Ultrastructural Changes Associated with Development of Pin Nematode, *Gracilacus* sp., with Special Reference to Its Survival¹

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The 4th stage larvae (L_4 s) of *Gracilacus* sp.² prior to lethargus withstand unfavorable conditions for a longer time than lethargic L_4 s and adults; for more than 42 months in soil kept in polythene bags and at least 80 days in water-saturated soil. For all thicknesses of cuticle, the ratio of the external cortical and basal layer of the quiescent L_4 s was ca. 10% and 55%, respectively, being the largest in all stages examined. With the development of L_4 s to adulthood, the permeability of cuticle increased. The intestinal cells of L_4 s were filled with lipid droplets, which decreased in amount and some of which changed into the age pigment granules in adults. In the course of the formation of adult cuticle, electron dense balls appeared in the lateral hypodermis of the lethargic L_4 s. Changes in survivability with development is discussed from an ultrastructural viewpoint.

INTRODUCTION

The starvation survival of the 4th stage larvae (L_4 s) of pin nematodes in a non-host and/or dried soil has been studied from an ecological viewpoint (RHODES and LINFORD, 1961). According to the mobility, posture, and integrity of internal digestive and reproductive organs, the L_4 s can be divided into 3 substages; quiescent, moving, and lethargic. The former two L_4 s withstand unfavorable conditions such as dryness, and also reversible each other depending on the soil moisture level. Once the moving L_4 s develop into the lethargus, they can no longer return to the quiescent substage, becoming susceptible to environmental stresses (ISHIBASHI et al., 1975). The initiation of such development of L_4 s in pin nematodes is stimulated by root diffusate of the host plants (RHODES and LINFORD, 1959; FISHER, 1960).

Many reports on the fine structure of nematodes are now available. However, an ultrastructural study of pin nematodes, one of the smallest plant parasitic nematodes, has not been conducted so far.

The present report is concerned with the ultrastructural changes of cuticle and lipid droplets in the pin nematode, *Gracilacus* sp., in the metamorphosis from the quiescent L_4 s to adults. The anatomical view will be considered together with some information covering the survivability obtained from the present investigation.

¹ Ecological significance of dormancy in plant parasitic nematodes. VIII.

² This nematode is the same as that previously reported by ISHIBASHI et al. (1975) as *Paratylenchus aciculus*.

MATERIALS AND METHODS

The quiescent L₄s of *Gracilacus* sp. were collected by the centrifugal floatation technique from mulberry field soil (sandy loam), which had been sampled in early October, and stored in polythene bags for 2 months at room temperature. The moving L₄s, lethargic L₄s, and adults were prepared by incubating the quiescent L₄s at 25°C in the mulberry root diffusate for 6, 72, and 144 hr, respectively. The original root diffusate used was prepared by the method described in the previous paper (ISHIBASHI et al., 1975) and stored at 5°C until use.

Survival in soil at different moisture levels.

The mulberry field sandy soil sampled on Oct. 1 was mixed thoroughly through a 20 mesh sieve. Fifty grams each of soil sample were put in a plastic container (dia 7.5 cm, height 4.5 cm). Three groups were made with 20 replicates each according to the soil moisture conditions; moist, water-saturated, and dry. For the moist soil, the moisture level was kept at about 11% on dry weight base throughout the experimental period. The containers of these two groups were tightly sealed to keep moisture constant. For the 3rd group, the soil material was allowed to dry during the experimental period. All containers were kept at ambient room temperatures (22–25°C). On the 10th, 20th, 40th, and 80th day after the onset of experiment, the number and developmental composition of surviving nematodes were investigated with 4 containers randomly sampled from each group.

The heavily infested mulberry field soil (volcanic loamy soil), which had been stored in the polythene bags at room temperatures for 42 months, was used to observe long term survival in the moderately dried soil (22.6%) under starvation.

Survival in glutaraldehyde fixative

Two hundred each of moving L₄s, lethargic L₄s, and adults were incubated in 5% glutaraldehyde fixative at 20°C. Twenty individuals in each developmental stage were cut with a surgical knife at 15 min intervals for up to 6 hr after commencement of incubation. The nematodes that could be cut without leaking the body contents were considered to have been completely fixed.

