restricted and occurs mainly due to plant-to-plant infestation via root networks in the soil (Perry et al., 2009), often resulting in an aggregated distribution. Run-off water from root-knot nematode-infested fields could infest other fields with the eroded soil and water (Palomares-Rius, 2022).

b) **Human Dispersal:** *Meloidogyne* spp. are dispersed passively over long distances through propagation material (seedlings, tubers, etc.), machinery (soil adhering to machinery can create secondary foci within the same field and primary foci in previously non-infested fields), soil, water (rainwater can transport soil and eggs/juveniles of *Meloidogyne* spp. to ditches; using the water from these ditches for irrigation can further spread these nematodes) (Been & Schomaker, 2006).

2. Detection survey

A detection survey is conducted in an area to determine if pests are present (FAO, 1990; revised FAO, 1995). These surveys are more frequently carried out to determine pest status in an area, and they follow a definite survey plan, which is approved by NPPO-Nepal. These surveys are carried out either seasonally or annually and / or following the eradication measures applied to a pest in a given area or production sites. These surveys are organized following a definite survey methodology based on statistical sampling, which are determined after taking into account the biology of the pest and employing appropriate detection techniques such as field diagnostic kits, traps etc. The results of the survey are documented and communicated (PPD/NPPO-Nepal, 2071 BS).

2.1 Purpose and scope of detection survey

The purpose of the detection survey is to determine the presence or absence of *Meloidogyne* spp. in a given area or production sites. The scope will be limited to maize and other defined crops to be grown for haylage/silage production for export to China and other concerned countries.

2.2 Timing of survey

Conduct the survey twice during the cropping season of maize:

- **Pre-planting:** To establish baseline data on nematode presence (Hafez, 1997).
- **Vegetative Growth Stage (V6-V8):** To detect the presence of nematodes and assess the impact of their infestations on plant growth and yield potential, conduct the detection survey during the middle of the growing season (Grabau & Vann, 2024), such as the sixth leaves stage (V6) and eighth leaves stage (V8).

2.3 Selection of survey area

Field plots of maize and concerned crops in the target areas.

2.4 Materials required for survey

- Soil auger or hand corer for soil sampling
- Gloves
- Hand lens
- Sample collection bags
- Zip lock plastic bags
- Plastic sampling containers
- GPS device
- Field notebook for recording observations
- Microscope for nematode identification
- Nematode extraction kit (Baermann funnel, sieves of different mesh sizes, including Whitehead tray, or centrifuge setup)

- Labels/tags
- Data sheets (with different formats for field recording and lab recording separately)
- Permanent markers
- Rubber bands

2.5 Identification of *Meloidogyne* spp.

Classification and identification of *Meloidogyne* species depends largely on morphological characters. Morphological identification of *Meloidogyne* nematodes is based on known reference specimens, literature descriptions and taxonomic keys and descriptions. Available molecular data can be helpful for species determination. However, until methods can be simplified and made more uniform, they are of limited value for phytosanitary purposes.

2.5.1 Morphological identification

Meloidogyne species can be challenging to identify with certainty due to their inherent variability. Adult females and infective juveniles should be examined to ensure accurate identification (Eisenback, 2020). The characters used for identification of *Meloidogyne* species using light microscopy include the appearance of adult females (body shape, stylet length, knob shape and perineal pattern shape), males (head shape, stylet length, knob shape and DGO-stylet knob length), and second-stage juvenile (body length, tail and hyaline tail length, DGO-stylet knob length, hemizonid position, and shape of tail and hyaline tail) (Karssen et al., 2013; CABI, 2021b).

2.5.1.1 Female Nematodes

Females of *Meloidogyne* species are pearly white with a rounded to pear-shaped body, size varies from 350 µm to 3 mm in length and 300-700 µm in width, with the stylet length ranging from 10-25 µm and the shape of stylet knob from rounded to transversely elongated and posteriorly sloping (Karssen et al., 2013). *Meloidogyne arenaria* females are pearly white and pear-shaped, measuring 500-1000 µm in length and 400-600 µm in width, with a 13-17 µm long robust stylet which is characteristically shaped with large, posteriorly sloped, tear-drop-shaped stylet knobs (CABI, 2021b).

