Journal of Nematology 18(4):479–487. 1986.

A Comparison of Techniques Useful for Preparing Nematodes for Scanning Electron Microscopy[1]

J. D. EISENBACK[2]

Abstract: Second-stage juveniles of *Meloidogyne incognita* were prepared by several different techniques for scanning electron microscopy (SEM). Sequential fixation in the cold (4–8 C) was superior to rapid fixation at room temperature, glutaraldehyde and glutaraldehyde–formalin were better fixatives than formalin alone, and critical point drying with carbon dioxide or Freon gave similar results that were only slightly better than air drying with Freon. Freeze drying sequentially fixed nematodes from 100% ethanol in liquid propane produced the best preserved specimens with the fewest artifacts. Specimens of various free-living and plant-parasitic nematodes were prepared for SEM by freeze drying. This technique was adequate for most genera but unsatisfactory for a few. Although each genus may require a different procedure for optimum preservation of detail, sequential fixation with glutaraldehyde and freeze drying are comparable and often superior to commonly used techniques for preparing nematodes for SEM.

Key words: critical point drying, fixation, fixatives, freeze drying, methods, morphology, *Acrobeles* sp., *Belonolaimus longicaudatus*, *Criconemella* sp., *Hemicycliophora* sp., *Heterodera glycines*, *Hoplolaimus* sp., *Meloidogyne incognita*, *M. pini*, *Mononchus* sp.

The scanning electron microscope (SEM) is a useful tool in the study of nematodes. Morphologic and taxonomic studies of nematodes have been greatly enhanced by the use of SEM, and species descriptions increasingly include scanning electron micrographs. Unfortunately, morphological details are often obscured as a result of poor preservation and by artifacts produced by preparative techniques. Specimen preparation remains a limiting factor in the quality of information provided by SEM.

Initial SEM investigations of plant-parasitic nematodes compared several different methods of specimen preparation (3–5). More recently reviews of techniques (7–9) as well as specific details of techniques (2) have been published. Early comparisons of preparative techniques used nematodes from several different genera and began with specimens that had been killed with hot formaldehyde-based fixatives. Killing with heat and formalin fixation subsequently has been shown to be inadequate for SEM. Each step in the preparation is important to the success of the remaining steps (2); distortions occurring in the initial stages of preparation generally become magnified in steps that follow. Some techniques regarded as unsuitable in previous investigations (4) may actually be quite useful if the nematodes are properly killed and fixed.

Successful preparation of nematodes for SEM consists of several steps. Killing, fixing, and drying are perhaps the most important. Widely used killing and fixation techniques follow two generalized patterns. Rapid fixation emphasizes the need to quickly stop cellular activity and thus prevent destruction of tissues through autolytic activities. Sequential fixation emphasizes the effect of the concentration of the fixative on tonicity of the buffer. Differences in tonicity between the buffer and tissue can cause swelling or shrinkage (2).

Primary fixation of protein molecules is important in preparation of nematodes for SEM. The initial comparison of preparative techniques used formaldehyde-based fixatives (4) which generally have been replaced by glutaraldehyde. Much better results have been obtained with glutaraldehyde because it is a dialdehyde with two reactive groups capable of cross-linking proteins (2). Unfortunately the earlier fixation techniques with formaldehyde also included heat (4), and poor preparations may have resulted from an inferior fixative, or heat, or both. Mixtures of aldehydes, particularly formalin and glutaraldehyde, are more and more commonly used for preparing biological materials for SEM. A comparison of the effectiveness of formalin, glutaraldehyde, and glutaralde-

Received for publication 6 February 1986.

[1] Partial support provided by U.S. Agency for International Development Contract No. ta-C-1234 to Dr. J. N. Sasser.

[2] Assistant Professor, Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

The author thanks S. M. Curi for inspiration and technical assistance.

TABLE 1. Techniques tested for preparing nematodes for scanning electron microscopy.

Fixation schedule	Temperature	Fixative(s)	Modification	Drying	Figure
Rapid	21 C	2% glutaraldehyde–1% formalin	None	Critical point dried with CO_2	1A
Sequential slow	4–8 C	2% glutaraldehyde–1% formalin	None	Critical point dried with CO_2	1B
Sequential fast	4–8 C	2% formalin	None	Critical point dried with CO_2	1C
Sequential fast	4–8 C	2% glutaraldehyde	None	Critical point dried with CO_2	1D
Sequential fast	4–8 C	2% glutaraldehyde–1% formalin	None	Critical point dried with CO_2	2A
Sequential fast	4–8 C	2% glutaraldehyde–1% formalin	None	Critical point dried with Freon	2B
Sequential fast	4–8 C	2% glutaraldehyde–1% formalin	None	Air dried with Freon	2C
Sequential fast	4–8 C	2% glutaraldehyde–1% formalin	OTO	Critical point dried with CO_2	2D
Sequential fast	4–8 C	2% glutaraldehyde–1% formalin	None	Freeze dried with 100% ethanol	3A
Sequential fast	4–8 C	2% glutaraldehyde–1% formalin	Glycerin infiltration	Critical point dried with CO_2	3C
None	21 C	None	Living material	Freeze dried with water	3B

hyde–formalin as primary fixatives is needed.

