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Formulation of an Insect Medium for Thrips Monolayer Cell Cultures (Thysanoptera: Thripidae: *Frankliniella occidentalis*)

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A thrips tissue culture system was developed from embryonic eggs from *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). Cell lines that have been established for other insects offer a wide range of usages for studies of insect physiology, toxicology, and pathology. This study examined the efficiency of different insect cell culture media for suitability in establishing a thrips cell culture system. Final development of a medium for thrips cell culture allowed cells to be cultured up to 7 months. Within 1–2 days after being explanted, tissue fragments and individual cells attached to the substrate; at this stage, fibroblast-like cells were the dominant cell type. Monolayers of epithelial-like cells were observed at 10–20 days. This is the first cell culture system established which provides a method for the examination of thrips cell growth and metabolism and which provides a method for the study of virus infection and replication within the thrips vector *F. occidentalis.*

KEY WORDS: Thrips; *Frankliniella occidentalis;* tissue; cell; tospovirus; vector.

INTRODUCTION

From the first reports of short-term survival and growth of silkworm cells by Trager in 1935 to the subsequent success of the establishment of a continuous insect cell line, from diapausing pupae in 1962 by Grace, insect cell lines have been of great assistance to researchers working with insect transmitted viruses (Hink and Bezanson, 1985). Thrips are the only known vectors of tospoviruses, causing widespread economic losses worldwide (Allen and Matteoni, 1988; Best and Gallus, 1953, Peters *et al.,* 1991). With no insect cell line suitable for the growth and study of tospoviruses, and to increase our understanding of the virus–vector relationships, a thrips tissue culture system was established.

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MATERIALS AND METHODS

Collection of eggs. Embryonic eggs of *Frankliniella occidentalis,* 3 days old, were the best source for obtaining tissues and were collected as in Murai (1982). Red eyespots were visible at this stage and the larvae were almost completely formed. Plexiglas cylinder cages, about 10 cm in diameter, which had two pieces of Parafilm stretched over the open ends were used. Before both ends of the cage were covered with parafilm, about 500–1000 adult thrips were added, along with some pinetree pollen. Distilled water was sealed between two sheets of Parafilm at the top of the cage in which the thrips oviposited their eggs. Cages were kept at 21°C and a photoperiod of 16:8 (L:D) hr. Eggs were collected every 2 days by peeling away the top layer of Parafilm and washing the eggs into a dish with distilled water. When the eggs had settled to the bottom, the old water was replaced with fresh distilled water.

Surface disinfection of eggs. When eggs reached the red eye stage, just prior to hatching, eggs were sterilized in a 15-ml conical centrifuge tube in 70% ethanol for 3–5 min. The eggs were then centrifuged (125*g*, 3 min) with an IEC Clinical centrifuge. All subsequent work took place in a sterile Laminar flow hood. Following sterilization by ethanol, the eggs were transferred to a sterile 15-ml centrifuge tube with 10 ml of distilled water containing 0.1 ml of Nystatin and centrifuged as mentioned above. Next, the eggs were again transferred to a new sterile tube, with 10 ml of filtered distilled water, and again centrifuged as already stated.

Homogenization and trypsinization. The disinfected eggs were covered with 1 ml of Tyrode's salt solution (Reddy, 1977) and centrifuged. The Tyrode's solution was removed and the eggs were homogenized in the 15-ml vial by tapping with a rounded-tip glass rod. Then 0.5 ml of 0.05% trypsin was added to the homogenized tissue and let sit for 15 min. Trypsinization was stopped by adding 6 ml of one of the media listed below. The tissue fragments and medium were then centrifuged at approximately 600*g* for 5 min. The

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TABLE 1
Hunter's Medium for Thrips Cell Culture