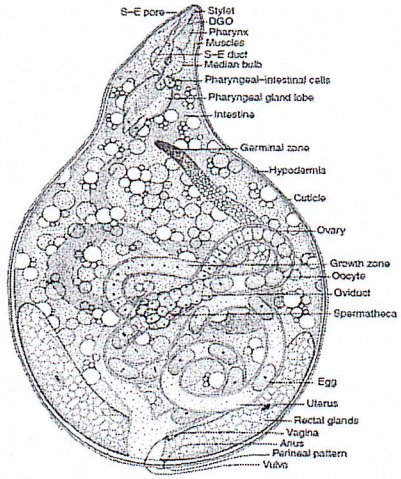


Figure 6. A female root-knot nematode (Eisenback & Triantaphyllou, 1991)

According to Karssen et al. (2013), the cuticle annulation is only visible in the head region and the posterior part of fully mature females, where a distinct cuticular or perineal pattern can be observed around the perineum (the vulva–anus region). The shape of this pattern can vary and is influenced by various developmental factors. This pattern is observed by creating perineal slides (e.g., after killing and staining the nematode). Features such as striations, wavy lines, and dorsal arch height help differentiate species. The cuticle's structure varies slightly between species and may be observed using high magnification (usually 400x or more). Look for annulations (rings) or reticulation patterns. The perineal pattern of *M. arenaria* usually has a low and rounded dorsal arch, lines in the lateral areas of the dorsal arch that sharply curve toward the tail terminus and meet the ventral striae at an angle (CABI, 2021b). Whereas, the perineal pattern of *M. javanica* has a round or oval to pear-shaped, dorsal arch varying from rounded to moderate height, sometimes flattened dorsally (CABI, 2021c).

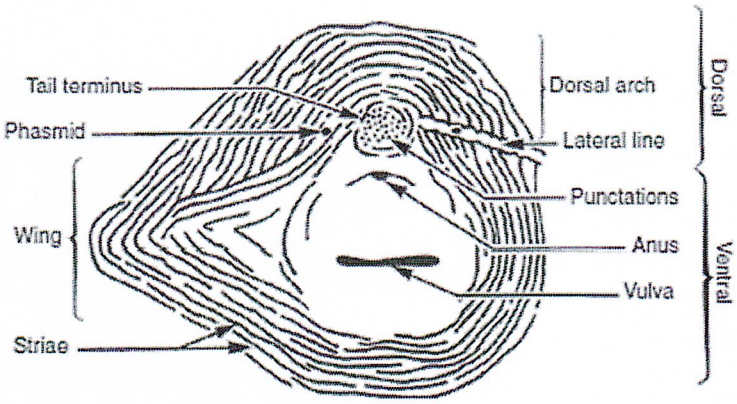


Figure 7. A root-knot nematode perineal pattern (Eisenback & Triantaphyllou, 1991)

2.5.1.2 Male Nematodes:

Identifying the male of *Meloidogyne* species (root-knot nematodes) is more challenging than identifying the female due to its smaller size and less distinctive features. Males are often present in low numbers, which makes their identification less frequent in samples. Head shape, stylet length, knob shape and DGO-stylet knob length are examined to identify the males of *Meloidogyne* species. Males are vermiform (worm-like), annulated, with a body length of 600-2500 µm, stylet length of 13-33 µm and DGO-stylet knob length of 2-13 µm (Karssen et al., 2013).

