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# EMBRYONIC TISSUE CULTURES OF PLANTHOPPER AND LEAFHOPPER VECTORS OF PLANT PATHOGENIC VIRUSES

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## Introduction

In recent years, tissue culture of insect vectors of plant pathogenic viruses has attracted the attention of investigators in the field of plant pathology and entomology. Several species of leafhoppers were used for *in vitro* cultivation (VAGO and FLANDRE 1963; HIRUMI and MARAMOROSCH 1964 a, b, c; MITSUHASHI and MARAMOROSCH 1964; MITSUHASHI 1965; CHIU, REDDY and BLACK 1966). CHIU and BLACK (1967) succeeded in establishment of cell lines from the embryonic culture of *Agallia constricta* VAN DUZEE, a vector of wound tumor virus. Preliminary success was obtained with the tissue culture of the pea aphid, *Acyrtosiphum pisum* (HARRISON), a vector of pea enation mosaic virus (TOKUMITSU and MARAMOROSCH, 1967).

The present report describes the first successful attempt to cultivate the embryonic tissues of a planthopper, *Laodelphax striatellus* FALLÉN, a vector of rice stripe, rice black-streaked dwarf and northern cereal mosaic viruses; two leafhoppers, *Inazuma dorsalis* MOTSCH., and *Nephotettix apicalis* MOTSCH., vectors of rice dwarf and rice transitory yellowing viruses. Embryonic tissues of these three insects have been maintained and proliferated growing cells *in vitro*.

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### Materials and Methods

The culture techniques described by MITSUHASHI and MARAMOROSCH (1964) for leafhopper tissue cultures were used in this experiment. The sitting drop technique (HIRUMI and MARAMOROSCH 1964 a, MITSUHASHI and MARAMOROSCH 1964) in microslide rings of 25 mm in diameter with cover glasses were employed in the present studies to maintain embryonic tissue of the insects, *L. striatellus*, *I. dorsalis*, and *N. apicalis*.

The basic media used were MITSUHASHI's medium for *Nephotittix cincticeps* Uhler tissue culture (MITSUHASHI 1965), the medium for *Agallia constricta* VAN DUZEE tissue culture (CHIU and BLACK 1967) and the NCM-4A (MITSUHASHI, personal communication) for the improved medium for *N. cincticeps*. The NCM-4A medium consists of 560 mg NaCl, 16 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 8 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 16 mg  $\text{NaHCO}_3$ , 400 mg glucose, 400 mg sucrose, 640 mg lactalbumin hydrolysate, 6.4 mg L-tryptophane, 1.6 mg L-cystein, 160 mg TC-yeastlate, 10 mg fetal bovine serum, 10 mg dihydrostreptomycin sulfate and distilled water to make 100 ml. Cell growth media were prepared by adding 4 ml of haemolymph of *Philosamia cynthia* PRYERI and crayfish *Procambarus clarkii* GIRARD to the basic media. The medium had a pH 6.5 without adjustment. The haemolymph from *P. cynthia* and crayfish was heated at 56°C for 30 min. before incorporation, and used after centrifugation at 2,000 rpm for 10 min. and clarification through Millipore filters having a pore diameter of 0.45  $\mu$ .

The eggs were collected 4 to 6 days after oviposition and surface sterilized in 70% ethanol for 10 seconds in a centrifuge tube, and then transferred into Maximow slides. Embryos were squeezed out through an opening in the chorion in Rinaldini's salt solution. After removing the yolk, the embryos were cut into several pieces. The embryonic tissues were trypsinized for 1 to 2 minutes at room temperature in 0.1 percent trypsin (Kanto Chemical Co. Inc.) in Rinaldini's salt solution. The trypsinized tissue fragments were transferred into a modified Ringer-Tyrode solution (CARLSON 1946) in a culture vessel in which they almost instantaneously attached to the glass

surface. The Ringer-Tyrode solution was then replaced by the culture medium that was changed several times.

The cultures were incubated at 27°C and the medium was changed once a week.