Penetration of vital stains through cuticle

Two kinds of experiments were designed to observe the penetration rates of stains into the bodies through cuticle. In the 1st experiment, one hundred specimens of each developmental stage were placed in a Syracuse watch glass filled with 2 ml of 50 ppm aqueous solution of eosin Y. The penetration rate was investigated 6, 24, and 48 hr after the onset of the experiment with 5 replications. The 2nd experiment was carried out by using the quiescent L₄s alone for distilled water, mulberry root diffusate, 50-ppm eosin Y in the diffusate, and 50-ppm methylene blue in the diffusate. Rates of nematode growth and stain penetration were investigated at 12 hr intervals up to 144 hr with 5 replications. After being incubated at 25°C in the staining solutions described above, the nematodes were examined under a microscope with the aid of strong transparent illumination. They were judged to be stained by eosin Y or methylene blue when the lipid droplets in the bodies showed brilliant pink or blue, respectively.

Lipid droplets

More than 20 individuals were arbitrarily chosen from each developmental stage and a drawing of the total body area and the dark lipid area demonstrated by Suddan black B was made on chart paper by using a Nikon drawing apparatus. The ratio of the lipid to total body area was calculated for each developmental stage.

Electron microscopy

For electron microscopy, the nematodes were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 hr for the adults and 12 hr for the L₄s at 2–3°C. After thoroughly rinsing in the cold phosphate buffer, the specimens were post-fixed by 1% OsO₄ for 2 hr at 2–3°C. The fixed materials were dehydrated in a graded ethanol series and finally embedded in Spurr's low viscosity epoxy resin. The ultrathin cross sections of nematode were stained by uranyl acetate for 1 hr and counter-stained with lead citrate for 5 min at 20°C. The sections were observed with a JEM 100B electron microscope operated at 80 kV.

RESULTS

Survivability in soil at different moisture levels

As the storage time increased, the number of surviving nematodes drastically decreased in the air-dried soil, followed by the water-saturated and the moist soil (Fig. 1). Eighty days after the commencement of experiment, the population decreased to 69%, 29%, and 6% of the initial population having *ca.* 650 nematodes per 50 g soil, in the moist, water-saturated, and air-dried soil, respectively. The ratios of the quiescent and moving L₄s in the composition increased with time for all moisture preparations. Under the air-dried and water-saturated conditions, the only nematodes recovered after 80 days were the quiescent and moving L₄s. From the air-dried soil, the 2nd and 3rd

Fig. 1. Changes in number and ratio of developmental stages of *Gracilacus* sp. in air-dried (A), moist (B), and water-saturated soil (C) kept at room temperature for 80 days.

Open column : Female adults. Striped column : Male adults. Densely dotted column : Lethargic L₄s. Thinly dotted column : Quiescent and moving L₄s. Closed column : 2nd and 3rd stage larvae.

Fig. 2. Times required to the complete fixation by 5% glutaraldehyde at 20°C for adults (A), lethargic L₄s (B), and moving L₄s (C) of *Gracilacus* sp.

stage larvae were not recovered on even the 10th day.

In the soil stored for 42 months, the quiescent L₄s were predominant in the composition and reached up to 98% of all surviving worms. These nematodes uniformly developed into adults in the presence of mulberry root diffusate. Neither male adults nor the 2nd to 3rd stage larvae were recovered from this soil.

Survival in glutaraldehyde fixative

As shown in Fig. 2, the percentage of nematodes fixed by 5% glutaraldehyde varied significantly according to their developmental stages. The adults were completely fixed in 30 min, while more than 40% of the moving L₄s were not fixed even after 6 hr.

Penetration of stains through cuticle

The ratio of nematodes stained by 50-ppm aqueous solution of eosin Y was higher in male adults, followed by female adults, lethargic L₄s, and moving L₄s. Only 1.5% of moving L₄s were stained in 48 hr (Table 1).

In the mulberry root diffusate, almost 100% of the quiescent L₄s developed into lethargic L₄s in 72 hr, and then developed steadily to adulthood (Fig. 3). The ratios of nematode development and stain penetration varied according to the kind of stains used. The development of nematodes treated by eosin Y did not differ from those incubated in root diffusate alone, while normal development was inhibited by methylene blue. Regardless of the kind of stain used, it penetrated into the nematode body just before the lethargus.

Table 1. PENETRATION RATE (%) OF EOSIN Y 50-PPM AQUEOUS SOLUTION INTO THE BODY OF *Gracilacus* SP. AT 25°C

Developmental stage	Incubation time (hr)		
	6	24	48
Moving L ₄ s	0.0	0.0	1.5
Lethargic L ₄ s	0.0	9.1	35.3
Male adults	0.0	83.3	100.0
Female adults	0.0	45.5	58.3

Fig. 3. Changes in the rates of pin nematodes stained either by eosin Y or methylene blue dissolved in mulberry root diffusate (broken line).

