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Low-Temperature Scanning Electron Microscope Observations of the *Meloidogyne incognita* Egg Mass: The Gelatinous Matrix and Embryo Development

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Abstract: The root-knot nematode *Meloidogyne incognita* was cultured monoxenically on excised tomato roots. Galls and egg masses were observed daily using a light microscope. Two phases were distinguished in the gelatinous matrix of the egg mass: a translucent, amorphous material on the surface of the egg mass and a denser, layered phase in which nematode eggs were deposited. Egg masses were also cryofixed, fractured, and observed as frozen, hydrated specimens on a cold stage in a scanning electron microscope (SEM). In the SEM, the layered phase appeared as a meshwork of fibrils that became more loosely associated as the gelatinous matrix aged. Small pearl-like bodies were observed along the fibers of gelatinous matrix. The egg shell surface and several stages of embryo development, including the one-cell stage, initial cleavages, blastula, gastrula, tadpole stage, elongation, and molt of the first-stage juvenile within the egg shell, were observed and photographed with this technique. The developmental events observed were consistent with those described in other nematode species with different techniques.

Key words: development, egg, egg mass, embryogenesis, gelatinous matrix, low-temperature scanning electron microscopy, *Meloidogyne incognita,* root gall, nematode, root-knot nematode, scanning electron microscopy, ultrastructure.

The embryogenesis of animal-parasitic nematodes was described over a century ago and reviewed in 1950 by Chitwood (6). More recently, comprehensive examination of embryonation in *Caenorhabditis elegans* traced the origin of every cell in the adult nematode (3,19). Embryogenesis has been studied less comprehensively for plant-parasitic nematodes. Raski (14) described embryo development of a tylenchid nematode, *Heterodera schachtii,* and more detailed descriptions of this process for *Meloidogyne javanica* and *Anguina agrostis* were published by Bird (1) and Bird and Stynes (5). All these descriptions were based on light microscopy and were illustrated with drawings or photomicrographs.

Root-knot nematodes (*Meloidogyne* spp.) lay eggs into a gelatinous matrix (GM). The eggs and the GM form the egg mass, which generally is found at the interface between the gall surface and the soil. The GM is produced by six rectal glands arranged radially around the female anal opening and is secreted through the anus before and during egg laying (9). The GM is secreted in a volume greater than that of the entire female body and may incorporate hundreds of eggs. Although the GM was first described many years ago, only a few studies have focused upon GM function and structure (4,7,17). In a comprehensive review on root-knot nematode morphology and ultrastructure, Bird (2) noted that no definitive experimental evidence exists regarding the function of the GM.

In light and electron microscopic studies, Orion et al. (12) and Orion and Franck (10) showed that the GM altered host cells to form a canal through which eggs were forced outside the gall. The lysis of the host cells suggested that the GM contained cellulytic or pectolytic enzymes. The GM was also suspected to protect the nematode against soil-borne microorganisms. Indeed, Orion and Kritzman (11) showed that the GM from *M. javanica* reduced reproduction of a bacterium and a yeast and that other microorganisms directly contacting the GM were agglutinated. Furthermore, Sharon et al. (15) demonstrated that contact with the GM surface caused binding of several microorganisms as well as red blood cells.

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The current study was initiated because the GM appears to have significant importance in the life history of root-knot nematodes. Our objective was to study the ultrastructure of the GM and eggs of *M. incognita* with the new technique of low-temperature SEM. This technique is useful for examining nematodes (18) because it allows cryo-fixation and observation of material directly, thereby avoiding artifacts from the structural and chemical changes that occur following chemical fixation, dehydration, and critical point drying—procedures that must be used in a conventional SEM investigation.

Materials and Methods

Meloidogyne incognita was cultured monoxenically on excised roots of tomato (*Lycopersicon esculentum* cv. Rutgers) on chemically defined medium (8) modified to contain 100 mg/liter ammonium nitrate and 0.8% Phytagel instead of agar. Procedures for seed surface sterilization, germination, transfer of root tips, and inoculation with the nematodes have been described previously (13). Cultures were kept in the dark at 26 C. During the third and fourth weeks following inoculation, light microscope observations were made daily on developing egg masses in the culture plates.

