CHAPTER III
MATERIAL AND METHODS

The present studies were focused on molecular characterization of *Meloidogyne* species of Assam, morphological and morphometric variations among the population of Assam and effect of abiotic factor namely, temperature on their biology. Experiments were carried out at Department of Nematology, Department of Agricultural Biotechnology and Department of Crop Physiology, Assam Agricultural University, Jorhat, during 2012-2015.

3.1 Molecular identification and characterization of root-knot nematodes of Assam

Soil as well as root samples were collected from 9 districts of Assam from the rhizosphere of different vegetations, particularly from vegetable crops. Root-knot nematode were extracted separately from each district and inoculated in susceptible tomato plants raised in sterilized soil for pure culture. Egg masses of root-knot nematode were collected from pure cultures, DNA were extracted from egg masses, then amplified it and gel electrophoresis was run.

3.1.1 Collection of soil sample and extraction of nematodes from soil

Soil samples were collected from around rhizosphere of various crops from nine districts of Assam (Jorhat, Golaghat, Sibsagar, Lakhimpur, Nagaon, Dhubri, Dibrugarh, Kokrajhar and Kamrup) as per standard procedure. Information on name of standing crop, previous crop, locality, date of collection, etc. was recorded at the time of sampling. Samples were mostly processed without delay.

Nematodes from the soil were extracted by Cobb’s modified sieving and decanting technique (Christie and Perry, 1951) using 20-, 60-, 150-, 250- and 325 mesh sieve. Each soil sample was thoroughly mixed and 250cc of soil was drawn from homogenous mixture of processing. The representative soil sample was placed in a plastic bucket and soaked in a liter of water. The soil lumps were broken to enable them to go into a suspension. The soil suspension was stirred well and allows standing for 5 seconds for the heavy soil particles to settle at the bottom of the bucket. This muddy suspension was then pour through 20 mesh sieve to discard other coarse materials. The
suspension from previous bucket was collected in a second bucket and was allowed to settle for about 15-30 seconds and then passed through a 60 mesh leaving the heavy particles in bucket. Washings from the 60 mesh sieve were collected in a 250 ml beaker. The whole aliquot obtained from 60 mesh sieve was then serially passed through 150-, 250- and 325 mesh sieve, collecting the residue left over on each sieve in a 250 ml beaker. The residues collected from different sieves were finally rinsed in 325 mesh sieves with a gentle jet of water and then collected in a beaker. The suspension containing nematodes was then poured gently over a single layered tissue paper placed over an aluminum wire gauge support and placed as such on the petridish containing filter water. After 24 hours, the nematode suspension was collected and examined under a stereozoom binocular microscope. The juveniles of root-knot nematode were picked using a bamboo splinter and were kept for inoculation to maintain pure culture.

3.1.2 Collection of root samples and extraction of nematode

Along with soil samples, root samples were also collected from nine districts of Assam. Root system of plants, those showing symptoms of yellowing, stunting were carefully observed. Root systems with characteristic gall were collected in polythene bags.

Labeling of collected plant samples were done as that of soil sample collected. The collected samples were washed carefully under tap water. The egg masses (clearly seen by naked eyes) that were attached to the infected root system were collected with the help of a forcep. The collected egg masses were kept for hatching at room temperature in water for 24 hours.

3.1.3 Raising pure nematode culture

3.1.3.1 Collection and sterilization of soil

For filling up the experimental pots, top soil was collected from Instructional cum Research (ICR) farm, AAU, Jorhat. The soil was cleaned thoroughly by removing unwanted materials like stones and roots, and then soil was mixed homogenously with dried cow dung and sand in the ratio of 2: 1: 1, respectively. The mixed soil was filled in a gunny bag and sterilized in an autoclave at 15 pound pressure per square inch for half an hour.
3.1.3.2 Sowing of seeds

Two seeds of tomato (var. Bioseed) were sown in each pot (1 kg size) filled with autoclaved soil at a depth of about 1 cm. A little amount of water was sprinkled over the pots. After one week of germination, seedlings were thinned out to one healthy seedling in each pot.