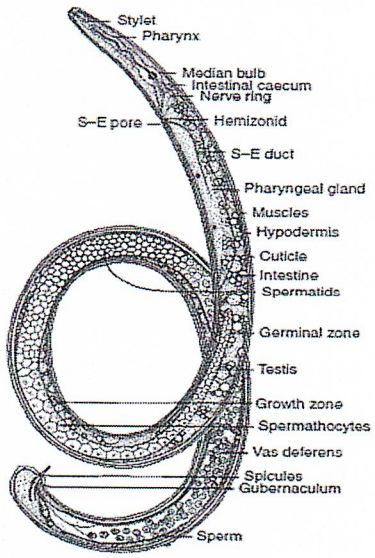
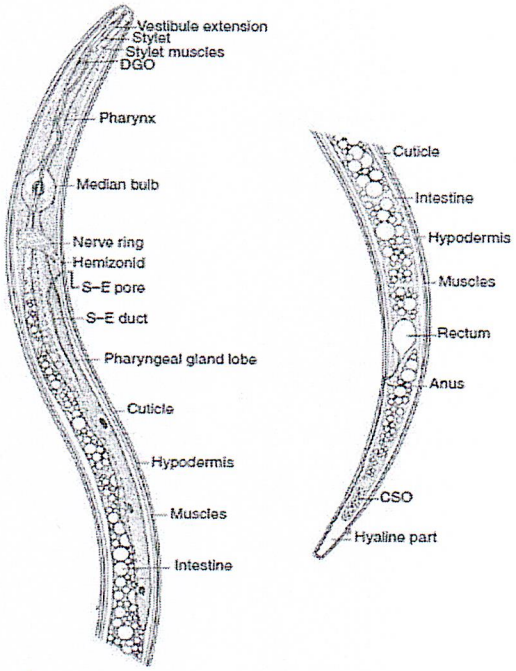


Figure 8. A male root-knot nematode (Eisenback & Triantaphyllou, 1991)

M. incognita males are vermiform, 1-2 mm in length with a bluntly rounded tail, terminus unstriated and slightly curved spicules (Eisenback, 2020). While males of *M. arenaria* are long (0.9-2.3 mm) and narrow (27-48 µm) with a longer stylet (20-28 µm) that is robust with a bluntly pointed tip (CABI, 2021b).

2.5.1.3 Second stage juveniles (J2s):

Karssen et al. (2013) described the infective second stage juvenile (J2) as vermiform and annulated, with a body length ranging from 250-600 µm, and a 15-100 µm long tail, tapering towards the tail tip which ends in a hyaline tail part. While the stylet is 9-16 µm long and the DGO-stylet knob length ranges from 2-



12 µm. Second-stage juveniles of *M. incognita* are vermiform, less than 0.5 mm long, tail tapering to subacute terminus, annulations coarsening posteriorly (Eisenback, 2020). Second-stage juveniles of *M. arenaria* are long (398-605 µm) and slender (13-18 µm), with a moderately long tail (44-69 µm) and a poorly defined hyaline tail terminus, which is moderately long (6-13 µm) and has a finely rounded to pointed tip (CABI, 2021b).

Figure 9. Second juvenile stage of root-knot nematode (Eisenback & Triantaphyllou, 1991)

2.6 Sample size and sampling methods

Select 5-10 random sampling points per maize field, ensuring that sampling spreads across the entire field while avoiding the field edges or areas with obvious damage. Collect samples in a zigzag pattern, W-pattern or Z-pattern from the root zone of the maize crop to account for field variability (DCS, 1996) (Fig. 10).

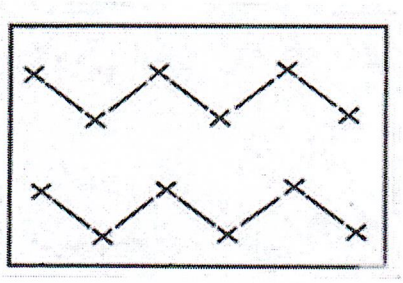


Figure 10. Zigzag sampling pattern for annual crops for nematode analysis (DCS, 1996)

2.6 Collection of samples and nematode extraction

Collect soil and/or root samples to confirm the presence of nematodes in the field and send to laboratory (Grabau & Vann, 2024) for further morphological and molecular identification.

2.6.1 Soil Samples

Soil sampling is one of the primary methods for detecting nematode presence, especially in the absence of clear visual symptoms.

