In Vitro Cultivation of the Embryonic Tissues of the Green Rice Leafhopper, *Nephotettix cincticeps* UHLER (Homoptera: Cicadellidae)

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Leafhopper tissue culture has recently been given special attention by investigators in the field of plant virology and entomology. There are many leafhopper species which are known as vectors of certain plant pathogenic viruses, and the cultivation of leafhopper vector tissues may provide a useful tool for the study of plant virus-insect vector cell interrelationships in vitro.

In general, insect tissue cultures have so far been made successfully only on lepidopterous ovariole tissues (GAW, LIU and ZIA, 1959; GRACE, 1962; GRACE and DAY, 1963), and very few other insect tissues have been cultivated up to the present. In leafhoppers, VAGO and FLANDRE (1963) reported that growing cells were obtained from some organs, and MITSUHASHI and MARAMOROSCH (1964) obtained growing cells from embryonic, nymphal and imaginal tissues of three species, *Macrosteles fascifrons* Stål, *Agallia constricta* (VAN DUZEE) and *Dalbulus maidis* Del. & W., which are vectors of aster yellows virus, wound tumor virus and corn stunt virus respectively. In the present study, attempts were made to cultivate the embryonic tissues of the green rice leafhopper, *Nephotettix cincticeps* UHLER, which is known as the vector of rice dwarf virus and also rice yellow dwarf virus.

**MATERIALS AND METHODS**

A stock colony of *N. cincticeps* was maintained in glass tubes containing rice seedlings. Mated female insects were confined to small rice seedlings in glass tubes and transferred every two days, so as to provide embryos at about the same stage of growth. Eggs were excised from the leaf tissues under a binocular dissecting microscope by means of needles, and put into a glass tube filled with water. The eggs were then surface-sterilized by placing in 70 per cent ethyl alcohol for 1 minute. The following procedure for setting up the cultures was the same as that employed in the tissue culture of three other leafhopper species (MITSUHASHI and MARAMOROSCH, 1964). The sitting drop culture method was used in the present study. Culture vessels consisted of cover glasses and micro-slide rings (MITSUHASHI and MARAMOROSCH, 1964). The culture medium consisted of 280 mg NaCl; 8 mg KCl; 8 mg CaCl₂·2H₂O; 4 mg MgCl₂·6H₂O; 8 mg NaH₂PO₄; 5 mg NaHCO₃; 160 mg D-glucose; 520 mg lactalbumin hydrolyzate; 520 mg Bacto-peptone; 200 mg TC-yeastolate; 20 ml TC-199 medium; 20 ml fetal bovine serum; 5 mg streptomycin;  

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and distilled water added to make 100 ml. The pH was adjusted to 6.5 with KOH. The fetal bovine serum and the TC-199 medium were purchased from Microbiological Associates, Inc., Bethesda, Md., U.S.A., the TC-yeastolate and the Bacto-peptone from Difco Detroit, Mich., U.S.A., the lactalbumin hydrolyzate from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A., and the dihydrostreptomycin sulfate from Takeda Chemical Industries Ltd., Osaka, Japan. All glassware was sterilized by dry heat at 180°C for 2 hours and the media as well as the salt solutions were sterilized by passage through Seitz or fritted glass filters. Observations on the status of growing cells were made with a Nikon MD inverted phase contrast microscope. Microphotographs were taken with this microscope. The movement of cells was recorded by time-lapse cinematography with the same microscope. Some cultures were permanently preserved by fixing with BOUIN's fluid and staining with DELAFIELD's haematoxylin for histological examination. Various histochemical techniques were also applied when necessary.

RESULTS

Cells grown from embryonic tissues At 25°C cell migration from the explanted embryonic tissues occurred within 24 hours after the culture was set up. Usually fibroblasts appeared before epithelial cells. The former formed networks while the latter formed cell sheets. In addition, some types of wandering cells migrated from the explants. They never formed a network or cell sheet but spread around the explants by amoeboid movement. Of all the types of cells, epithelial cells were most abundant. The epithelial cells were further divided into five types, distinguished from each other by their shape, size and behavior.