Drying nematodes is necessary because of the physical requirements of the SEM. The surface tension of water drying from the specimen exerts tremendous pressures that cause severe distortions (2). Several different drying techniques have been used to reduce these pressures. Critical point drying (CPD) with Freon or carbon dioxide (CO_2) is most commonly used. Air drying with volatiles, infiltrating with a low surface tension fluid, and freeze drying are also used (4,7). A comparison of several drying techniques is needed to evaluate the usefulness of each technique.

The purpose of this study was to evaluate the details of common preparative techniques and to reevaluate methods useful for biological tissues but not regularly used for nematodes. All tests were run simultaneously with specimens from the same population of nematode. Second-stage juveniles of *Meloidogyne incognita* (Kofoid and White) Chitwood were selected for the comparative studies because they are readily available in large quantities and are often difficult to prepare without distortion and artifacts. Juveniles of root-knot nematodes were desirable because freshly hatched specimens are in a similar nutritional and physiological state. The best preparative procedure used for second-stage juveniles was selected and tested with nematodes from several different genera including both free-living and plant-parasitic forms.

MATERIALS AND METHODS

A population of *Meloidogyne incognita* cultured on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a greenhouse maintained at 22–28 C was selected. Newly hatched second-stage juveniles were obtained by incubating egg masses on wet tissue paper supported by a plastic screen in a moist chamber. Specimens were simultaneously processed by various techniques (Table 1). Most of the techniques involved the same basic steps of fixation, postfixation, dehydration, drying, mounting, coating, and viewing (2). Differences in techniques generally included only type of primary fixative, rate of fixation, and method of drying.

Fixation schedule: The first and most important step in preparing nematodes for SEM is fixation. As mentioned previously, two commonly used methods of fixing nematodes are rapid and sequential fixation.

Rapid fixation: Specimens were immersed in 2% glutaraldehyde–1% formalin (buffered in 0.1 M sodium cacodylate adjusted to a pH of 7.2) for 2 hours at room temperature. They were stored overnight

(15–20 hours) in the fixative in a refrigerator (4–8 C).

Sequential fixation: Specimens were chilled in 0.5 ml (12 drops) of tap water in a refrigerator (4–8 C) for 30 minutes. They were sequentially fixed by adding 4 drops of cold 4% glutaraldehyde–2% formalin (buffered with 0.1 M sodium cacodylate, pH 7.2) every half hour until the final concentration of fixative reached 2% glutaraldehyde–1% formalin (12 drops of fixative). Fixation continued overnight (15–20 hours) in the cold.

One group of specimens was chilled for 30 minutes in a refrigerator and slowly sequentially fixed by adding a single drop of fixative every half hour until the desired concentration was achieved. The nematodes continued fixing as they were kept in a refrigerator overnight (15–20 hours).

Fixatives: Primary fixation of protein molecules is usually achieved by an aldehyde or a mixture of aldehydes. Most specimens in this study were fixed with a combination of 2% glutaraldehyde and 1% formalin. For comparative purposes some nematodes were sequentially fixed with 2% glutaraldehyde or 2% formalin. All fixatives were buffered with 0.1 M sodium cacodylate adjusted to a pH of 7.2.

Drying: After fixation by various procedures and fixatives, all specimens were postfixed in 2% osmium tetroxide (buffered in 0.1 M sodium cacodylate, pH 7.2) for 8 hours in the cold (4 C), dehydrated in a seven-step ethanol series, and dried by several different means. Drying is necessary for successful observation with the SEM. Unfortunately evaporating liquids exert extreme pressures on the surface of the specimens. Three drying techniques that are useful in avoiding the damaging effects of surface tension pressures are CPD; drying from a volatile, low surface tension liquid; and freeze drying.

Critical point drying and drying from a volatile liquid: Most nematodes in this study were CPD with CO_2 using standard procedures (2). Some specimens were infiltrated with Freon-113 and CPD with Freon-13 (2). For comparison, some specimens were allowed to air dry from the transitional fluid, Freon-113, which is a volatile, low surface tension fluid.

Freeze drying: Fixed, dehydrated nematodes were frozen in 100% ethanol by immersing them in liquid propane cooled by liquid nitrogen. The specimens were evacuated overnight to a pressure of 10^{-1} Torr at −80 C and freeze dried. For comparison, some nematodes were immersed live and not fixed into liquid propane and subsequently freeze dried.

Drying specimens infiltrated with glycerin: Nematodes are routinely infiltrated with glycerin for the preparation of permanent slides. Sometimes it may be desirable to examine these specimens with the SEM. They can be observed while infiltrated with glycerin, or the glycerin can be removed and the nematode becomes rehydrated (2). For this study, second-stage juveniles were processed into glycerin to be used as permanent mounts (1), and some of them were rehydrated and CPD as previously described.