Solid lines indicate the rates of stages of nematodes developing at 25°C in four solutions: eosin Y in the diffusate (A), methylene blue in the diffusate (B), diffusate only (C), and distilled water (D). Open circle: quiescent L₄s. Closed circle: lethargic L₄s. Closed square: adults.

Cuticle

The bodies of both L₄s and newly formed adults were observed to be covered with the external cortical layer of shed cuticle of the previous stages; one in the former and two in the latter (Figs. 4B and 4F). The thickness of the shed cuticle was about 21 nm on average.

The average thickness of cuticle at the interchordal area was 240 nm in the quiescent and moving L₄s, and 230 nm in adults just after the final molt. In the lethargic state, the nematodes had two sheets of cuticle; one was degenerating cuticle of L₄s, the other generating adult cuticle showing gradual development during the lethargus (Figs. 4C and 4D). The contents of L₄'s cuticle are probably absorbed into the newly forming adult cuticle (Fig. 5D). The adult cuticle gradually increased in thickness after the final molt.

Fundamentally, the cuticle consisted of 4 layers: external cortical, internal cortical, median, and basal layers (Fig. 4G). Of all thicknesses of cuticle, the ratio of external cortical layer was about 10% in both the quiescent and moving L₄s, and less than 9% in the newly formed adults. The basal layer was also thicker in the quiescent and moving L₄s than in the newly formed adults; about 55% of cuticle in the former two and about 35% in the latter. The striations of the basal layer in the quiescent L₄s were not as clearly observed as those in adults. The median layer was variable in thickness according to the developmental stages, and hardly recognizable in the quiescent and moving L₄s (Fig. 4B). In the quiescent L₄s recovered from the soil stored for 42 months, the electron density of the median layer of the interchordal

Fig. 4. Ultrastructural changes of cuticle of *Gracilacus* sp. with development from quiescent L_4 s to adults through the lethargus.

A : Lateral chord of quiescent L_4 stored for 42 months in non-host soil, showing an increased electron density in the median layer of cuticle. $\times 23,500$. B : Thicker basal layer (BL) of cuticle in the lethargic L_4 . $\times 80,000$. C : Formation of adult cuticle (CA) in lethargic L_4 . $\times 70,000$. D : Infolding of hypodermis into annuli under the shed cuticle of lethargic L_4 (C_4). $\times 20,000$. E : Degenerated materials (DM) in the interspace between the adult cuticle (CA) and boundary layer (arrow) of hypodermis in which many glycogen granules (G) appeared. $\times 36,250$. F : Newly formed adult cuticle (CA) covered with 2 shed cuticle (SC) of the previous stages. $\times 47,000$. G : Oblique section of adult cuticle showing 4 layers : external cortical layer (ECL), internal cortical layer (ICL), median layer (ML), and basal layer (BL), MF : miofilament of somatic muscle. HY : hypodermis. $\times 70,000$.

Fig. 5. Cross section of lateral hypodermis of quiescent, moving, and lethargic L_4 s of *Gracilacus* sp.

A : Moderately electron-dense lipid droplets (L) in the lateral hypodermis of moving L_4 . $\times 20,000$. B : Very large lipid droplet (L) in the intestine of quiescent L_4 stored for 42 months in non-host soil. $\times 9,400$. C : Electron-lucent lipid droplets (L) deposited in the intestine. $\times 10,000$. D : Electron-dense balls (DB) appeared in the lateral hypodermis of the lethargic L_4 in which the formation of adult cuticle (CA) is advancing under the shedding cuticle of L_4 (C4). $\times 14,000$.

Fig. 6. Cross section of lateral hypodermis and intestine of newly formed adults of *Gracilacus* sp.

A : Electron-dense balls (DB) and many glycogen granules (G) observed in the lateral hypodermis. $\times 30,000$. B : Boundary layer (arrow) of hypodermis separated from the newly formed adult cuticle (CA). $\times 12,000$. C : Intestine of female adult showing the age pigment granules (AP) and presumed site of glycogen mobilization (arrow) in the lipid droplet. $\times 9,400$.

Table 2. CHANGES IN THE RATIO OF LIPID AREA TO TOTAL BODY AREA WITH DEVELOPMENT FROM 4TH-STAGE LARVAE TO ADULTS.