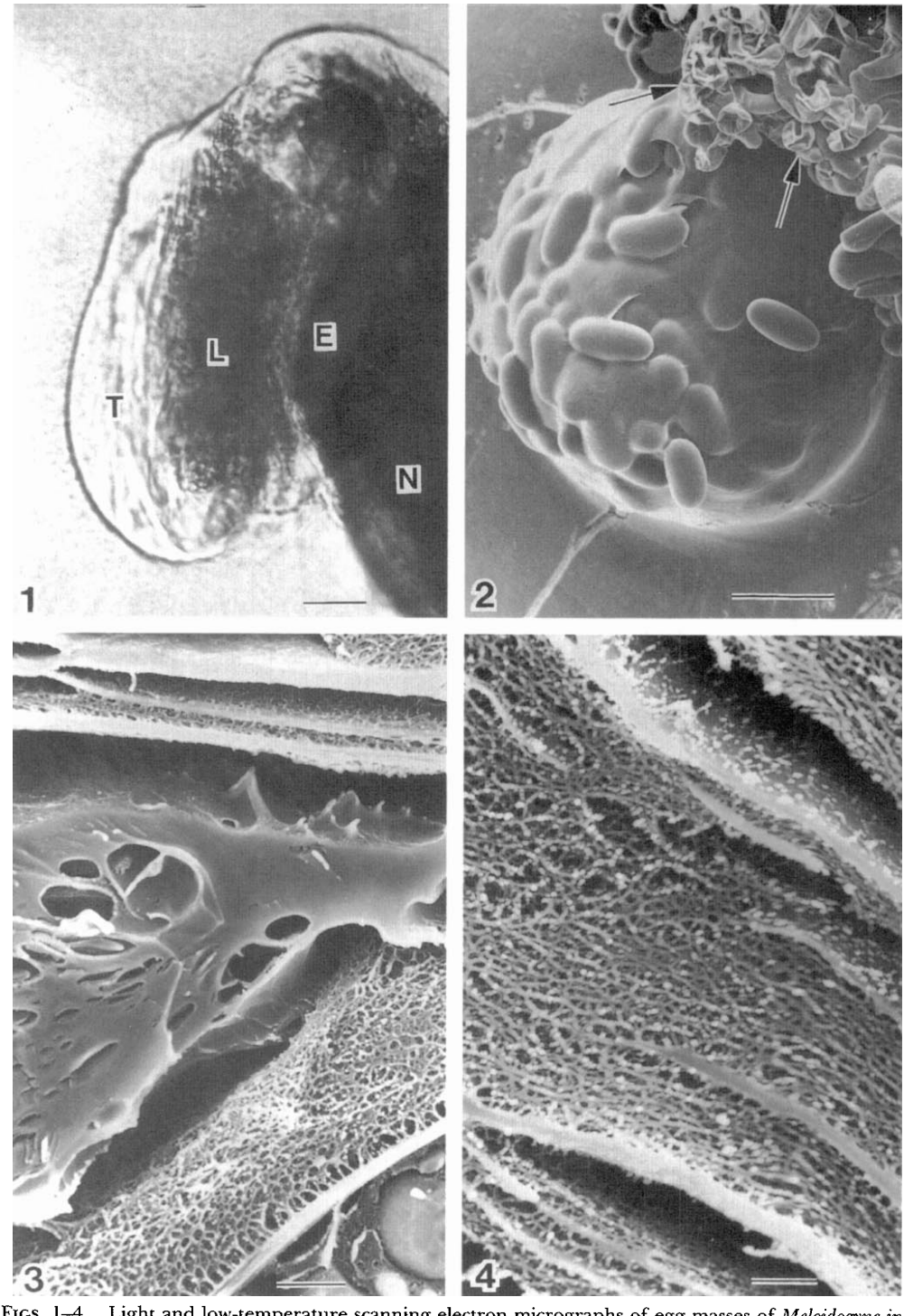
SEM observations were performed on a Hitachi S-4100 field emission scanning electron microscope equipped with an Oxford CT-1500 Cryotrans System. Specimen preparation consisted of removing young (1–3 days old) and mature (7–10 days old) egg masses from the surface of the galls and placing them in gold-hinged holders mounted on a Denton complementary freeze-etch specimen cap as previously described (18). Briefly, the specimens were cryofixed by submerging the cap assembly in the Oxford nitrogen slush chamber, evacuating, and withdrawing the cap into a cryo-transfer arm for transfer to the Oxford prechamber. A precooled pick in the prechamber was then used to fracture the samples by lifting and rotating the fracture arm of the complementary cap 180°. The specimens were then either sputter coated with platinum in the prechamber and inserted onto the cryostage of the microscope or etched for 8 minutes at −90 C, coated in the prechamber, and moved to the cryostage for observation. Accelerating voltages of 10 kV were used to observe or record images onto Polaroid Type 55 P/N film. For comparative purposes, a few specimens were prepared for SEM examination using conventional procedures (13) consisting of chemical fixation in 3% glutaraldehyde, dehydration in a graded ethanol series, and critical point drying from liquid CO_2.

