Bull. zool. Surv. India, 1 (2) : 115-128, 1978

CULTURING AND LIFE HISTORY STUDIES OF *ROTYLENCHULUS RENIFORMIS* LINFORD AND OLIVEIRA ON BRINJAL (NEMATODA)

R. V. SINGH AND S.KHERA

Zoological Survey of India, Calcutta

ABSTRACT

Embryonic, post-embryonic and post-infection developments of the nematode, *Rotylenchulus reniformis* Linford and Oliveira, parasitizing the egg plant (*Solanum melongena* L. var. purple round) in West Bengal are described. Four moults including the one within the egg shell are observed. Second stage larvae utilizes the reserve food received from the egg during development. Heteromorphism of the tails of the second and third stage larvae is reported for the first time. The immature female is the infective stage of this nematode. Females complete its development in 20-25 days and males in 12-16 days at an average temperature of 24-35°C.

INTRODUCTION

A good amount of work has been carried out on the life cycle of *Rotylenchulus reniformis* amongst others by Linford and Oliveira (1940), Nakasono (1966), Birchfield (1962) and Rebois (1973). In India Nath *et al.* (1969), Khan and Khan (1969), and Sivakumar and Seshadri (1971) have also contributed to the study of the life cycle of *R. reniformis.*

Life history studies of *R. reniformis* were taken up because of the facts that (1) survey carried out by us revealed that *R. reniformis* was one of the commonest and dominating nematodes parasitizing vegetable crop of West Bengal, and (2) whereas three moults have been reported by Linford and Oliviera (1940) in *R. reniformis*, Nakasono (1966) and Sivakumar and Seshadri (1971) have reported four moults in the same nematode. Moreover, its life history has not been studied on egg plant. Hence, it was decided to study the embryonic, post-embryonic and post-infection development of *R. reniformis* on egg plant (*Solanum melongena* L. var. purple round) under laboratory conditions.

MATERIAL AND METHODS

Culturing : *Rotylenchulus reniformis* was cultured on tomato (*Lycopersicon esculentum* Mill. var. best in all) by single egg mass technique. Field soil and sand were mixed in proportion of 1 : 1 and autoclaved at 1.4 kg. cm.^{2} pressure up to two hours. About 10 days old tomato seedlings were transplanted singly in sterilized soil filled in 9 x 3 cm. diameter glass tubes. Initially five tubes were prepared. Single egg mass was collected from around the female of *R.*

reniformis infesting roots of okra collected from field. This egg mass was placed for hatching at room temperature in petri dish, 6 cm. in diameter, containing distilled water. About 20-25 larvae were inoculated with the help of a fine pipette in the vicinity of roots by making three small holes. The holes were closed with sterilized soil and irrigated lightly.

About 40 days after inoculation each tube with plant was placed in inverted condition in a 500 ml. beaker containing water. After about 15 minutes each tube was tapped gently at its bottom, so that the plants along with fine roots came out intact. These roots were again washed carefully taking care to prevent any loss of egg masses and nematodes. Roots were observed under a stereoscopic binocular microscope and egg masses were collected from the roots. These egg masses were placed in petri dishes for hatching of larvae and sub-culturing was done by inoculating these larvae on tomato, okra (*Abelmoschus esculentus* (L.) Moench. var. pusa sawani) and brinjal (*Solanum melongena* L. var. purple round) seedlings growing in sterilized soil filled in 15 cm. earthen pots. These pots were kept on fire bricks so as to prevent any inter-pot contamination, the entire experiment being designed on cemented floor. Whenever *R. reniformis* nematodes were required for different experiments these were obtained from the pure cultures thus established in the laboratory.

Life history : Nematodes used for these studies were obtained from pure cultures developed vide *supra*. Adult females were carefully removed from roots or left attached to the fine roots and kept separately in small glass cavity blocks containing tap water for laying eggs. Some uncleavaged eggs were selected for studying embryonic development. Eggs were mounted singly in a small drop of tap water on a 22 mm. square coverglass which was then inverted over a cavity slide containing very thin layer of water at the bottom. Each coverglass was sealed on the slide with petroleum jelly along the margin so as to keep the coverglass in position and to check the evaporation of moisture. These cavity slides were stored at room temperature in petri dishes with a moist filter paper at the bottom of the latter to reduce evaporation from hanging drops. During the experiment maximum temperature range was 31—38.8°C (Average maximum temperature 35°C) and minimum temperature was 20—27.4°C (Average minimum temperature 24°C).

Egg masses recovered from pure cultures were dissected to remove males and previously hatched larvae if present inside them. These egg masses were placed for hatching in 10 cm. x 10 cm. petri dishes containing tap water. The hatched larvae (second stage larvae) of uniform age were placed in tap water in the glass cavity blocks for studying post-embryonic development. The water in the cavity blocks was changed every day.

Sex differentiation could be observed only in the third stage larvae ; female and male larvae were separated for further development. Some larvae were fixed daily, starting just after hatching till the male larvae became adult and female larvae developed into immature females. The larvae were stained in 0.1% cotton blue lactophenol or acid fuchsin lactophenol and mounted on glass slides.

Post-infection development was studied simultaneously by using second stage larvae hatched out from the same lot. Two egg-plant seeds were grown in sterilized sand-filled, 9 x 3 cm. glass tubes. Second stage larvae of uniform age were inoculated in these tubes by making small holes near the plants, when plants were at two leaf stage. Observations were started after 24 hours of

inoculation. Five tubes were washed and processed daily in the same manner as for culture studies. Roots were stained in 0.1% acid fuchsin or cotton blue lactophenol and cleared in pure lactophenol. Nematodes were carefully dissected out from the roots and mounted in lactophenol or glycerine. Some small pieces of fine roots with nematodes *in situ* were also mounted. These observations were continued till the females started laying eggs. Several adult females were also mounted on glass cavity slides. Body dimensions were taken and camera lucida drawings of different stages made.