	g/liter
Inorganic salts	
Calcium chloride, anhydrous	1.1
Calcium chloride, $2H_2O$	0.0925
Magnesium chloride, $6H_2O$	0.1
Magnesium chloride, anhydrous	0.5341
Magnesium sulfate	2.535
Potassium chloride	2.92
Potassium phosphate, monobastic	0.9185
Sodium bicarbonate	0.575
Sodium chloride	6.1
Sodium phosphate, dibasic	0.795
Amino acids	
β-Alanine	0.7
L-Alanine	0.225
DL-Alanine	0.225
L-Arginine	1.55
L-Asparagine, anhydrous	0.35
L-Aspartic acid	0.75
L-Cysteine, 2HCl	0.2767
L-Cysteine	0.12
L-Glutamic acid	1.4
L-Glutamine	2.55
Glycine	0.5
L-Histidine	3.025
DL-Isoleucine	0.125
L-Isoleucine	0.2
L-Leucine	0.2875
L-Lysine HCl	2.31
DL-Methionine	0.075
L-Methionine	0.2
DL-Phenylalanine	0.275
L-Proline	2.05
L-Serine	1.45
DL-Threonine	0.3
L-Threonine	0.525
L-Tryptophan	0.21
L-tyrosine · 2Na · $2H_2O$	0.9422
DL-Valine	0.1
L-Valine	0.4
Vitamins	
p-Aminobenzoic	0.00001
D-Biotin	0.00001
Choline chloride	0.0006
Flavine mononucleotide · Na	0.00005
Folic acid	0.00051
myo-inositol	0.00101
Niacin	0.00051
D-Pantothenic acid (Hemicalcium)	0.00051
Pyridoxine HCl	0.00051
Riboflavin	0.00001
Thiamine monophosphate · HCl	0.0005
Other	
D(−)Fructose	0.2
Fumaric acid	0.0875
Galactose	0.45
D(+)Glucose	2.35
α-Ketoglutaric acid	0.535
L(−)malic acid	0.935
Phenol red · Na	0.0055
Sodium pyruvate	0.275
Succinic acid	0.09
Sucrose	13.34
D(+)Trehalose	2.0
Tryptose phosphate broth	1.475
Yeast Extract	2.0
Added components	
Histidine solution: L-histidine HCl monohydrate	8.0
+L-Histidine free base	10.0
Medium 199, 10×, with Earle's salts	40 ml/liter
CMRL 1066, with L-glutamine	40 ml/liter
Fetal bovine serum, sterilized	60 ml/liter
Antibiotics	
Penicillin–streptomycin, 120,000 units/ml	9.6 ml/liter
Gentamycin	100 mg/liter

Note. Osmolality, 290–310; pH 6.4–6.5 at room temperature.

medium and trypsin solution were then removed, leaving the tissue fragments and cells at the bottom of the tube. Two milliliters of fresh medium was added to resuspend the tissue and cells and dispensed at 1 drop per well (about 40 μl) into a 96-well Corning tissue culture plate using a Pasteur pipette (Fisher controlled-drop Pasteur pipets, borosilicate glass, 22–26 drops/ml) or dispensed into a Corning 25-cm^3 flask with 2 ml additional media added. In the 96-well plates, the outside wells had three drops of filtered distilled water to help maintain the desired vapor pressure. The plate was then sealed with Parafilm. One drop of fresh media was added to each well containing tissue 3–4 days after initial dispension into the 96-well plate. Primary cultures were kept at 24°C in an incubator and fresh media added or changed thereafter at 4- to 7-day intervals. In these cultures, most explants from embryonic fragments would attach to the substrate within 24–48 hr if kept on a stationary nonvibrating surface. Cell growth around the explants or from single cells could be observed after 48 hr of incubation. The primary cultures could be maintained for 4–5 months by changing the medium every 4–7 days. The 1-day-old medium was drawn off, centrifuged to remove debris, and mixed with fresh media (ratio 1 ml fresh:2 ml conditioned media).

Culture media. Several commercially available media were evaluated for their potential use in starting primary thrips cell cultures (Sigma Co., St. Louis). All media were made with 10% heat-inactivated fetal bovine serum (FBS) with pH adjusted to a final 6.4–6.5. A final medium was developed which gave the best results, herein called Hunter's Thrips medium (Table 1), successfully starting primary cultures. Attempts to start cell cultures using individual commercially available media alone resulted in slow growth and reduced longevity, as with Liu and Black's medium as modified