Results

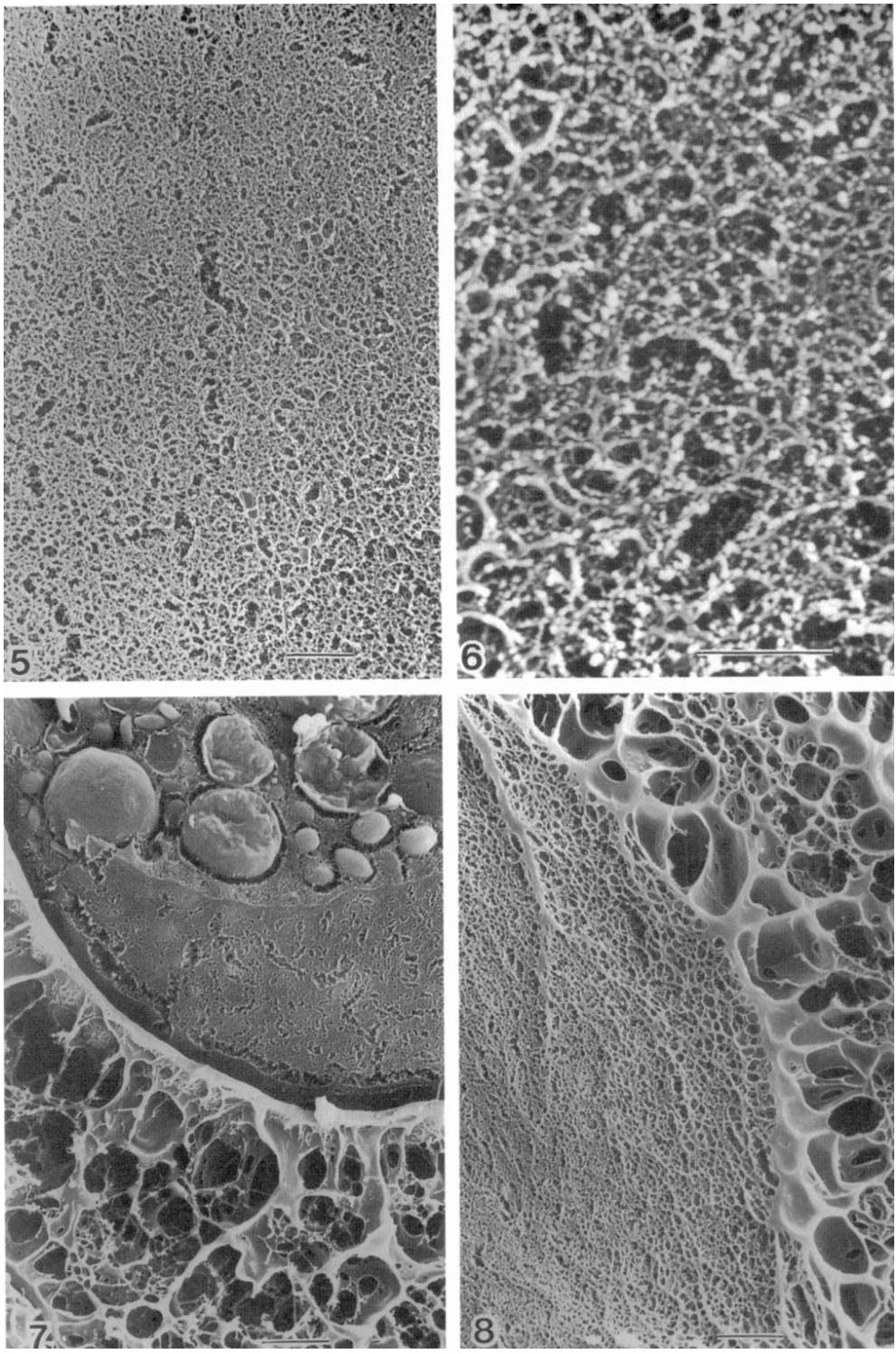
Gelatinous matrix: Light microscopic observations revealed two distinct structural phases in the GM (Fig. 1). The outer portion of the GM, which apparently was secreted before egg laying, appeared as a transparent, colorless substance. The second structural phase of the GM consisted of a yellowish brown material occurring in loosely organized layers. Most of the eggs were deposited into this phase, which was more abundant than the transparent, colorless phase.

Low-temperature SEM revealed that the epidermal cells on the gall surfaces in the root cultures enlarged, became loosely associated with each other, and resembled callus tissue (Fig. 2). The two phases of the GM observed in the light microscope (LM) were resolved further in the SEM. The phase that appeared transparent and colorless in the light microscope appeared dense and amorphous in the frozen SEM preparations (Fig. 3). The phase that appeared layered in the LM appeared in the SEM as concentric layers with a fibrillar or meshed fine structure (Fig. 4).

The density of the layered material in the GM appeared to change with age. In the newly formed egg masses, the mesh-like material was very dense but interspersed with small spaces, ca. 0.5 μm in diameter (Fig. 5). At high resolution, spherical pearl-like bodies, 100–150 nm in diameter, appeared randomly distributed along the fibrillar component (Fig. 6). In



FIGS. 1–4. Light and low-temperature scanning electron micrographs of egg masses of *Meloidogyne incognita*. 1) Light micrograph of a 2-day-old egg mass, showing the relative positions of the nematode (N), eggs (E), and the translucent (T) and layered (L) phases of the gelatinous matrix; bar = 40 μm. 2) SEM micrograph of a frozen, intact egg mass with callus-like root tissue on the surface of the gall (arrows); bar = 100 μm. 3) SEM micrograph of a fractured egg mass, illustrating the outer amorphous phase of the gelatinous matrix (GM) and a portion of an egg (lower right); bar = 2.0 μm. 4) SEM micrograph of fractured GM showing the fibrillar nature of the mesh-like phase, which appears to occur in successive concentric layers; bar = 1.0 μm.



FIGS. 5–8. Scanning electron micrographs of freeze-fractured egg masses of *Meloidogyne incognita*. 5) Meshwork characterizing the layered phase of the GM; bar = 2.0 μm. 6) Spherical, pearl-like bodies that lie along the fibrils; bar = 1.0 μm. 7) Fractured face of a mature GM (lower portion of the figure), with part of an egg in the upper right; bar = 2.0 μm. 8) Adjacent layers of a mature GM illustrating the newly secreted layer (lower left) and an older layer consisting of a more mature matrix (upper right); bar = 2.0 μm.