RESULTS

Female deposited 5-9 eggs within 24 hours in glass cavity blocks containing tap water. To begin with a fully formed egg was held in the anterior part of the uterus then it passed slowly to the mid region of uterus (Plate 1, A). From uterus the egg was expelled through vagina by contraction and retraction of vaginal walls. The eggs were laid singly and were in single-celled (Fig. 1, A), rarely two-celled, stage. Eggs measured 78-102 x 36-52 μum and were more or less oblong in shape. Each egg consisted of an outer smooth shell or chorion lined internally by a thin vitelline membrane and completely filled with protoplasm. Protoplasm comprised a large nucleus in the middle and cytoplasmic globules of variable size.

EMBRYONIC DEVELOPMENT

Durations of different stages of embryonic development are given in table 1.

After 1-2 hours of deposition, the egg underwent first transverse cleavage and divided into two equal blastomeres a and b (Fig. 1, B). A polar body was visible in between the two cells on lateral side. The second and third divisions were also transverse and took place first in the anterior cell (a) Fig. 1, C)

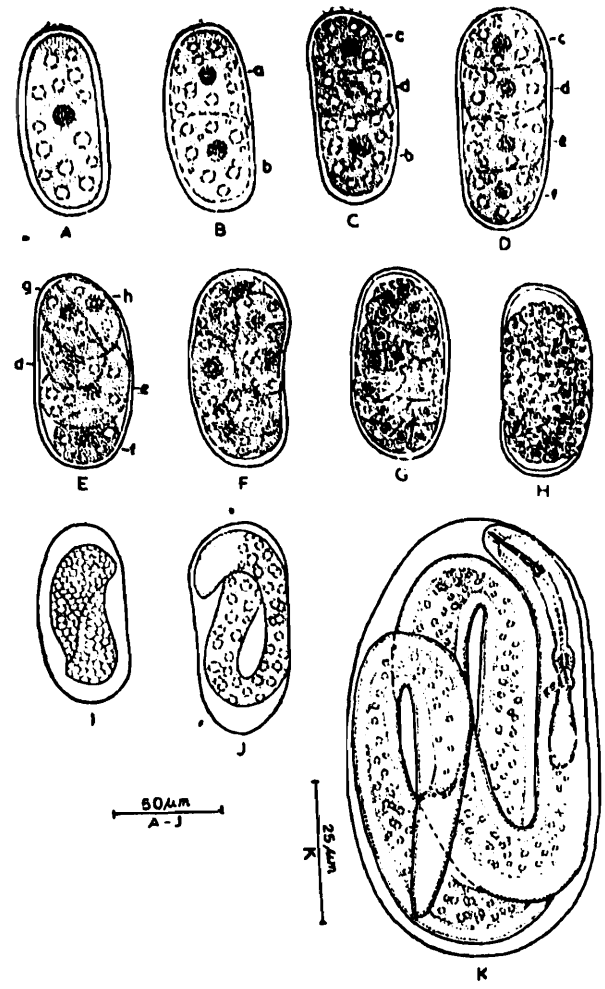

Fig. 1. Embryonic development of *Rotylenchulus reniformis* A—Single celled stage, B—2—celled stage, C—3—celled stage, D—4—celled stage, E—5—celled stage, F—8—celled stage, G & H—Multicellular stages, I—Tadpole stage, J—First stage larva, K—Second stage larva within egg.

and then in the posterior cell (b). The embryo now consisted of four cells or blastomeres c, d, e and f. (Fig. 1, D). The fourth division was a longitudinal cleavage, by which the anterior most cell (c) divided and gave rise to five cell stage g, h, d, e, f (Fig. 1, E). After this the cleavage was very rapid and gave rise to eight cell stage (Fig. 1, F) ; exact sequence could not be observed. Thereafter the cell divisions were very fast and were in different planes, giving rise to multicellular structure (Fig. 1, G) ; later on these cell differentiated in two layers (Fig. 1, H).

This was followed by the tadpole stage (Fig. 1, I). When the embryo reached the tadpole stage, there was a vigorous movement and condensation of protoplasm resulting in the formation of a vermiform structure. This was the first stage larva showing movement inside the egg shell (Fig. 1, J). After sometime the larva stopped movement and became quiescent—a prelude to first moult. During the first moult, the prorhabdion appeared first, then the body annulations and cephalic framework made their appearance. Oesophagus and intestine became more prominent. First moult cuticle was observed by puncturing the shell with the help of a needle.

The second stage larva (Fig. 1, K) made movements by coiling and uncoiling its body inside the egg shell. Lips of the second stage larva were observed pressing against the egg shell. The anterior half portion of the larva came out with slight jerk and then slowly left the egg shell.