## Results

### 1) Embryonic tissue culture of *Laodelphax striatellus*.

i) Media for cultivation. In preliminary tests, five different kinds of culture media were used for the cultivation of embryonic tissues of *L. striatellus*. a) In MITSUHASHI's medium for *N. cincticeps*, cell migration was poor and no cell growth was observed, although contraction of the explanted tissues in this medium was active. b) When MITSUHASHI's medium was supplemented with haemolymph of *P. cynthia*, active cell migration and contraction of the tissues were observed within 24 hours after the culture was set up. However, no cell growth was observed in this medium. c) In MITSUHASHI's improved medium NCM-4A for *N. cincticeps* tissue culture, cell migration occurred after 24 hours, but no cell growth was obtained. d) When this third medium was supplemented with haemolymph of *P. cynthia*, active cell migration was observed without any contraction of the tissues, and cells started to grow to form a cell sheet around the explants. e) The fifth medium employed was the NCM-4A supplemented with haemolymph of crayfish. Poor cell migration took place and no contraction occurred in this medium.

Evidently, best cell growth was obtained in NCM-4A medium supplemented with haemolymph of *P. cynthia*. Therefore, further embryonic tissue cultures of *L. striatellus* were carried out by using this medium.

ii) Cell types. Six distinct cell types were observed in the embryonic tissue cultures of *L. striatellus*. Most predominant cells grown in this medium were epithelial cells forming compact cell sheets, migrating within 5 to 6 days after the fibroblast cells which started growing 24 hours after cultivation. They consisted of two types, the first and second types.

The first cell type was represented by small epithelial cells, which were polygonal in shape with dense cytoplasm around the nuclei, and the border line between each cell was not clearly delineated. No cytoplasmic granules or oil droplet were observed in these cells. Fig. 1 shows the cell sheet of such cells after 28 days of cultivation. The size of these cells was about 25  $\mu$  in diam. and nuclei were round in shape, approximately 7.5  $\mu$  in diam.

The second cell type was represented by epithelial cells with clear cell

border, free from cytoplasmic granules or oil droplets, with round nuclei surrounded by thin cytoplasmic layers. The border line of each cell was clearly delineated. The cells usually formed fairly compact cell sheets and thus each cell appeared polygonal in shape, having almost same size or being larger than that of the first type. They multiplied by active mitoses as shown in Fig. 2 and 3.

The third cell type consisted of vacuolated epithelial cells. They grew at the periphery of the cell sheets in the old cultures. The characteristic large vacuoles were abundant around the nuclei within the cytoplasm, as shown in Fig. 4. The size of the cells was about  $75\ \mu$  long,  $50\ \mu$  wide with nuclei of  $12.5\ \mu$  in diameter.

The fourth cell type was made of long fibroblast cells forming network structures. They migrated within 24 hours after the cultures were set up. The cells were slender and elongated, as shown in Fig. 5, having elliptical nuclei. The cells were about 150 to  $200\ \mu$  long,  $10\ \mu$  wide with nuclei  $7.5\ \mu$  in diameter. Mitoses were also observed.

The fifth and sixth cell types obtained were either smaller or larger wandering cells as shown in Fig. 6. They appeared usually within 48 hours after the culture was set up, and gradually increased in number after fifteen days. The small wandering cells appeared at the beginning almost round in shape, then developed dipolar or tripolar pseudopodia when they attached to the glass surface. Sometimes they expanded to form rough networks. The cells were free from cytoplasmic granules or droplets. Their average size was about  $17.5$  to  $30\ \mu$  in diam. Their nuclei were round, about  $10\ \mu$  in diam. The large wandering cells had round nuclei of about 15 to  $20\ \mu$  in diam. with very thin and widely spread cytoplasm and an average diameter of 100 to  $150\ \mu$ .

Hollow spherical vesicles were found around the explants. They swelled up by increasing in the number of the surface cells, as shown in Fig. 7.

iii) Cell growth. Although tissues attached well to the glass surface of the culture vessels irrespective of trypsinization, active cell migration was usually obtained from the trypsinized tissues. Cell migration from the explants occurred usually within 24 hours after the culture was set up. In most cases, fibroblast-like cells migrated first and formed network structures (Fig. 5). Migration began also in the wandering cells within 24 hours (Fig. 6).