GM from mature egg masses, the sizes of the spaces were quite variable and were as wide as 2 μm (Fig. 7). In the older egg masses, the GM appeared less fibrillar, and the pearl-like spherical bodies occurred less frequently (Fig. 8).

Each fractured egg mass often contained considerable structural variation (Fig. 8). Successive zones differing in fine structure probably produce the layered appearance observed with the LM. Because egg laying may continue for 2 weeks, the successive layers may be formed by intermittent secretion of the GM, with the newly secreted material being more dense and the older material less dense, with large gaseous spaces (Fig. 8).

Embryogenesis: In SEM observations, the oblong egg of *Meloidogyne incognita* measured about 35 μm by 80 μm (Fig. 9). Although the surface of the shell appears relatively smooth in the light microscope, under SEM the shell had a textured appearance because of two distinctive topographical structures (Fig. 10).

Low-temperature SEM images of freeze-fractured egg masses revealed tightly clustered eggs containing embryos at various developmental stages (Fig. 11). In contrast, images obtained with conventional procedures (Fig. 12) contained considerable artifacts in the developing embryo; neither cell numbers nor early formed tissues could be recognized.

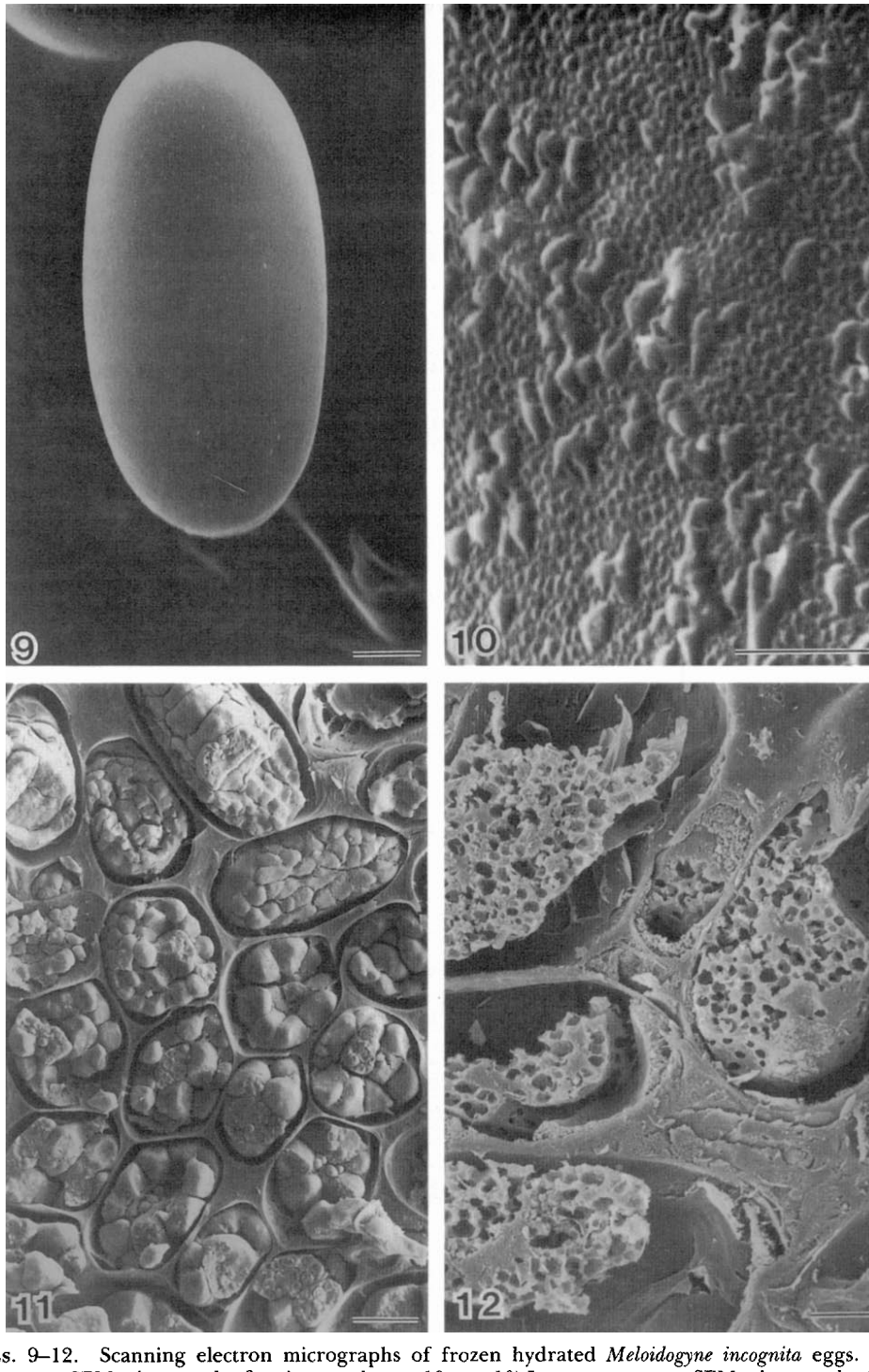
Low-temperature SEM images of the periphery of the egg mass revealed individual eggs surrounded by an abundance of GM and tending to have a characteristically oblong shape (Fig. 13). In contrast, eggs in the center of the mass were much closer to one another and often were surrounded by only a thin layer of GM. These eggs were frequently more angular in cross section than eggs in the periphery and appeared to be slightly deformed by the tightly packed arrangement (Fig. 11).