Post-embryonic Development

On no occasion any larval or moulting

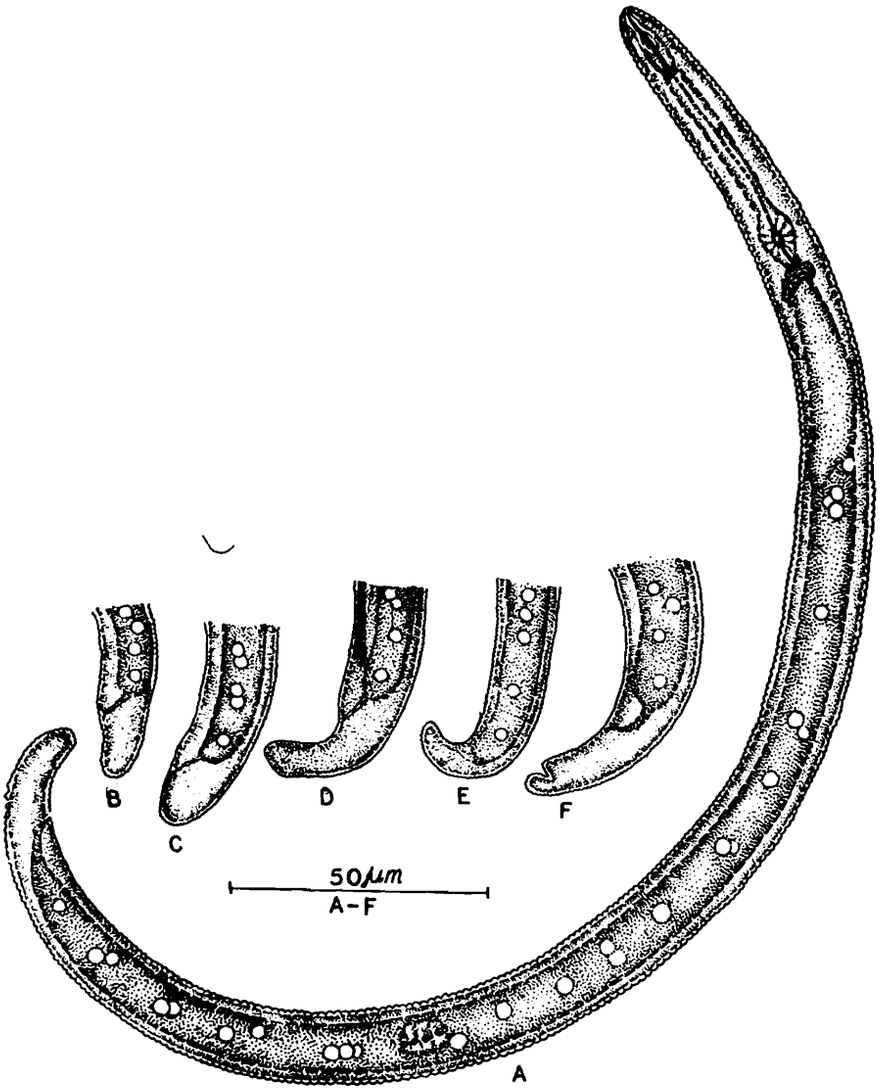

Fig. 2. Post—embryonic development of *Rotylenchulus reniformis* A—Second stage larva, B—F—Heteromorphic tails of stage larva.

stage was found in or attached to the roots of the host plant. These studies are based on development of *R. reniformis* in water. Dimensions and duration of different stages of post-embryonic development are given in tables 2 and 3 respectively.

Second stage larva ;

Second stage larva (Fig. 2, A) hatched out from the egg ; it was very active. Head truncate with 4-5 annules, cephalic framework well developed 3-5 µm high and 6-7 µm in diameter, stylet well developed 13-16 µm long with rounded basal knobs, latter 2-3 µm wide. Dorsal oesophageal gland orifice 10-16 µm posterior to stylet base. Procorpus tubular, median bulb 8-11 x 6-8 µm in diameter with well developed crescentic valve plates. Oesophageal gland overlapping intestine lateroventrally or laterally. Nerve ring encircling short narrow isthmus, about 49—64 µm from anterior end. Excretory pore 63—78 µm from anterior end. Hemizonid 2—3 annules wide and 1—6 body annules above the excretory pore. Tail 13—24 µm long comprising 15—25 annules and generally with rounded terminus. However, heteromorphic tail observed in this stage (Fig. 2, B-F).

Genital primordium 11—15 µm long oval in shape and 64—73% of body length, comprising three cells in the larva forced out after puncturing of the shell and in the larva just hatched out normally. Later on, it became a four celled structure and remained as such during the complete durotion of second stage larva. Anterior and posterior cells of genital primordium were smaller than the two central cells.

This larva did not show any sex differentiation.

Second moult :

On the fifth day of hatching the larva ceased its activity and assumed an arcuate shape. Moulting started on the sixth day after hatching when basal part of the stylet (knob + shaft) disappeared first. Procorpus, median bulb with valve became indistinct. Cuticle of the second stage larva started separating from anterior end and gradually extended to the posterior end. Conus (anterior part of stylet) was cast off with moulting cuticle and remained attached to it. Cephalic framework of second stage larva was also visible in the moulting cuticle.

Third stage larva :

Third stage larva was enveloped within the second moult cuticle and was very inactive. Sex of the third stage larva could be identified by seeing male third stage larva, which had a bulging on ventral side of the tail around the anus (cf. plate IIA & B).

Female larva (Fig. 3, A)—Head truncate, with 4—5 annules and marked with slight depression in the middle at the location of buccal orifice. Cephalic framework weakly developed. Stylet absent. Oesophagus with less organised procorpus, latter expanded slightly at anterior end. Median bulb showing only a suggestion of valve ; true crescentic plates in median bulb absent. Nerve ring around short narrow isthmus, 48—64 µm from anterior end. Lumen of the oesophagus and the oesophago-intestinal valve not visible. Tail 17—24 µm long comprising 20—26 annules with rounded terminus.