- Select 6 random sampling points per field. At each sampling point, collect soil cores around 200 g from the surface to a depth of 15-20 cm, close to the plant roots (root zone), with the help of a soil auger. Mix the soil samples from all sampling points to prepare a final composite sample of 200 g (Baidya, 2023).
- Take about 20 soil cores from an area of 10 acres or less and cores within a single area. Mix them thoroughly to make a composite sample. Soil should not be overly wet or dry when sampling (Grabau & Vann, 2024).
- Place soil samples in sterile plastic bags, close them tightly with rubber bands to prevent moisture loss and label each sample with field information (e.g., field ID, GPS coordinates and location, date of sampling, name of crop).

- Store the soil samples in a cool, dry place to avoid direct sunlight and transport them to the laboratory as soon as possible for nematode extraction.

2.6.2 Root Samples

- Carefully uproot 1-2 maize plants (V6-V8 growth stage) per sampling point with the tops discarded and the soil shaken from the root systems, ensuring minimal damage to the roots (DCS, 1996).
- Check the roots for the characteristic galling caused by root-knot nematodes.
- Collect 5-10 cm segments of roots from each plant to get a representative sample from each sampling point to make a composite sample of 50-100 g (Pretorius, 2018).
- Place the root samples in a clean container or plastic bag and label each root sample with the same information as the soil sample (e.g., field ID, GPS coordinates and location, date of sampling, name of crop).
- Ensure roots are kept cool (but not frozen) and transported to the lab within 24-48 hours for nematode extraction and analysis.

2.6.3 Nematode extraction

a) Nematode extraction from soil: Modified Baermann Funnel Method

Extract the juvenile and adult nematodes from soil using the modified Baermann funnel method (Figure 11) described by Hooper et al. (2005) as follows:

- Crumble each sample finely by hand. Spread the tissue paper inside a plastic sieve placed on extraction plates (plastic plate, extraction tray, plastic tray). Take 100 g of soil from the composite sample and spread uniformly over the tissue paper.
- Add clean tap water carefully from one side of the extraction tray until the soil layer gets totally wet. Label the extraction tray and the sieves to identify the soil samples.
- Leave the extraction sets for 24 hours and move motile juveniles from soil to the extraction tray.
- After 24 hours, lift the plastic sieves with soil to drain the water into the extraction tray, and remove the soil.
- Pour the water with nematodes from the extraction tray into a labeled beaker. Use a wash bottle to rinse the tray and also add the water into the beaker.

- Take this volume of water (300 ml) with nematode juveniles as a stock sample or suspension. Leave the suspension for an hour to settle down the nematodes.
- Pipette out 10 ml suspension from the stock sample after stirring well and keep into empty petri-plates for isolation/extraction of juveniles.
- Use a nematode fishing needle for manual fishing and collect them in the cavity blocks. Count the nematode numbers and identify genera/species under compound stereo-microscope and compound microscope (Baidya, 2023).

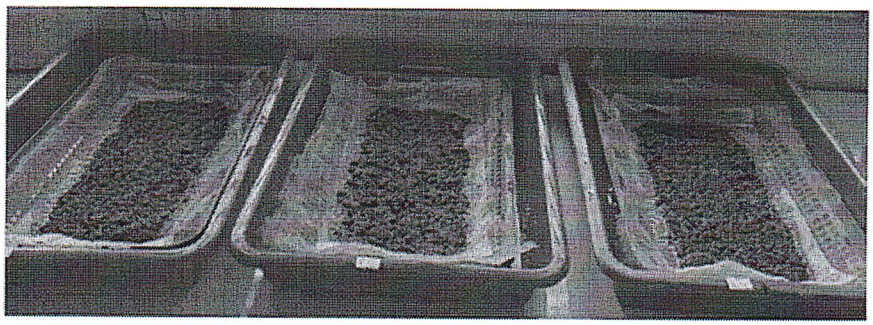


Figure 11. Modified Baermann funnel method for extraction (Baidya, 2023)

b) Nematode extraction from roots: NaOCl extraction method

- Wash roots to remove adhering soil and take 5 g root from a composite sample of 50-100 g.
- Cut the galled-root into 1-2 cm pieces.
- Put these root pieces in a 250 ml conical flask containing 200 ml of 0.5% sodium hypochlorite (NaOCl) solution then shake vigorously for 4 mins to extract nematode eggs by sieving method (Barker et al., 1985).
- Sieve the solution into a 30 µm mesh sieve for the extraction of eggs.
- Collect juveniles by pouring the egg suspension onto a filter paper and incubate at 28±2 °C to obtain freshly hatched juveniles (J2). Collect juveniles within 2-4 days of incubation (Baidya, 2023).