The earliest stage of embryo development observed was the single-cell stage, with one cell filling the entire volume of the egg (Fig. 13). Transverse cleavage of this cell resulted in two approximately equally sized cells (Fig. 14); asynchronous cleavage of each of these cells resulted in the four-celled stage (Fig. 15). In other eggs, the four cells had proliferated to become a cluster of isodiametric, undifferentiated cells characteristic of the blastula stage (Figs. 16,17). Organization and cellular differentiation appeared to continue in the central portion of the egg along the longitudinal axis, thereby forming the gastrula (Fig. 18). With continued differentiation, the large cellular mass in the center of the egg became surrounded by the small peripheral cells of the ectoderm (Figs. 19,20). In other eggs, further morphogenesis produced the tadpole stage (Fig. 21). More mature eggs contained embryos in a twice-folded, easily recognizable early juvenile stage (Fig. 22). Other juveniles had matured to the late juvenile stage, where they were folded four times (Fig. 23). During this stage, the cuticle of the first-stage juvenile could be observed clearly (Figs. 23,24).

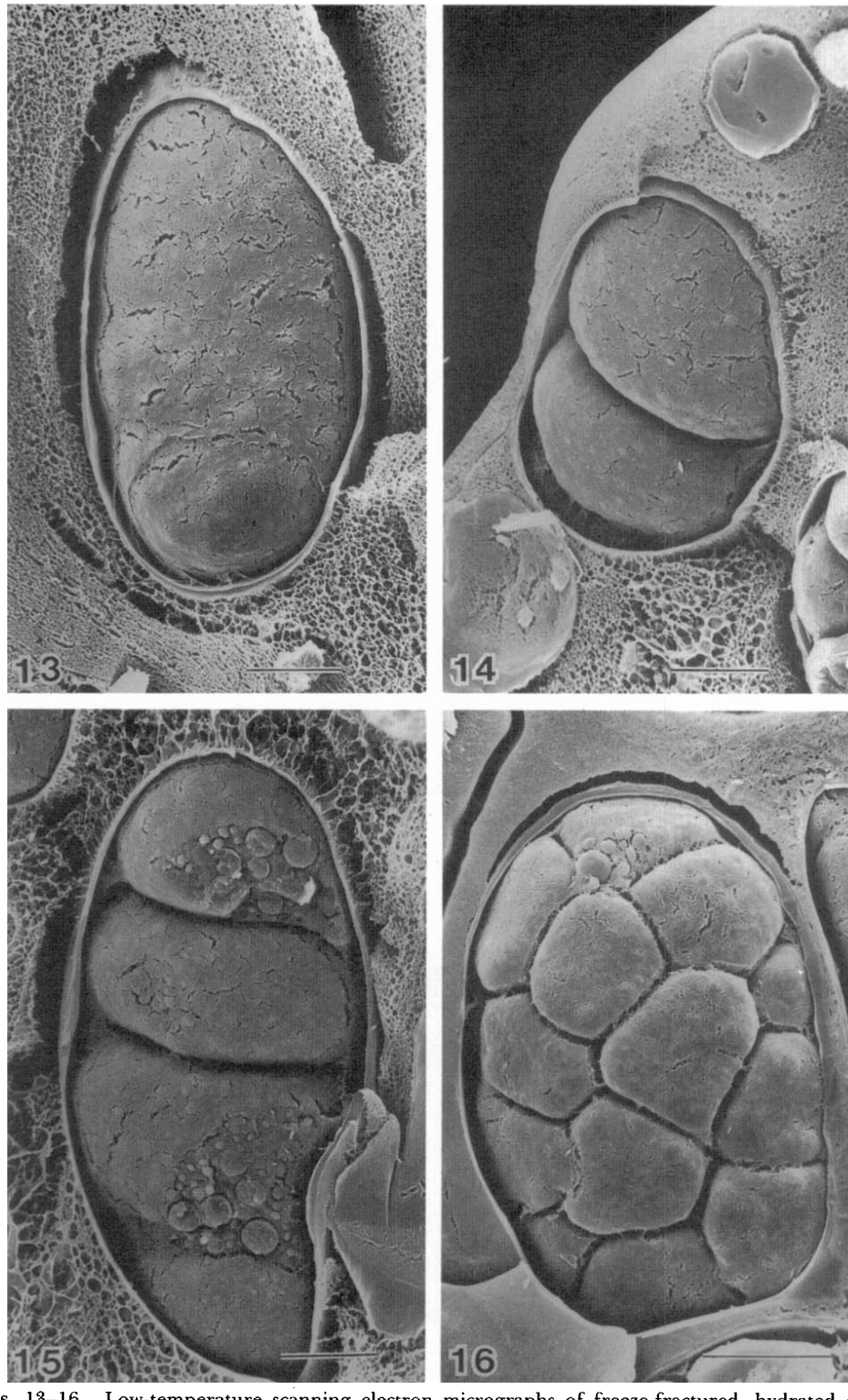
Discussion

The light and electron micrographs show that the root-knot nematode GM is a complex material composed of amorphous, fibrillar, and spherical macromolecular structures that probably have different functions. The amorphous, hyaline component may have enzymatic or hormonal activity, may induce enlargement of the root epidermal cells, or may cause lysis and separation of the root cells. The latter process appears to form the previously described canal or cavity through which the egg mass passes to the outside of the gall (10,12).