Genital primordium 21—40 µm long of 10—12 cells and situated at 69—74% of body length from anterior end. Two cells

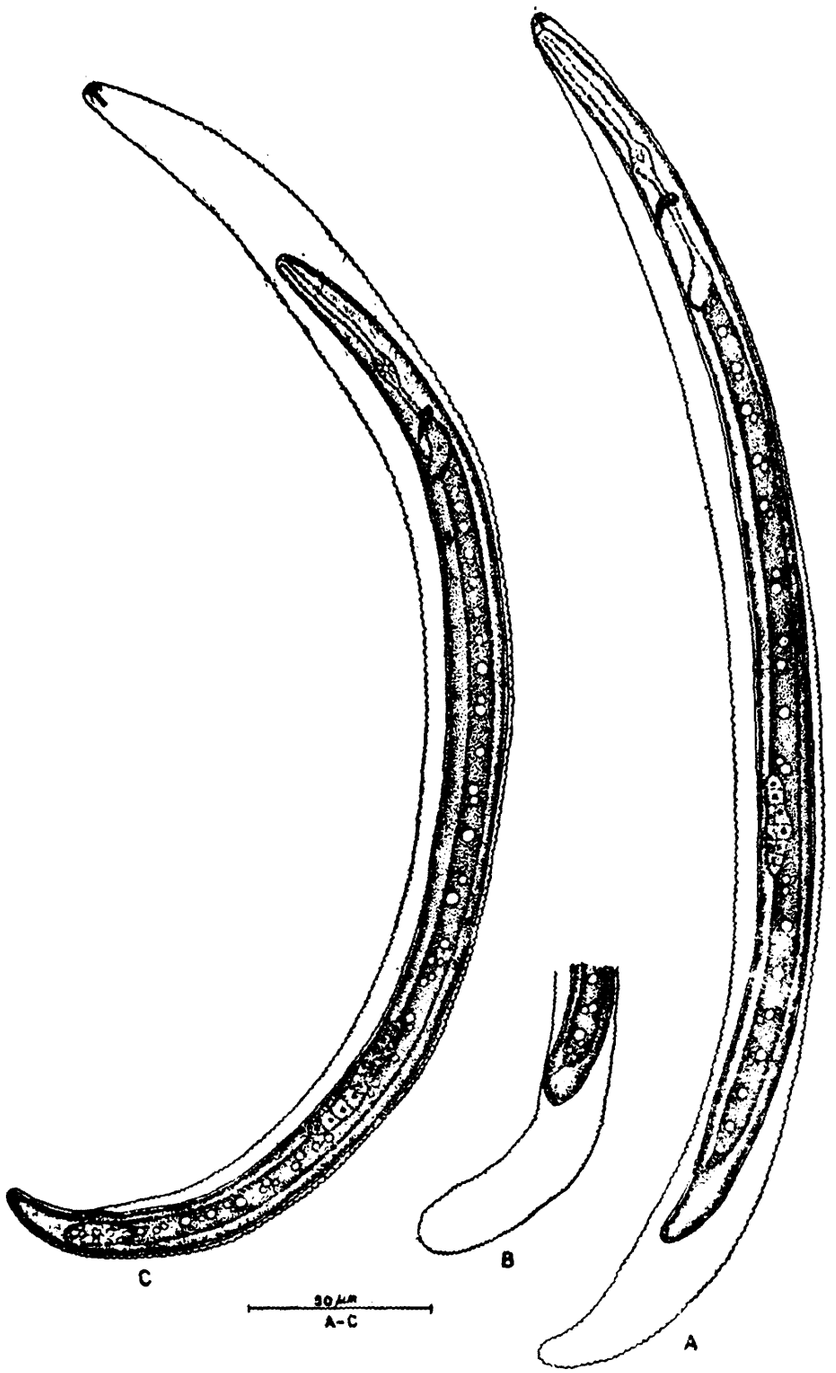

Fig. 3. Post—embryonic development of *Rotylenchulus reniformis* A—Third stage female larva, B—Heteromorphic tail of third stage female larva, C—Third– stage male larva.

of genital primordium arranged on either end and cells in the middle in two rows with 3—4 cell in each row.

One third stage female larva was observed with cylindrical arcuate tail with broadly rounded terminus. Anal pore of this larva was situated much posterior towards tail terminus in comparison with other (normal) larvae. Moulting cuticle

enveloping third stage heteromorphic larva indicates that this variability of tail shape continues from second stage larva (Fig. 3, B).

Male larva (Fig. 3, C)— The tail portion near the anal region was slightly bulged (plate II, B). A mass of cells was formed in the rectal region - the spicule primordium. These two characters differentiated the male third stage larva from the female third stage larva. The genital primordium, 25—48 µm long, consisted of 12—14 cells, a single anterior cell followed by 3 cells arranged in two rows in the middle and then 5—7 cells in a single row. Other morphological characters were similar to those of female third stage larva.

Third moult :

Third moulting cuticle appeared inside the second moult cuticle. Third moulting cuticle was very smooth and finer than the second moulting cuticle. Conus part of stylet of of second stage larvae was siill attached to second moult cuticle. Cephalic framework was not visible in third moulting cuticle. Fourth stage larva could be seen within the two moulting cuticles. Movement of larva was very sluggish during moulting.