Enhancing electrical conductivity: Dehydrated nematodes are poor conductors of electricity. Electrons accumulate in nontreated specimens, and charging artifacts destroy the image. Most of the nematodes in this study were sputter-coated with a thin layer (200 Å) of gold to enhance conductivity. For comparison, an alternative without coating was used for some nematodes. Thiocarbohydrazide ligates osmium to tissues which increases electrical conductivity. Sequentially fixed nematodes were treated with osmium–thiocarbohydrazide–osmium (OTO) (6) and CPD with CO_2.

Mounting and viewing: Specimens were mounted on SEM stubs and all treatments except OTO were sputter-coated with approximately 200 Å of gold. At least 50 specimens of each treatment were viewed and photographed with a Jeol T200 SEM operating at 25 kV. All variables in viewing the specimens were adjusted to produce the best possible image, and all observations were made within 8 hours of each other.

Preparation of various other genera: The best processing procedure was selected from the tests with second-stage juveniles of *Meloidogyne incognita* and used on several nematode genera including both plant parasites and free-living forms. Specimens were obtained from routine soil washings collected in nematode assays. Genera were prepared by freeze drying sequentially fixed specimens including *Acrobeles* sp., *Be-*

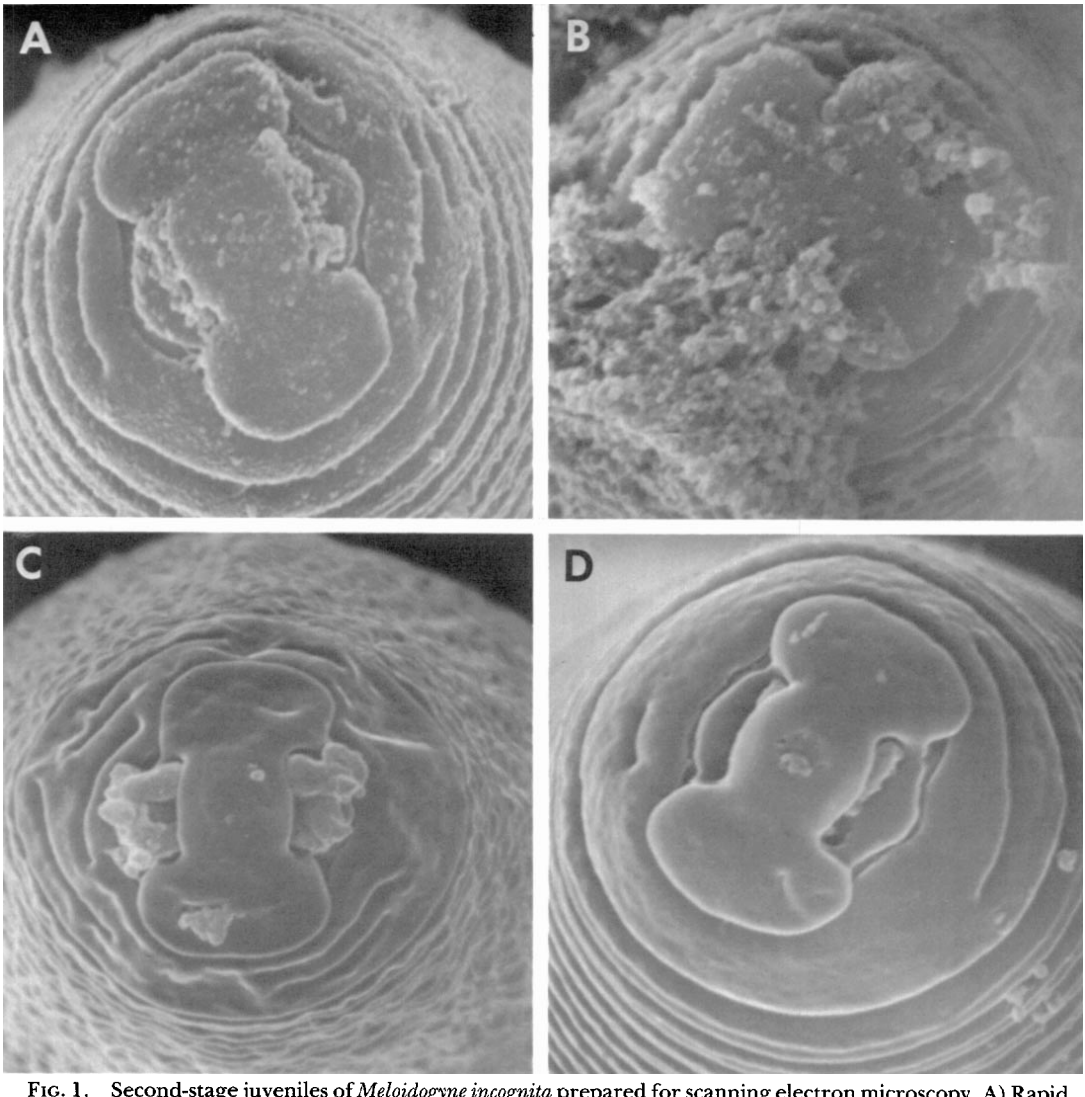

