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## International Organization of Citrus Virologists Conference Proceedings (1957-2010)

### Title

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### Permalink

<https://escholarship.org/uc/item/01t93041>

### Journal

International Organization of Citrus Virologists Conference Proceedings  
(1957-2010), 12(12)

### ISSN

2313-5123

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### Publication Date

1993

### DOI

10.5070/C501t93041

Peer reviewed

# Purification and Serology of the Organism Associated with Citrus Huanglungbin

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**ABSTRACT.** A modified method was used to purify the bacterium-like organism (BLO) from periwinkle plants infected with citrus huanglungbin. Large numbers of BLOs were purified by homogenization of infected periwinkle tissue in an isolation medium followed by cellulase digestion and density gradient centrifugation. Electron microscopy revealed that morphology and internal cellular structure were well maintained. The purified BLOs were used to immunize common and BALB/c mice for the preparation of polyclonal and monoclonal antibodies which were shown to have specific response, and were able to detect the BLOs in diseased tissue by immunoelectron microscopy and immunofluorescence.

Citrus huanglungbin (greening), associated with a bacterium-like organism (HBLO), is one of the most devastating diseases of citrus in China (3). In recent years, new citrus plantings in southern China have all become infected soon after establishment. For good disease control, a reliable and simple detection method is urgently needed. Garnier *et al.* (1, 2) successfully produced monoclonal antibodies against greening organisms from Poona (India) and Fujian (China), using sieve tube extracts of infected periwinkle as immunogen. In this paper we report the preliminary results of purification of the huanglungbin BLO from infected periwinkle using Percoll density gradient centrifugation, the production of polyclonal and monoclonal antibodies to it, and their use for its detection in plant tissues.

## MATERIALS AND METHODS

**Plant tissues.** Infected Ponkan mandarin plants were obtained by feeding infected *Diaphorina citri* individuals on them. The diseased periwinkles were then obtained by firstly transmitting the HBLOs from the Ponkan by dodder, and then transmitting them to other periwinkle plants by top grafting. All these plants, as well as healthy controls, were grown in a screenhouse maintained at 21-28 C in winter and 23-32 C in summer.

**Purification of the BLO.** Leaf midribs from infected periwinkles were homogenized in an isolation medium

containing 0.5M D-mannitol, 0.3M glycine, 0.1% PVP, 0.03M MOPS and 0.001M EDTA (pH 5.5). After digestion with cellulase at 37 C for 3 hr, the preparation was centrifuged at 4000 rpm for 10 min. The supernatant was then centrifuged at 20,000 *g* for 40 min and the resultant resuspension was centrifuged through a Percoll discontinuous gradient at 16,000 *g* for 30 min. The pellet was resuspended in a medium containing 0.5M D-mannitol, 0.3M glycine, 0.001M EDTA and 0.03M MOPS (pH 7.4), and used as test antigen. The leaf midribs of healthy periwinkle were similarly treated as controls.

**Production of polyclonal antibodies.** Common mice were injected with 0.5 ml of purified HBLO emulsified with an equal volume of Freund's complete adjuvant. The second and third injections using 0.5 ml antigen emulsified with an equal volume of Freund's incomplete adjuvant were given at intervals of 2-3 weeks. Ehrlich ascites tumor cells (0.2 ml) were injected 7 days after the final boost injection, and 6-7 days later ascites fluid was collected, centrifuged (2000 *g*/15 min) and stored with 0.1% sodium azide at -20 C.

**Production of monoclonal antibodies.** Purified HBLO was used as antigen to immunize 8-week-old BALB/c mice. Intraperitoneal injections (0.2 ml) emulsified with an equal volume of Freund's complete adjuvant were given on days 1 and 22, and on day 36, 0.3 ml antigen emulsified with Freund's incomplete adjuvant was intravenously injected. Mice were killed 3-5 days after

the last injection. Murine myeloma cells (SP2/0) were fused with spleen cells from the immunized mice in the presence of PEG 1000. Hybridomas were selected after incubation in HAT medium.