(A) Small epithelial cells. They were the most common type of epithelial cells and were identical with type A epithelial cells obtained in the tissue culture of *M. fascifrons* (MITSUSHASHI and MARAMOROSCH, 1964). The diameters of the cell and the nucleus were 20 μ and 10 μ respectively (Plate I, Fig. 1). These cells multiplied rapidly forming a typical cell sheet around the explant (Plate I, Fig. 2). Mitoses were observed frequently in these cells.

(B) Elliptical epithelial cells. They were identical with the cells described as type B epithelial cells in *M. fascifrons* tissue culture. The cells were elliptical and their nuclei were round. Sometimes these cells were binucleate (Plate I, Fig. 3). The long and the short diameters of the cells were 75 μ and 25 μ respectively, and the diameter of the nucleus was 20 μ. These cells were mainly distributed at the periphery of the growing cell sheets and also formed a typical cell sheet (Plate I, Fig. 4). Mitoses were common in these cells.

(C) Epithelial cells with clearly delineated cell border. These cells were identical with type C epithelial cells observed in *M. fascifrons* tissue culture. The sizes of the cell and the nucleus were 50 μ and 20 μ respectively. These cells had rounded nuclei and very thin cytoplasm. They formed a cell sheet mostly at the periphery of the growing cell sheet, and multiplied by mitoses (Plate I, Fig. 5).

(D) Giant epithelial cells. The cells of this type were identical with type D epithelial cells of *M. fascifrons*. The sizes of cells
varied between 100 $\mu$ and 200 $\mu$. Usually their nuclei were round (Plate I, Fig. 6), but sometimes irregular (Plate I, Fig. 7). The average size of nuclei was 20 $\mu$. Polyploid cells were common in these giant cells, and binucleate cells were often seen (Plate I, Fig. 8). These cells were distributed on the outside of the growing cell sheet, and rarely formed cell sheets. They multiplied by mitoses.

(E) Epithelial cells with blister-like structure in the cytoplasm. These cells were similar to type E epithelial cells obtained from the culture of alimentary tracts of *M. fascifrons*. These cells were found primarily in old cultures. The sizes of the cells and nuclei were 80 $\mu$ and 15 $\mu$ in diameter. Their cytoplasm swelled up to form a blister-like structure probably due to pinocytosis (Plate II, Fig. 9). No mitoses were seen in these cells.

Fibroblast type cells obtained from the embryonic tissues of *N. cincticeps* were slender spindle shaped cells and were identical with type A fibroblasts obtained in tissue culture of *M. fascifrons*. The sizes of cell and nucleus were 30 $\mu$ and 8 $\mu$ respectively (Plate II, Fig. 10). These cells sometimes formed networks (Plate II, Fig. 11), and multiplied by mitoses. This type of cell usually came out from the explants prior to any other and was the most common type during the initial period of cultivation. Later, however, the epithelial cells predominated. Cells similar to type B fibroblasts of *M. fascifrons* culture were not seen in the embryonic tissue culture of *N. cincticeps*.

Three types of wandering cells were obtained.

(A) Small wandering cells. Usually these cells came out from the explants after the formation of epithelial cell sheets. While these cells were migrating on the epithelial cell sheets, they were almost spherical in shape, whereas they became flat and developed pseudopodia when they came into contact with the glass surface (Plate II, Figs. 12, 13). The average sizes of the cell and the nucleus were 15 $\mu$ and 5 $\mu$ respectively. These cells migrated outside the growing cell sheet by amoeboid movement. They multiplied by mitoses.

(B) Large wandering cells. These cells were distributed outside the growing cell sheet. The cytoplasm was very thin and spread widely. Usually the cytoplasm contained some liquid droplets which may have been formed as the result of pinocytosis, and sometimes it also contained extrinsic materials which may have been taken into the cytoplasm by phagocytosis (Plate II, Fig. 14). Sometimes cells were binucleate. The diameters of the cell and the nucleus were 100 $\mu$ and 15 $\mu$ respectively. Mitoses were seen in these cells, but not frequently.

