*Fundam. appl. Nematol.*, 1995, **18** (3), 235-239

# Chitin in *Meloidogyne javanica*

Alan F. BIRD and Peter G. SELF

*C.S.I.R.O. Division of Soils, P. B. 2, P.O. Glen Osmond, South Australia, 5064. Australia.*

Accepted for publication 15 june 1994.

**Summary** – The gelatinous matrix (GM) exuded from living females of *Meloidogyne javanica*, which ultimately with the eggs forms the egg mass, was tested for the reported presence of chitin. Freshly exuded GM dissolved rapidly when 5 % KOH was added to a perfusion slide containing the nematode at 22 °C. The GM in egg masses was tougher but rapidly dissolved in a solution of sodium hypochlorite. Further treatment of GM-free eggs with 5 % KOH at 105 °C over 24 h led to breakdown of the eggs. Egg shells resisted this treatment and gave a chitin-type electron diffraction pattern when examined under the electron microscope and gave a positive test for chitosan. Chitin is not found in the GM and reports of its presence are probably due to egg shells or contaminating fungal hyphae which can be detected by the use of fluorochromes such as Calcofluor White under UV light.

**Résumé – *Chitine chez* Meloidogyne javanica –** La gangue gélatineuse extrudée par les femelles vivantes de *Meloidogyne javanica* et formant avec les œufs la masse d'œufs, a été analysée en vue de la présence de chitine. A 22 °C, la gelée fraîchement extrudée se dissout rapidement lorsque du KOH à 5 % est ajouté à une lame de perfusion contenant le nématode. La gelée des masses d'œufs est plus dure mais se dissout en 24 h dans la potasse à 5 % portée à 105 °C. Les coques des œufs résistent à ce traitement et produisent un spectre de diffraction électronique de type chitine lorsque examinées en microscopie électronique. La présence de chitine n'a pas été détectée dans la gelée et sa signalisation est probablement due à des hyphes de champignons contaminants, lesquels peuvent être détectés en lumière ultra-violette à l'aide de fluorochromes, tel le Calcofluor White.

**Key-words :** Chitin, egg shells, fungal hyphae, gelatinous matrix, *Meloidogyne javanica*.

It is generally accepted that " the eggshell is the only structure in nematodes in which the presence of chitin has been conclusively demonstrated " (Bird & Bird, 1991). However, some reference continues to be made to a publication in this journal nine years ago (Spiegel & Cohn, 1985) in which chitin was reported to have been detected in the gelatinous matrix (GM) of the root-knot nematode *Meloidogyne javanica*. These workers did not find chitin in the GMs of two other genera of nematodes tested namely, *Tylenchulus semipenetrans* and *Meloidoderita kirjanovae* but, as might be expected, did find it in the eggs of all three genera.

Chitin is a nitrogenous polysaccharide (a polymer of N-acetylglucosamine) that is insoluble in hot solutions of KOH, gives a positive chitosan colour test and has a characteristic X-ray diffraction pattern in its purified form (Richards, 1951; Muzzarelli, 1977). The chitin layer of the egg shell of *M. javanica* has been shown to be insoluble in hot (160 °C) concentrated KOH and to give a positive van Wisselingh colour test for chitosan (Bird & McClure, 1976). However, the GM of this nematode dissolves when heated in 0.2 M NaOH (Bird, 1958).

Chitin, first discovered in mushrooms by Braconnot (1811), is widely distributed in nature, particularly in forms such as the fungi and insects where it is a common constituent of their walls and cuticles respectively (Richards, 1951; Muzzarelli, 1977).

The GM of the egg-mass is very sticky and it is extremely difficult to obtain clean material free of contaminants and debris. The presence of fungal hyphae and a few egg shells could remain undetected even by the most careful worker and their presence would give a positive result for chitin. In this paper we report on the testing and comparison of GM freshly exuded from the female nematode with that found in mature egg masses. These GMs and their contaminants were compared with egg shells from *M. javanica*.

