CHAPTER IV
EXPERIMENTAL FINDINGS

4.1 Molecular characterization of root-knot nematodes of Assam

Nine root-knot nematode populations from nine different districts (Jorhat, Golaghat, Sibsagar, Lakhimpur, Dhubri, Nagaon, Dibrugarh, Kokrajhar and Kamrup) of Assam were collected for molecular characterization.

4.1.1 Genomic DNA isolation

Genomic DNA from root-knot nematode pure culture was isolated as described in 3.1.4.

4.1.2 Quantitative analysis by Spectrophotometre

The isolated DNA was quantified and its purity was estimated with the help of Spectrophotometric method. Results of Spectrophotometric analysis were presented in Table 1. Results revealed that all the samples were having \( A_{260}/A_{280} \) ratio above 1.8 with DNA concentration as higher as 289.45 ng/µl and as low as 159.90 ng/µl.

4.1.3 Meloidogyne incognita specific PCR and DNA sequence Analysis

PCR analysis of \( M. \) incognita revealed a PCR product of 399bp against \( M. \) incognita specific primer pair MiF - MiR for all the \( M. \) incognita isolates viz., Mi1-Mi9 (Fig. 3).

Altogether 4 isolates from different parts of Assam were selected and sequenced at Bioserved Biotechnology (I) Pvt. Ltd, Hyderabad. They were designated as Mi1 (Jorhat), Mi4 (Lakhimpur), Mi6 (Nagaon) and Mi9 (Kamrup). DNA sequences were assembled and aligned using MultiAlin software (Appendix VI). Sequenced similarities of Mi isolates from Assam population were presented in Table 2. Results revealed that sequenced similarity of Mi isolates from four districts showed 89 per cent to 99 per cent homology. The isolates sequenced were found to be unmatched with NCBI Blast.
4.1.4 Molecular variability by using RAPD technique

RAPD analyses were done with 12 different primers for all the nine population of root-knot nematode to detect the molecular variation among the isolates of root-knot nematodes collected from nine districts of Assam. All the bands produced in the present study were scored for the analysis (Fig. 5-16).

All the 12 primers used in RAPD analysis were found to be polymorphic to nine root-knot nematode population (Table 3). Data analysis based on the result of amplification with 12 primers revealed a total of 56 bands among which RAPD 1, 2 and 7 amplified the highest amount of bands (6 bands). The number of fragments generated by each primer varied from 3 to 6. The primers RAPD 1, 2, 3, 6, 7, 8, 9, 10, 11, 12 and 13 gave the highest polymorphism percentage (100 per cent), lowest being (80 per cent) produced by RAPD 4. Out of the 56 fragments generated by 12 RAPD primers, 55 fragments were polymorphic while 1 was monomorphic.

4.1.5 Cluster analysis of nine root-knot nematode isolates

Binary data obtained were used for cluster analysis leading to dendogram and simple matching coefficients. Cluster analysis of the nematode isolates was performed by arithmetic average (UPGMA-method) using NTSYS-PC programme and their dendogram was presented in Fig. 17.

The simple matching coefficient values were generated using Jaccard’s similarity coefficient and the data in Table 4, represent similarity values between the isolates. The similarity coefficient among RAPD profile of root-knot nematode was higher than 0.375 (37.5 per cent). The maximum similarity value of 0.804 (80.4 per cent) was observed between the isolates Kokrajhar and Sibsagar, and Kamrup and Kokrajhar, followed by similarity value of 0.768 (76.8 per cent) between isolates of Kokrajhar and Dhubri. The lowest similarity value of 0.375 was recorded between isolates of Dhubri and Nagaon. The rest of the isolates showed values ranging from 0.393 to 0.750.

The dendogram generated with the similarity data grouped the isolates into three clusters with most bifurcation at 0.47 similarities (Fig. 17). The first cluster had five isolates viz., Jorhat, Sibsagar, Kokrajhar, Kamrup and Dhubri. The second cluster comprised of three isolates viz., Golaghat, Lakhimpur and Dibrugarh, while the third cluster comprises of Nagaon isolates which was found to be totally different from other isolates.
Grouping of clusters may be due to the presence of three races of *M. incognita* in nine districts of Assam.