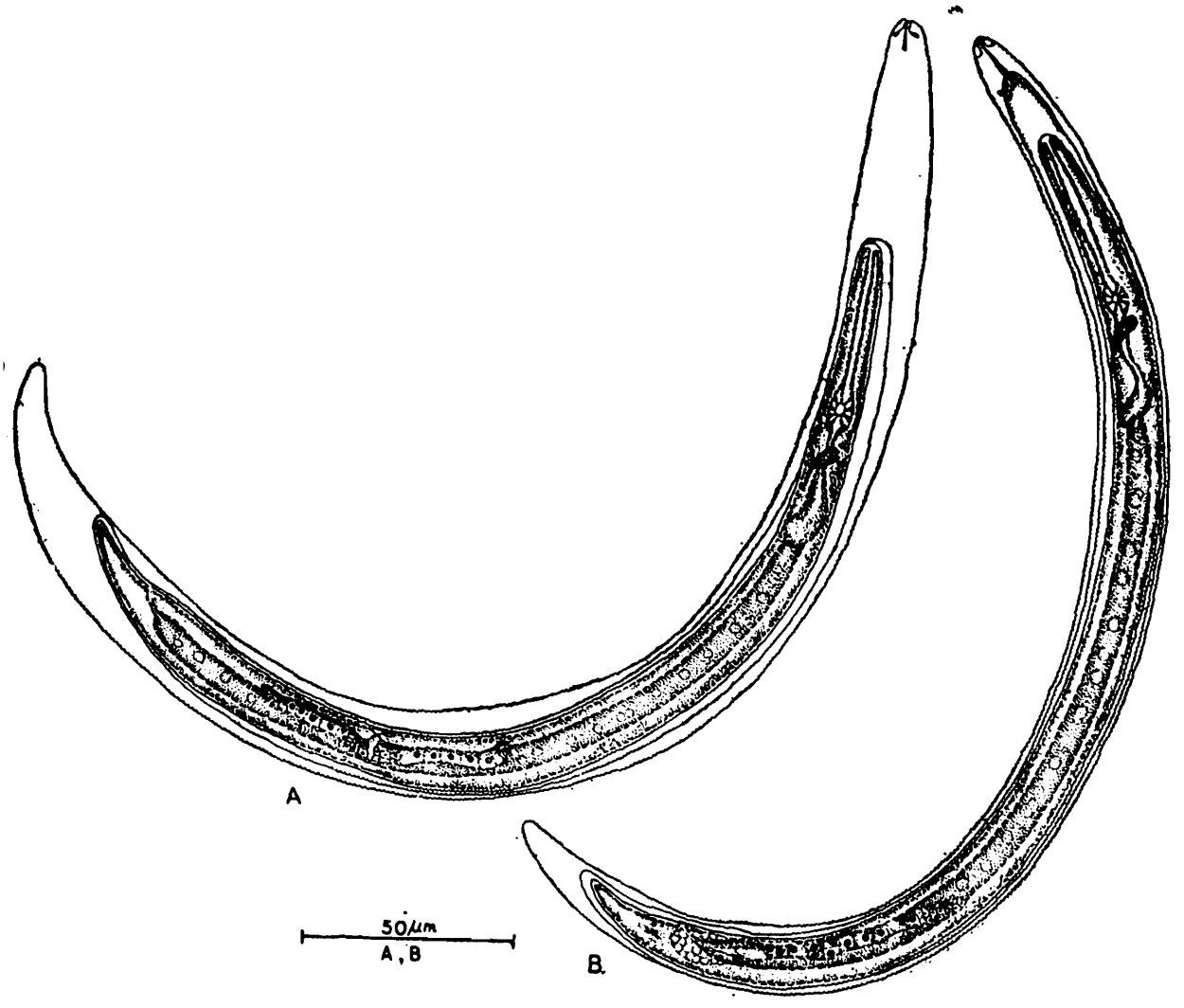

Fig. 4. Post-embryonic development of *Rotylenchulus reniformis* A—Fourth stage female larva, B—Fourth stage male larva.

During the third moult gonad of female and male larvae were differentiated into ovary and testis.

Fourth stage larva :

Female larva (Fig. 4, A)— The fourth stage female larva resembled the third stage female larva in general morphological characters except in the development of gonad. Stylet absent ; median bulb without crescentic valve plates. Excretory pore 42—60 µm from anterior end. Tail 20—24 µm long with roundcd terminus.