(C) Giant wandering cells. These cells were characterized by a very thinly spread cytoplasm within which was a radially streaked cytoplasmic network (Plate II, Fig. 15). The average sizes of the cell and the nucleus were 180 $\mu$ and 20 $\mu$ in diameter respectively. These cells showed amoeboid movement, but the movement was not very active. No mitoses could be observed in these cells. Cells similar to type A and type B wandering cells obtained in the tissue culture of *M. fascifrons* were not observed in the embryonic tissue culture of *N. cincticeps*. Cell multiplication by mitoses Following
the initiation of cell migration, the migrated cells started to multiply by mitoses. Usually mitoses occurred frequently at the periphery of the growing cell sheet. Since cells which multiplied along the glass surface became very flat, most of the cell divisions occurred parallel to the glass surface. Cell divisions vertical to the glass surface rarely occurred. The side view of mitoses was, therefore, seen readily. The course of the mitoses could be followed easily after late prophase. The following mitotic stages are described according to CARLSON (1946). Late prophase was recognized when the chromosomes were scattered in the nucleus (Plate III, Figs. 17, 18). Prophase lasted 30 minutes or longer at 25°C. At the end of prophase, the nuclear membrane disappeared almost instantaneously (Plate III, Fig. 19), and chromosomes began to move into the equatorial plane (Plate III, Figs. 20~22). From late prophase to metaphase, the cell appeared to shrink and gradually increased in thickness. Flatly spread cytoplasm moved to the center of the cell leaving a fiber-like cytoplasmic structure around the cell membrane (Plate III, Figs. 18~24). The time taken from the disappearance of the nuclear membrane to the completion of chromosome arrangement in the equatorial plane varied from 10 minutes to 150 minutes at 25°C. In polyploid cells, the chromosomes took a much longer time to complete their arrangement than in diploid cells. At metaphase the chromosomes in the equatorial plane were recognized as a short straight line (Plate III, Figs. 23, 24). The duration of metaphase varied from 10 minutes to 60 minutes at 25°C, but once the chromosomes started to divide, anaphase was completed within 10 minutes (Plate III, Figs. 25~29). At telophase the daughter cells gradually reduced their thickness and became flat by spreading their cytoplasm (Plate III, Figs. 30, 31). The nuclear membrane reformed usually 30 minutes after the cell was completely divided (Plate III, Fig. 31). Sometimes, the cells did not divide at anaphase and this resulted in the production of binucleate cells. This type of mitosis was often observed in type B epithelial cells.

When mitoses occurred in binucleate cells, both nuclei changed simultaneously. Usually the formation of chromosomes and the breakdown of the nuclear membrane occurred at the same time in both nuclei, but sometimes one nucleus lost its membrane a few minutes earlier than the other nucleus. At prometaphase, chromosomes from the two nuclei came together and moved into an equatorial plane. But, chromosomes from the two nuclei occasionally moved separately and lined up in two separate planes in early prometaphase. These chromosomes in separate planes later united in an equatorial plane. Anaphase and telophase occurred normally, and finally two cells each with a nucleus were produced.

Multipolar, most often tripolar, mitoses were occasionally observed, especially in the epithelial cells. The time required for the completion of these types of cell division was about the same as that for normal mitoses. Multipolar mitoses could be followed easily after metaphase (Plate IV, Figs. 32~39), but they could not be predicted before metaphase.

Cell multiplication by mitoses continued throughout the cultivation period of over 4 months, the medium being changed once a week. In old culture, the developed cell
sheets from different explants met each other and covered a large area of the glass surface. Although mitoses were frequently observed at the periphery of the growing cell sheets, the cells near by the explants also survived for a long period and sometimes mitoses were observed even after 4 months.

