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Methodology for Root-knot Nematode Isolation, Morphological Identification and Analysis of Qualitative Parameters of Mulberry Foliage

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Abstract
A comprehensive, step-by-step methodology for isolating, identifying and estimating root-knot nematode populations in mulberry roots and soil has been meticulously compiled, providing a robust and authoritative framework for researchers and practitioners. Furthermore, a detailed approach for analyzing the qualitative parameters of mulberry foliage including biochemical and elemental constituents has been rigorously established. The paper also offers unparalleled insights into the complex interactions between root-knot nematodes and mulberry plants.

Keywords: Root-knot nematode, Root incubation method, Modified Baermann's funnel, Perineal patterns, Mulberry, Biochemical and elemental constituents

1. Introduction

Mulberry (*Morus alba* L.) is a highly adaptable perennial plant that thrives in diverse climatic conditions, ranging from temperate to tropical regions, and can grow well in various soil types. The foliage of mulberry is the primary economic product, as it is the sole source of nutrition for silkworms, *Bombyx mori* L.), which feed exclusively on mulberry leaves to support their growth and productivity. Consequently, the quantity of quality leaves produced per unit area has a direct impact on cocoon production and the quality of raw silk.

Among various factors influencing successful cocoon crop production, mulberry leaf quality stands out as a crucial contributor, accounting for approximately 38.20%. This highlights the significance of high-quality foliage in cocoon production. However, mulberry leaf quality is susceptible to degradation due to various biotic and abiotic stress factors, in addition to soil parameters. Biotic stresses like pests, significantly impact mulberry leaf quality. The perennial, fast-growing and lush green nature of mulberry plants makes them vulnerable to attracting pests and diseases [1]. These biotic stressors affect both the above-ground and below-ground parts of the mulberry plant, with symptoms often manifesting in the foliage.

The rhizosphere of mulberry is marked by a higher population of nematodes [2]. A total of 42 nematode species, spanning 24 genera, are associated with mulberry. This association has been reported in almost

all countries where sericulture is practiced, highlighting the widespread presence of these nematodes in mulberry cultivation [3]. Among the reported nematodes, the root-knot nematode (RKN), *Meloidogyne* spp. Pose severe threat to mulberry cultivation. The RKN, *Meloidogyne incognita* (Kofoid and White) Chitwood, was first identified as a pest of mulberry in India by Narayanan *et al.* in 1966 [4]. Infestations are more commonly observed in sandy to loamy soils and under irrigated conditions. As RKNs parasitize underground roots, damage symptoms often go unnoticed until severe stunting, marginal necrosis, and yellowing of leaves become apparent [5]. Characteristic knots or galls form on the roots, impairing water and nutrient uptake, and resulting in poor plant growth [6]. *M. incognita* is a significant pest, causing substantial damage to mulberry crops, resulting in a 20-50% reduction in leaf yield and a decline in leaf quality [7].

The RKNs undergo three life stages: egg, larva (with four juvenile stages: J1, J2, J3, and J4) and adult. The J2 larva infects host plants by entering the roots and feeding on parenchymatous cells, causing hypertrophy and hyperplasia, which leads to the formation of characteristic knots [8]. The larvae moult four times within the roots, developing into mature, oval or spherical, egg-laying females. Each female lays 200-300 ellipsoidal eggs, coated with a gelatinous substance, which hatch and release larvae into the soil under favorable conditions. The life cycle is completed in 30-40 days, with 2-3 cycles occurring annually. Optimal conditions for RKN growth and development include temperatures between 15-30°C and soil moisture levels of 40-60% [9]. As generations repeat, the size and number of galls increase, damaging parenchyma tissue and creating cracks and holes that invite secondary root infections. Consequently, root-knot infested plants exhibit symptoms of nutrient deficiency and other root diseases, such as root rot [10].

The accurate identification and characterization of RKNs are crucial for developing effective management strategies, as different species and populations may exhibit varying levels of virulence and response to control measures. This paper outlines a step-by-step approach for isolating RKNs from mulberry roots, identifying them based on morphological characteristics and a detailed methodology to analyze qualitative parameters of mulberry foliage affected by RKN infestation. By following the methodology presented here, researchers and practitioners can obtain reliable data on RKN populations, including their density, distribution and species composition.

Isolation of RKN

To isolate the RKN, five grams of infested root sample should be processed following the root incubation method [11]. The root samples should be washed with distilled water to remove any debris. The washed roots should be cut into 2.5 cm segments and sliced longitudinally. The cut segments must be placed on tissue paper spread over a wire gauge in a Petri plate, and the water level must be maintained to prevent desiccation. The Petri plate is then incubated undisturbed for 48 hours. Post incubation, the water in the Petri plate is transferred to a suspension column and the volume should be made to 200 ml, out of which 10 ml should pipetted out and must be observed under a stereo-binocular microscope for enumerating and identifying the isolated nematodes.