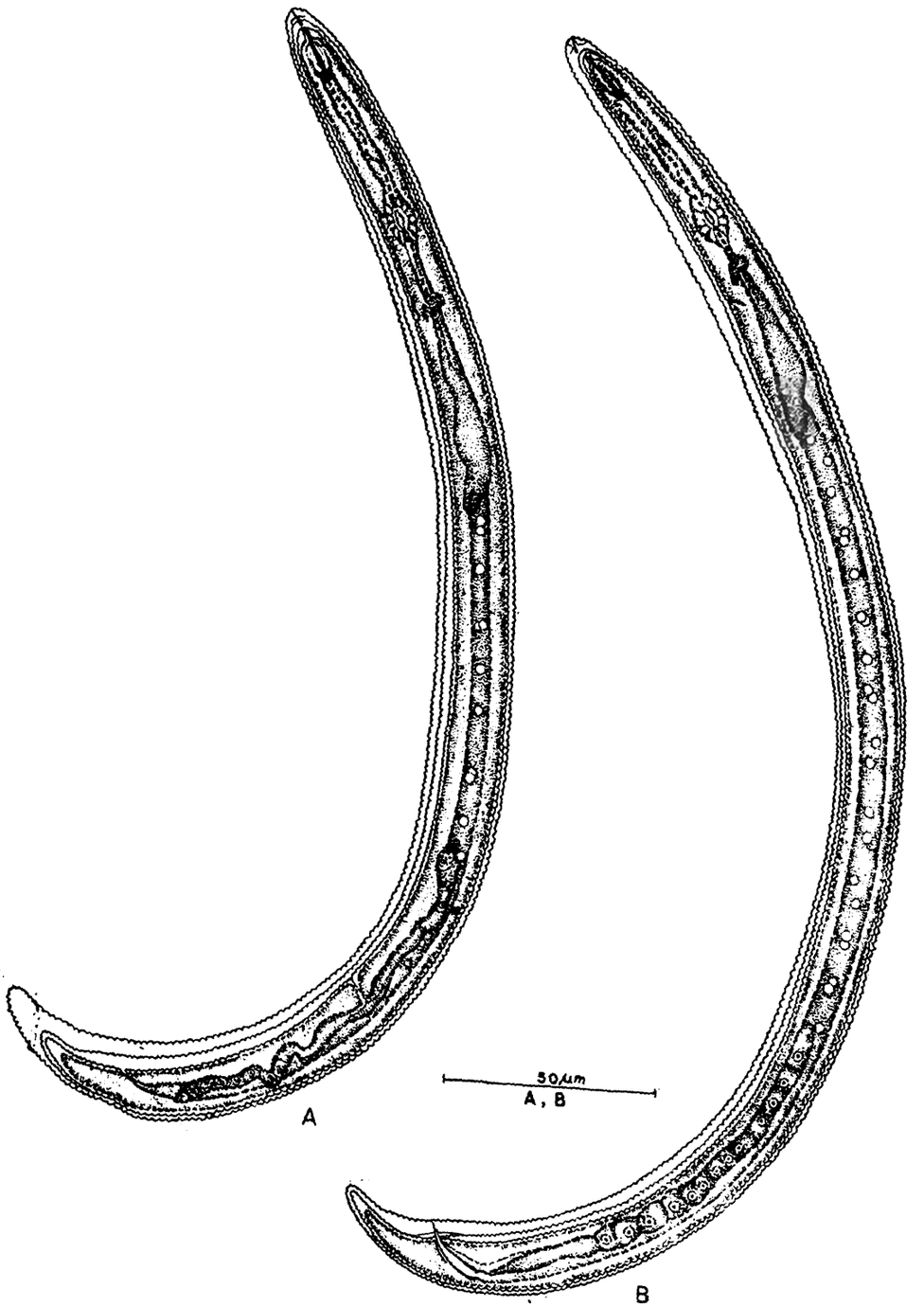

Fig. 5. Post-embryonic development of *Rotylenchulus reniformis* A—Immature female within moulting cuticles, B—Adult within moulting cuticles.

Anterior and posterior gonads were now discernible because of a slight suggestion of vagina and a hyaline area corresponding to vulva having appeared. Each gonad consisted of cells arranged in a single row except in the middle, in front of vagina, where three rows of 3—4 cells each occurred. Spermatheca not formed. This stage, after moulting, developed into immature females.

Male larva (Fig. 4, B)— The fourth stage male larva developed after 2—3 days of third stage male larva and lasted for 1—2 days and then underwent fourth moult. Larva could be identified by the bulging portion of tail around anus caused by the spicule primordium ; faintly cuticularised markings of spicula and gubernaculum were located in the spicule primordium. Cells of the gonad were arranged in a single row except for a few cells in two rows in the middle. Posterior end of gonad reached up to the vicinity of spicule primordium.

Fourth moult :

Fourth moult started 24 hours after the appearance of the fourth stage larva. The fourth moult cuticle was also smooth and slightly thicker than the third moult cuticle. During this moult the stylet and the crescentic plate of median bulb appeared again. There was an increase in size of the gonads. Both the ovaries started forming loops and the vulva was fully formed ; this stage is referred to in literature as immature female. In the male spicula and gubernaculum were fully formed. To begin with, the male and immature female were covered by three moulting cuticles for 1—2 days (Fig. 5, A & B) and then escaped out of these cuticles (Fig. 6 A & B).

Immature female (Fig. 6, A) :

This is the most active stage of the nematode. Immature female possessed well developed cephalic framework, stylet procorpus, median bulb with sclerotized crescentic plates and oesophageal gland overlapping intestine laterovventrally. Anterior and posterior ovary started getting looped.

POST-INFECTION DEVELOPMENT

Only the immature or young females were observed penetrating the roots. Male was not observed penetrating the roots. About one third to half the anterior portion of young female was seen penetrating the roots. Several immature young females were dissected out within 24 hours of penetration. These females were observed with offset head, $\frac{1}{4}$th part of stylet mostly coming out from the oral aperture ; procorpus. median bulb and oesophageal gland enlarged. Female started gradually bulging on ventral side near the vicinity of vulva after 24—48 hours of penetration. Both the ovaries increased in size, each forming one loop (Fig. 7, A & B). There was a slight increase in diameter of the tail.

Development of female was continued till it attained a typical reniform shape. Swollen female body was filled up with developing gonads. Secretion of gelatinous matrix was observed on the fifth day of infection. One to three eggs were laid on sixth to seventh day of infection.

Males were observed in the gelatinous matrix before the eggs were laid in it. Cleavaged eggs were observed inside the female body (plate I, B & C). In the females which could be easily detached from the roots, there were more cleavaged eggs as compared to those females which

Fig. 6. Post-embryonic development of *Rotylenchulus reniformis*
A—Immature female, B—Adult male.

could not be detached easily from the roots.