2.6.4 Fixing and mounting technique

Fix the nematodes with a hot 4% formaldehyde solution and transfer to anhydrous glycerin (De Grisse, 1969). Prepare microscope slides using a paraffin wax ring to mount the nematodes. Position a paraffin ring in the center of the glass slide followed by a small drop of glycerin. Place the nematodes on the

glycerin drop, and position a cover slip carefully on top of the ring. Heat the slide on a hot plate until the paraffin melts, allowing the cover slip to settle securely in place (Ryss, 2017).

2.7 Data recording and mapping

- Data should be recorded in several respects like
  - ✓ Date of collection
  - ✓ Collection number
  - ✓ Locality
  - ✓ GPS coordinates
  - ✓ Elevation
  - ✓ Host plant type and infestation severity
  - ✓ Plant growth stage
  - ✓ Local name(s)
  - ✓ Habit and habitat
- Use mapping tools like GIS to create infestation distribution maps

2.8 Diagnostic laboratories

- National Plant Pathology Research Centre, Nepal Agricultural Research Council, Khumaltar, Lalitpur
- Central Agricultural Laboratory, Department of Agriculture, Hariharbhawan, Lalitpur
- National Herbarium and Plant Laboratories, Department of Plant Resources, Godawari, Lalitpur
- Natural History Museum, Swayambhu, Kathmandu
- Private laboratories – Center for Molecular Dynamics Nepal (CMDN), Thapathali, Kathmandu; Nepal Plant Disease and Agro Associates (NPDA), Balaju, Kathmandu, and others if any.

3. Reporting

The responsible or concerned organizations (diagnostic laboratory) or an independent surveyor, after analysis and identification, should submit a report to the NPPO-Nepal for the reporting/declaration of nematode. The reports should include infestation maps, photographs and specimen vouchers. If specimens cannot be identified morphologically, they should be identified by molecular methods.

4. Record keeping

NPPO-Nepal, in collaboration with responsible laboratories, will preserve the specimens and keep all the records safely. The documentation system should be well maintained by the NPPO-Nepal and the collaborating institutions will have access to it.

5. Molecular identification of *Meloidogyne* spp.

While morphological identification can provide initial clues, *Meloidogyne* species identification often requires molecular techniques like PCR (Polymerase Chain Reaction) or DNA barcoding for confirmation, as many species are morphologically similar (Riva, 2023).

5.1 Extraction of deoxyribonuclease (DNA) and polymerase chain reaction (PCR)

Isolate the eggs and/or J2 of the *Meloidogyne* populations obtained with a micropipette (eggs) or fished out with a fine-tip needle (J2) from the respective samples using a stereo-microscope (40 × magnification). Transfer the eggs and/or J2 from each individual sample to a 1.5 ml Eppendorf tube containing approximately 20 µl, double-distilled water.

Extract the DNA of the eggs/J2 from each field separately using the following procedure (Pretorius, 2018):

- Crush the nematodes using a melted, glass pipette tip.
- Add 20 µl chelex® 100 (5 %) solution and 3 µl proteinase K (20 mg/ml) to each sample.
- Centrifuge the J2 homogenate at 12000 rpm for 20 seconds and incubate at 56 °C for 2 hours.
- Incubate at 95 °C for 10 minutes to deactivate the proteinase K and store at -20 °C until it is used for the polymerase chain reaction (PCR).