### 4.2 Morphological and morphometric variation among the population of root-knot nematode(s) of Assam

During the present investigation, morphological and morphometric variations of root-knot nematode, collected from 9 districts of Assam (Jorhat, Golaghat, Sibsagar, Lakhimpur, Dhubri, Dibrugarh, Nagaon, Kokrajhar and Kamrup) were studied. Variations and similarities among the populations were illustrated below:

**Second stage juvenile (J₂) of Meloidogyne incognita**

Body slender, lip region continuous with the body contour. Cephalic framework well developed. Stylet slender with distinct round small basal knobs. Metacorpus distinct with basal bulb extension overlapped ventrally, genital primodia developed, rectum and anus clearly visible. Tail with hyaline terminus with conoid shaped.

Morphological characters of *M. incognita* second stage juvenile population collected from nine districts of Assam exhibited similarity in head portion (Plate 3) and variations in tail shape (Plate 4). Tail shape of second stage juveniles of *M. incognita* collected from Golaghat, Kokrajhar, Nagaon and Sibsagar district was similar in shape. Likewise, tail shape of second stage juveniles of Dibrugarh, Jorhat and Lakhimpur, and Dhubri and Kamrup population showed similarity.

Studies on morphometric variations (body length, stylet length, lip height, lip width, MB, a, b, b’, c and c’) among the populations of *M. incognita* from nine districts of Assam showed no distinct variations in the major characters, except in MB value. MB value of Kamrup population was higher (61.79) than other populations. Results presented in Tables (5 and 6) showed the morphometric characters of *M. incognita*.

**Body length:** Body length of *M. incognita* second stage juvenile from all the nine populations ranged from 0.360-0.478 mm in total, with population means varying from 0.390-0.440 mm. Longest juveniles were found in Kamrup population (0.478 mm) while, shortest being found in Golaghat population (0.360 mm).
**Stylet length:** Stylet well developed in second stage juvenile of *M. incognita* with small rounded basal knobs. Stylet length of *M. incognita* second stage juveniles from all the nine populations were found to be in the same range (10-12 \( \mu \text{m} \)).

**Lip height and lip width:** Lip height and lip width of *M. incognita* second stage juvenile of all the nine populations of Assam were also found to be in the same range of 2-3 \( \mu \text{m} \) and 4-5 \( \mu \text{m} \), respectively.

**MB:** Length from the anterior end to the centre of median bulb in relation to esophagus length (MB) showed variation among the nine populations of *M. incognita* second stage juvenile. This value ranged from 36.36-61.79 in total populations, with population means varying from 43.83-51.87. Highest value of MB was recorded in Kamrup population followed by Jorhat population while, lowest was recorded in Sibsagar population followed by Dibrugarh population (Table 5).

**a ratio:** Total length of nematode body in relation to body width (a ratio) for all the nine populations of Assam ranged from 26.07-33.38 in total, with population means varying from 29.02-30.71. Highest a ratio was recorded in Sibsagar population followed by Jorhat population while, lowest was recorded in Golaghat population (Table 5).

**b ratio:** Ratio b of *M. incognita* second stage juvenile ranged from 3.81-5.61 in total populations, with population means varying from 4.41-5.05. Highest value of b was recorded in Golaghat population followed by Jorhat population while, lowest was recorded in Sibsagar population followed by Golaghat population (Table 5).

**b’ ratio:** Ratio b’ ranged from 3.01-4.48 in total population, with population means varying from 3.44-3.87. Highest b ratio was recorded in Lakhimpur population while, lowest being found in Jorhat population (Table 5).

**c ratio:** Length of the tail in relation to total body length, ranged from 7.73-10.66 in total population, with mean varying from 8.47-9.90. Highest c ratio was recorded in Kokrajhar population followed by Sibsagar population while, lowest was recorded in Lakhimpur population (Table 5).

**c’ ratio:** c’ ratio was found to be in ranged of 3.00-4.90 in total population, with mean varying from 3.57-4.39. Highest c’ ratio was recorded in Lakhimpur population followed by Golaghat population while, lowest was recorded in Kokrajhar population (Table 5).
Mature female of *M. incognita*


Perineal pattern of *Meloidogyne* spp. collected from nine districts of Assam were found to be similar with the perineal pattern of *M. incognita* with high dorsal arch, distinct lateral lines absent. Vulva, anus and phasmid present in perineum (Plate 5a and 5b). No variation on perineal pattern was observed in size and shape of perineal pattern of *M. incognita* collected from nine districts of Assam.