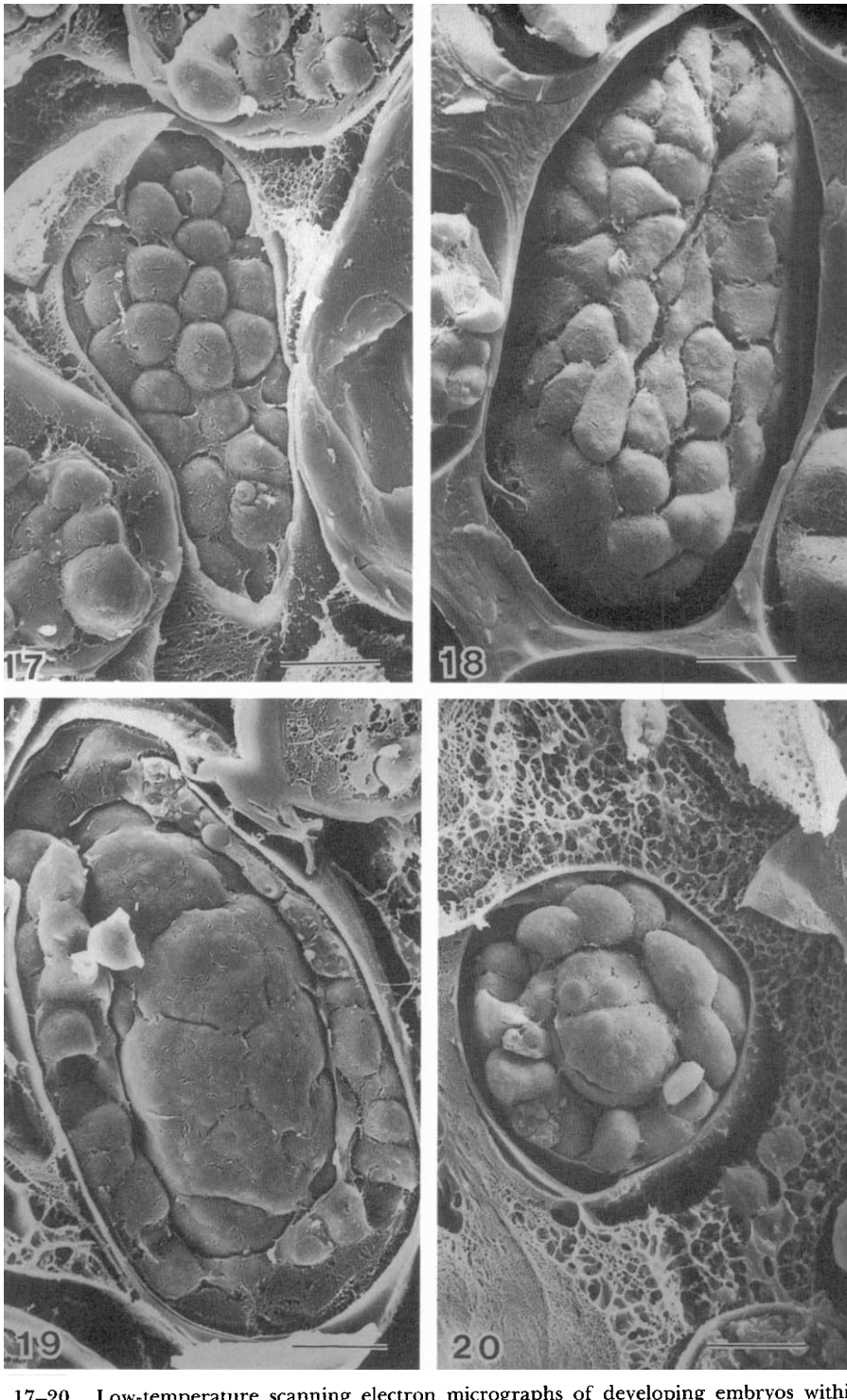
The more mature region of the GM appears fibrillar or mesh-like under the SEM. A granular or mesh-like structure was also described by Bird and Soeffky (4), who found that the matrix consisted of an irregular meshwork when hydrated but was a uniform granular mass with greater density when dehydrated. They concluded that the meshwork of the GM may inhibit water loss from the eggs, as had been sug-



