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Cryofixation and cryosubstitution as a method for preparing *Meloidogyne javanica* eggs for electron microscopy

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Summary – Transmission electron microscopy (TEM) of nematodes eggs is difficult because the lipid layer of the egg shell prevents penetration of the chemicals used for fixation and embedding. Conventional chemical fixation of intact eggs does not allow observation of the internal egg components as they are damaged during processing. Cryofixation and cryosubstitution was used as an alternative method for the preparation of intact nematode eggs for sectioning and resulted in sections of eggs that were well fixed and free from the artefacts caused by chemical fixation. The integrity of embryos and larvae within eggs was maintained. The nematode eggs can now be studied as an intact system by using this method.

Résumé – ***Cryofixation et cryosubstitution : une méthode de préparation des œufs de* Meloidogyne javanica *pour l'examen en microscopie électronique*** – L'étude en microscopie électronique à transmission des œufs de nématodes est rendue difficile par la présence de la couche lipidique de la coque qui empêche la pénétration des produits chimiques utilisés pour la fixation et l'inclusion. La fixation d'œufs intacts avec les produits chimiques usuels ne permet pas en effet l'observation des composants internes de l'œuf, ceux-ci étant détériorés durant le processus. La cryofixation/cryosubstitution est utilisée comme une méthode de remplacement pour la préparation d'œufs de nématodes intacts en vue de coupes fines. Cette méthode permet d'obtenir des coupes correctement fixées et dépourvues des artefacts causés par les fixateurs chimiques. Les embryons et les larves contenus dans l'œuf sont conservés intacts. En utilisant cette méthode, les œufs de nématodes peuvent maintenant être étudiés sans que leur intégrité ne soit atteinte.

Keywords : Cryofixation, cryosubstitution, electron microscopy, *Meloidogyne javanica,* nematode eggs.

Nematode eggs are difficult to fix for electron microscopy because the inner lipid layer of the shell provides an impermeable barrier to chemical fixatives (Bird, 1971; Wharton & Jenkins, 1978). This problem has been overcome partially by using long incubations in fixatives (Bird, 1968), by cracking the eggs open to allow penetration of the fixatives (Bird & McClure, 1976; Perry *et al.,* 1982; Perry & Trett, 1986; Wharton & Bone, 1989), or by freezing, sectioning on a cryotome and then fixing (Wharton, 1979). Recently a microwave fixation technique has been used to aid the penetration of chemical fixatives into nematode eggs with suitable results for examining the ultrastructure of unhatched nematodes (Jones & Gwynn, 1991). Cryofixation and cryosubstitution procedures have been successfully used as a way of observing living material without the use of chemical fixatives (Menco, 1986). Freezing samples rapidly in a cryogen cooled by liquid nitrogen, instantaneously arrests all activity and captures cell components without the artefacts associated with chemical processing, such as shrinkage and dissolution of metabolites. Cryofixation has been used to study fungal-infected eggs under scanning electron microscopy (SEM) with good results (Lopez-Llorca & Duncan, 1988, 1991) but these eggs were damaged through fungal penetration of the egg shell and this may have aided processing. The aim of this work was to compare chemical fixation with cryofixation and cryosubstitution as methods for studying whole, intact nematode eggs.

Materials and methods

NEMATODE EGGS

Eggs of *Meloidogyne javanica* were inoculated onto tomato plants grown in pots and 6 to 8 weeks later, eggs from the next generation of nematodes were harvested by gently agitating roots in 0.5 % NaOCl (modified from McClure *et al.*, 1973).

CONVENTIONAL CHEMICAL FIXATION FOR ELECTRON MICROSCOPY

Nematode eggs were fixed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 h at room temperature and the pelleted eggs were then washed twice in the same buffer. The eggs were enrobed in 2 % agarose Type VII (low gelling temperature, Sigma), washed again with 0.1 M sodium cacodylate buffer and then post-fixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at room temperature.