3.1.3.3 Inoculation of nematodes

Second stage juveniles of root-knot nematode, already kept for hatching from different locations of Assam, were inoculated separately to the one week old tomato seedling separately. These inoculated seedling were maintained in the Culture House and subsequently used as a source of root-knot nematode eggs for DNA extraction.

3.1.4 Molecular identification and characterization of root-knot nematode

3.1.4.1 Extraction of genomic DNA

Genomic DNA was extracted from nematode egg masses following the procedure given by Karajeh et al. (2010) with modifications.

100 numbers of egg masses (Karajeh et al., 2010 used 10-15 egg masses) were collected from pure culture and were placed in 1.5 ml microtube. The egg masses were disinfested with 500 µl of 0.5 per cent sodium hypochlorite for 5 minutes. The pellets were washed thoroughly with sterile water to remove the traces of sodium hypochlorite in the egg masses. To the extraction buffer (250 mM Tris-HCL, pH 8.0, 250 mM sodium chloride, 50 mM Ethylene Diaminetetraacetic Acid and 0.5% Sodium dodecyl Sulfate), 10 µl of β-mercaptoethanol was added. The eggs were crushed mechanically with the help of conical grinder that fits exactly to the tube for 5 minutes. After crushing, 0.5 volume of 3M sodium acetate at pH 5.2 was added to the lysate and kept in -20°C freezer for 30 minutes. The tubes were centrifuged at 13000 rpm for 5 minutes and the supernatant was transferred to another sterile microtube. To the supernatant, cool isopropanol of equal volume was added and were kept in -20°C for 1 hour. Later on, the microtube from -20°C was taken out and centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded, and residue was washed the with 70 per cent ethanol, air dried until there was no trace of ethanol and then suspended in 30 µl of sterile water.
3.1.4.2 Spectrophotometric analysis of isolated genomic DNA

One µl of genomic DNA sample were placed in the Nano Drop for quantification. OD values of the samples were recorded at OD\textsubscript{260} and OD\textsubscript{280}. OD\textsubscript{260}/OD\textsubscript{280} ratio was calculated. A ratio between 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids. A ratio lower than 1.8 indicates the presence of protein and/or other UV absorbers. A ratio higher than 2.0 indicates that the sample may be contaminated with phenol. The concentration of DNA was calculated using the formula concentration of DNA= (A\textsubscript{260}x50 x Dilution factor) µg/ml.

3.1.4.3 DNA quality confirmation by gel electrophoresis

A 1.0 per cent agarose gel (Appendix V) was prepared using 1X TBE and ethidium bromide (0.5μl/ml). 2 µl of 6X gel loading dye, 2 µl of DNA and 8 µl of distilled water were mixed together before loading the wells of the gel. 1 µl of 1kb ladder was placed in one well as molecular weight standards. The electrophoretic gel was run at 70 mAmp till the dye had migrated one-third of the distance in the gel. Migrated DNA was visualized using a UV transilluminator and the gel images were captured using Gel Documentation.

3.1.4.4 PCR Amplification

3.1.4.4.1 Primer designing

A pair of \textit{Meloidogyne incognita} specific primer was used to amplify the DNA of four \textit{Meloidogyne} spp. isolates. The sequences are as follows:

MI-F 5´ GGGATGTGTAAATGCTCCTG3´
MI-R 3´ CCCGCTACACCCTCAACTTC5´ and the expected product length was 399bp

3.1.4.4.2 Polymerase Chain Reaction for \textit{M. incognita} specific primer

The PCR reaction was performed in a 20 µl PCR mix containing 2 µl 10X \textit{Taq} buffer A, 0.5 µl dNTPs 10 mM, 0.2 µl \textit{Taq} DNA polymerase, 15.3 µl double distilled water, 0.5 µl forward primer, 0.5 µl reverse primer and 1 µl template respectively. Constituents were mixed well by vortexing and were run in a thermo cycler with the following program: at 94°C for 5 minutes followed by 30 cycles with a denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for 7 minutes.
The PCR products were analyzed in 1.5 per cent agarose gel electrophoresis in 1X TBE buffer containing Ethidium Bromide (0.5μl/ml).

