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Development of a Continuous Whitefly Cell Line [Homoptera: Aleyrodidae: *Bemisia tabaci* (Gennadius)] for the Study of Begomovirus[1]

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The whitefly *Bemisia tabaci* (Gennadius) is a widely distributed pest of many important food and fiber crops. This whitefly is also a vector of more than 70 plant-infecting viruses. A cell line was established *in vitro* using embryonic tissues from the eggs of *Bemisia tabaci* (Gennadius), B biotype (pseudonym *B. argentifolii* Bellows & Perring), and referred to as 'Btb(Ba)97, Hunter-Polston'. Cell cultures were successfully inoculated with begomovirus (BGMV and ToMoV)-infected tomato plant sap. Embryonic tissues were seeded into Kimura's modified medium and kept at a temperature of 24°C. Continuous cell cultures were established and have since undergone 92 passages in 25-cm^2 flasks. Cell doubling time is approximately 3 days and the cells have been successfully revived after 1 year after storage at −80°C. The cell population is monolayers of predominately fibroblast with some epithelial cells. Begomoviruses (bean golden mosaic begomovirus, BGMV, and tomato mottle begomovirus, ToMoV) were inoculated to the cell cultures independently and detected by labeling by an indirect immunofluorescence technique. The viruses were detected bound to the cell membranes and within the cell cytoplasm. This is the first report of a continuous cell line established from a species of whitefly and its inoculation with two begomoviruses. The successful inoculation of whitefly cell cultures with begomoviruses shown in our results represents great promise for the development of systems that allow researchers to achieve a better understanding of the complex relationship between begomoviruses and their whitefly vectors.

***Key Words: Bemisia tabaci; B. argentifolii;* begomovirus; cell culture; geminivirus; insect vector; whiteflies.**

INTRODUCTION

The whitefly *Bemisia tabaci*(Gennadius), B biotype (pseudonym *B. argentifolii* Bellows and Perring) (Bellows *et al.*, 1994), is a polyphagous pest which feeds on over 500 plant species, including many important agricultural crops, such as tomato, bean, cotton, squash, melon, crucifers, ornamental crops, and many others. Losses due to this whitefly in 1991 and 1992 in only four United State states (Arizona, California, Florida, and Texas) exceeded $200 and 500 million, respectively (Henneberry and Toscano, 1996). This whitefly has rapidly spread around the world and is devastating crops due to its polyphagous nature, the toxic effects of its saliva, its ability to quickly develop large populations, and its resistance to chemical insecticides (Schuster *et al.*, 1996; Polston and Anderson, 1997).

B. tabaci is also a vector of plant viruses and over the past 10 years has emerged as one of the most important vectors of these pathogens. This species is capable of transmitting plant viruses from six families, though the majority of viruses belongs to Geminiviridae and Closteroviridae (Markham *et al.*, 1994). Over 60 species of begomoviruses and 7 species of criniviruses (family Closteroviridae) are transmitted by this whitefly (Markham *et al.*, 1994; Wisler *et al.*, 1997). Begomoviruses cause economic losses in numerous crops worldwide (Polston and Anderson, 1997). The recent introduction of the highly pathogenic tomato yellow leaf curl virus (TYLCV-Is) into the Western Hemisphere has increased the need to develop new management strategies for both the disease and the whitefly vector (Polston and Anderson, 1997; Polston *et al.*, 1999).

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Research was undertaken to develop a whitefly cell culture from the vector species, *B. tabaci* (Gennadius). The *in vitro* cultivation of whitefly cells would provide a valuable tool for investigations of whitefly pathology, genetics, and physiology. Whitefly cell cultures will also provide a system for analysis of the biological and cellular interactions of the whitefly vector with the viruses it transmits.