Identification of RKN species

The isolated nematodes must be transferred to a Petri plate containing distilled water kept under a stereo-binocular microscope. The posterior portion of the nematode should be cut with a perineal pattern knife [12] and the body contents should be cleaned. Transfer the cleaned posterior portion of the female

nematode to a drop of glycerin on a clean glass microscope slide. Carefully place glass wool fragments around the trimmed portion to prevent damage and ensure optimal viewing conditions. Place a cover slip over the specimen and seal with nail polish to prevent moisture loss. Finally, observe the prepared slide under a stereo binocular microscope and confirm species identification based on the perineal pattern characteristics described by Chitwood [13].

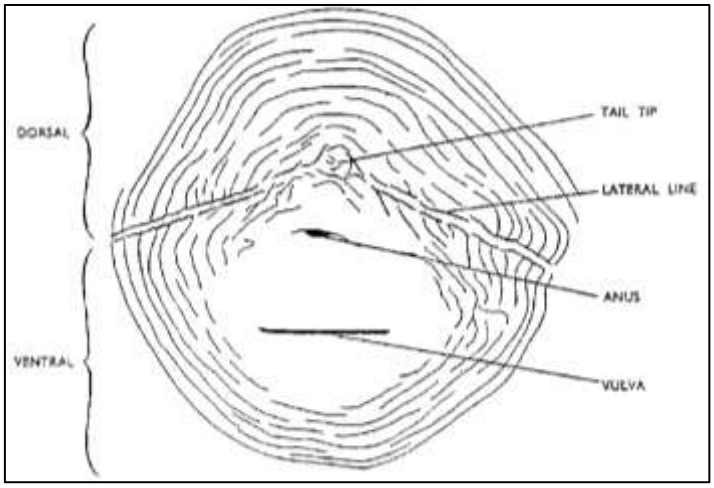

Fig 1: Perineal pattern in the female nematode (*Meloidogyne* sp.)

RKN species based on the perennial pattern

a) *Meloidogyne incognita*

1. Striae are smooth, wavy and sometimes in a zig-zag pattern
2. Lateral lines are absent
3. Squarish high dorsal arch containing a distinct whorl around the tail terminus

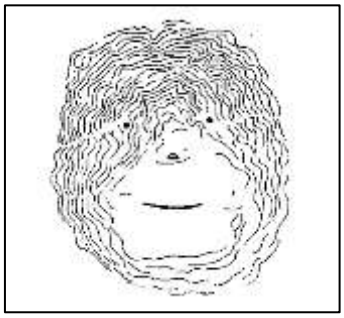

Fig 2: Perineal pattern of *Meloidogyne incognita*

b) *Meloidogyne javanica*

1. Striae are smooth and somewhat wavy
2. Unique distinct lateral ridges across the pattern and fade away around the tail terminus
3. The dorsal arch is often low and rounded, frequently possessing a whorl in the tail terminus area

Fig 3: Perineal pattern of *Meloidogyne javanica*

c) *Meloidogyne arenaria*

1. Striae are smooth and slightly wavy, often extended laterally, forming wings on one or both lateral sides of the patterns
2. Distinctive lateral ridges are absent and the pattern is forked, irregular lateral fields
3. The dorsal arch is low and intended near the lateral fields, forming rounded shoulders

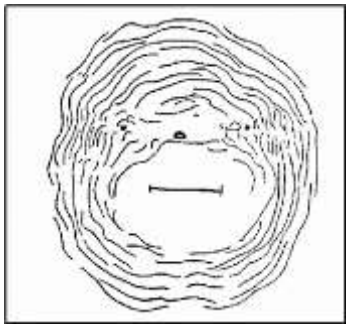

Fig 4: Perineal pattern of *Meloidogyne arenaria*

d) *Meloidogyne hapla*

1. Striae are close, smooth and wavy, some patterns form wings on one or both lateral side
2. The region of perennial pattern between the anus and tail terminus is stippled with subcuticular markings
3. Lateral ridges are absent, but irregularities in the striae mark the lateral fields and the dorsal arch is usually low and rounded
4.

