Quick Detection of Rice Dwarf Virus Infection Employing Its Vector Cell Monolayers

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Rice viruses, such as dwarf,[5] gall dwarf[14] and transitory yellowing,[4] are known to be transmitted by their leafhopper vectors to host plants, but not mechanically transmissible by any other methods. The infectivity of these viruses has been assayed only by artificial injection of small quantities of virus suspensions into abdomens of the leafhoppers.[11] However, this method required a long period, about 45–60 days, before final results were obtained. Therefore, the studies of these viruses were not advanced compared with that of sap-transmitted plant viruses.

These viruses are shown to be propagative in their insect vectors as well as in plant hosts, following Fukushi's discovery[6] in 1940. From this point of view, one can expect the establishment of monolayer cell cultures of the insect vectors to be an effective new experimental system. Chiu and Black[2] first succeeded in culturing cell monolayers of *Agallia constricta*, a vector of clover wound tumor[1] viruses, and demonstrated infection of the insect cell monolayers with virus.

The present paper describes cell cultures derived from *Nephotettix cincticeps* (NC) and *N. nigropictus* (NN), leafhopper vectors of rice dwarf (RDV) and rice gall dwarf (RGDV) viruses in field, respectively, and the inoculation method of RDV to the cultured cell monolayers.

Monolayer cell cultures of leafhopper vectors

Embryonic fragments, dissected from eggs of NC and NN leafhoppers, were used as primary tissue culture explants. Primary cultures and sub-cultures were carried out by the method of the preceding report.[9] Monolayered cells were usually composed of 2 types of epithelial cells. The majority were spindle shaped cells, 7–10 μ wide and 15–60 μ long. The minority were round shaped cells, 6–20 μ in diameter. Liu and Black's medium,[12] slightly modified, was used as the growth medium in the experiments (Table 1). When these cells were cultured and trans-

Table 1. Medium composition for *Nephotettix* leafhopper cells

1.	Schneider's Drosophila medium (revised)	500 m*l*
2.	Medium 199 (10X concentrate) with Hanks' salt and glutamine, without sodium bicarbonate	50 m*l*
3.	Medium CMRL 1066 with glutamine	25 m*l*
4.	Fetal bovine serum*	150 m*l*
5.	0.05 M histidine solution[13]	500 m*l*
6.	Penicillin G potassium	120,000 unit
7.	Streptomycin sulfate	120 mg
8.	Neomycin sulfate (10,000 μg/m*l*)	6 m*l*
9.	Fungizone (250 μg/m*l*)	4 m*l*

Total volume of the medium was about 1,240 m*l*, and the pH value of the medium was adjusted to 6.50–6.60 with 2N HCl.
* Heat treatment at 56°C for 30 min

ferred to new flasks, eventually 6 NC and 4 NN cell lines were established, and these have been passed through 85–147 and 81–121 subcultures respectively, at intervals of 4–10 days (Plate 1a & 1b, Table 2).