FIG. 1. Second-stage juveniles of *Meloidogyne incognita* prepared for scanning electron microscopy. A) Rapid fixation at room temperature with 2% glutaraldehyde–1% formalin and critical point dried (CPD) with carbon dioxide (CO_2). Preservation is good but fine precipitate obscures details. B) Slow sequential fixation in the cold with 2% glutaraldehyde–1% formalin and CPD with CO_2. Preservation is poor and much precipitation covers surface. C) Sequential fixation with 2% formalin in the cold and CPD with CO_2. Morphology is distorted with wrinkles even though precipitation is absent. D) Sequential fixation in the cold with 2% glutaraldehyde and CPD with CO_2. Morphology is well preserved and relatively free from artifacts.

lonolaimus longicaudatus Rau, *Criconemella* sp., *Hemicycliophora* sp., *Heterodera glycines* Ichinohe, *Hoplolaimus galeatus* (Cobb) Thorne, *Meloidogyne pini* Eisenback, Yang and Hartman, and *Monochus* sp.

Results and Discussion

Processing techniques were evaluated on the basis of the final image obtained with the SEM. The quality of preservation of the nematode and the interaction of electron beam with the surface of the specimen were the most important factors affecting the final image. Generally, specimen preservation was the most important aspect, because beam–specimen interaction was controlled by adequately coating the dried nematodes with a layer of electrically conductive metal. Poor preservation of tissue usually resulted in wrinkles caused by shrinkage of the specimen. Some techniques were associated with a surface precipitate which often obscured morphological details.