Follow any of these methods to identify *Meloidogyne* spp. present in the sample:

a) NADH dehydrogenase subunit 5 (NADH5) technique and DNA sequencing (Pretorius, 2018)

- Prepare the DNA homogenate of eggs/J2 from each field in individual PCR tubes for sequencing by adding 12.5 µl Master mix, 8 µl nuclease-free water, 1 µl forward primer, 1 µl reverse primer and 8 µl template DNA.

- Dilute the primers to 10 p.mol before use. Centrifuge all samples for 10 seconds and place in the C1000™ Thermal Cycler (BioRad) under the conditions described in Table 1.
- Analyze the PCR product by electrophoresis on a 1.5 % agarose gel with 1x TAE buffer.
- Load an O'GeneRuler™ 1kb DNA Ladder in the first well to establish the size of the DNA bands for the eggs/J2 present in each sample.
- Mix 4 µl template DNA of the eggs/J2 from each field with 2 µl GelRed and add to individual wells in the agarose gel.
- Run the gels then for 30 minutes at 120 V in an electrophoresis chamber and visualize with ultraviolet (UV) illumination.
- The size of the NADH5 band is visible at the 610 bp fragment length.
- Send the remaining DNA template of eggs/J2 from each field for sequencing in Genbank.

Table 1. PCR: Thermocycling conditions

Steps	Temperature (°C)	Time (min)	Cycles
Initial denaturation	94	2	
Denaturation	94	30	
Annealing	61	30	35x
Extension	72	60	
Final Extension	72	5	

b) Sequence-derived amplified region – polymerase chain reaction (SCAR-PCR)

- Specific primers are commercially available to amplify the DNA of *Meloidogyne* spp. life stages. Primer sets are available for species such as *M. arenaria*, *Meloidogyne enterolobii* Yang & Eisenbach, 1983, *Meloidogyne hapla* Chitwood, 1949, *M. incognita* and *M. javanica*.
- The primers each constitute eight to ten nucleotides (Table 3).

The egg/J2 DNA is subjected to the following protocol (Pretorius, 2018):

- Each PCR tube contains 12.5 µl Master mix, 8.5 µl nuclease-free water, 1 µl forward primer, 1 µl reverse primer and 2 µl template DNA. Dilute the primers to 10 p.mol before use.
- Include a 'no template' control (NT-no DNA, only containing nuclease free water) and a known reference sample containing DNA of monoculture populations of each *Meloidogyne* spp. in the SCAR-PCR amplification process (Table 2).
- Centrifuge the content of the PCR tubes and place in the C1000™ Thermal Cycler (BioRad) under the conditions described in Table 2.
- After PCR amplification, analyze the products via gel electrophoresis to confirm the species' presence of the expected DNA fragment size.
- Sequence the PCR products to obtain the nucleotide sequence using a Sanger sequencing method.
- Compare the sequences obtained against available genetic databases (e.g., GenBank) using sequence alignment tools such as BLAST (Basic Local Alignment Search Tool) to identify the species based on their genetic similarities.
- Confirm the species of *Meloidogyne* based on the similarity of the obtained DNA sequence with known sequences from *Meloidogyne* species (Table 3). The comparison may involve phylogenetic analysis to establish a closer relationship among the identified species.

Table 2. *Meloidogyne* spp. populations used as references during the identification of root-knot nematode species contained in maize roots

***Meloidogyne* spp. and life stage**	**DNA fragment size (bp)**
Meloidogyne arenaria; second-stage juveniles (J2)	420
Meloidogyne enterolobii, second stage juveniles	200
Meloidogyne incognita; mature females	1200
Meloidogyne javanica; mature females	670

Table 3. Primer codes used for the identification of *Meloidogyne* spp. for both NADH5 and SCAR-PCR