MATERIALS AND METHODS

Cell culture medium components. The medium used for the culturing of whitefly cells was Kimura's modified medium (Kimura, 1984). Components were mixed as follows: 100 ml of Schneider's Drosophila medium (Gibco BRL, No. 11720-034, Grand Island, NY), 100 ml of L-histidine hydrochloride, monohydrate, pH 6.2 (1.3 g/100 ml dH_2O) (Gibco BRL, No. 11062-015), 10 ml of medium 199, 10× (Gibco BRL, No. 11181-039), 5 ml of CMRL medium 1066 (Gibco BRL, No. 11530-037). After these solutions were mixed together, the pH was adjusted to 6.35 with 1 M NaOH or 1 M HCl. Penicillin–streptomycin solution (10,000 U/ml) was added to the medium (2.5 ml/250 ml medium) (Gibco BRL, No. 15140-122). The medium was then filter sterilized (0.2 μm). Finally, 30 ml of sterile fetal bovine serum which had been heat inactivated (57°C for 30 min) was added. (Gibco BRL, No. 26140-079). Cells were subcultured using cell dissociation solution, nonenzymatic. Using 1 ml/flask, let sit 10 min (Sigma Co., St. Louis, MO, No. C-1544).

Tissue culture. Embryonic eggs, distinguished by clearly visible double red eye spots, were the source of the tissue culture. Eggs from nonviruliferous whiteflies were collected using a small brush to scrub the underside of cabbage leaves (*Brassicae oleracea* L. var. *capitata*) while leaves were submerged in 70% ethanol. When the eggs had settled to the bottom, the ethanol along with adults, nymphs, and debris was drawn off and replaced with fresh 70% ethanol. Eggs were in ethanol no more than 15 min. Subsequent steps were conducted in a sterile Laminar-flow hood. The eggs were transferred to a sterile 15-ml centrifuge tube and centrifuged at 600*g* for 3 min. The ethanol was drawn off and the eggs were transferred to a 1.5-ml microcentrifuge tube. Eggs were rinsed three times in 1 ml sterile water and then centrifuged at 4000 rpm (1310 rcf) in an Eppendorf centrifuge for 3 min. The eggs were finally rinsed with histidine solution (L-histidine, monohydrate 13 g/L, pH 6.2) and centrifuged for 3 min at 4000 rpm. The histidine solution was replaced with 200 μl of insect medium. Eggs were homogenized in insect medium using a sterilized pestle. More insect medium was added until the egg tissue was suspended in 1 ml of liquid. The homogenate was then dispensed into the inner wells of a 48-well tissue culture plate using a sterilized Pasteur pipet (approx 100 μl/well). Additional medium was added to cover the bottom of each well (approx 6 to 7 drops). The outside wells were filled with sterile water. Parafilm was wrapped around the lid and plate to prevent vapor loss. Explanted tissues were examined daily with a Bausch and Lomb inverted microscope. Wells which contained floating "debris" had 3/4 of the medium replaced with fresh medium the day following transfer. Medium was replenished by exchanging half the medium in each well with fresh medium at 7- to 10-day intervals.

Cell inoculation. Cells were transferred to an 8-well, cell-culture slide system (Sigma, St. Louis, MO) and allowed to grow until nearly confluent. Two wells per slide were inoculated with purified bean golden mosaic virus (BGMV)(0.25 g/L) (Cancino *et al.,* 1995) syringe filter sterilized through 0.2 m membrane. The next two wells were inoculated with filter-sterilized tomato sap from a tomato mottle virus (ToMoV)-infected plant. Under sterile conditions, 0.2–0.3 g of young leaf tissues was surface-sterilized with 70% ethanol, rinsed in sterile water three times, rinsed once in L-histidine buffer, pH 6.3, and then ground with a sterilized mortal and pestle. The sap was then transferred into sterile 1.5-ml centrifuge tubes, which were centrifuged for 2 min at 6000 rpm, and kept on ice until used. Two healthy plant sap control wells were inoculated with healthy, uninfected tomato sap processed the same as for the virus-infected tomato sap inoculum. The last two wells were inoculated with L-histidine buffer as buffer control. The inoculation period was 1 h, and then cells were washed twice with L-histidine buffer. Fresh medium was then applied in all wells. All treated cell cultures were covered and allowed to sit at room temperature for 24 h before processing.