**Change of the explant** During the cultivation of embryonic tissues, the explants underwent some morphological changes. When the culture was set up, the explants still retained a general outline of embryo fragments, but with advancing cultivation, they gradually lost their original shape owing to continuous cell migration and histolysis. Explants became dark in color in old cultures, due to the formation of a melanin-like substance. This pigment formation did not interfere with the growth of cells. The explants also formed internal hollow spherical vesicles (Plate II, Fig. 16). The vesicles usually appeared 3 or 4 days after the culture was set up. In the early stages of vesicle formation, they consisted of a mono-layer of cells. The vesicles continued to swell up and became larger and larger. They were filled with liquid, probably from the medium that surrounded them. The vesicles, sometimes collapsed when the medium was being changed. The collapsed vesicles sometimes swelled up again, healing the lesion over, and continued to grow. Occasionally collapsed vesicles became attached to the glass surface and produced cells that proliferated around the vesicles. Embryos of *N. cincticeps* before blastokinesis did not show any movement when they were taken out of their chorions, whereas the embryo fragments explanted at this stage usually began to contract 2 or 3 days after the culture was set up. The interval and the intensity of the contraction movement were different in different cases. Sometimes the developed cell sheets were also moved by the contractions of the explants. Contraction movement of the explants lasted for almost the entire period of cultivation.

**DISCUSSION**

Primary cultures of *N. cincticeps* embryonic tissues were carried out successfully. The growth of the cells from embryonic tissues of this leafhopper occurred almost in the same manner as that in other leafhopper species (Mitsushashi and Maramorosch, 1964). In the process of setting up the cultures, the use of mercuric chloride for the surface-sterilization of eggs was avoided, because the egg of *N. cincticeps* had been found to be very sensitive to such treatment (Mitsushashi, 1965). If the eggs were treated with 0.1 per cent mercuric chloride for 5 minutes, the embryos were severely affected and from such embryos, only a few fibroblast-like cells migrated in the beginning of cultivation and there was no migration of epithelial cells at all. Such an effect of surface-sterilization of eggs on the growth of cells was not observed in the embryonic tissue cultures of other leafhopper species (Mitsushashi and Maramorosch, 1964).

Nine types of cells were obtained from the embryonic tissues of *N. cincticeps*. The five types of epithelial cells already described in the *M. fascifrons* tissue culture (Mitsushashi and Maramorosch, 1954) were all observed in the *N. cincticeps* tissue culture. In the present study, only one type of fibroblast was obtained, and the large fiber-like cells described as type B fibroblast in the *M.*
fascifrons tissue culture were not observed. The three types of wandering cells obtained in the present study were all different from those obtained in M. fascifrons tissue culture. The origin of these types of cells could not be determined, although the cell types were easily distinguished from each other by their shape, size and behavior.

Development in the explanted embryonic tissues occurred at both the cellular and tissue levels. At the cellular level, various types of cells migrated from the explants and they multiplied by mitoses. Mitoses were very common at the periphery of the growing cell sheet. Usually mitosis was accompanied by cell division, but sometimes mitosis was completed without cell division, resulting in the production of binucleate cells. Binucleate cells were common in the large epithelial cells and the large wandering cells, but they were scarcely found in the small epithelial cells, small wandering cells and fibroblasts. Multipolar mitoses were sometimes observed. Tripolar mitoses and tetrapolar mitoses were easily recognized by the Y-and X-shaped arrangement of chromosomes at metaphase. Stillwell (1947) reported that the frequency of multipolar mitoses in the heart muscle cells of chick embryos grown in vitro increased when the cells were exposed to high temperature. The same was true in N. cincticeps embryonic tissue cultures. When the cultures were maintained at 32°C, tripolar mitoses could often be observed. Multipolar mitoses occurred both in the small and large epithelial cells, but not in the fibroblast and wandering cells. In the sitting culture of leafhopper tissues, cell divisions occurred almost always parallel to the glass surface. The polar view of mitoses was, therefore, rarely obtained by this culture method. In the course of the present study, mitotic figures of leafhopper cells previously incorrectly described as the polar view of metaphase (Mitsushashi and Maramorosch, 1964) were identified as the side view of late prophase as illustrated in Plate III.