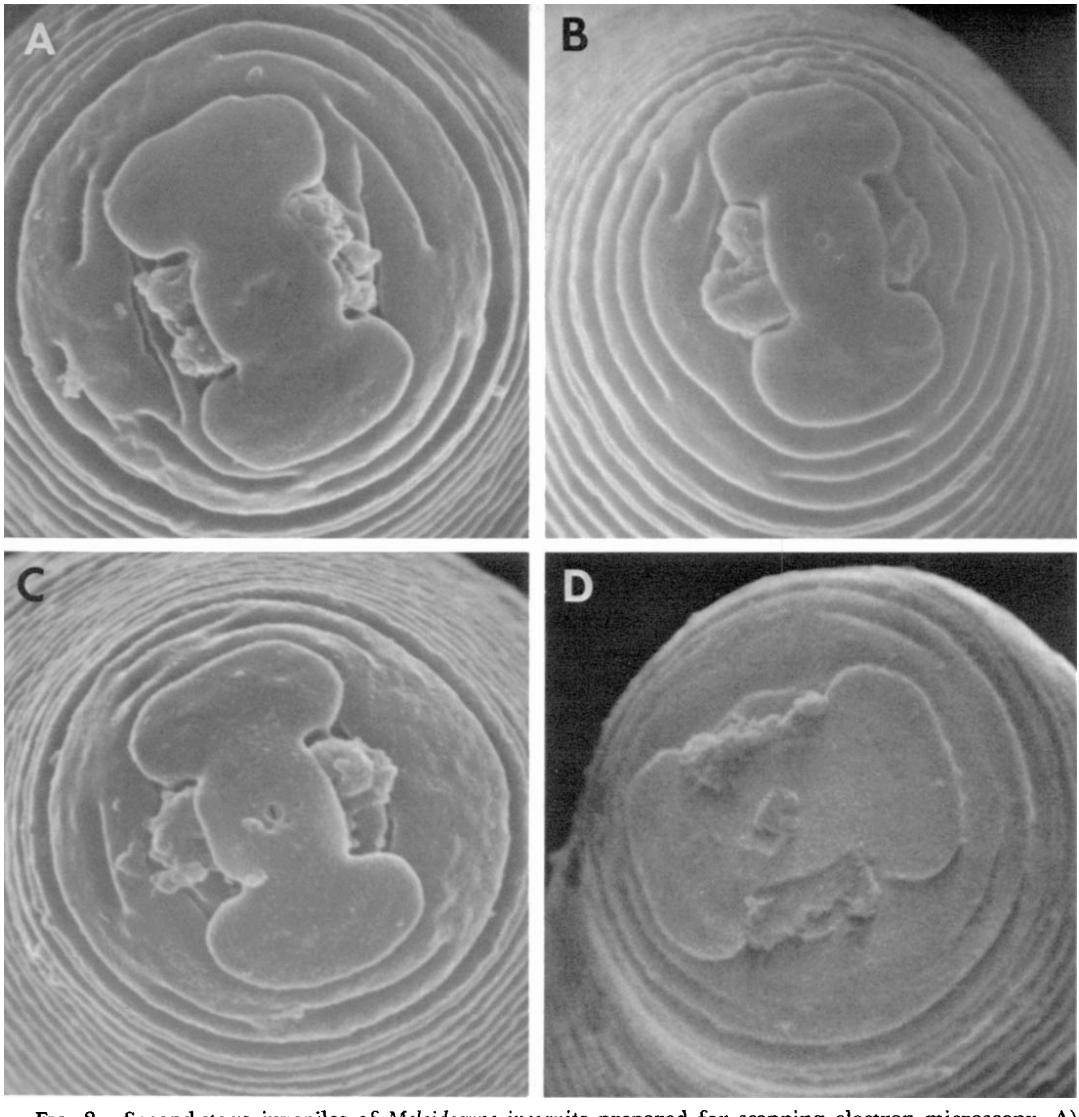

FIG. 2. Second-stage juveniles of *Meloidogyne incognita* prepared for scanning electron microscopy. A) Sequential fixation in the cold with 2% glutaraldehyde–1% formalin and critical point dried (CPD) with Freon. Preservation is similar to specimens CPD with carbon dioxide (CO_2). B) Sequential fixation in the cold with 2% glutaraldehyde–1% formalin and CPD with CO_2. Preservation is similar to specimens CPD with Freon. C) Sequential fixation in the cold with 2% glutaraldehyde–1% formalin and air dried with Freon. Even though the morphology is relatively free from distortion, the surface is covered with a fine precipitate. D) Sequential fixation with 2% glutaraldehyde–1% formalin in the cold, subsequently treated with osmium–thiocarbohydrazide–osmium, CPD with CO_2, and viewed and photographed uncoated. Image is distorted by charging artifacts.

Fixation schedule: The schedule of fixation was very important to the success of the preparative technique. Nematodes that underwent rapid fixation at room temperature did not shrink excessively; however, a precipitate formed on the surface and ruined the quality of the preparation (Fig. 1A). Sequential fixation in the cold produced the best preservation of tissue (Figs. 1D, 2A–C, 3A), but if fixation took too long shrinkage occurred and surface precipitates formed (Fig. 1B).

Fixatives: The type of fixative used for primary fixation of protein molecules was critical to the successful preservation of morphology. Glutaraldehyde and glutaraldehyde–formalin gave satisfactory results (Figs. 1D, 2A–C, 3A). Formalin alone