**Electron microscopy (EM).** Purified HBLOs were negatively stained with 2% phosphotungstic acid (pH 6.0) for 2-3 min., and were then embedded in 1% agarose. After fixing and embedding in resin (5), ultra-thin sections were cut with an LKB III ultramicrotome and examined under a JEM 100 CX II electron microscope.

**Immunoelectron microscope (IEM).** The collodion-covered grids with different concentration of antibody IgG were incubated at 25 C for 30 min. After washing with saline solution, they were put on a drop of antigen and incubated at 32 C for 4 hr. Then, they were covered with corresponding concentration of antibody IgG for 15 min. They were washed with PBS and stained with 2% PTA (pH 6.0) before EM examination.

**Immunofluorescence.** Sections from periwinkle (30  $\mu$ m) and citrus (10  $\mu$ m) midribs of diseased and healthy

plants were cut with a freezing microtome as described by Martin-gros *et al.* (6). The sections were incubated in the hybridoma supernatant for 30 min at room temperature, and then in a 10,000-fold dilution of antimouse sheep IgG labelled with fluorescein isothiocyanate (IgG-FITC) for 30 min. After washing with PBS-Tween, they were observed under a Zeiss epifluorescence microscope.

## RESULTS

**Purification.** The purification method used yielded large numbers of HBLOs, with a concentration of 40,000 to 150,000 per EM grid. In ultrathin sections, the purified HBLOs were round, oval or elongated, ranging in size from 30-600 nm in width to 500-1400 nm long. An envelope 20-38 nm thick was observed and was composed of three layers: an outer layer with high electron density and uneven surface, an inner, thinner, electron dense layer, and an intermediate layer of lower electron density (Fig. 1).

The morphology and structure of the purified organisms were similar to

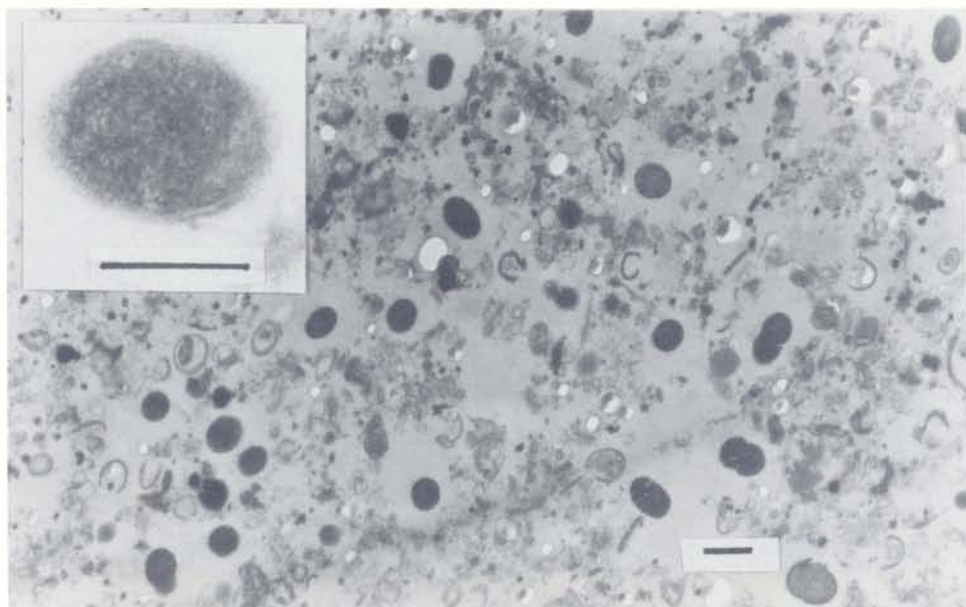


Fig. 1. Negatively-stained bacteria-like organisms partially purified from huanglungbin-infected periwinkle by Percoll density gradient centrifugation. (Bar represents 250  $\mu$ ).



organisms observed *in situ*, and were only obtained from huanglungbin-affected periwinkle.

**Ascites antibody and immunoelectron microscopy (IEM).** The purified IgG from ascites antibody preparation was absorbed with an equal volume of healthy periwinkle extract at 37 C for 2 hr or overnight at 4 C. Similarly, the healthy control immunized ascites IgG was absorbed with huanglungbin-infected periwinkle material. In IEM tests, ascites antibodies reacted at a titer of 1/320 (Fig. 2). Ascites prepared from healthy controls were negative.