4.3 Effect of temperature on biology (embryogenesis, penetration and multiplication) of root-knot nematode, *Meloidogyne incognita*

4.3.1 Effect of temperature on embryogenesis of *Meloidogyne incognita*

The embryonic development of *M. incognita* was studied at three different temperature. One at the ambient temperature where, temperature fluctuates everyday with 30.8°C being maximum and 19.3°C being minimum (Appendix III) during the period of study. The other two were 28°C and 31°C, which were maintained in BOD at Dept. of Nematology, AAU, Jorhat.

**Embryonic development at Ambient temperature**

*M. incognita* laid eggs in the gelatinous matrix. They laid egg at single cell stage. Just after laying eggs, the cytoplasm covers the volume of the egg (Plate 6A). The single cell egg started to retract from the shell boundary, producing a gap at the pole on both the sides. At the same time, the cytoplasm showed some activities and these activities led the egg cell to divide into two approximately equally sized blastomeres (S1 and P1), within 23 hrs of deposition. The nucleus of each blastomere was prominent with two distinct nuclei (Plate 6B). After 24 hrs of the first cleavage, the second cleavage, transverse to the longitudinal axis of the egg, took place. Second cleavage occurred in both S1 and P1 blastomere, each producing two equal sized cells with prominent nuclei resulting in four-celled stage (Plate 6C). After 3 hrs 20 minutes of second cleavage, third cleavage (8 cells) took place (Plate 6D). Cells within the egg started to become larger producing a distinct gap at pole with pointed hyaline structure.
towards the cells. The cells that were distinct within them became dissociates and pointed gap at the pole disappeared within 19 hrs 15 minutes after third cleavage. After 5 hrs, one cell became prominent and could be seen separately at the edge of the clusters of cells, within the egg shell. 1½ hrs later, the prominent cell that appeared earlier loosens up little with demarcation line within the protoplasm along the posterior side. After 1½ hrs, round hyaline structure appeared at the posterior side of the egg shell. Later on, within 11 hrs 15 minutes, the round hyaline structure disappeared again. After 4 hrs 30 minutes, cells dissociation took placed, blastula stage took placed (6E) after 28 hrs.

Gastrulation stage (6F) occurred within 25 hrs 45 minutes after blastula stage where organization and cellular differentiation appeared to continue in central portion of the egg along the longitudinal axis. With continued differentiation, the larger cellular mass in the centre of the egg become surrounded by the small periphery cells of the ectoderm (Plate 6F). Cells at the anterior region of the embryo multiplied rapidly entering the tadpole stage after 20 hrs 45 minutes of gastrula stage where the first movement of embryo was noticed (Plate 6H). Developing embryo continued to grow and reached first stage juvenile after 9 hrs of tadpole stage (Plate 6I). Anterior region of the first stage juvenile was transparent, devoid of stylet and esophagus, but with a clear cut of lumen of the esophagus. It continued to elongate, became slender with pointed tail region that lay close to the head region. The juvenile continued to grow. The juvenile lay inside the egg compressed with two fold (Plate 6K). After 7 hrs of formation, first stage juvenile, the late juvenile stage folded four times and during that stage the cuticle of the first-stage juvenile could be observed clearly (Plate 6L). Movement of juvenile within the egg shell became faster and well developed stylet, median bulb were noticed. Juvenile continued to move inside the egg with pauses in between, exerting pressure on the eggshell at regular intervals. In the mean time, eggshell became thinner, flexible and occasionally bulged out due to pressure exerted out by the moving juvenile. The juvenile attempted several times to puncture the eggshell by stylet thrusts accompanied by pressure exerted by lip at different places. Selecting a place for hatching towards the narrower end of eggshell, the juvenile pressed its lip region at that site and punctured the eggshell with continuous stylet thrusts (Plate 6M). Ultimately, the eggshell was broken and juvenile hatched out from it. Complete embryogenesis took 183.50 hrs (7.645 days).
From Table 7, it was observed that total time taken for embryogenesis of *M. incognita* was found to be 4.468 days at 28°C while, it took 5.168 days at 31°C.

### 4.3.2 Effect of temperature on penetration of *Meloidogyne incognita*

Effect of temperature on penetration of root-knot nematode, *Meloidogyne incognita* was studied under four different temperatures (21, 23, 27 and 31±1°C).