## Materials and methods

Tomatoes (*Lycopersicon esculentum* cv. Tiny Tim) were grown in pots containing a commercial potting mixture in a temperature-controlled glass house. When the seedlings had grown to a height of approximately 10 cm, they were infected with freshly hatched larvae of *Meloidogyne javanica*. The rate of growth of these nematodes was monitored by harvesting plants at regular intervals and dissecting out and examining the nematodes. Young female nematodes were dissected out and placed in 0.1 M phosphate buffer (pH 7.4) in a perfusion slide for observation of the exudation of GM at 22 °C (Dropkin & Bird, 1978). Egg masses were dissected several weeks later from the roots of plants containing similarly aged nematodes. The GM as an exudate from the female in a perfusion slide and as a component of the egg mass was tested with chemicals used to detect the absence or presence of chitin. These chemicals included

5 % potassium hydroxide, sodium hypochlorite containing 3 % available chlorine and 25 $\mu g\ ml^{-1}$ of the fluorescent dye Calcofluor White in phosphate buffer (Brydon *et al.*, 1987). This material was either photographed under transmitted light with the bright field or differential interference contrast optics of an Olympus Vanox AHBT microscope or under blue or UV incident light using the AH2-2FL fluorescent attachment of this microscope.

Egg shells for observation under the transmission electron microscope (TEM) were obtained as follows. Egg masses were carefully dissected from roots keeping them as free from debris as possible. They were centrifuged in distilled water at 50 g for 10 min. The supernatant was removed and sodium hypochlorite containing 3 % available chlorine was added (this dissolves the GM within 10 min), the centrifuge tube was shaken and allowed to sit for 5 min after which it was centrifuged at 1000 *g* for 5 min to compact the eggs. The supernatant was removed, distilled water was added, the tube was shaken and centrifuged at 1000 *g* for 10 min. This was repeated three times to wash the eggs. Then 5 % KOH was added and the centrifugation at 1000 *g* repeated. The supernatant was removed, the contents of the tube were shaken, placed in a screw capped bottle and heated at 105 °C for 24 h. The bottle was shaken and decanted into a centrifuge tube where the egg shells were cleaned by centrifugation and washing with distilled water. Some of these egg shells were examined under the light microscope and others were placed in drops on carbon-coated TEM support grids, allowed to dry and examined and photographed in a Philips EM 400 TEM. Selected area electron diffraction (SAED) was used to examine specific areas of the egg shell material. X-ray diffraction patterns of the whole sample were obtained by allowing water droplets containing egg shells (as per TEM preparation) to dry on a low-background silicon plate and then collecting a $\theta$-$2\theta$ scan on a Philips PW 1710 powder X-ray diffractometer. Chitin was detected chemically in eggs by means of the van Wisselingh colour test for chitosan. Initially eggs were heated in sealed tubes at 160 °C in a solution of KOH that was saturated at 22 °C. Insoluble material was separated by centrifugation, washed in a series of alcohols, of decreasing concentration, to water and then treated on a microscope slide with a drop of 0.2 % iodine in potassium iodide followed by a drop of 1 % sulphuric acid. If chitosan is present the egg shell turns a red-violet colour. When 75 % sulphuric acid is added the material goes into solution.

## Results

Prior to egg laying in *M. javanica* the GM starts to be secreted from the six large rectal gland cells. This secretion can be enhanced by a number of chemicals, including monovalent phosphate ions (Dropkin & Bird, 1978), so that when placed in a perfusion slide in phosphate buffer, the female nematode soon starts to exude GM. When 5 % KOH is perfused at room temperature around the nematode, the GM is rapidly dissolved. Thus this pure and egg-free form of GM does not contain chitin. The GM in egg masses appears tougher and does not break down completely over a period of 24 h in 5 % KOH at 40 °C (Fig. 1 A, B).

However, when these egg masses are washed in distilled water and a solution of sodium hypochlorite (*ca* 3 g available chlorine/100 ml) is added, the GM starts to dissolve immediately and within ten minutes the eggs are completely free of adhering GM (Fig. 1 C). Further treatment of these egg masses, after washing in distilled water by centrifugation, with 5 % KOH at 105 °C over 24 h leads to the break down of the egg leaving only the chitin-containing egg shell which, although transparent, is readily detected under Nomarski optics (Fig. 1 D). Chitin was not detected in X-ray diffraction patterns of whole samples although a number of contaminating materials (quartz, illite, montmorillonite and kaolinite) were detected. However, electron diffraction patterns of areas on the egg shells free of mineral contamination (Fig. 2 A) showed, albeit weakly, an annular ring corresponding to the 0.45-0.47 mm spacing characteristic of chitin (Rudall, 1955) (Fig. 2 B, C).