3.1.4.5 Molecular identification of root-knot nematode through gene sequencing

Four selected isolates (Jorhat, Lakhimpur, Nagaon and Kamrup) were sent for sequencing to Bioserve Biotechnology (I) Pvt. Limited, Hyderabad.

3.1.4.5.1 Sequence analysis

The sequences were aligned in a global multiple sequence alignment programme, MultiAlin. Sequences were assembled and edited with BioEdit and aligned. Obtained sequences were subjected to BLAST using NCBI GenBank database to compare with other Meloidogyne species.

3.1.4.6 RAPD Analysis

Twelve (12) different RAPD primers were used for DNA finger printing. The primers were OPA1, OPA2, OPA3, OPA4, OPA6, OPA7, OPA8, OPA9, OPA10, OPA11, OPA12 and OPA13.

3.1.4.6.1 Polymerase Chain Reaction

The PCR reaction was performed in a 15 μl PCR mix containing 1.0 μl of the template DNA, 1.5 μl 10X Taq buffer A, 1 μl of 10mM dNTPs, 1 μl of Taq DNA polymerase, 1 μl of primer and 9.5 μl of double distilled water. Constituents were mixed well by vortexing and were run in a thermo cycler with the following program: 94°C for 1 minute followed by 35 cycles with a denaturation at 94°C for 1 minute, annealing at 32°C/34°C for 1 minute (annealing temperature varies with different primers, Appendix VII), extension and final extension at 72°C for 1 minute.

The RAPD products were analyzed by electrophoresis in 1.5 per cent agarose gel with 1X TBE buffer stained with ethidium bromide (0.5 μl/ml). 100bp was used as standard molecular weight. Amplified bands were visualized under Gel Documentation system and captured the image for record.

RAPD bands were recorded as a binary matrix of 0 and 1 corresponding to the absence and presence of bands, respectively and analyzed based on Jaccard’s Coefficient similarity matrix using NTSYS (Numerical Taxonomy and Multivariate Analysis System) software (Rohlf, 1993).
3.2 Morphological and morphometric variations among the population of root-knot nematode(s) of Assam

Soil along with root samples were collected from around the rhizosphere of vegetable crops from nine districts of Assam. Root-knot nematode population collected from these nine districts were studied for morphological and morphometrics variation.

3.2.1 Extraction of nematodes

Nematodes were extracted from soil as well as from root by the following procedure as described in 3.1.1 and 3.1.2.

3.2.2 Killing and fixing of nematodes

Nematode suspension, concentrated in few ml of water were killed and fixed simultaneously by pouring equal amount of boil 8 per cent formalin (8ml formaldehyde solution + 92 ml distilled water). The fixed nematodes were stored in Mc Cartney bottle with proper label until further processing.

3.2.3 Clearing of nematodes

Nematodes were cleared / processed by Seinhorst’s slow method (Seinhorst, 1962). Fixed nematodes were transferred to cavity block containing 1 ml of Seinhorst solution I (96% ethanol 20 ml + glycerine 1 ml + distilled water 79 ml) and kept in an oven for 12 hour at 40°C with its lid slightly open. The cavity block was then filled with Seinhorst solution II (96% ethanol 95 ml + glycerine 5ml) and again kept in an oven at 40°C for 4-6 hour.

3.2.4 Mounting and sealing

A small drop of dehydrated glycerine was placed at the middle of the cleaned and dried glass slide. Nematodes were transferred from the cavity block to the glycerine drop with the help of a splinter. Three small cubes of equal sized of paraffin wax were placed radially around the nematodes. A clean round cover slip (18 mm, No. 1) was placed carefully over the paraffin wax and was heated in slide warmer to melt the paraffin wax.

3.2.5 Preparation of perineal pattern

The entire knotted root system was cut into a small pieces and stained by NaOCl-Acid Fuchsin method (Byrd et al., 1983). After staining, root sample was
allowed to cool at room temperature. The excess stain was removed by rinsing in running water and finally the stained root was wrapped into tissue paper for removing water from the root surface. This was then, preserved in glycerin in a small petriplate (60 mm). Female nematode was dissected from the stained galls of the root with the help of a fine needle. Ten mature females from each district were taken for preparation of perineal patterns. The posterior half of the female body was cut on 45% lactic acid on the transparent glass slide and cleared away the body contents. The posterior cuticle was then transferred to a drop of glycerol on a glass slide where, it was trimmed to a size slightly greater than the pattern. The cuticular pattern was finally mounted in a drop of anhydrous glycerin on clean slide with cover slip of 18 mm (No.1) keeping their outer surface uppermost and then, sealed with nail varnishes.