After three 10 min washes in 0.1 M sodium cacodylate buffer the eggs were dehydrated through a graded ethanol series (50 %, 70 %, 85 %, 95 % and 100 %) for 20 min each.

Infiltration, embedding and sectioning

Eggs were infiltrated with Spurr's resin (Spurr, 1969) over a 7 day period on a rotator at room temperature, with 24 h in each of the following ethanol:Spurr's resin mixtures; 3:1, 2:1, 1:1, 1:2 and 1:3. The last two incubations were in pure Spurr's resin for 24 h each, with fresh resin being added every 12 to 16 h. Samples were placed under vacuum for 10 min and transferred to a rotator at 37 °C. After 2 h, fresh resin was added and the specimens were placed at 37 °C for another 2 h before blocking. Polymerisation took 16 h at 50 °C. Blocks were trimmed and sectioned with a diamond knife on a Reichert-Jung Ultracut E ultramicrotome and placed onto formvar coated copper slot grids. Sections were stained using lead citrate (Reynolds, 1963) and uranyl acetate (Daddow, 1986).

Cryofixation and cryosubstitution

A drop of nematode eggs suspended in distilled water was placed onto a thin layer of 2 % agarose and allowed to dry until the eggs adhered to the agarose. Small blocks of agarose, about 3 mm in diameter were cut and placed onto a metal pin for plunge freezing. Liquid nitrogen was used to cool propane to – 187 °C in a Reichert-Jung KF80 immersion cryofixation system. When the temperature of the propane was between – 187 °C and – 180 °C the eggs were plunged into the cryogen, then stored under liquid nitrogen. A dry-ice/acetone bath was used to cool 2 % osmium tetroxide in acetone (dried over molecular sieve) of which 1.5 ml was added to each cryotube containing only one or two blocks of agarose with eggs. The eggs were kept in the osmium-acetone solution for 3 days at – 79 °C using a dry-ice/acetone bath to maintain the temperature. Samples were allowed to warm up to – 20 °C overnight, then brought to 4 °C for 4 h before being placed at room temperature for three 20 min washes in dry acetone. The specimens were then infiltrated, embedded and sectioned as previously described using acetone instead of ethanol for infiltration. Grids were stained and examined under TEM.

Scanning electron microscopy

Samples for SEM were prepared following the cryofixation and cryosubstitution procedure until the last acetone wash at room temperature. Specimens were subjected to critical point drying using a Polaron critical point dryer, mounted and coated with platinum before observation.

Light microscopy

Thick sections were cut from polymerised blocks and heat fixed onto a glass microscope slide. Sections were stained with toluidine blue on a hot plate for 2 min, rinsed and viewed under bright field microscopy.

Examination of specimens

Samples prepared for transmission electron microscopy were viewed using a Hitachi H-800 transmission electron microscope. Scanning electron microscopy was performed using a JEOL G400F scanning electron microscope and an Olympus phase contrast microscope with camera was used for light microscopy.

Results

Chemically fixed eggs

Eggs that were processed using a standard chemical fixation protocol were unsuccessfully fixed, surviving for 24 h in glutaraldehyde and 1 h in osmium tetroxide. Sections of eggs stained with toluidine blue showed loss of internal contents that also resulted in distortion and shrinkage of the egg shape (Fig. 1 A). Blocks were difficult to cut as the eggs fell out of embedding material. Only the egg shell remained partially intact as was evident when viewed under TEM (Fig. 1 B).

Cryofixed and cryosubstituted eggs

Cryofixed and cryosubstituted egg sections viewed under the light microscope showed that eggs had remained intact and internal contents had been successfully fixed (Fig. 1 C). Eggs observed under the scanning electron microscope displayed a smooth surface of the egg shell (Fig. 1 D). Both embryonic and larval stages of eggs could be clearly identified within intact shells as observed under electron microscopy (Fig. 2 A, C). Sections of whole eggs containing embryonic cells showed the developing cells and nuclei, containing nucleoli, very clearly (Fig. 2 B). The DNA within the nuclei did not form aggregates, which can occur when chemical fixatives are used. The layers of the egg shell could be clearly identified. Sections of larvae within eggs showed the cuticle striations and internal contents of the larvae. Closer examination showed the layers of the cuticle (Fig. 2 D).