Staining of egg masses, from which eggs had been dissected away, with the chitin staining fluorochrome Calcofluor White did not reveal any chitin in the GM itself but did reveal fungal hyphae inhabiting the GM (Fig. 2 D). Both these hyphae and the transparent egg shells are difficult to detect under normal bright field optics. Egg shells gave a positive colour test for chitosan (Fig. 3) and these dissolved when 75 % sulphuric acid was added.

## Discussion

Because freshly exuded and uncontaminated GM from *M. javanica* is rapidly dissolved at room temperature (22 °C) in a solution of 5 % KOH, it is clear that it does not contain chitin. However, the GM that surrounds the eggs and makes up the egg mass can be very sticky and a number of contaminants can adhere to it. Furthermore, we have shown in this paper that the GM of egg masses can be inhabited by chitin-containing hyphae of various fungi. These hyphae and the shells of hatched eggs are not always easy to see under normal bright field optics. It is these structures that we think were responsible for the previous report of chitin in the GM of *M. javanica* egg masses. The egg shell, which remains insoluble after treatment with 5 % KOH at 105 °C for 24 h and which shows an electron diffraction pattern characteristic of chitin and gives a positive test for chitosan, is the only part of *M. javanica* that contains chitin.

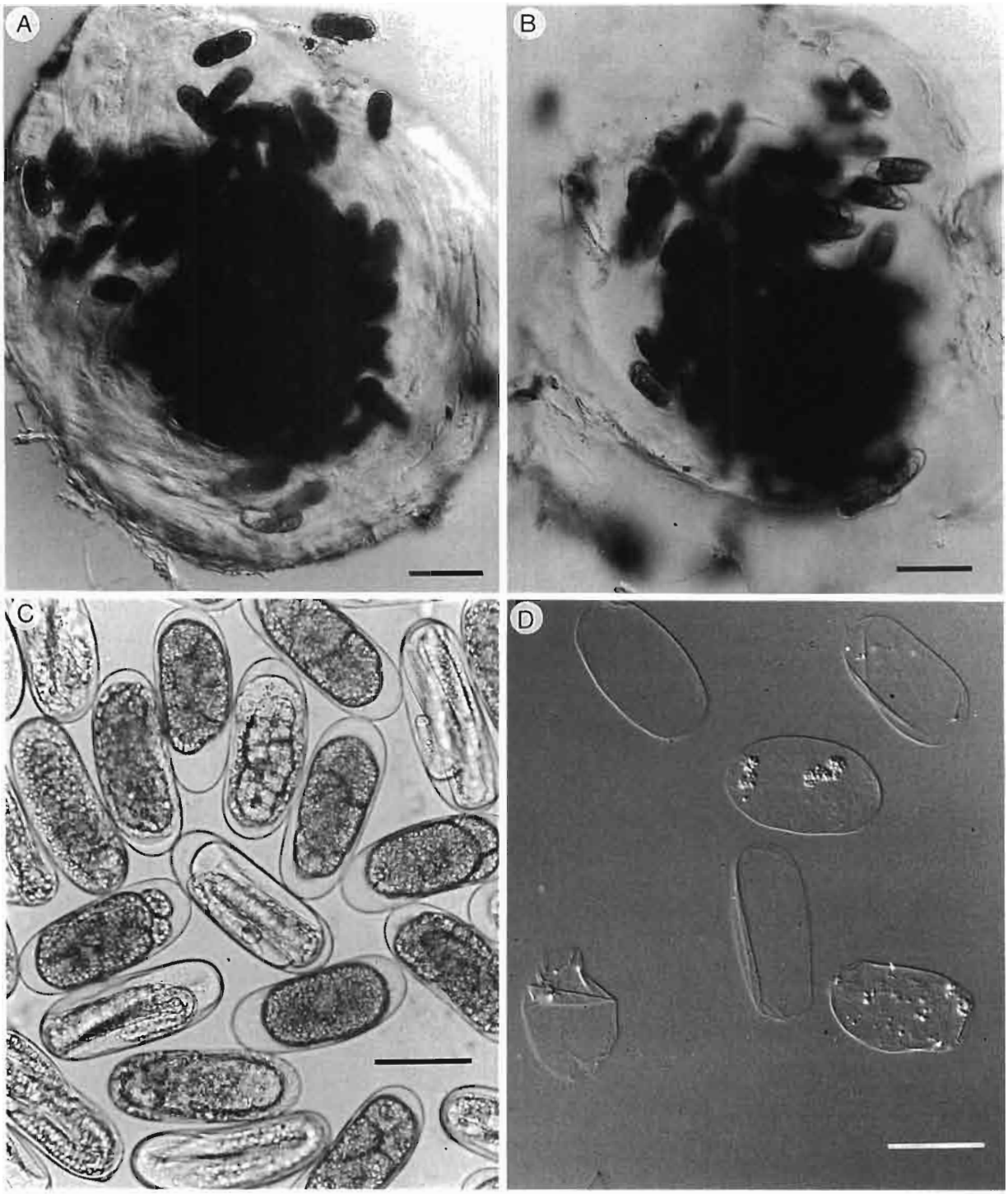


***Fig. 1.*** *Responses of* Meloidogyne javanica *egg masses to various chemical treatment. A : Freshly dissected egg mass prior to treatment; B : The same egg mass after exposure to 5 % KOH at 40 °C for 24 h; C : Clean eggs after treatment in sodium hypochlorite for 10 min; D : Egg shells after treatment in 5 % KOH at 105 °C for 24 h.* (Scale bars : A, B = 100 µm; C, D = 50 µm).