On the fifth and sixth days, small epithelial cells and epithelial cells with a clear cell border started to migrate and continued growing. The hollow

spherical vesicles were sometimes formed on the explants in this medium after about six days and continue swelling up to enlarge (Fig. 7).

After fifteen days, most fibroblast cells (Fig. 5) formed networks and epithelial cells grew to form cell sheets. Mitoses were frequently observed within such growing epithelial cells, mostly at the periphery of the sheets. There were many individual wandering cells around the explants, but no further cell growth was obtained (Fig. 6).

After about 25 days, the epithelial cell sheets developed further, showing active mitoses. They sometimes included vacuolated epithelial cells (Fig. 4). Some of the epithelial cell outgrowth continued expanding for more than 100 days.

Attempt was made to transfer the original explants and the hollow spherical vesicles to another culture vessel. After being transferred, cell migration from the explants was considerably poorer and no mitosis was observed, although the tissues survived for additional four weeks.

## 2) Embryonic tissue cultures of *Inazuma dorsalis*.

i) Media for cultivation. In preliminary studies, three culture media were examined for embryonic tissue culture of *I. dorsalis*. a) MITSUHASHI's NCM-4A with *P. cynthia* haemolymph and b) without haemolymph. At the beginning, migration of fibroblast-like and epithelial cells occurred actively but the cells degenerated within 15 to 20 days of migration. c) Satisfactory cell growth was obtained in CHIU and BLACK's medium with lobster haemolymph for *A. constricta* tissue cultures. Thus further experiments were carried out with this medium.

ii) Cell types. Five distinct types of cells were recognized in the *I. dorsalis* tissue cultures. Most predominant cells were epithelial cells with a clear cell border, polygonal in shape, and forming compact cell sheets (Fig. 8). Their average size was about 25 to 30  $\mu$  in diam. with nuclei 10  $\mu$  in diam. They migrated within 48 hours after the culture was set up and grew actively, forming cell sheets. Frequent mitoses were observed in these cells. Elliptical cells sometimes formed cell sheets as shown in Fig. 9. The size of the cells was approximately 40 to 50  $\mu$  in diam., with nuclei 15  $\mu$  in diam. These cells developed by active mitotic cell division, clearly observable under the light microscope.

Two distinct types of fibroblast cells were identified. One was represented by long fibroblast cells of slender elongated shape (Fig. 10), about 75  $\mu$  in length, 5  $\mu$  in width, with elliptical nuclei of  $5 \times 10 \mu$ . The other cell type consisted of short fibroblast cells, with a diameter of about  $25 \times 5 \mu$ , and

nuclei about  $5\mu$  in diam. (Fig. 11).

Vacuolated giant wandering cells which included numerous large vacuoles around the nuclei, as large as  $75$  to  $125\mu$  in diam. and nuclei about  $25\mu$  in diam., migrated within 48 hours, increased in number, and accumulated around the explants after 10 days (Fig. 12). The movement of these cells was not active and they degenerated within 30 days.

In addition, characteristic canal-like tubules were observed, with cells arranged on both sides of the tubules (Fig. 13). Hollow spherical vesicles were also formed on the periphery of the explants.

iii) Cell growth. Active cell migration occurred within 48 hours after the cultures were set up. First of all, vacuolated giant wandering cells migrated, and then epithelial cells gradually developed within six days to form compact epithelial cell sheets around the explants. Frequent mitoses were observed at the periphery of these cell sheets. Contractive movement of the explants was active in this medium. Fibroblast cells migrated on the sixth day and started growing after 10 days. The hollow spherical vesicles were formed on the explants after about 10 days. Epithelial cells continued active outgrowth to form cell sheets for more than 60 days after migration began.