Discussion

Our results confirmed those of others who have shown that intact nematode eggs cannot be prepared for electron microscopy by conventional chemical fixation. It has always been necessary to damage eggs in some way to allow penetration of the chemical fixatives (Bird & McClure, 1976). However, in the present study intact nematode eggs could be readily examined using cryofixation and cryosubstitution. Samples were easily processed, and procedures for preparation were less invasive than for conventional chemical fixation.

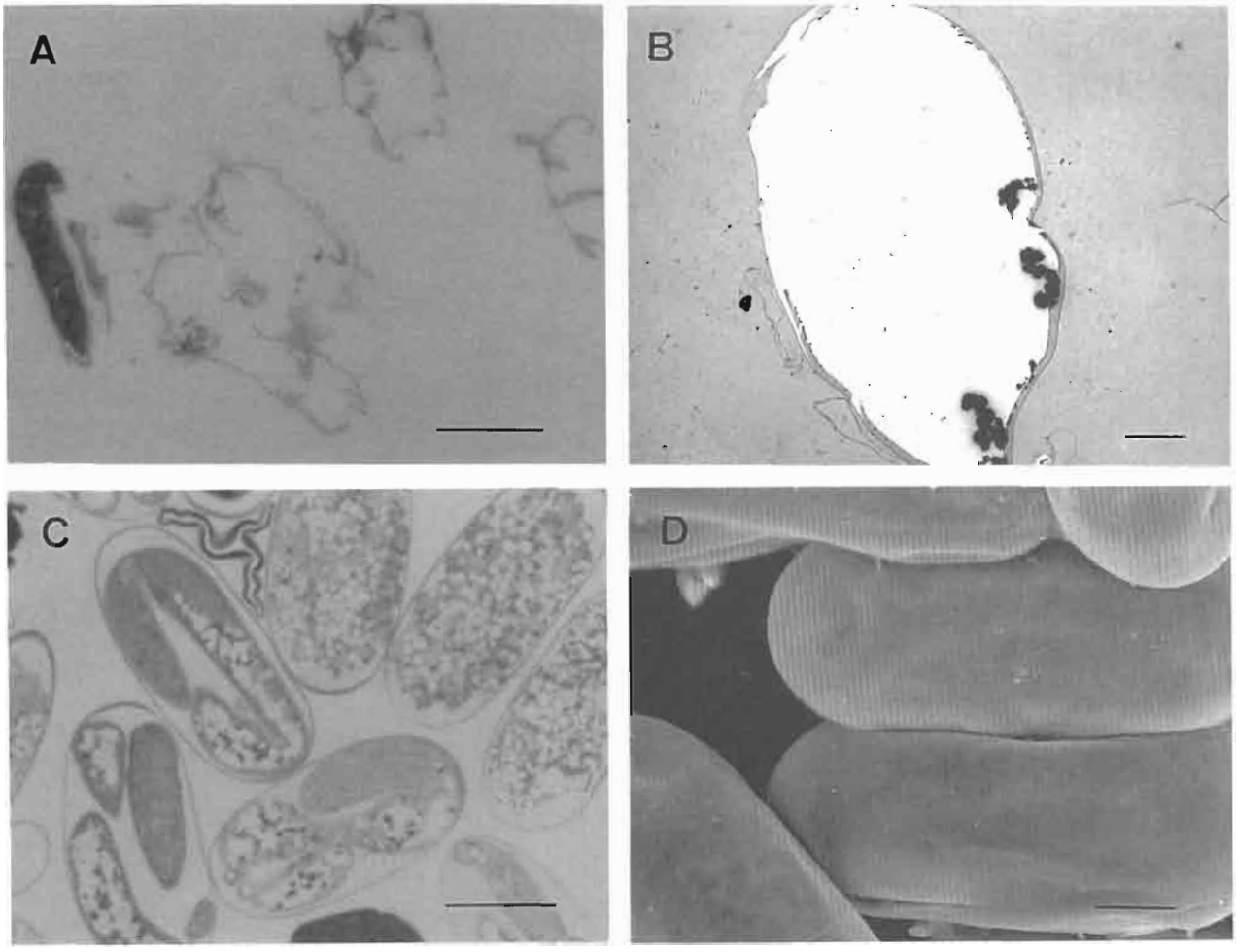

Fig. 1. A : Light microscopy of a toluidine blue stained section of a chemically fixed egg; B : TEM of a chemically processed egg; C : Light microscopy of a toluidine blue stained section of cryo-processed nematode eggs; D : SEM of cryoprocessed eggs. *(Bars equivalent : A, C = 40 μm; B = 5 μm; D = 15 μm.)*

Jones and Gwynn (1991) suggested that methods with quick fixation times were best because artefacts from physiological processes such as autolysis could occur in larvae that were still living in the fixative. Our own observations have shown that larvae within eggs can survive 24 h in glutaraldehyde and 1 h in osmium tetroxide. The fixation time can be substantially reduced by cryofixation, because only fractions of a second are required for fixation. This allows the technique to be applied to the capture of very rapidly occurring physiological or metabolic processes.

The major disadvantage of cryofixation is the formation of ice crystals which can cause major structural damage to cells. Ice crystal damage may occur if the sample is left to warm at any time before substitution is complete. Care must therefore be taken to maintain the temperature of the samples below – 40 °C for more than 10 min (Steinbrecht, 1985). Ice crystals may form in the specimens while freezing as only the leading edge of the sample is instantly frozen, producing vitrified water without crystal growth. However, minimal damage was found in the nematode egg system and cryofixation was superior to chemical fixation because ultrastructural detail was apparent and membranes and other structures were better preserved. A cryogen such as propane was used because the transfer of heat between the specimen and the cryogen was much faster, cooling the specimen more rapidly than if a less efficient medium were used.

Substitution is used to dehydrate a specimen, and serves as a valuable link between cryofixation and further processing at room temperature (Steinbrecht & Müller, 1987). Acetone is used to dissolve the ice and serves to remove the water from the specimen. The