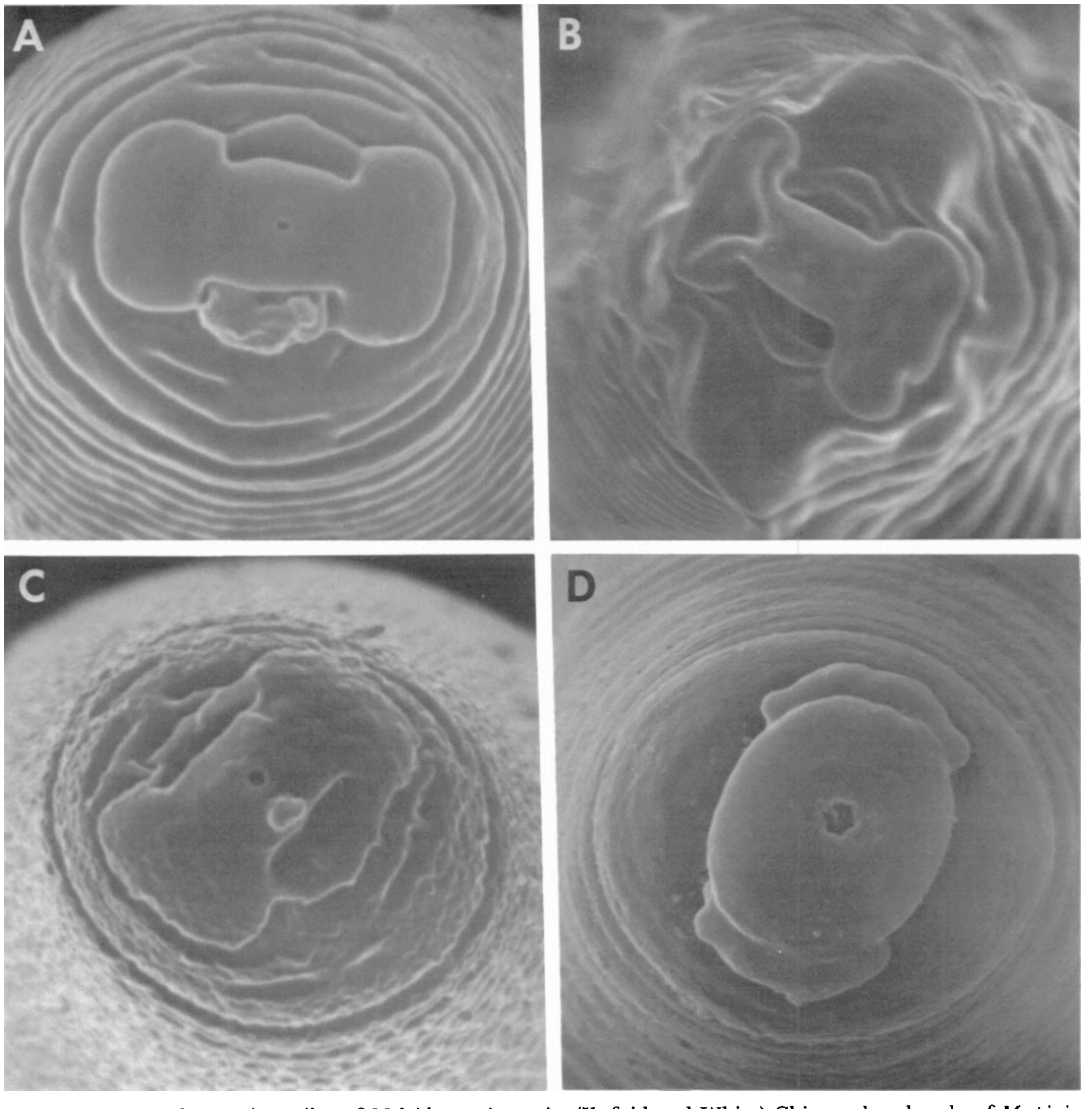

FIG. 3. Second-stage juveniles of *Meloidogyne incognita* (Kofoid and White) Chitwood and male of *M. pini* Eisenback, Yang, and Hartman prepared for scanning electron microscopy. A) Sequential fixation in the cold in 2% glutaraldehyde–1% formalin and freeze dried with 100% ethanol. Morphological details well preserved and free of artifacts. B) Freshly hatched live specimen freeze dried with water. Morphology is severely distorted. C) Specimen infiltrated with glycerin, rehydrated, and critical point dried with carbon dioxide. Preservation is poor. D) *M. pini* male prepared for SEM by freeze drying of fixed material with 100% ethanol.

did not adequately preserve tissues, and shrinkage was evident as surface wrinkles (Fig. 1C).

Drying: Preparation of nematodes for SEM generally requires drying the specimens. The most commonly used method for drying nematodes and other biological tissues is CPD with CO_2 or Freon. If the specimens were properly fixed, drying with CO_2 or Freon gave comparable results (Figs. 1D, 2A, B). Proper fixation even allowed the nematodes to air dry from Freon without much shrinkage of tissues; however, a fine precipitate or residue covered the surface of the specimens (Fig. 2C). Nematodes processed into glycerin for permanent mounts and then rehydrated and critical point dried were not well preserved (Fig. 3C). Freeze drying properly fixed specimens produced the most stable, best preserved nematodes (Fig. 3A). Freeze drying live, unfixed specimens resulted in the worst preparations (Fig. 3B).