DISCUSSION

That the second stage larva of *Rotylenchulus reniformis* after passing through the usual moults and stages, developed into mature males or immature females in water without feeding showed that egg of *R. reniformis* contains ample amount of reserve food materials which is passed on to the larva and consumed during development. The experiment conducted also confirms that it is only the immature female which is the infective stage of this nematode. Linford and Oliveira (1940) stated that *R. reniformis* completed its (egg to egg) development on cowpea in 25 days. Nath *et al.* (1969) reported that this nematode developed (egg to egg) in 29 days on castor at 30-32°C. Sivakumar and Seshadri (1971) reported that *R. reniformis* completed its egg to egg life cycle on okra var. pus sawani within 24—29 days. However, they did not record, or, at least state, the temperature at which their studies were carried out. Rebois (1973 stated that *R. reniformis* developed rapidly at 29.5°C on soyabean and the female completed its life cycle in 19 days. In the present experiment females of *R. reniformis* completed their development on egg plant (*Solanum melongena* var. purple round) in 20—25 days at an average temperature of 24—35°C. Males of *R. reniformis* completed their development in 12—16 days.

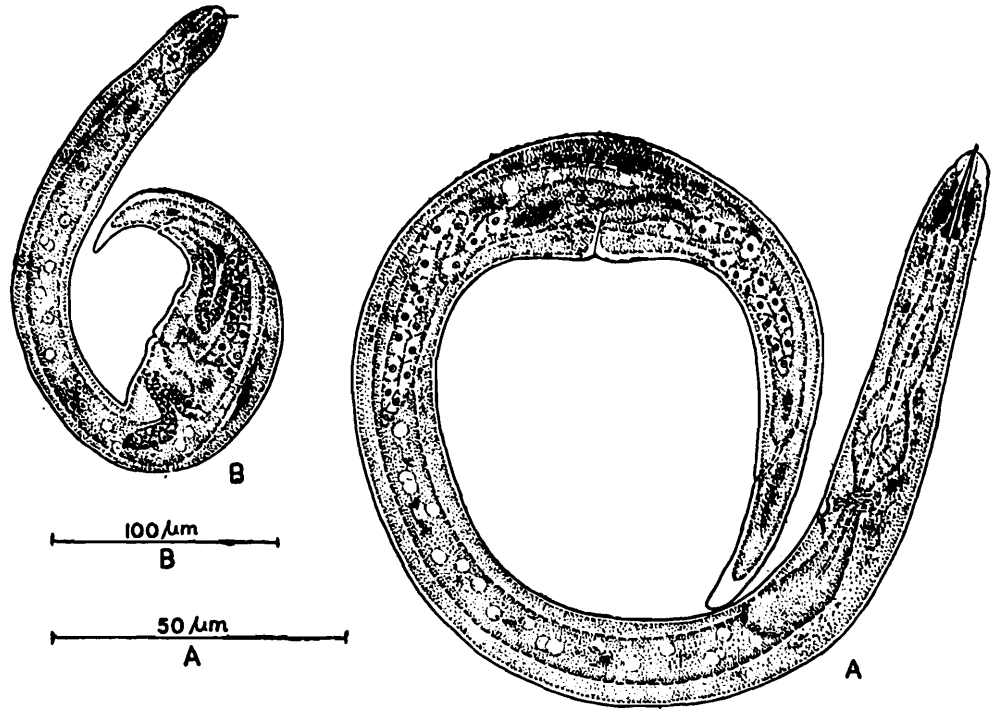

Fig. 7. Post-infection development of *Rotylenchulus reniformis* A—Young female after 48 hours, B—Female at matrix secreting stage.

Embryonic development of *R. reniformis* was found basically similar to that of other plant parasitic nematodes. During the present observations two cells resulting after first cleavage were observed to be somewhat equal and are consistent with the findings of Sivakumar and Seshadri (1971). However, Khan and Khan (1969) reported both cells

unequal (anterior larger and posterior smaller). Linford and Oliveira (1940) did not notice one moult inside the egg shell and reported only three moults in *R. reniformis*. The present study confirms the findings of Nakasono (1966) and Sivakumar and Seshadri (1971) that there are four moults in *R. reniformis*, one of which is inside the egg shell.

Heteromorphism in the shape of the tails of the second and third stage female larva is being reported for the first time in this genus. This phenomenon has been reported by Minton and Golden (1968), and Gupta and Edward (1974) in *Hoplolaimus* spp. and Mathur and Prasad (1972) in *Hirshmanniella oryzae*.

Linford and Oliveira (1940) found that sex of the third stage larva could be identified by the bulging tail of male larva. The spicule primordium, as described by Hirschmann (1962) in *Ditylenchus triformis*, and Sivakumar and Seshadri (1971) in *R. reniformis*, was observed in the present case in the third and the fourth stage male larva. Sivakumar and Seshadri (1971) also illustrated this structure in third and fourth stage larva. However, they did not mention anything about spicule primordium in the text ; perhaps they did not attach much importance to these characters in the differentiation of sex.

Steiner and Buhrer (1964) postulated that the mating took place in pre-parasitic life of the nematode and doubted this for a case of paedogenesis. In the present study it was observed that the spermatheca was not formed in immature females. Spermatheca filled with sperm was observed only in mature females. Moreover, several males were observed in the gelatinous matrix near the vulva in mature females before egg laying ; probably these were attracted by females for copulation. However, copulation was not observed. It thus seems that mating occured only when female attained maturity. The present findings are consistent with those of

TABLE 1. Duration of embryonic stage of *R. Reniformis*.