Developmental stage	Sexes	Area (μm^2)		B/A $\times 100$ (%)
		Body (A)	Lipid (B)	
Moving L ₄	Mixed	3575	2614	73.1
Lethargic L ₄	Female	4110	2756	72.9
	Male	3606	2598	72.1
Adults	Female	4205	2425	57.5
	Male	3795	1526	40.2

and chordal areas was higher than that of other stages (Fig. 4A). The boundary layer of the hypodermis of the newly formed adults was separated from the basal layer of the cuticle, and degenerated materials were observed in the interspace between the two layers (Figs. 4E and 6B).

Lipid droplets

The ratio of the lipid area to the total body area in L₄s was 72–73%, with no differences between the moving and lethargic ones. In adults, however, the ratio was reduced to 57.5% in females and 40.2% in males. No difference between the two was observed in the lethargic L₄s (Table 2).

The intestinal cells of the quiescent and moving L₄s were filled with large electron lucent lipid droplets without obvious intestinal lumen or microvilli (Fig. 5C). The lipid droplets in the hypodermal cells under the lateral chords moderately increased in their electron density in these stages (Fig. 5A). Besides the deposition of electron lucent lipid droplets in the intestinal cells, electron dense balls appeared in the lateral chordal cells of lethargic L₄s in which the formation of adult cuticle was advancing (Fig. 5D). In the newly formed adults, the dark inclusions of age pigment appeared in the intestinal cells (Fig. 6C) and electron dense balls in the lateral hypodermis (Fig. 6A). During and after the final molt, glycogen granules increased in quantity both in the lateral hypodermis and in intestinal cells (Figs. 5D and 6A). As shown in Fig. 6C, some of these appeared in lipid droplets, indicating the presumed site of the lipid mobilization to glycogen.

DISCUSSION

It has been reported that the drying rates affect the survivability of nematodes; slower drying resulted in high survival (ELLENBY, 1968a; EVANS and PERRY, 1976). From the fact that the cuticle of quiescent and moving L₄s of *Gracilacus* sp. used in this experiment is low-permeable to vital stains as well as being highly resistant to glutaraldehyde fixative, the low permeability of cuticle to water may account for the high survival rate of these larvae under desiccation. In this case, the shed cuticle of the previous stage may play an important role in decreasing the rate of water loss from the L₄'s body. ELLENBY (1968b) also demonstrated that the ensheathed L₃s of *Haemonchus contortus* survived desiccation for a longer time than exsheathed ones. In addition to shed cuticle, the external cortical layer of the L₄s was the thickest in all stages examined in the present study. However, the lethargic L₄s were susceptible

to environmental stresses, although they had two sheets of cuticle. In *Aphelenchus avenae*, the molting stage was the most susceptible to nematicides (EVANS and THOMASON, 1969). BIRDS and ROGERS (1965) suggested the incorporation of the inner materials of the old cuticle in the newly forming cuticle during molting in *Meloidogyne javanica*. From these observations as well as Fig. 5D showing the degenerated materials between the shedding and forming cuticles in the lethargic L₄s, it may be concluded that the decreased survivability of lethargic L₄s stems from the enhanced permeability of the cuticle, through which the nematodes reabsorb degenerated substances of the old cuticle.

The long term survival of L₄s of pin nematodes has been reported for *P. dianthus* and *P. projectus* (RHODES and LINFORD, 1961). In the present study, L₄s of *Gracilacus* sp. survived at least 42 months without feeding. We have observed that the surviving stages of nematodes are filled with lipid droplets in many cases, and it is true for L₄s of pin nematodes in which electron-lucent lipid droplets are deeply deposited. Such a deep deposition of large lipid droplets was also shown in the dispersal 3rd-stage larvae of *Bursaphelenchus lignicolus* surviving within wilted pine trees or on an agar without food (KONDO and ISHIBASHI, 1978). The surviving 4th-stage larvae of pine wood or pin nematodes have no functional feeding or digestive organs. This leads us to believe that they consume the lipid materials for the formation of adult organs during the final molt. The stored lipid droplets in the intestinal cells decreased with development to adults through the lethargus. Instead, glycogen granules, as reported on *Meloidogyne incognita* (DROPKIN and ACEDO, 1974), began to increase in the lateral hypodermal and intestinal cells. High metabolic activity during metamorphosis may account for the mobilization of lipid to glycogen in these cells. Considering all of the above items, the lipid droplets stored in surviving L₄s of pin nematodes may be important as an energy source not only for long term survival under starvation but also for the regeneration of organs such as the stylet, esophagus, intestine and cuticle, and for the formation of the adult gonad after the final molt.

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