Enhancing electrical conductivity: Sputter-

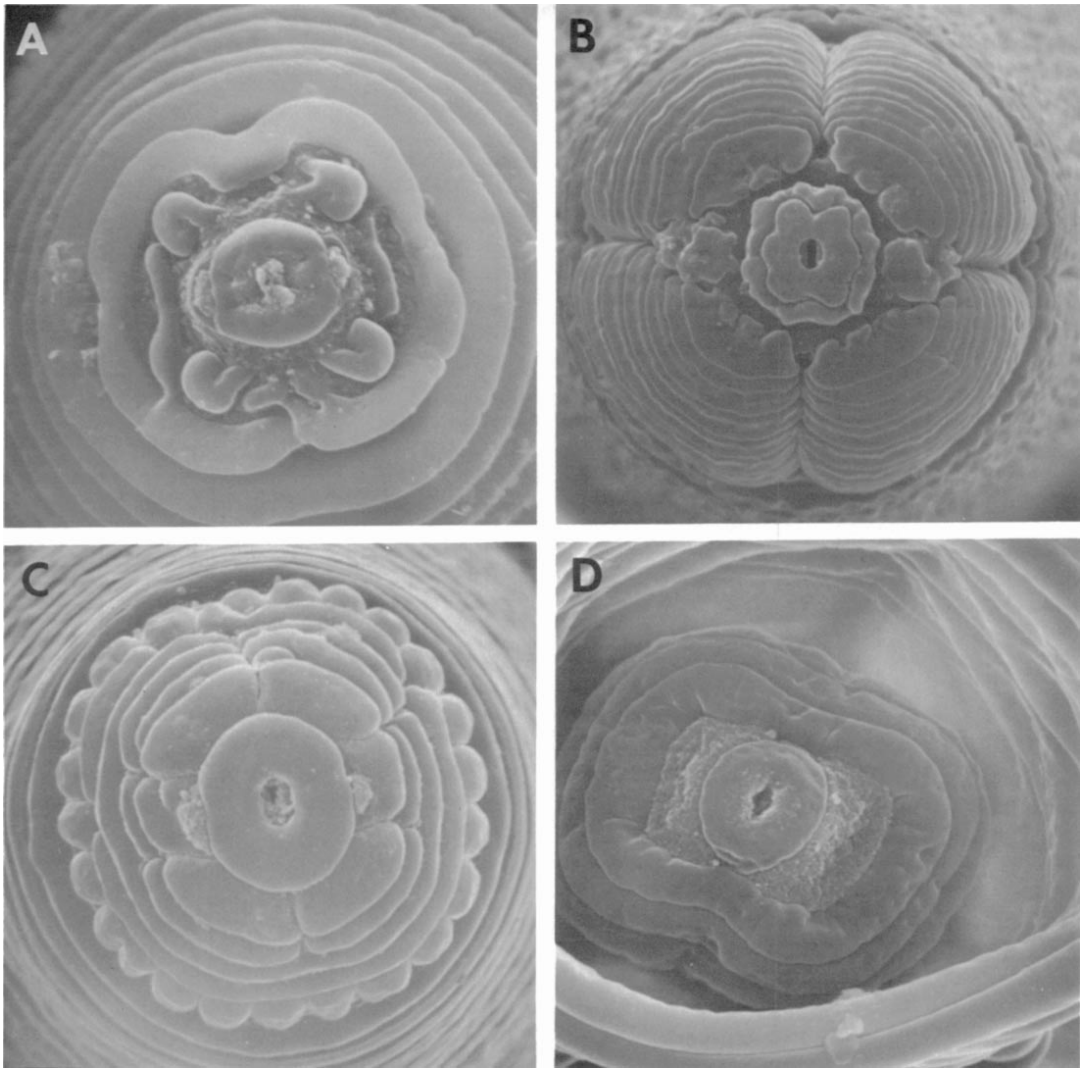

FIG. 4. Specimens of various nematode genera prepared for scanning electron microscopy by freeze drying fixed material with 100% ethanol. A) *Criconemella* sp. B) *Belonolaimus longicaudatus* (Cobb) Thorne. C) *Hoplolaimus* sp. D) *Hemicycliophora* sp.

coating dried and mounted nematodes with approximately 200 Å of gold prevented the build-up of image-destroying electrons. The specimens were quite stable under the high voltage of the electron beam, and the image was free of charging artifacts. Surface morphology was enhanced, and morphological details appeared well preserved. Treatment with OTO did not enhance electrical conductivity enough to prevent charging artifacts from destroying the final image (Fig. 2D).

Preparation of various other genera: Freeze drying sequentially fixed specimens of several other plant-parasitic and free-living nematodes was satisfactory for most, but not all, specimens. Good results were obtained with *Meloidogyne pini* males (Fig. 3D), *Criconemella* sp. (Fig. 4A), *Belonolaimus longicaudatus* (Fig. 4B), *Hoplolaimus* sp. (Fig. 4C), *Hemicycliophora* sp. (Fig. 4D), *Mononchus* sp. (Fig. 5A), and *Acrobeles* sp. (Fig. 5B). Unsatisfactory results were obtained with *Xiphinema* sp. (Fig. 5C) and *Heterodera glycines* males (Fig. 5D).

The primary fixative, fixation schedule, and drying methods are important in preparing nematodes for SEM. Glutaraldehyde or a mixture of glutaraldehyde and formalin was superior to formalin alone.