Code	Primer sequence 5'-3'	Specificity and reference source
[1]FNAD5	TATTTTTTGTTTGAGATATATTAG	*Meloidogyne* spp. identification (mtDNA gene) (Janssen et al., 2016)
[2]RNAD5	CGTGAATCTTGATTTTCCATTTTT	
[1]Far	TCGGCGATAGAGGTAAATGAC	*Meloidogyne arenaria*-specific SCAR (Zijlstra et al., 2000)
[2]Rar	TCGGCGATAGACACTACAACT	
[1]Finc	CTCTGCCCAATGAGCTGTCC	*Meloidogyne incognita*-specific SCAR (Zijlstra et al., 2000)
[2]Rinc	CTCTGCCCTCACATTAGG	
[1]Fjav	GGTGCGCGATTGAACTGAGC	*Meloidogyne javanica*-specific SCAR (Zijlstra et al., 2000)
[2]Rjav	CAGGCCCTTCAGTGGAACTATAC	
[1]Fent	AACTTTTGTGAAAGTGCCGCTG	*Meloidogyne enterolobii*-specific SCAR (Long et al., 2006)
[2]Rent	TCAGTTCAGGCAGGATCAACC	

[1]F=Forward primer, [2]R=Reverse primer

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Annex #. Standard sample label for sampling of plant parasitic nematodes

Date of sampling	Sampling time
Farmer's name	Contact number
Location	
Altitude of the locality	GPS coordinates of locality
Total area sampled	Sample type: Soil Root
Crop history	Sampling unit: g

ANNEXES

Annex- 1: Field datasheet

1. Name of field/Site visited:
2. Date/Time of visit:
3. GPS reference point Latitude:
   Longitude: Altitude:
4. Province: District:
   Municipality: Ward no./Place:
5. Climate data of locality: Average min. temp (in °C):
Average max. temp (in °C): Rainfall (in mm)
6. Survey/Field plot no.
7. Host plant species inspected: Variety:
8. Phenological stage of the plant:
7.1 Description of habitat (such as aspect, slope, vegetation type, soil type)

7.2 Alternate host plant species found infected, if any:
9. Sampling method:
10 Contact details of the local informant involved in the survey:
11. Details of pest recorded

SN	Scientific name	Common name	Plant parts affected	Symptom & Sign	Disease incidence	Severity % / Score

10. Any additional information (including collection of specimens for investigation):

11. Name/Signature of surveyor with date:

Annex 2: Format for forwarding specimens

1. Collection number: 2. Date of Collection:
3. Submitting organization:
4. Name/Address/Contact no. of the sender:
5. Locality of collection (Province / District / Municipality / Ward No. / Place):

6. Reasons for identification:
7. Name of the host plant species (Scientific name / Common name / Variety:

8. Origin of host/commodity (Source of seed/planting materials, if applicable):

9. Plant parts affected:	[ ] roots; [ ] stems; [ ] leaves; [ ] inflorescence; [ ] fruits; [ ] seeds/nuts [ ] others (_____)
10. Category of pest specimen/organism submitted:	[ ] insects; [ ] mites; [ ] nematodes; [ ] fungi; [ ] bacteria; [ ] virus; [ ] others (_____)
11. Life stage of the pest (Applicable to insects):	[ ] egg; [ ] larvae; [ ] pupae; [ ] adult; [ ] nymphs; [ ] juveniles; [ ] anamorphic [ ]; cysts; [ ]others (_____)
12. Type of pest specimen/organism submitted:	[ ] preserved specimen; [ ] pinned/card board mounted specimen; [ ] dry specimen with host; [ ] culture; [ ] disease specimen (fresh); [ ] disease specimen (partially dry); [ ] slide mount; [ ] others (_____)

14. Number of specimens submitted per each collection:
15. Signature/stamp/office seal of the sender with date:

For identifier use

16. Name &address of Diagnostic/Referral Laboratory:
17. Remarks of identifier (condition of receipt of specimens):
18. Pest identification (Common/Scientific name/Taxon):
19. Description notes, if any:

Place: _____

Date: _____

_____

(Signature/Name/Designation of Identifier)

Note: This form should be prepared in duplicate by the sender and forwarded to the identifier/referral laboratory along with each collection of specimens. The identifier should return the original copy after entering the particulars of the pest identified along with description notes and remarks if the identifier will retain any to the sender of the specimen and duplicate the copy.