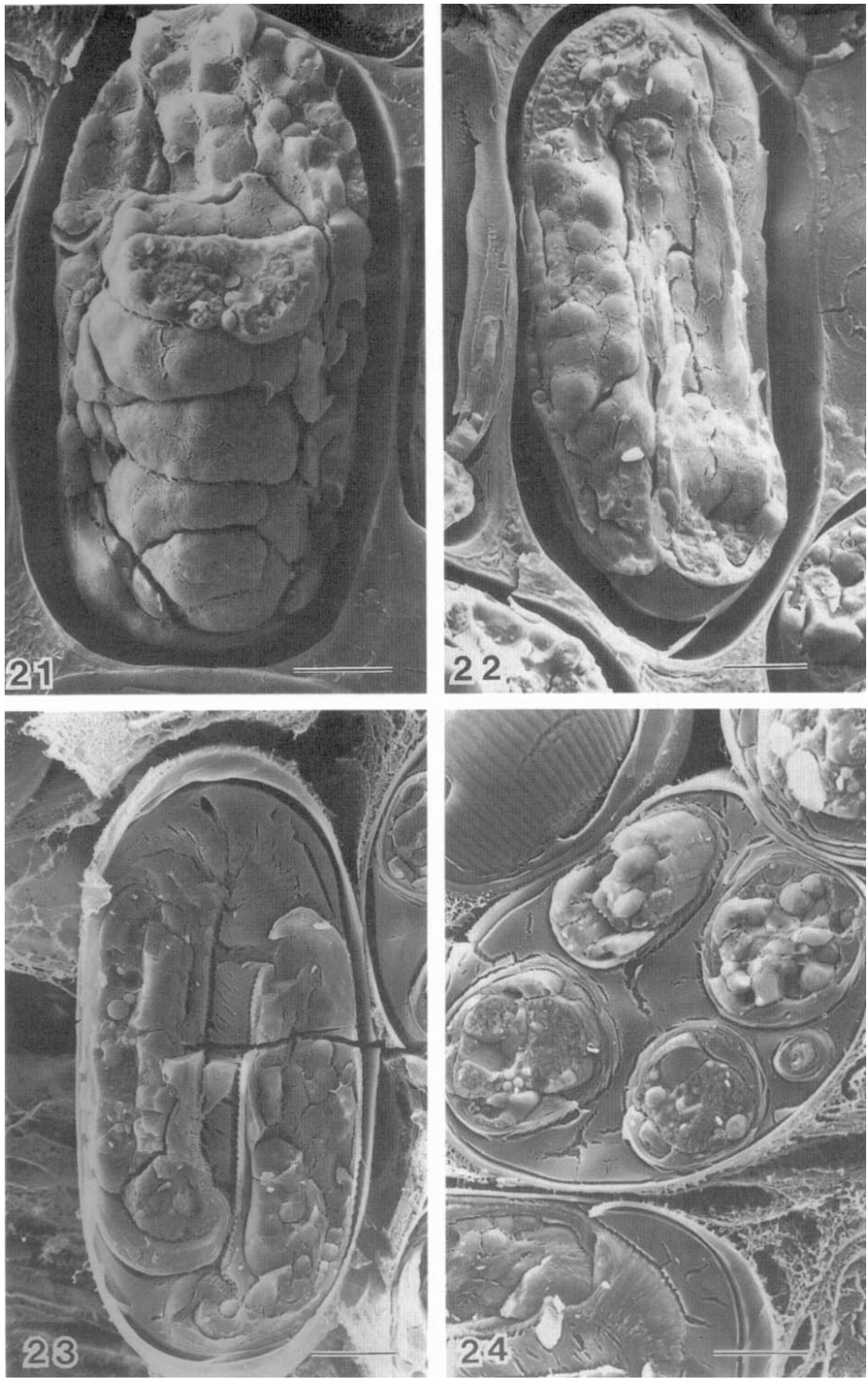
FIGS. 9–12. Scanning electron micrographs of frozen hydrated *Meloidogyne incognita* eggs. 9) Low-temperature SEM micrograph of entire egg; bar = 10 μm. 10) Low-temperature SEM micrograph of surface of egg, showing two distinctive structures resulting in a textured appearance to the egg; bar = 0.2 μm. 11) Low-temperature SEM micrograph of fractured egg mass illustrating several embryos in early developmental stages; bar = 20 μm. 12) Ambient temperature scanning electron micrograph of a fractured egg mass that had been fixed, dehydrated, and critical-point dried by conventional procedures; no distinct cells or other features were observed with this technique; bar = 10 μm.



FIGS. 13–16. Low-temperature scanning electron micrographs of freeze-fractured, hydrated eggs of *Meloidogyne incognita*. 13) Cell membrane of one-cell stage; bar = 10 μm. 14) Two-celled stage resulting from the first cleavage; bar = 10 μm. 15) Second cleavage has been completed in the anterior end, resulting in two distinct cells and appears to be in progress in the posterior end; bar = 10 μm. 16) Blastula possibly containing 16 isodiametric undifferentiated cells; bar = 10 μm.



FIGS. 17–20. Low-temperature scanning electron micrographs of developing embryos within freeze-fractured, hydrated eggs of *Meloidogyne incognita*. 17) Blastula, possibly the 32-cell stage; bar = 10 μm. 18) Early gastrula stage, with cells tending to organize along the longitudinal axis of the egg; bar = 10 μm. 19) "Tadpole" stage, with large cells in the central region surrounded by smaller peripheral cells that constitute the ectoderm; bar = 10 μm. 20) Cross section of another egg in tadpole stage; bar = 10 μm.