Fig 5: Perineal pattern of *Meloidogyne hapla*

Taxonomical status of RKN

Phylum	-	Nematoda
Class	-	Secernentea
Sub class	-	Diplogasteria
Order	-	Tylenchida
Sub order	-	Tylenchina
Super family	-	Tylenchoidea
Family	-	Heteroderidae (Meloidogynidae)
Sub family	-	Meloidogyninae
Genera	-	*Meloidogyne*
Species	-	*incognita, javanica, arenaria, hapla*

Estimation of RKN population in soil

To estimate the RKN population in soil, a 200 cc soil sample is processed using a combination of Cobb's sieving and decantation method, followed by the Modified Baermann's funnel method [11]. Initially, the soil sample is added to a 1000 ml beaker with sufficient water to create a soil solution, which is then stirred thoroughly and allowed to settle for heavier particles. The solution is subsequently passed through a series of sieves with mesh sizes of 100, 250, 325 and 400, respectively. The residue collected from the 325 and 400 mesh sieves is then poured over tissue paper spread on a wire gauge, placed on Baermann's funnel, and maintained with a consistent water level to keep the tissue paper wet. The setup is left undisturbed for 48 hours, after which the suspension volume is adjusted to 200 ml, and a 10 ml subsample is pipetted out for nematode counting using a stereo-zoom binocular microscope. Finally, the nematode population is estimated for the original 200 cc soil sample.

Estimation of RKN population in mulberry roots

To estimate the RKN population in mulberry roots, a 5 g root sample is processed using the root incubation method [11]. Initially, the root samples are washed with water to remove any debris. The washed roots are then cut into 2.5 cm bits and sliced longitudinally to facilitate nematode emergence. The cut bits are placed on tissue paper spread over a wire gauge in a Petri plate, and the water level is maintained to prevent desiccation. The Petri plate is then incubated undisturbed for 48 hours, allowing nematodes to emerge from the root tissue. After incubation, the suspension in the Petri plate is collected and examined for nematodes using a stereo-binocular microscope. The counted nematodes are finally estimated to represent the population present in the original 5 g root sample.

Analysis of the qualitative parameters of mulberry foliage

A composite sample of forty leaves should be collected from every plant, it must be air-dried and then dried in the hot-air oven at 60 °C for 18 hours. The dried samples must be powdered and stored in polythene bags. These samples are used to analyze total carbohydrates, proteins, crude fibre, ash, nitrogen, phosphorus, potassium, calcium, magnesium and sulphur contents.

Biochemical constituents

i. Leaf moisture content (%)

The moisture content of mulberry leaves in each plant is determined by the hot air oven method, where

the fresh weight of the samples is recorded and then the samples are placed in the hot air oven for 24 hours at 105±1°C, again the dry sample weight is recorded. The per cent moisture content is determined by the formula:

$$\text{Leaf moisture content (\%)} = \frac{\text{(Fresh weight of sample)} - \text{(Dry weight of sample)}}{\text{Fresh weight of the sample}} \times 100$$

ii. Moisture retention capacity (%)

To determine the leaf moisture retention capacity after six hours of harvest, a composite sample of thirty leaves is harvested from each plant and the fresh weight is weighed immediately (ensure no water droplets/dew present). The leaves should be kept open under laboratory conditions and the weight must be recorded after 6 hours of harvest. The per cent moisture retention capacity is calculated by using the formula [14]:

$$\text{Moisture retention capacity (\%)} = 100 - \text{Moisture loss (\%)}$$

where, fresh weight is considered to be 100 per cent

$$\text{Moisture loss (\%)} = \frac{(\text{A} - \text{B})}{\text{A}} \times 100$$

where, A - fresh weight of leaf; B - leaf weight after six hours of harvest

iii. Chlorophyll content (mg/g)

Chlorophyll content in mulberry leaf is determined by the following procedure described by Hiscox and Isrealstam [15]. The leaf's chlorophyll 'a', 'b' and total chlorophyll contents are computed using the formula suggested by Arnon [16].

$$\text{Chlorophyll "}a\text{" (mg/g)} = \frac{(12.7\ \text{A663} - 2.69\ \text{A645}) \times \text{X}}{1000 \times \text{n}}$$

$$\text{Chlorophyll "}b\text{" (mg/g)} = \frac{(22.9\ \text{A645} - 4.68\ \text{A663}) \times \text{X}}{1000 \times \text{n}}$$

$$\text{Total chlorophyll (mg/g)} = \frac{(20.2\ \text{A645} - 8.02\ \text{A663}) \times \text{X}}{1000 \times \text{n}}$$

where, A663 - Absorbance at a wavelength of 663 nm; A645 - Absorbance at a wavelength of 645 nm; X – Total volume of the filtrate; n – Sample weight.

iv. Total carbohydrates (%)