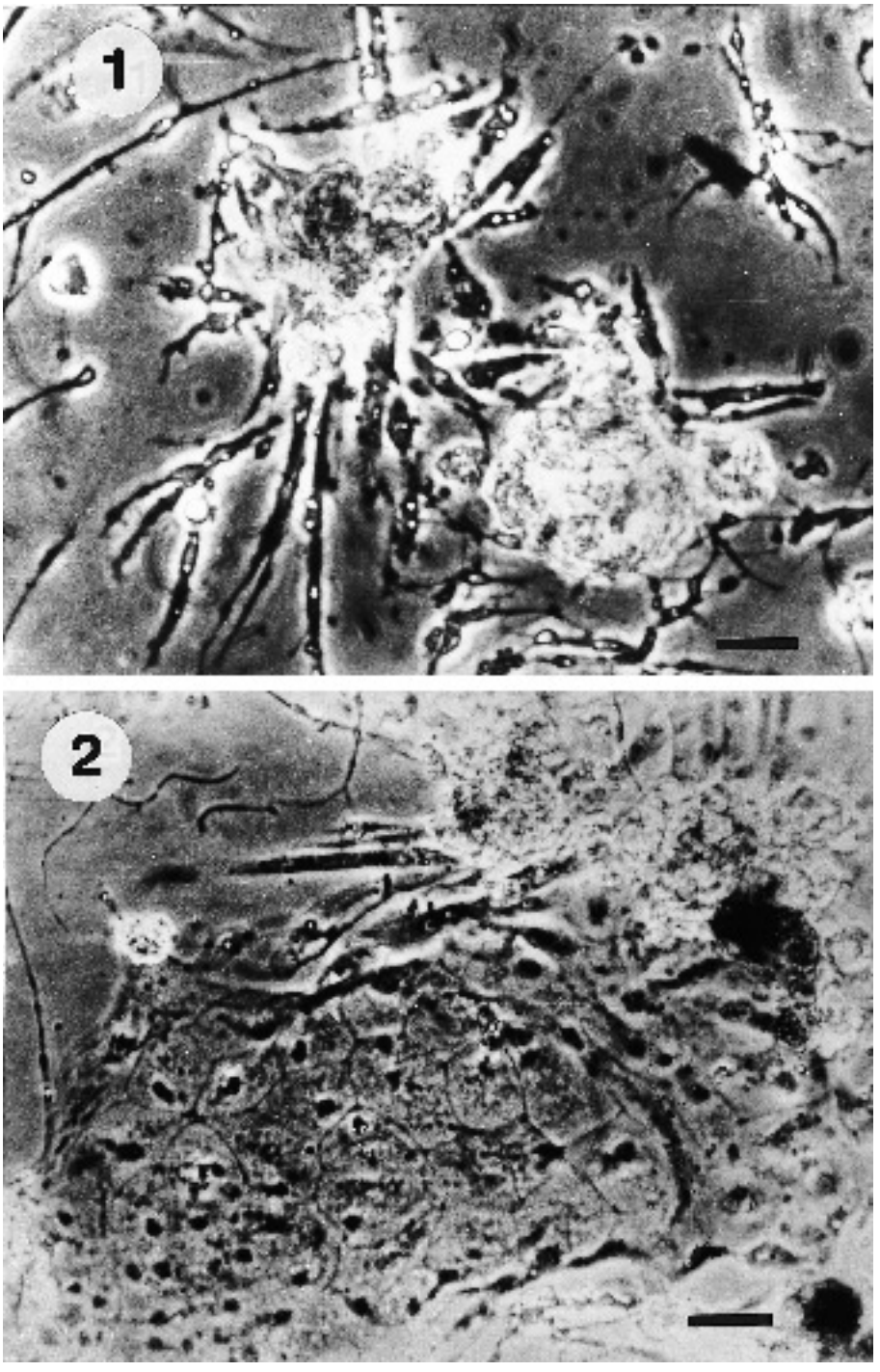

FIG. 1. Light micrograph of thrips tissue fragment showing the outward growth of fibroblast-like cells. 10 days. Bar, 10 μm.
FIG. 2. Light micrograph of monolayers of epithelial-like cells growing between thrips tissue fragments, at 20 days. Bar, 10 μm.

by Kimura (1984), Grace's, or Leibovitz's medium supplemented with 10% FBS. Several media evaluated, Chiu and Black's, HY-Q serum free, Mitsuhashi and Maramorosch's, and Shield and Sang's (Sigma, Co.), failed to support cell growth, even when supplemented with 10% FBS.

Primary tissue cultures were from tissues of unknown origin as they stemmed from seeded embryonic fragments. However, it is suggested that these tissue fragments differentiate from muscle, nerve, and epithelial cells as described by Kuroda and Shimada (1989). Supportive evidence attributed to the cell morphology and activity such as tissues which twitched when placed under strong light or tissues which could

be observed contracting quite strongly, and the presence of cells growing in monolayers, were similar to descriptions of cells from other established insect cell lines. Some tissues were still reactive to light after 60 days.

At 24–48 hr after cultivation, the development of fibroblast-like cells attaching to the plate was observed and they were the dominant cell type (Fig. 1). After 10–20 days, monolayers of epithelial-like cells were observed (Fig. 2).

CONCLUSIONS

Considerable progress has been made in establishing a thrips cell culture system for the study of tospoviruses. Even though cells can be maintained for up to 7 months, more work needs to be done to develop a continuous thrips cell line. In general the specific criteria necessary for thrips tissue cell line establishment are just beginning to be understood.

Successful development of many other primary cultures and insect cell lines (Adam and Sander, 1976; Chiu and Black, 1967; Mitsuhashi, 1989) has allowed the virus infection of several insect monolayers, which have proved to be invaluable in the study of the molecular biology of plant viruses (Hsu *et al.,* 1983). Insect tissue culture is a valuable tool in studies of virus acquisition and transmission, viral components, and the processes of virus replication within insect hosts and provides clues to the control of these diseases.

The development and research of thrips primary tissue cultures are the critical first steps necessary, which will provide the information needed to develop permanent cell lines. Furthermore, the development and use of these primary thrips cell cultures may provide the much-needed *in vivo* system for the study of tospoviruses. Studies on the cycle of *Tospovirus* replication and infection in these primary cell cultures should yield valuable information about the cellular interactions between these viruses and vectors.

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