Stage	Time in hours	
	Duration of stage	Duration from egg deposition
1—2 cells	1— 2	1— 2
2—3 cells	3	4— 5
3—4 cells	1— 2	5— 7
4—5 cells	3— 4	8—11
5—8 cells	14—18	22—29
8 to multi celled	16—20	38—49
Multi-celled to first stage	32—39	70—88
First stage to second stage	21—25	91—113
Second stage to hatching	29—34	121—147 (5-6 days)

TABLE 2. Dimensions of post-embryonic stages of *R. reniformis*

Sl. No.	Stage	No. of specimens measured	Length in µm	a	b	c	Length of genital primordium or gonad in µm	Body width at genital primordium or vulva in µm	Stylet in µm	Remarks
1	Second stage larva	20	313—390	19—23	3.6—4.3	14—30	11—15	14—17	13—17	
2	Third stage larva	10 ♀ ♀	265—370	19—24	3.3—4.2	16—19	21—40	13—16	—	Stylet absent
		10 ♂ ♂	287—382	21—23	3.9—4.4	17—20	25—48	—	—	Stylet absent
3	Fourth stage larva	10 ♀ ♀	260—368	20—27	3.6—4.4	14—17	60—98	13—15	—	Stylet absent
		10 ♂ ♂	270—380	22—24	3.9—4.5	14—18	70—130	—	—	Stylet absent
4	Immature female encased in moulting cuticle	5	310—340	22—24	2.6—3.2	14—16	86—98	11—18	14—20	
5	Mature male encased in moulting cuticle	5	350—380	27—28	3.7—3.8	15—18	115—125	—	12—16	
6	Immature female free from moulting cuticle	10	380—425	22—26	2.9—3.4	13—18	86—115	13—16	16—20	
7	Mature male free from moulting cuticle	10	335—410	25—29	3.7—4.2	15—20	100—165	—	12.6—14	
8	Mature female	10	434—510	—	—	—	—	98—130	12.6—14	

TABLE 3. Duration of different stages of life history of *R. reniformis*.

Stage	Duration in day	
	Male	Female
Egg	5—6	5—6
Second-stage larva	4—5	4—5
Third stage larva	2—3	2—3
Fourth stage larva	1—2	3—4
Pre-oviposition period	—	6—7
	12—16 days	20—25 days

Linford and Oliveira (1940), Nakasono (1966) and Sivakumar and Seshadri (1971).

Some females of *R. reniformis* retained cleavaged eggs inside the body just as some *Heterodera* spp. lay some eggs in the egg mass and retain others in their body ; the females with eggs in their body later form the cyst. However, as female of *R. reniformis* does not form cyst, one reason for this phenomenon that can be suggested is that the females age or under certain ecological conditions loose, at least in part, the muscular tonicity of uteri or vagina or both. Under such circumstances expulsion of eggs is delayed and embryonic development starts inside the female body. This explanation, however, needs further elucidation. Or, it is possible that embryonic development inside the body of the mother is just a case of adaptive (parallel) evolution in the development of the phenomenon of endoparasitism.

REFERENCES

BIRCHFIELD, W. 1962. Host-parsite relations of *Rotylenchulus reniformis* on *Gossypium hirsutum*. *Phytopathology*, **52** (9) : 862-865.

GUPTA, P. and EDWARD, J. C. 1974. Heteromorphism in tail shapes of females of *Hoplolaimus* sp. *Indian J. Nematol.*, **4** : 100-101.

HIRSCHMANN, H. 1962. The life cycle of *Ditylenchus triformis* (Nematoda : Tylenchida) with emphasis on post-embryonic development. *Proc. helminth. Soc. Wash.*, **29** : 30-43.

KHAN, F. A. and KHAN, A. M. 1969. Studies on pre-embryonic development of the reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, 1940. (Abstr.). *All India Nematology Symposium, New Delhi* : 37-38.

LINFORD, M. B. and OLIVEIRA, J. M. 1940. *Rotylenchulus reniformis* nov. gen., nov. sp., a nematode parasite of roots. *Proc. helminth. Soc. Wash.*, **7** (1) : 35-42.

MATHUR, V. K. and PRASAD, S. K. 1972. Embryonic development and morphology of larval stages of the rice root nematode, *Hirschmanniella oryzae*. *Indian J. Nematol.*, **2** : 146-157.

MINTON, A. N. and GOLDEN, A. M. 1968. Heteromorphic larvae of an undescribed *Hoplolaimus* species. *Nematologica*, **14** (1) : 11.

NAKASONO, K. 1966. Role of males in reproduction of reniform nematode *Rotylenchulus* spp. (Tylenchida : Hoplolaimidae). *Jap. J. appl. Ent. Zool.*, **1** : 203-205.

NATH, R. P., SWARUP, G. and RAMA RAO, G. V. S. V. 1969. Studies on the reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, 1940. *Indian Phytopath.*, **22** : 99-104.

REBOIS. R. V. 1973. Effect of soil temperature on infectivity and development of *Rotylenchulus reniformis* on resistant and susceptible soyabeans *Glycine max*. *J. Nematol.*, 5 (1) : 10-13.

SIVAKUMAR, C. V. AND SESHADRI, A. R. 1971. Life history of the reniform nematode, *Rotylenhculus reniformis* Linford and Oliveira, 1940. *Indian J. Nematol.*, 1 (1) : 7-20.

STEINER, G. AND BUHRER, E. M. 1964. The plant nematode problem of the American tropics. *J. Agric. Univ. P. Rico*. 48 : 69-100.