### 3) Embryonic tissue culture of *Nephotettix apicalis*.

i) Media for cultivation. Three kinds of culture media were used for the embryonic cultures of *N. apicalis*. In the medium of CHIU and BLACK without lobster haemolymph, cell migration occurred within 24 hours after the cultures were set up. However, the cell growth was very poor and the cells degenerated after 20 days. When the medium was supplemented with lobster haemolymph, cell migration occurred within 24 hours, but no cell outgrowth was observed. On the other hand, in the NCM-4A medium with no supplementation of insect and lobster haemolymph, remarkable cell migration and cell growth occurred. Thus this medium was used for the further cultivation.

ii) Cell types. Five cell types were identified in the cultures. Most predominant were epithelial cells with clear cell border, dense nuclei and thin cytoplasm, forming compact cell sheets. Their size was polygonal in shape, about  $17.5\mu$  in diam., with nuclei about  $12.5\mu$  in diam. (Fig. 14). These cells multiplied by mitoses.

As indicated in Fig. 15, tripolar mitoses were sometimes observed in the elliptical epithelial cells. The average size of the cells was  $120 \times 40\mu$  with round nuclei about  $30\mu$  in diam.

Giant epithelial cells were also found in this medium. They were some-

times binucleate (Fig. 16) and their average size was  $100 \times 75 \mu$ , with nuclei about  $10 \mu$  in diam.

Vacuolated large epithelial cells were irregular in shape including large vacuoles or droplets as shown in Fig. 17. Their average size was 50 to  $65 \mu$  in diam. and the nuclei about  $10 \mu$  in diam.

Only one type of fibroblast cells developed in this medium. The cells were short and their average size was  $37.5 \mu$  in length,  $6.2 \mu$  in width with elliptical nuclei about  $12.5 \times 6.2 \mu$ .

Hollow spherical vesicles (Fig. 19) were found in this medium. They swelled up as the number of cells increased through mitotic divisions.

iii) Cell growth. The epithelial cells with clear cell border started to migrate within 24 hours in this medium. Within 6 days, active cell out-growth was observed and compact cell sheets were formed, as shown in Fig. 14 and 15. Mitoses were frequently observed in these cells at the periphery of the sheets. Contractile movement of the explants was active. After 10 days, giant epithelial cells, vacuolated large epithelial cells and fibroblast cells developed and the epithelial cell sheets continued to grow actively. After about 20 to 30 days of *N. apicalis* tissue culture, the epithelial cells with clear cell border were most predominant. Hollow spherical vesicles were also formed. Vacuolated large epithelial cells usually degenerated within 30 days.

### Discussion and Conclusions

Primary culture of embryonic tissues of *L. striatellus*, *I. dorsalis* and *N. apicalis*, insect vectors of certain plant pathogenic viruses, were successfully carried out.

The best growth of *L. striatellus* was obtained in medium NCM-4A supplemented with haemolymph of *P. cynthia*, while *I. dorsalis* cells grew best in CHIU and BLACK's medium with lobster haemolymph. The addition of insect haemolymph or egg extract has been found to enhance cell growth in several earlier studies on insect tissue cultures (GRACE 1958, AIZAWA et al. 1961, TAKAMI 1963). Probably *P. cynthia* and lobster haemolymph have some essential components stimulating the growth of insect tissues. However, MITSUHASHI's NCM-4A medium for *N. apicalis* was found adequate for the cultivation of *N. apicalis* tissues without supplementation of haemolymph.

It was somewhat surprising to find that the three species of vectors required different compositions of tissue culture media for optimal cell proliferation, although BLACK (1969) suggested that the lobster haemolymph could

be replaced by fetal bovine serum in the *A. constricta* cultures.