**Monoclonal antibody (MA) and immunofluorescence (IF).** Two hybridomas (CF1 and CF2) secreting MAs against the HBLO were selected using IF tests on sections of leaf midribs from healthy and infected periwinkles. Both reacted specifically with infected periwinkle and citrus showing symptoms, but not with asymptomatic or healthy plants (Table 1).

TABLE 1  
DETECTION OF HUANGLUNGBIN BLOs  
BY IMMUNOFLUORESCENCE

Antigen	monoclonal antibodies	
	CF1	CF2
Infected periwinkle		
with symptoms	+++ <sup>z</sup>	+++
no symptoms	-	-
Healthy periwinkle	-	-
Infected citrus		
with symptoms	+	+
no symptoms	-	-
Healthy citrus	-	-
MLO infected periwinkle	-	-

<sup>z</sup>+++ = strong immunofluorescence; + = weak immunofluorescence; - = no immunofluorescence.

## DISCUSSION

Our results indicate that it is possible to obtain HBLOs in high concentration from infected periwinkle tissue, and can be used as antigen for the preparation of ascites and monoclonal anti-

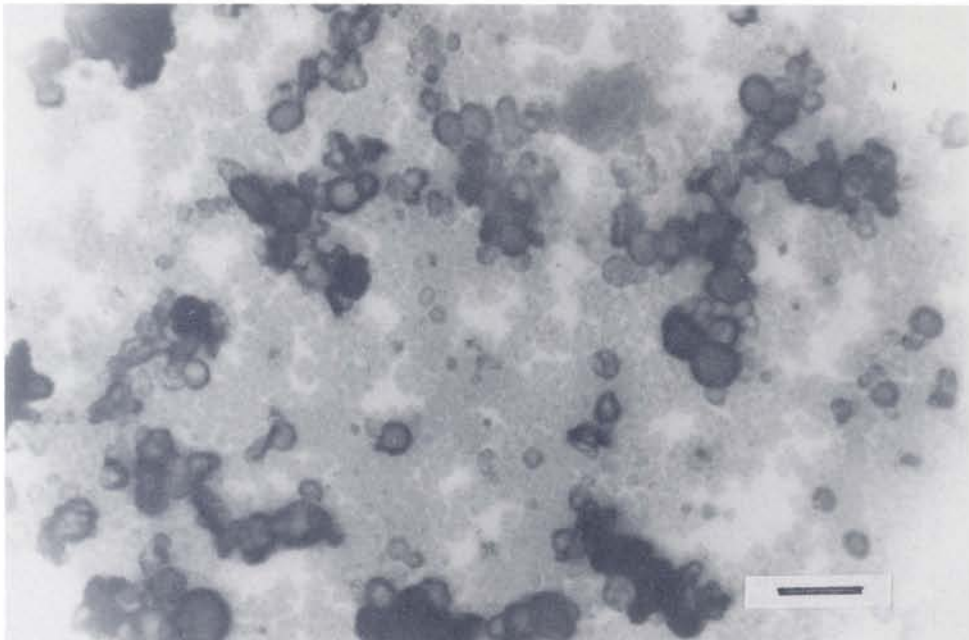


Fig. 2. Detection of HBLOs purified by immunoelectron microscope using ascites antiserum. (Bar represents 700  $\mu$ ).

bodies. The purification procedure maintained the morphological integrity of the HBLO and its antigenic activity. This is probably due to the appropriate osmolarity in the isolation medium (535-645 mosm/kg). Previous work showed that the osmolarity in the extract from infected periwinkle midribs was higher than from healthy plants (5). The reasons for this are unknown. The

titer of the anti-ascites antiserum was low and interference from non-specific reaction was obvious. The further study to improve the production of polyclonal antibody is progressing. The stronger IF reaction of MAs with infected periwinkle (Table 1) may be due to a higher concentration of HBLO in this host, as has been previously demonstrated by electron microscopy (4).

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