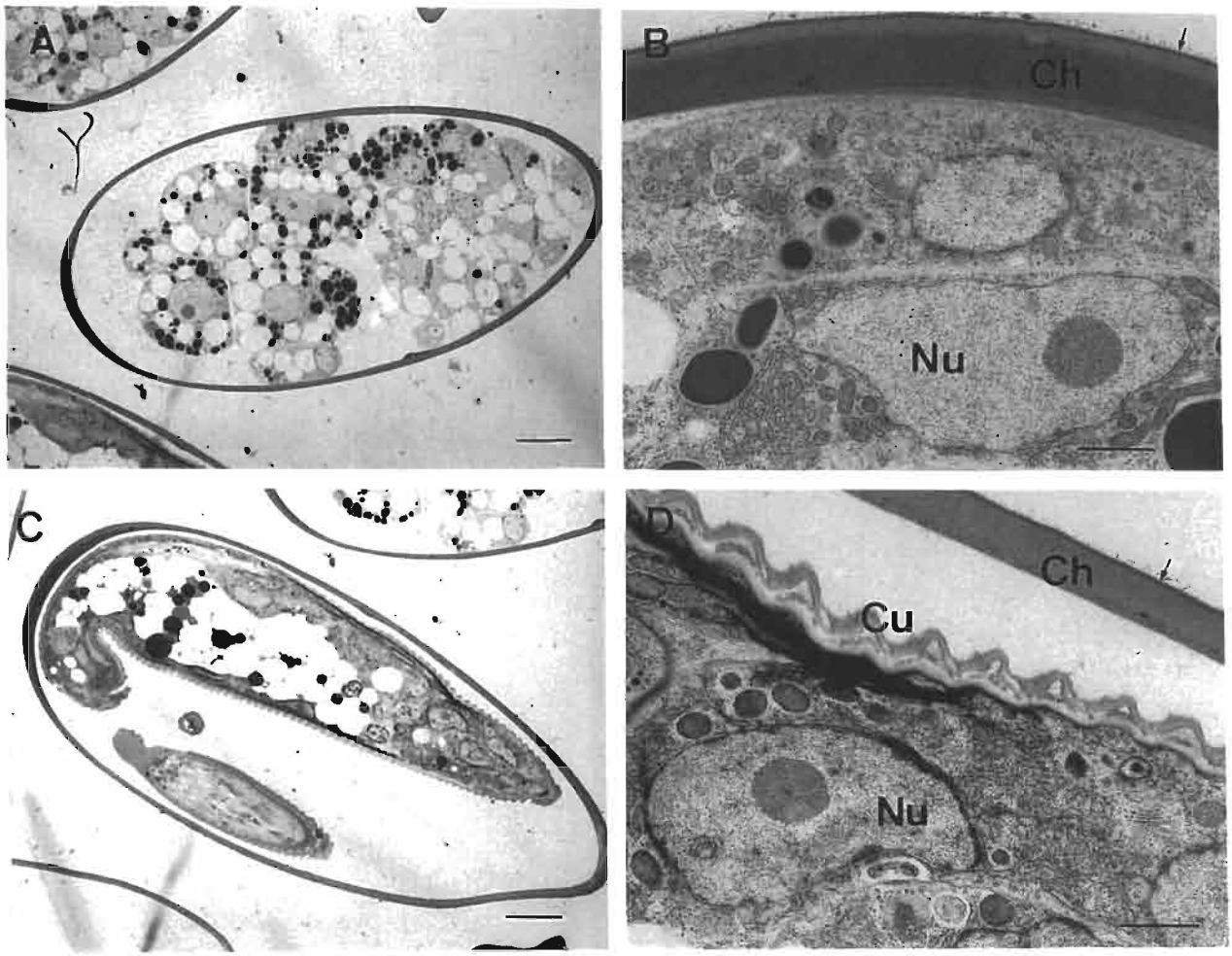

Fig. 2. Cryoprocessed nematode eggs. A : An early developmental stage egg showing a cluster of embryonic cells; B : Higher magnification of an embryonic cell showing well preserved cellular components and nucleus (Nu), as well as the chitin (Ch) and vitelline (arrow) layers of the egg shell; C : A larva within an egg; D : Higher magnification of C showing well preserved contents and developing cuticle (Cu). *(Bars equivalent : A, C = 5 μm; B, D = 1 μm.)*

chemical fixative, osmium tetroxide is included to aid in stabilisation of ultrastructure, which can begin at the lowest temperature in which the fixative is reactive (Steinbrecht & Müller, 1987) usually around subzero temperatures. The substitution was left for three days, so there was time for the osmium tetroxide to penetrate the egg shell and prevent deterioration of tissue. The penetration of this fixative may have been aided by the solvent which may have penetrated the egg shell better than water in which most chemical fixatives are dissolved. Less material is washed out of sections with cryosubstitution (Menco, 1986) so the processing does not affect the immunoreactivity of the specimens (Ichikawa *et al.*, 1989), allowing specific components to be traced.

The embedding protocol used was long, but successful. Shorter times were tried and tended to give similar results, but best results were obtained over the longer time periods. Cryofixation and cryosubstitution does not involve expensive and complex equipment or harmful chemical fixatives such as the aldehydes, and is a very useful method for material that is difficult to fix. We developed the method to study the interaction between nematode eggs and bacterial antagonists that do not damage the egg sufficiently for chemical processing.

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