At the tissue level, the explants formed hollow spherical vesicles. This vesicle formation seemed to be one of the ways in which cells multiplied without keeping contact with the glass surface. However, the cells usually multiplied along the glass surface. Cells which were not in contact with the glass surface, could multiply on the surface of the spherical vesicles. The size of vesicles increased as the cells multiplied. When the cells on the surface of the spherical vesicles happened to come into contact with the glass surface after accidental collapse of the vesicles, they began to migrate and multiplied by mitoses along the glass surface. Similar vesicle formation of explants has been reported in cockroach tissue culture (Larsen, 1964; Marks and Reinecke, 1964), and leafhopper tissue culture (Mitsushashi and Maramorosch, 1964). Explants seem to play some role in cell growth, for, cells from explants, after migration and multiplication, began to degenerate when the explants were removed from the developed cell sheets.

In the present study, subculturing was not tried, but the epithelial cell sheets grew large enough for use in virus inoculation experiments. However, the establishment of cell lines by subculturing leafhopper tissues and the application of such cultured cells to the study of virus multiplication is the
ultimate goal of this work.

SUMMARY

Embryonic tissues of green rice leafhopper *Nephotettix cincticeps* UHLER were cultivated for over 4 months *in vitro*. Development in the explanted embryonic tissues occurred at both the cellular and tissue levels. Nine types of cells, 5 of epithelial cells, 1 fibroblast and 3 wandering cells, grew from explanted tissues. Epithelial cells formed cell sheets, and fibroblasts formed networks. Wandering cells spread widely outside the epithelial cell sheets. Mitoses were frequently observed at the periphery of growing cell sheets. Multipolar mitoses were occasionally observed. Explanted tissues showed contraction movements during the entire period of cultivation. Considerable morphological changes including the formation of hollow spherical vesicles, occurred in the explanted tissues during cultivation.

REFERENCES


EXPLANATION OF PLATES

Plate I. *Nephotettix cincticeps* embryonic tissue culture. Bright phase contrast.

Fig. 1. Type A epithelial cells (X 350).
Fig. 2. A cell sheet of the type A epithelial cells (X 100).
Fig. 3. Type B epithelial cells (X 350).
Fig. 4. A cell sheet of the type B epithelial cells (X 100).
Fig. 5. Type C epithelial cells (X 350).
Fig. 6. Type D epithelial cells with round nucleus (X 350).
Fig. 7. Type D epithelial cell with irregular nucleus (X 350).
Fig. 8. Binucleate type D epithelial cell (X 350).

Plate II. *Nephotettix cincticeps* embryonic tissue culture. Bright phase contrast.

Fig. 9. Type E epithelial cell (X 350).
Fig. 10. Fibroblasts (X 350).
Fig. 11. Network of fibroblasts (X 100).

Fig. 12. Migrating small wandering cells on a epithelial cell sheet (white spots around the explant) and the same type of cells with pseudopodia developed after attachment to the glass surface (left) (X 100).

Fig. 13. The same type of wandering cells as in Fig. 12 (X 350).

Fig. 14. Large wandering cells (X 350).

Fig. 15. Very large wandering cells (X 350).

Fig. 16. Hollow spherical vesicles formed in an explanted tissue (X 100).

Plate III. Side view of normal mitosis observed in the type B epithelial cell. Dark phase contrast (X 400).

Figs. 17 and 18. Late prophase.
Figs. 19~22. Prometaphase.
Figs. 23 and 24. Metaphase.
Figs. 25~29. Anaphase.
Figs. 30 and 31. Telophase.

Plate IV. Side view of tripolar mitosis observed in the type B epithelial cell. Dark phase contrast (X 700).