Processing cells for indirect immunofluorescent staining. Cell culture medium was removed and the cells were washed twice with 10 mM phosphate buffer solution (PBS) (Sigma). Cells were fixed in 3.7% formaldehyde in PBS which was added to all cells for 15 min at room temperature. The cells were then washed gently with five changes of PBS, 2–3 min each time. One well from each treatment, 4 wells per slide, was blocked with normal goat serum for 15 min (~4 mg/ml) as a control check for background levels. Cells were then incubated with the appropriate primary antibody (rabbit polyclonal antisera 1110, 1175, or 1200 were applied diluted 1:1000 in 10 mM PBS, pH 7.4, supplemented with 10% saponin for 30 min at room temperature (Cancino *et al.,* 1995; Hunter *et al.,* 1998). Cells were washed with PBS containing 0.1% saponin, 5 times for a total of 10 min. The cells were incubated with the secondary antibody (goat anti-rabbit) conjugated with fluorescein-isothiocyanate (FITC), diluted in PBS supplemented with 0.1% saponin and 4 mg/ml

of normal goat serum, for 20 min at room temperature. The cells were washed in PBS containing 0.1% saponin five times for 10 min total and then twice for 5 min total with only PBS. Processed cells were mounted in Aqua-Mount (mounting medium, antiquenching fluorescent extender, polyvinyl alcohol-vinylacetate, Polysciences, Inc., Warrington, PA) and examined using an Olympus inverted UV microscope at 100× (method from Willingham and Pastan, 1985).

RESULTS AND DISCUSSION

A continuous whitefly cell line was successfully established from embryonic cells of the whitefly, *Bemisia tabaci*, and was designated 'Btb(Ba)97 Hunter-Polston'. Cells were deposited in the permanent collection of the American Type Culture Collection as Accession Number CRL-12420. Development of primary cell cultures from whiteflies was difficult taking at least 30 different attempts to cultivate. Cells were first subcultured 6 months after the beginning of embryonic tissue culture. The established monolayer cells were subcultured at 7- to 10-day intervals at a 1:2 split ratios. The cells are firmly attached to the substrate and require either scraping or a cell dissociation solution for transfer. Monolayers of epithelial-like cells (Fig. 1a), which had a morphology distinct from that of the fibroblastoid cells, developed in the medium shortly after subculturing (cells at fifth passage, Fig. 1). Spindle-shaped, fibroblast-like cells were observed 72 h after cultivation. Subsequent passages revealed the fibroblast-like cells to be the dominant cell type (Fig. 1b). It was of interest that the cell types established using Kimura's insect medium were similar to those of other cells cultured using this medium (Kimura, 1984). It may be that this medium plays a role as a filter for these specific cell types to be established from embryonic cell tissues.