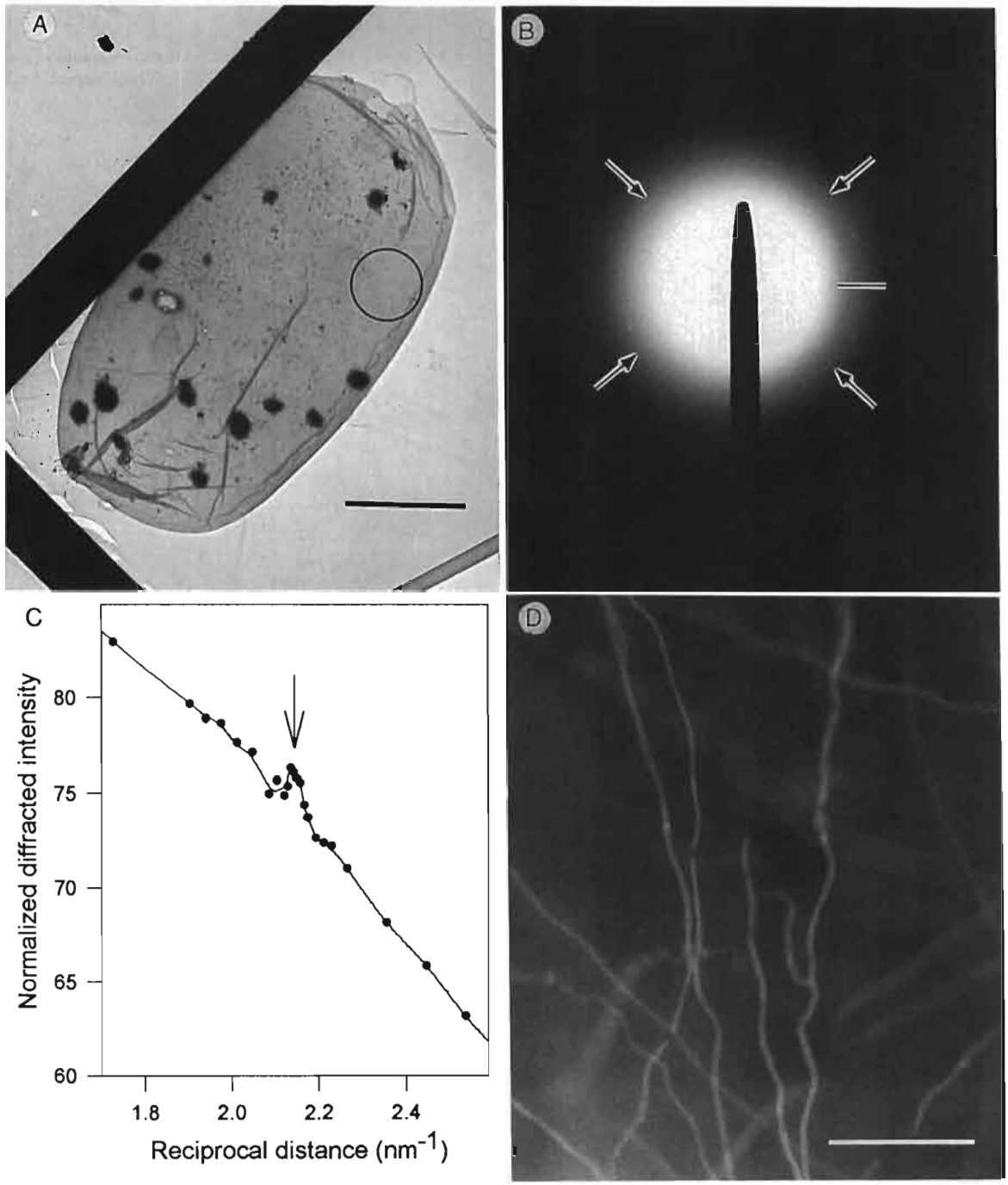


***Fig. 2.*** *Detection of chitin in* Meloidogyne javanica *egg shell by selected area electron diffraction (SAED) and fungal hyphae in the GM by Calcofluor White. A : Electron micrograph of an egg shell showing mineral contamination. The area chosen to be free of contamination and used for SAED is circled; B : SAED pattern of egg shell material. The faint 0.46 nm ring characteristic of chitin is arrowed; C : Photodensitometer trace along the line shown in B. The intensity increase at 0.46 nm is clearly seen (arrowed) in this trace; D : Calcofluor White-treated GM showing the presence of fungal hyphae by fluorescence under UV light* (Scale bars : A = 20 µm; D = 40 µm).

***Fig. 3.*** *Eggs exhibiting a positive test for chitosan* (Scale bar = 50 µm).

**References**


BIRD, A. F. (1958). The adult female cuticle and egg sac of the genus *Meloidogyne* Goeldi, 1887. *Nematologica,* 3 : 205-212.

BIRD, A. F. & BIRD, J. (1991). *The structure of nematodes, 2nd edition.* San Diego & London, Academic Press, 316 p.