The total carbohydrates in the leaf sample is estimated using the Anthrone method [17]. The amount of carbohydrates in the sample is determined by plotting a standard curve of absorbance at a wavelength of 630 nm (A630) on the Y-axis and concentration of glucose on the X-axis. The total carbohydrates is expressed in percentage.

v. Total protein (%)

The total proteins in the leaf samples is estimated using Lowry's method. The amount of protein in the sample is determined by plotting a standard absorbance curve at a wavelength of 660 nm (A660) along the Y-axis and protein concentration along the X-axis. The total protein is expressed in percentage

vi. Crude protein (%)

The per cent crude protein is estimated using the nitrogen content determined by the micro-Kjeldahl method involving digestion, distillation and titration of the sample using the formula:

$$\text{Crude protein (\%)} = \text{Nitrogen (\%)} \times 6.25$$

vii. Crude fibre (%)

The crude fibre of the sample is estimated by using a moisture and fat-free sample in Fibra plus apparatus. 2 g of moisture and the fat-free sample is transferred to fibre estimating thimbers and placed in the

digestion chamber. 200 ml of sulphuric acid must be added and the temperature is set at 500 °C for 10 min., then the temperature should be reduced to 400 °C and left for 30 min., after that the acid must be drained by using a suction pump and washed with boiled distilled water. After acid wash, the digestion chamber must be filled with 150 ml of boiling sodium hydroxide solution for about 30 min at 400°C. Then the alkali solution must be drained out using the suction pump and again the tubes must be washed with boiled distilled water. After that, the crucibles must be placed in an oven at 100 °C for about one hour and the weight of the crucible is taken after which the crucible should be placed in a muffle furnace at 550 °C for 3 hours or until the white ash is formed and the final weight is recorded.

The fibre content of the sample is calculated by:

$$\text{Crude fibre (\%)} = \frac{[100 - (\text{Moisture} + \text{Fat content of the sample})] \times \text{We} - \text{Wa}}{\text{Weight of the sample}} \times 100$$

where, We - Pre-weighed ash (g); Wa - Weight of dish after washing (g)

viii. Ash (%)

The ash content in the samples is determined using the formula [18]:

$$\text{Ash content (\%)} = \frac{(Z - X)}{Y} \times 100$$

where, X – Weight of empty crucible (g); Y – (Weight of sample) (g); Z – (Weight of crucible + ash) (g)

Elemental constituents

The powdered leaf samples (1 g) must be treated with 10 ml of concentrated HNO_3 and keep overnight for pre-digestion. Then the samples must be digested with 10 ml of the di-acid mixture (9:4 ratio of HNO_3 and $HClO_4$) until the snow-white residue remains. The residue should be cooled and diluted to 100 ml using distilled water, filtered and used to analyse all the elements except for nitrogen.

i. Nitrogen (%)

The nitrogen percentage in the leaf samples is determined by the Kjeldahl digestion-distillation method. The plant sample (1g) is digested using the digestion mixture and sulphuric acid in a digestion flask. After complete digestion, the digested material must be distilled in an alkaline medium and the liberated ammonia is trapped in a 2 per cent boric acid solution containing mixed indicator. The trapped ammonia must be then titrated against standard sulphuric acid [19].

$$\text{Nitrogen (\%)} = \frac{\text{TV} \times \text{Normality of acid} \times 0.014 \times \text{Volume of digested sample}}{\text{Weight of the sample} \times \text{aliquot taken}} \times 100$$

where, TV – Titration value

ii. Phosphorous (%)

Phosphorus content in the di-acid digested extract is estimated by the vanadomolybdo-phosphoric yellow colour method in nitric acid medium and the colour intensity is measured at 420 nm wavelength as described by Piper [19].

iii. Potassium (%)

Potassium in the plant sample is determined by atomizing the diluted di-acid extract in a flame photometer as outlined by Piper [19].

iv. Secondary nutrients

The calcium and magnesium contents in the mulberry leaves is estimated by the EDTA titration or the versanate-titration method. The sulphur content in the di-acid digested sample is estimated by the

turbidimetric method as outlined by [20].

v. Estimation of micronutrients

The micronutrients are estimated using the specified reagents in the atomic absorption spectrophotometer (AAS).

2. Conclusion

A systematic approach for isolating, identifying and quantifying RKN populations in mulberry roots and soil has been developed, providing a valuable tool for researchers and practitioners. Following morphological identification, molecular confirmation can be pursued to further validate the results. Furthermore, a comprehensive methodology for analyzing the qualitative parameters of mulberry foliage affected by RKN infestation has been outlined, encompassing biochemical and elemental constituents.

3. Conflict of Interest

None

4. References

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