Embryonic tissues of *L. striatellus* yielded fibroblast cells within 24 hours, afterwards epithelial cells started migrating. Wandering cells began to migrate within 48 hours from tissues of *I. dorsalis* and *N. apicalis*, while epithelial cells actively migrated within 24 hours from tissue cultures of these insects. Six embryonic cell types from *L. striatellus*, five cell types each from *I. dorsalis* and *N. apicalis* were recognized. Three types of epithelial cells, a fibroblast type and two wandering types of cells were obtained from *L. striatellus* tissues; two epithelial, two fibroblast and one wandering types of cells grew from *I. dorsalis*; four epithelial and one fibroblast types of cells from *N. apicalis* were identified. Most of the cell types were similar or identical with cell types described in other leafhopper tissue cultures such as cultures of *M. fascifrons* (HIRUMI and MARAMOROSCH 1964 c, MITSUHASHI and MARAMOROSCH 1964) and *N. cincticeps* (MITSUHASHI 1965). Small epithelial cells identical with Type II (HIRUMI and MARAMOROSCH 1964, c) and Type A (MITSUHASHI and MARAMOROSCH 1964) of *M. fascifrons*, and small epithelial cells of *N. cincticeps* (MITSUHASHI 1965) were obtained only in *L. striatellus* tissue cultures. Elliptical epithelial cells, similar to Type I and Type B epithelial cells (HIRUMI and MARAMOROSCH 1964 c, MITSUHASHI and MARAMOROSCH 1964) were common in *I. dorsalis* and *N. apicalis* cultures. The epithelial cells with clear cell border were actively growing in all three insect tissue cultures, which were described earlier in the leafhopper tissue cultures such as *M. fascifrons* (MITSUHASHI and MARAMOROSCH 1964) and *N. cincticeps* (MITSUHASHI 1965). Giant epithelial cells were obtained only in *N. apicalis* tissue cultures, which are similar to Type D from *M. fascifrons* (MITSUHASHI and MARAMOROSCH 1964) and giant epithelial cells in *N. cincticeps* tissue culture (MITSUHASHI 1965). Long fibroblast cells, identical with Type I and Type A from *M. fascifrons* by HIRUMI and MARAMOROSCH (1964, c) and MITSUHASHI and MARAMOROSCH (1964) respectively, were obtained in *L. striatellus* and *I. dorsalis* tissue cultures. Short fibroblast cells were observed in the tissue cultures of *I. dorsalis* and *N. apicalis*. Two types of wandering cells such as small and long wandering cells were obtained in the tissue culture of *L. striatellus*, which are similar to those described in *N. cincticeps* (MITSUHASHI 1965). Vacuolated giant wandering cells were characteristic in the tissue culture of *I. dorsalis*. The structure of the cells are similar to those of Type A wandering cells in *M. fascifrons* tissue culture (MITSUHASHI and MARAMOROSCH 1964). According to MITSUHASHI and MARAMOROSCH (1964), these cells are similar to large phagocytes in *M. fascifrons* (HIRUMI and MARAMOROSCH 1964 c). Hollow

spherical vesicles were formed on the explants from all three insect tissues. The cells which formed the vesicles seems to be identical with epithelial cells with clear cell border. This agrees with the assumption that the vesicles were formed by the cells which failed to attach to the glass surface, as pointed out by MITSUHASHI and MARAMOROSCH (1964). Canal like structures, similar to those of *M. fascifrons* tissue cultures (MITSUHASHI and MARAMOROSCH 1964) were found in cultures of *I. dorsalis* but not in those of the other two insects. The function of this structure is not known.

Since the insects used in this experiment are important vectors of certain plant pathogenic viruses such as rice stripe, rice black-streaked dwarf, northern cereal mosaic viruses for *L. striatellus*; rice dwarf virus for *I. dorsalis* and *N. apicalis*; rice transitory yellowing virus for *N. apicalis*. The *in vitro* tissue cultures may permit inoculation experiments with those plant viruses, as has been done by MITSUHASHI and NASU (1967). However detailed studies on virus-cell interactions will require monolayer cultures. Further attempts to obtain monolayer cultures and to establish cell lines are now under way.

### Summary

Embryonic tissues of three insect vectors of plant viruses, *Laodelphax striatellus* FALLÈN, *Inazuma dorsalis* MOTSCH. and *Nephotettix apicalis* MOTSCH. were cultivated for over 40 days *in vitro*.