FIGS. 21–24. Low-temperature scanning electron micrographs of developing embryos within freeze-fractured, hydrated eggs of *Meloidogyne incognita*. 21) Early embryo elongation stage. Lower portion of the egg illustrates surface of the embryo; in upper portion, the fracture plane has descended into the cells; bar = 10 μm. 22) Elongation stage with readily distinguishable juvenile; bar = 10 μm. 23) First-stage juvenile; bar = 10 μm. 24) Cross section of second-stage juvenile; bar = 10 μm.

gested by Wallace (17) based on the adverse effect of low moisture on the survival and hatching of eggs of *M. javanica.* We also believe that the mesh structure may retain or bind water, thereby maintaining the developing eggs in a constant and moist environment. This fraction of the GM, or the spherical bodies, may also be the source of the antimicrobial activity reported in egg masses of *Meloidogyne* spp. (11). A mesh-like composition of the GM was also described by Dropkin and Bird (7), who examined the GM secreted by young *M. javanica* females dissected from roots; the exuded material sometimes appeared clear under the light microscope but was granular or mesh-like under the electron microscope. They also attempted to stimulate the rectal gland cells to secrete GM with various molecules extracted from host plant roots; DNA was the most active stimulant.

The description of embryogenesis presented in this study is quite similar to that of animal-parasitic (6), microbivorous (19), or other tylenchid nematodes (1,5,8,14, 16). For example, the asynchronous division of the cells in the two-celled stage of the embryo has been observed in *C. elegans* (19). The images obtained with low-temperature SEM on frozen hydrated specimens were quite clear and showed extensive detail, unlike specimens that underwent conventional chemical fixation, dehydration, and critical point drying. Additional investigations performed with this technique should provide further structural and functional information about nematode development and the role of the gelatinous matrix.

Literature Cited

1. Bird, A. F. 1972. The influence of temperature on embryogenesis of *Meloidogyne javanica.* Journal of Nematology 4:206–213.

2. Bird, A. F. 1979. Morphology and ultrastructure. Pp. 59–83 *in* F. Lamberti and C. E. Taylor, eds. Root-knot nematodes (*Meloidogyne* species): Systematics, biology and control. London: Academic Press.

3. Bird, A. F., and J. Bird. 1991. The structure of nematodes, 2nd ed. San Diego: Academic Press.

4. Bird, A. F., and A. Soeffky. 1972. Changes in the ultrastructure of the gelatinous matrix of *Meloidogyne javanica* during dehydration. Journal of Nematology 4:166–169.

5. Bird, A. F., and B. A. Stynes. 1981. The life cycle of *Anguina agrostis:* Embryogenesis. International Journal for Parasitology 11:23–33.

6. Chitwood, B. G. 1950. Nemic embryology. Pp. 202–212 *in* B. G. Chitwood and M. B. Chitwood, eds. Introduction to nematology. Baltimore: University Park Press.

7. Dropkin, V. H., and A. F. Bird. 1978. Physiological and morphological studies on secretion of a protein-carbohydrate complex by a nematode. International Journal for Parasitology 8:225–232.

8. Lauritis, J. A., R. V. Rebois, and L. S. Graney. 1983. Development of *Heterodera glycines* Ichinohe on soybean, *Glycine max* (L.) Merr. under gnotobiotic conditions. Journal of Nematology 15:272–281.

9. Maggenti, A. R., and M. W. Allen. 1960. The origin of the gelatinous matrix in *Meloidogyne.* Proceedings of the Helminthological Society of Washington 27:4–10.

10. Orion, D., and A. Franck. 1990. An electron microscopy study of cell wall lysis by *Meloidogyne javanica* gelatinous matrix. Revue de Nématologie 13: 105–107.

11. Orion, D., and G. Kritzman. 1991. Antimicrobial activity of *Meloidogyne javanica* gelatinous matrix. Revue de Nématologie 14:481–483.

12. Orion, D., G. C. Loots, and T. Orion. 1987. Cell lysis activity of *Meloidogyne* gelatinous matrix. Revue de Nématologie 10:463–465.

13. Orion, D., W. P. Wergin, and B. Y. Endo. 1980. Inhibition of syncytia formation and root-knot nematode development on cultures of excised tomato roots. Journal of Nematology 12:196–203.

14. Raski, D. J. 1950. The life history and morphology of the sugar beet nematode *Heterodera schachtii.* Phytopathology 40:135–152.

15. Sharon, E., D. Orion, and Y. Spiegel. 1993. Binding of soil microorganisms and red blood cells by the gelatinous matrix and eggs of *Meloidogyne javanica* and *Rotylenchulus reniformis.* Fundamental and Applied Nematology 16:5–9.

16. Siddiqui, I. A. 1970. The biology of *Meloidogyne naasi.* Nematologica 16:133–143.

17. Wallace, H. R. 1968. The influence of soil moisture on survival and hatch of *Meloidogyne javanica.* Nematologica 14:231–242.

18. Wergin, W. P., R. M. Sayre, and E. F. Erbe. 1993. Use of low temperature scanning electron microscopy to observed frozen hydrated specimens of nematodes. Journal of Nematology 25:214–226.

19. Wood, W. B. 1988. Embryology. Pp. 215–241 *in* W. B. Wood et al., eds. The nematode *Caenorhabditis elegans.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.