**Temperature at 21±1°C**

The second stage juveniles were inoculated at the rate of 1 J₂/ gm of soil with the help of micro pipette around the feeder root of tomato plant (var. Bioseed). Second stage juvenile (J₂) started to penetrate root within 2 hours of inoculation, just behind the root tip (root cap). After 4 hours, second stage juvenile penetrated fully inside the root epidermis. The second stage juvenile reached stele region within 10 days resuming the vertical position with tail towards the cortex (Plate 7).

**Temperature at 23±1°C**

The second stage juveniles were inoculated at the rate of 1 J₂/ gm of soil with the help of micro pipette around the feeder root. Second stage juvenile (J₂) started to penetrate root within 2 hours of inoculation, just behind the root tip (root cap). After 4 hours, second stage juvenile penetrated fully inside the root epidermis. The second stage juvenile reached stele region within 7 days resuming the vertical position with tail towards the cortex (Plate 7).

**Temperature at 27±1°C**

The second stage juveniles were inoculated at the rate of 1 J₂/ gm of soil with the help of micro pipette around the feeder root. Second stage juvenile (J₂) started to penetrate root within 2 hours of inoculation, just behind the root tip (root cap). After 4 hours, second stage juvenile penetrated fully inside the root epidermis. The second stage juvenile reached stele region within 5 days resuming the vertical position with tail towards the cortex (Plate 7).

**Temperature at 31±1°C**

The second stage juveniles were inoculated at the rate of 1 J₂/ gm of soil with the help of micro pipette around the feeder root. Second stage juvenile (J₂) started to penetrate root within 2 hours of inoculation, just behind the root tip (root cap). After
4 hours, second stage juvenile penetrated fully inside the root epidermis. The second stage juvenile reached stele region within 7 days resuming the vertical position with tail towards the cortex (Plate 7).

4.3.3 Effect of temperature on multiplication of *Meloidogyne incognita*

The study on effect of temperature on multiplication of *Meloidogyne incognita* was carried out under controlled condition maintain at four different temperature levels (21±1°C, 23±1°C, 27±1°C and 31±1°C) within growth chamber of Department of Crop Physiology, AAU, Jorhat. The mean data on number of gall per root system, egg masses per root system, eggs per egg mass, final nematode population and reproduction factor in soil per pot was recorded at different temperature levels are presented in Table 8, Fig. 18, 19 and 20.

4.3.3.1 Number of galls per root system

Mean data on the number of galls per root system recorded at different temperature levels were presented in Table 8 and Fig. 18.

Data presented in Table 8 indicated that root-knot nematode, *M. incognita* produces maximum galls (119.6) at 27±1°C, while minimum number of galls per root system was produced (71.6) at 21±1°C. At 23±1°C and 31±1°C, number of galls produced by *M. incognita* per root system was 83.2 and 93.8, respectively. Numbers of galls produced by *M. incognita* per root system at different temperatures were significantly different.

4.3.3.2 Number of egg masses per root system

Mean data on the number of egg mass per root system recorded at different temperature levels are presented in Table 8 and Fig. 18.

Maximum number of egg masses per root system (275.2) were recorded at 27±1°C followed by 31±1°C (171.8), whereas minimum number of egg masses per root system (98.6) were recorded at 23±1°C followed by 21±1°C. Numbers of egg masses per root system produced by *M. incognita* at different temperatures were significantly different.

4.3.3.3 Number of eggs per egg mass

Mean data on the number of eggs per egg mass recorded at different temperature levels are presented in Table 8 and Fig. 18.
Data presented in Table 8 indicated maximum number of eggs per egg mass (343.6) observed in 27±1°C followed by 31±1°C, while minimum number of eggs per egg mass (246.4) was recorded in 21±1°C, followed by 23±1°C.

4.3.3.4 Final nematode population

Mean data on final nematode population recorded at different temperature levels are presented in Table 8 and Fig. 19.

Data presented in Table 8 indicated the final nematode population was highest (1335.2) in 27±1°C while lowest (687.6) in 21±1°C. Among the different temperature levels, 27±1°C was found to be more effective in increasing the *M. incognita* population followed 31±1°C. All the temperature levels differed significantly among each other.

4.3.3.5 Reproduction factor of nematodes

Mean data on reproduction factor of nematodes population recorded at different temperature levels are presented in Table 8 and Fig. 20.

Data presented in Table 8 revealed that all the temperature levels differed significantly from each other in reproduction. The reproduction factor of nematodes decreased significantly below the temperature level 23±1°C. The highest (2.67) and the lowest (1.37) reproductive rates were observed in the temperature levels of 27±1°C and 21±1°C, respectively.