Satisfactory cell growth of *L. striatellus* was obtained in medium NCM-4A (MITSUHASHI's improved medium for *N. cincticeps*, personal communication) supplemented with haemolymph of *Philosamia cynthia* PRYERI. The medium used for *Agallia constricta* by CHIU and BLACK supplemented with haemolymph of lobster was suitable for *I. dorsalis*. Medium NCM-4A without any insect or lobster haemolymph supplementation was adequate for *N. apicalis*.

Six distinct cell types were identified from the embryonic cultures of *L. striatellus*, five from *I. dorsalis*, and five from *N. cincticeps*. Most of the cell types were similar or identical with some of the cell types described earlier from *M. fascifrons* and *N. cincticeps* tissue cultures.

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### Explanation of Figures

#### PLATE I

- Fig. 1.** A cell sheet of epithelial cells in an embryonic tissue culture of *Laodelphax striatellus* FALLÉN after 28 days. Dark phase contrast.  $\times 400$
- Fig. 2-3.** Epithelial cells of clear cell border from *L. striatellus* tissue cultures after 28 days. Arrows show a mitotic cell division. Dark phase contrast.  $\times 900$
- Fig. 4.** Vacuolated epithelial cells from a *L. striatellus* tissue culture at 26 days. Dark phase contrast.  $\times 600$
- Fig. 5.** Long fibroblast cells obtained in an embryonic tissue culture of

*L. striatellus* at 40 days. Dark phase contrast.  $\times 800$ .

- Fig. 6.** Two types of wandering cells from a *L. striatellus* tissue culture at 18 days. Small (small arrows) and large (large arrows) wandering cells are shown. Dark phase contrast.  $\times 300$ .
- Fig. 7.** Hollow spherical vesicles from a *L. striatellus* tissue culture. An arrow shows a dividing cell in metaphase. Dark phase contrast.  $\times 1000$

#### PLATE II

- Fig. 8.** A cell sheet consisting of epithelial cells with a clear cell border in an embryonic tissue culture of *Inazuma dorsalis* MOTCHULSKY on the 6th day of cultivation. Dark phase contrast.  $\times 600$
- Fig. 9.** Elliptical epithelial cells from an *I. dorsalis* tissue culture at 6 days. Arrows indicate cell divisions. Dark phase contrast.  $\times 1200$
- Fig. 10.** Long fibroblast cells from *I. dorsalis* tissue culture at 20 days. Dark phase contrast.  $\times 600$
- Fig. 11.** Short fibroblast cells from an *I. dorsalis* tissue culture at 22 days. Dark phase contrast.  $\times 600$
- Fig. 12.** Vacuolated giant wandering cells from *I. dorsalis* tissue culture at 6 days. Dark phase contrast.  $\times 600$
- Fig. 13.** Canal like structure surrounded on both sides by cells, in a tissue culture of *I. dorsalis* at 24 days. Dark phase contrast  $\times 600$

#### PLATE III

- Fig. 14.** A sheet of epithelial cells with clear cell border from a *Nephotettix apicalis* MOTCHULSKY tissue culture at 25 days. Dark phase contrast.  $\times 500$
- Fig. 15.** Elliptical epithelial cells showing tripolar cell division in the tissue culture of *N. apicalis* at 25 days. Dark phase contrast.  $\times 1200$
- Fig. 16.** Giant epithelial cells in a *N. apicalis* tissue culture at 21 days. Note that some of the cells are binucleate. Dark phase contrast.  $\times 800$
- Fig. 17.** Vacuolated large epithelial cells in a *N. apicalis* tissue culture at 16 days. Dark phase contrast.  $\times 800$
- Fig. 18.** Short fibroblast cells from a tissue culture of *N. apicalis* at 18 days. Dark phase contrast.  $\times 800$ .
- Fig. 19.** Two hollow spherical vesicles which consist of epithelial cells with clear cell border, obtained in a *N. apicalis* tissue culture at 16 days. Dark phase contrast.  $\times 800$