BIRD, A. F. & MCCLURE, M. A. (1976). The tylenchid (Nematoda) egg shell : structure, composition and permeability. *Parasitology,* 72 : 19-28.

BRACONNOT, H. (1811). Recherches analytiques sur la nature des champignons. *Annals Chimie,* 79 : 265-304.

BRYDON, L. J., GOODAY, G. W., CHAPPELL, L. H. & KING, T. P. (1987). Chitin in egg shells of *Onchocerca gibsoni* and *Onchocerca volvulus. Mol. Biochem. Parasit.,* 25 : 267-272.

DROPKIN, V. H. & BIRD, A. F. (1978). Physiological and morphological studies on secretion of a protein-carbohydrate complex by a nematode. *Int. J. Parasit.,* 8 : 225-232.

MUZZARELLI, R. A. A. (1977). *Chitin.* Oxford, Pergamon Press, 309 p.

RICHARDS, A. G. (1951). *The integument of arthropods.* Minneapolis & London, University of Minnesota Press, 411 p.

RUDALL, K. M. (1955). The distribution of collagen and chitin. *Symp. Soc. exp. Biol.,* 9 : 49-71.

SPIEGEL, Y. & COHN, E. (1985). Chitin is present in gelatinous matrix of *Meloidogyne. Revue Nématol.,* 8 : 184-186.