Fig. 32. Metaphase.
Figs. 33~36. Anaphase.
Figs. 37~39. Telophase.
doi組織（in vitro）で4か月以上培養することができた。移植した胚が組織片から9種の異なった型の細胞が周囲へ移住し増殖した。上皮細胞はcell sheetを形成し、境界性細胞は膜状に広がった。また、上皮細胞のcell sheetの外側では多数の遊離細胞が増殖した。生長するcell sheetの外膜では盛んに有核分裂が行なわれた。正常の有核分裂のほか、しばしばその有核分裂がみられた。一方移植片全体を培養中なのか、形態の変化を示した。移植片は中空で球状の組織を形成し、それらは初期においては単一細胞層からなっていた。移植片は培養全期間を通じて収縮運動を続けた。

新刊紹介

ダニ類—その分類・生態・防除・佐々学書（1965）、B刊、486頁、東京大学出版会発行、5,000円

数年前、わが国のダニ学がようやく発展のきざしを見出しはじめた頃、東京大学生物部教授の佐々学さんが研究室にダニ学を学ばせたという人がそれなく集まって、ディスカッションをしたり、所属不明のダニを持ちよっては皆で調べたりする機会がしばしば持たれていた。その当時の佐々教授は、米国で催された第1回国際ダニ学学会に出席して帰国されたばかりの興奮を抑え、その頃すでに“ダニ学”出版の構想が練られていたように思われる。ところが幸運に恵まれ、半ばあきらかつつ申請した文部省の出版助成金が通り、急速に出版計画が具体的に迫り、全国に在するダニ研究者がこれに協力する運びとなった。

執筆者は合計13人、理学・医学・薬学・農学の各分野の出身者が当たられた。各執筆者の主な分担箇所は以下の通りである。（五十音順）佐藤淳一（ササダニ類）、浅沼晴（ヤドリダニ類、マダニ類、伝染病媒介虫としてのダニ類）、石井敬一郎（植物寄生虫に対するダニ類）、伊戸泰博（ホコリダニ類のその被害）、今村泰二（ミズダニ類）、内田亨（序、系統的配置、ミズダニ類補遺）、江原昭三（植物寄生ダニ類の分類と生態、ハダニ類およびフシダニ類の被害とその防除）、川島健治郎（タカラダニ上科）、熊田信夫（ツツガムシ科）、佐々学（形態と分類、生活史と習性、多気門類、ヤドリダニ類、前気門上図、ケアダニ類概説、ツツガムシ科を除くケアダニ類、ヒゼンダニ類、人体外部寄生虫としてのダニ類、伝染病媒介虫介としてのダニ類、食品・薬品に発生するダニ類の被害と被害の問題、私たちが、村木猛（衛生用殺ダニ剤の応用）、福井正信（家畜害虫としてのダニ類、衛生用殺ダニ剤の応用）、三浦昭子（実験方法および実験法—共著）のような実験者別に紹介すると、はなはだ難儀とした内容で論文集のような感を受けるが、実際には第1編総論、第2編各論、第3編応用と整電配列を示している。第1編ではどのダニのグループの研究者がもれず通すべき事項、すなわちダニ類の系統的位置、一般構造、分類体系、発育史や食性、採集、保存、標本作製、検査および同定の方法、それに飼育法などについてまとめられている。第2編は検索表と解説を主体とし、一世紀ものものを対称し、日本産のものについては解説の程度が詳しくないであろう。応用上あまり重要でないと考えられるグループについても最低限科までつけとめられるように構成され、医学・農学方面で重要視されるグループについてはややままたはその他の検索や解説が用意されている。このような点で理学書としてみた場合には記述に精粋の差があるが、応用で活用される書物としては弾力性に富んだ便利な編集方針といえよう。各所に挿入された300以上の豊かな図が理解を助け、読めているにも秀しい。更に第3編では一般にもなじみ深い衛生害虫とし

（科学博物館）