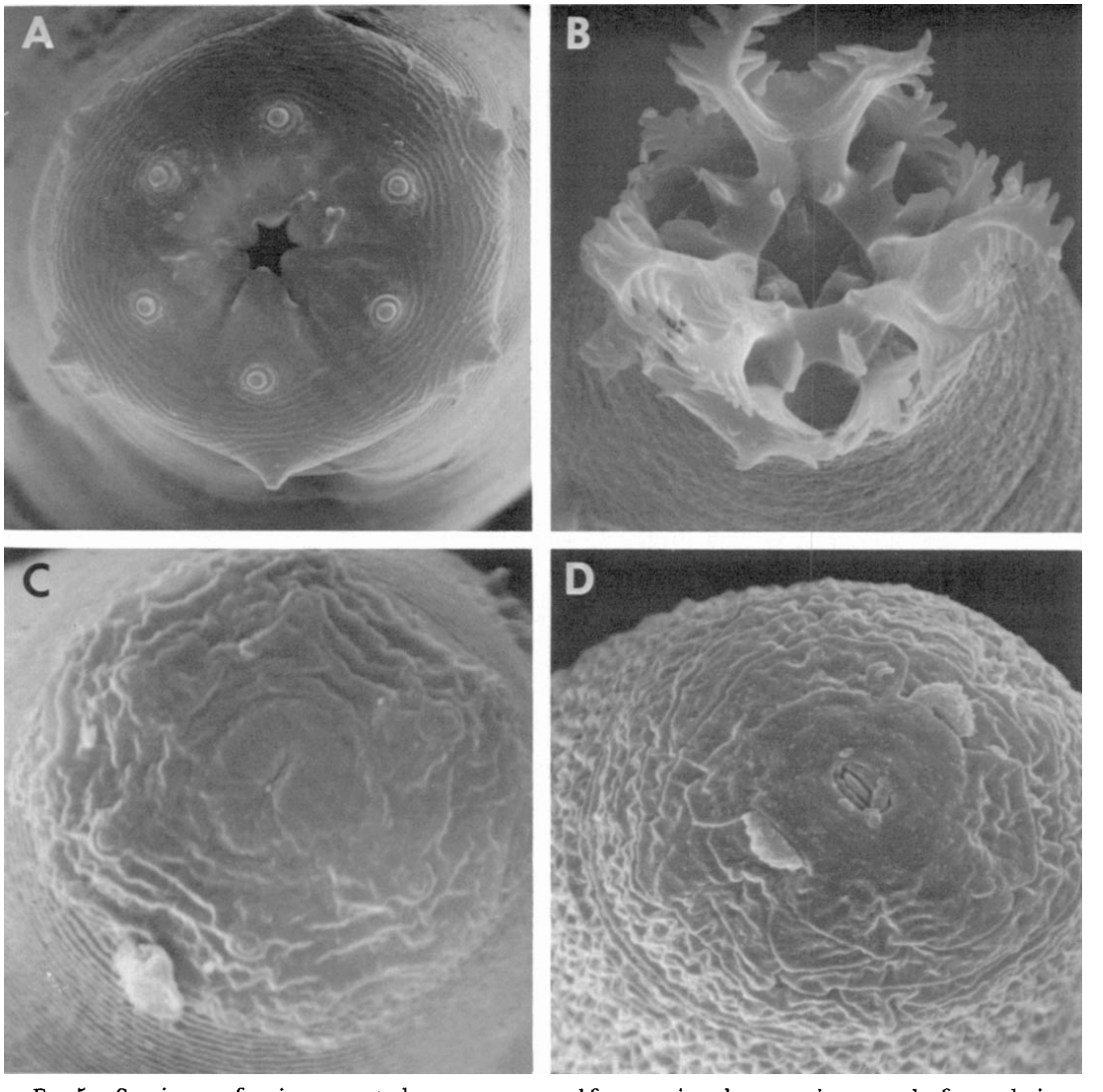

FIG. 5. Specimens of various nematode genera prepared for scanning electron microscopy by freeze drying fixed material with 100% ethanol. A) *Mononchus* sp. B) *Acrobeles* sp. C) *Xiphinema* sp. D) *Heterodera glycines* Ichinohe male.

Sequential fixation was superior to rapid fixation provided the sequential fixation schedule was not too slow. Critical point drying with Freon or CO_2 gave good results and was surprisingly similar to air drying from Freon. Freeze drying produced the best preservation of gross morphology and was associated with the fewest artifacts. Although each genus may require a different procedure for the optimum preservation of detail, sequential fixation with glutaraldehyde and freeze drying was superior to other commonly used fixation and drying techniques.

LITERATURE CITED

1. Eisenback, J. D. 1982. Description of the blueberry root-knot nematode, *Meloidogyne carolinensis* n. sp. Journal of Nematology 14:303–317.

2. Eisenback, J. D. 1985. Techniques for preparing nematodes for scanning electron microscopy. Pp. 79–105 *in* K. R. Barker, C. C. Carter, and J. N. Sasser, eds. An advanced treatise on *Meloidogyne*, vol. 2. Methodology. A cooperative publication of the North Carolina State University Department of Plant Pathology and U.S. Agency for International Development, Raleigh.

3. Green, C. D. 1967. Preparation of nematodes for examination under the stereoscan electron microscopy. Nematologica 13:279–282.

4. Green, C. D., A. R. Stone, R. H. Turner, and

S. A. Clark. 1975. Preparation of nematodes for scanning electron microscopy. Journal of Microscopy 103:89–99.

5. Hammon, R. A. 1969. The preparation of nematode material for scanning electron microscopy. Journal of Microscopy 90:273–274.

6. Murphy, J. A. 1978. Non-coating techniques render biological specimens conductive. Scanning Electron Microscopy II:175–194.

7. Wergin, W. P. 1981. Scanning electron microscopic techniques and applications for use in nematology. Pp. 175–204 *in* B. M. Zuckerman and R. A. Rohde, eds. Plant parasitic nematodes, vol. 3. New York: Academic Press.

8. Wergin, W. P. 1982. Bibliography on the use of the scanning electron microscope (SEM) in studies of plant parasitic nematodes. Scanning Electron Microscopy III:831–836.

9. Wergin, W. P., and A. R. Stone. 1981. Techniques for preparation and examination of plant parasitic nematodes in the scanning electron microscope. Scanning Electron Microscopy III:169–176.