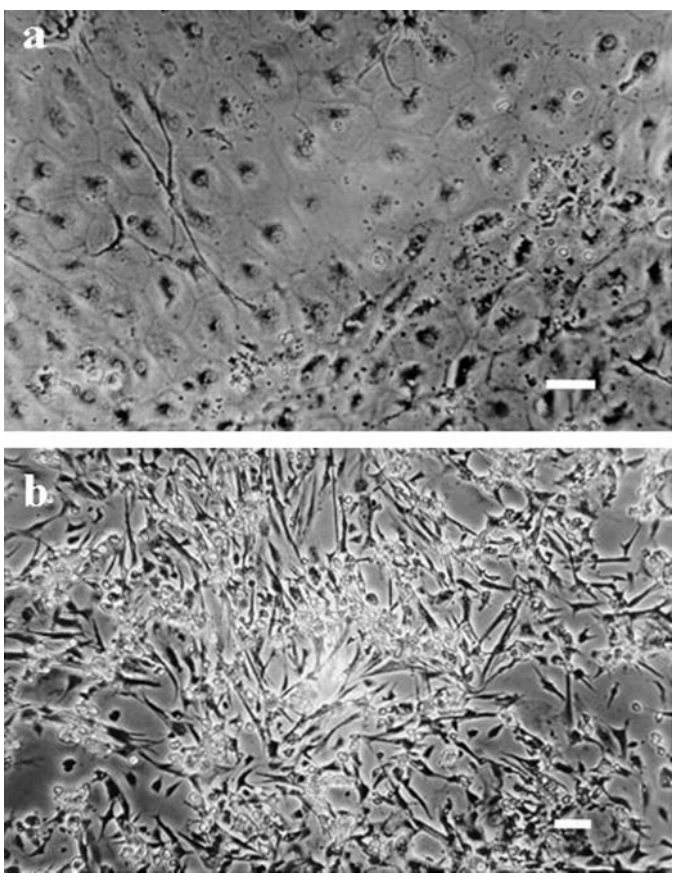

FIG. 1. (a) Phase contrast of cultured whitefly cell monolayer of Btb(Ba)97 Hunter-Polston cell line. (b) Phase contrast of whitefly fibroblasts in culture of *Bemisia* tabaci cultured cells. Bar = 100 μm in both figures.

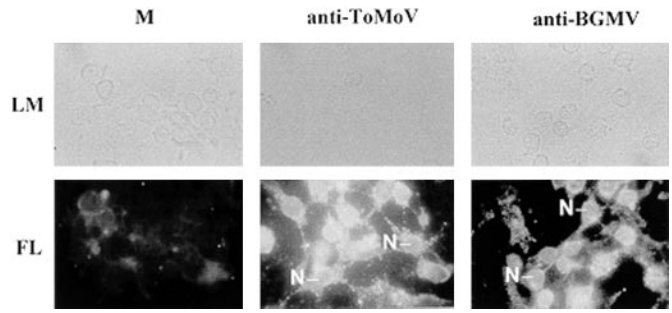

FIG. 2. Light (LM) and fluorescent (FL) microscope images of whitefly cell cultures, Btb(Ba)97, inoculated with begomoviruses for 1 h and processed 24 h postinoculation. (M, mock inoculation with healthy tomato sap). Primary antibodies to the appropriate begomovirus inoculum (Tomato mottle begomovirus-infected tomato sap, anti-ToMoV; (bean golden mosaic begomovirus-infected purified virus inoculum, anti-BGMV antibody). The same set of cells viewed by light microscopy was photographed using filter sets for Nikon FluorPhot system (N, nuclei darker than cytoplasm).

This is the first report of the establishment of a continuous whitefly cell line, and the inoculation of whitefly cells with two different Begomoviruses (BGMV) and (ToMoV). Both Begomoviruses were detected when individually inoculated to whitefly cells using antibodies made to whole virions in an indirect immunofluorescence technique (Fig. 2). When independently inoculated, both BGMV and ToMoV were detected bound to the outer cell membranes and appeared to be within the cell cytoplasm (Fig. 2). Nevertheless there was no labeling within cell nucleii (remained darker than cytoplasm). Previous studies have demonstrated and detected begomovirus within the cells of the whitefly midgut and salivary glands (Hunter *et al.*, 1998). Although, there are limited reports of begomovirus replication in whiteflies (Ghanim *et al.*, 1998) this evidence was based on the detection of the retention of viral nucleic acids within whiteflies over the life span of the insect. Our results did not show the presence of viral particles in the nucleii where the replication takes place. Although this observation can be due to the

fact that virus replication may occur at very low levels as has been proposed for tomato yellow leaf curl virus (Ghanim *et al.*, 1998). In order to provide conclusive evidence on whether begomoviruses replicate in whitefly cells, more sensitive techniques need to be used. Our future research goals include tracing the begomovirus replication in cultured whitefly cells by using engineered Begomoviruses incorporating a reporter gene such as green fluorescent protein (GFP).

Whitefly cell lines will provide a system for the intimate study of the interactions between begomoviruses and whitefly cells. The development of whitefly cell cultures also increases the number of cell lines within the Homoptera now available for research. Past primary cultures and established insect cell lines (Adam and Sander, 1976; Chiu and Black, 1967; Mitsuhashi, 1989) have provided valuable insights into virus-cell interactions and allowed detailed analyses on the molecular biology of some plant viruses (Hsu *et al.*, 1983). These advances may allow the development of novel viral gene expression systems for the control of insect pests. The successful inoculation of whitefly cell cultures with begomoviruses shown in our results represents great promise for the development of a system that allows researchers to achieve a better understanding of the complex relationship between begomoviruses and their whitefly vectors.

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