3.2.6 Measurements and photography

Measurements of the mounted nematode were taken with the help of an ocular micrometer, calibrated with a stage micrometer. For denoting the dimensions of nematodes, de Man’s (1880) formulae were used. Necessary photographs were taken with the help of a Digital camera 7.2 mega pixel fitted to Research Microscope Axiostar.

3.3 Effect of temperature on the biology of root-knot nematode, Meloidogyne incognita

Studies on the effect of temperature on embryonic development of M. incognita, penetration and multiplication of M. incognita on host plant were conducted under laboratory conditions.

3.3.1 Study on embryogenesis (hatching)

Egg masses of root-knot nematode were collected from the already maintained pure culture in the Net House of Department of Nematology, AAU, Jorhat. The egg masses were carefully teased under stereoscopic zoom binocular microscope at 4X to release eggs from the egg masses. A single egg at single cell stage were hand picked with the help of horse-tail hair splinter and placed in a small drop of distilled water on 18 mm round cover slip. At the edge of the cover slip, a ring was made using petroleum jelly. The cover slip was then inverted over the cavity of the cavity slide in such a way that water drop does not touch the surface of the slide. A gentle pressure was given around the cover slip by a blunt needle to seal it.
Developmental stages of egg were studied under a compound microscope at half hourly interval. Photomicrographs of different developmental stages were taken with the help of a camera attached to a Zeiss microscope.

To study the effect of temperature on embryogenesis, cavity slides of 18mm diameter with eggs were kept at ambient temperature (Appendix III), 28°C and 31°C temperature in BOD incubator and time required for hatching was recorded.

3.3.2 Study on penetration

Autoclaved soils containing mixture of soil, dried cowdung and sand at 2:1:1 respectively, was filled with pots of 150 gm capacity. Three seeds of susceptible tomato (var. Bioseed) were sown to each pot. One week after germination, seedlings were thinned out to one healthy seedling in each pot. Second stage juvenile of *M. incognita*, were inoculated with the help of micro pipette to the feeder root at a rate of 1J2 per gram of soil.

Inoculated pots were kept in growth chamber at 21±1°C, 23±1°C, 27±1°C and 31±1°C. Three seedlings were carefully uprooted on time interval starting from one hour after inoculation. The root system was washed very carefully under tap water and teased with the help of a fine needle under stereo-zoom binocular microscope to observe the penetration of juvenile(s) inside the root tissue.

3.3.3 Study on multiplication of *M. incognita*

Earthen pots of 500 gm capacity were filled with autoclaved soil mixture (soil, dried cow dung and sand at 2:1:1, respectively). To each pot, three seeds of tomato (var. Bioseed) were sown. Pots were sprinkled with water regularly. One week after germination of seeds, the pots were thinned to keep one healthy seedling per pot. Then, pots were inoculated with second stage juvenile of *M. incognita* at the rate of 1 J2/gm of soil. The inoculum were collected from the pure culture already maintained in the net house of Department of Nematology, AAU, Jorhat as per the procedure described in 3.1.3. Pots were kept at different temperature of 21±1°C, 23±1°C, 27±1°C and 31±1°C inside growth chamber in Department of Crop Physiology, AAU, Jorhat. After 45 days of inoculation, plants were uprooted carefully. 200cc of soil was processed from each pot to find out the final soil population of *M. incognita* by the procedure as described in 3.1.1. Roots were washed carefully under tap water and number of galls per root system, number of egg mass per root system, number of eggs per egg mass, final nematode population and its reproduction factor were recorded.
All the treatments were replicated 5 times.

Reproduction factor of *M. incognita* was calculated by using the following formula:

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\text{Reproduction factor} = \frac{\text{Final nematode population}}{\text{Initial nematode population}}
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