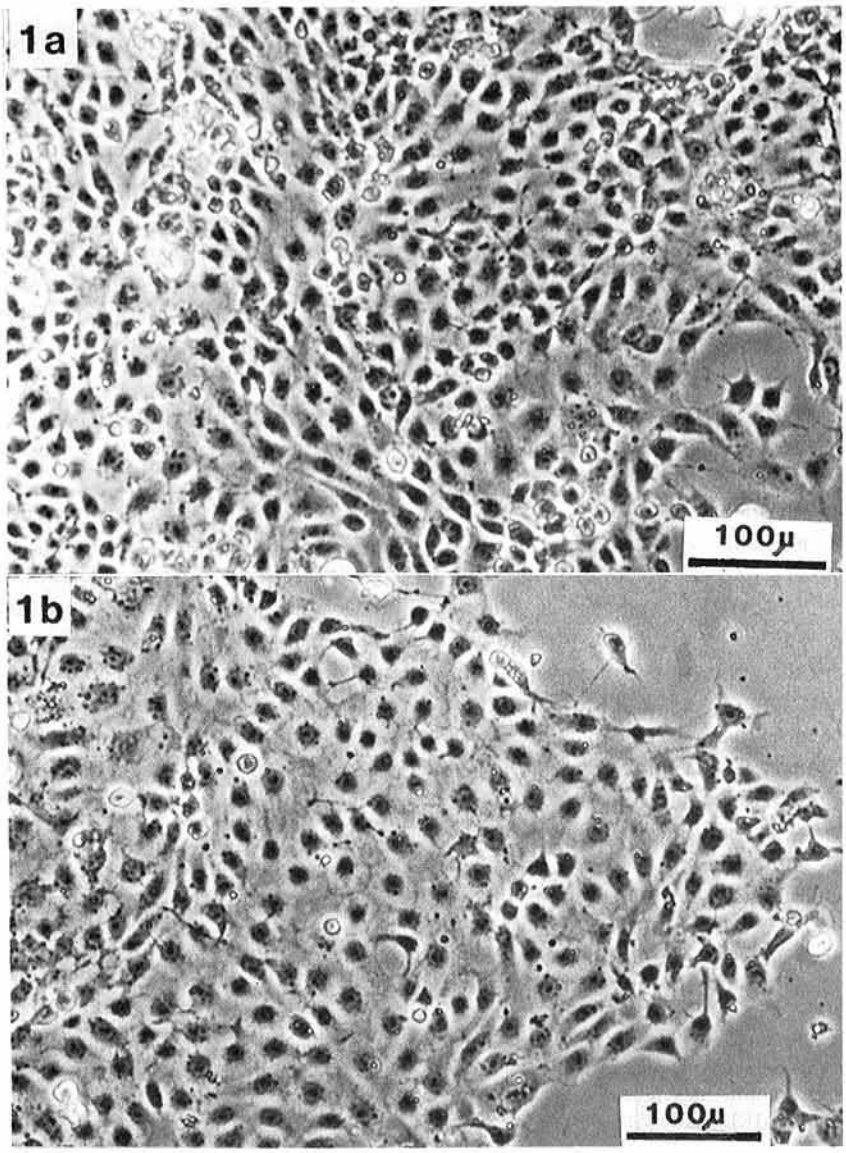

Plate 1. 1a: A portion of a monolayer culture of the NC-24 cell line grown in a disposable flask for 96 hr
1b: A portion of a monolayer culture of the NN-7 cell line grown in a disposable flask for 96 hr

Table 2. Establishment of some cell lines of *Nephotettix cincticeps* (NC) and *N. nigropictus* (NN)

Cell line	Date that set up primary culture	No. of employed eggs	Ages of eggs	Max. no. of passages*
NC-15	April 27, '78	30 eggs	8-10 days	93 passages
NC-19	Jan. 24, '79	40	8-11	85
NC-20	Feb. 14, '79	24	9-11	94
NC-21	Feb. 15, '79	40	7-10	93
NC-24	Feb. 28, '79	40	7- 9	147
NC-25	March 2, '79	40	6- 9	140
NN- 1	April 29, '80	36 eggs	8-10 days	81 passages
NN- 2	April 30, '80	25	5- 9	121
NN- 5	May 9, '80	36	7- 9	109
NN- 7	May 14, '80	27	6- 8	108

* Maximum number of passage in each cell line was checked on Oct. 30, 1984.

Inoculation of virus to cell monolayers of leafhopper vectors

1) Preparation of inoculum

All the established cell lines of NC and NN were subjected to inoculation with virus extracts from infected leaves of rice plants. The infected leaves were surface-sterilized with 70% ethanol for 3 min and then rinsed with sterile distilled water. The sterilized leaves were ground with 4 times the leaf weight of phosphate buffer solution (0.1 M, pH 7.3). The slurry was then clarified in a Hitachi 20PR centrifuge at 1,470 × *g* for 10 min. The supernatant was used as a source of inoculum. Further dilution of the inoculum was made in a solution containing 0.1 M histidine and 0.01 M $MgCl_2$ (His-$MgCl_2$, pH 6.0).[10]

2) Preparation of monolayers

Cell monolayers for infectivity assay were grown on coverslips of 15 mm diameter, previously washed in ethanol in an ultrasonicator. About 2–3 days after subculturing, cells were harvested from culture flasks, and sedimented by low speed centrifugation (about 250 × *g*). The cell pellets were resuspended in growth medium to give a dilution of about 3.5–5.5 × 10^6 cells/m*l* and thoroughly dispersed. Cell suspension of 0.12 m*l* were spread over the entire area of each of two or three coverslips in a plastic dish (60 mm in diameter) enclosed in a sealable glass dish sealed with parafilm M (American Can Company, Greenwich, CT, U.S.A.). The freshly seeded coverslips were allowed to remain undisturbed on a lab. bench for a minimum period of 3 hr to allow cell attachment before removal to a 28°C incubator. The cell monolayers on coverslips were used for virus inoculation less than 48 hr after seeding.

3) Virus inoculation and incubation of inoculated cell monolayers

Confluent cell monolayers on coverslips were used for infectivity assay. They were washed twice with His-$MgCl_2$ solution, and then inoculated with 0.05 m*l* of inoculum. Inoculated monolayers were incubated for 1–3 hr at 28°C to accomplish inoculation. At the end of the inoculation period, the inoculum was removed, the monolayers were washed twice with growth medium and then each coverslip was covered with about 0.12–0.2 m*l* of the medium. Inoculated monolayers were then incubated at 28°C for 40–48 hr.

4) Detection of virus infection

The direct fluorescent antibody technique was used for detection of virus infections. The cell monolayers on coverslips were washed by dipping in a beaker of phosphate buffered saline solution (PBS, pH 7.3) containing 0.01 M phosphate and 0.15 M NaCl. They were

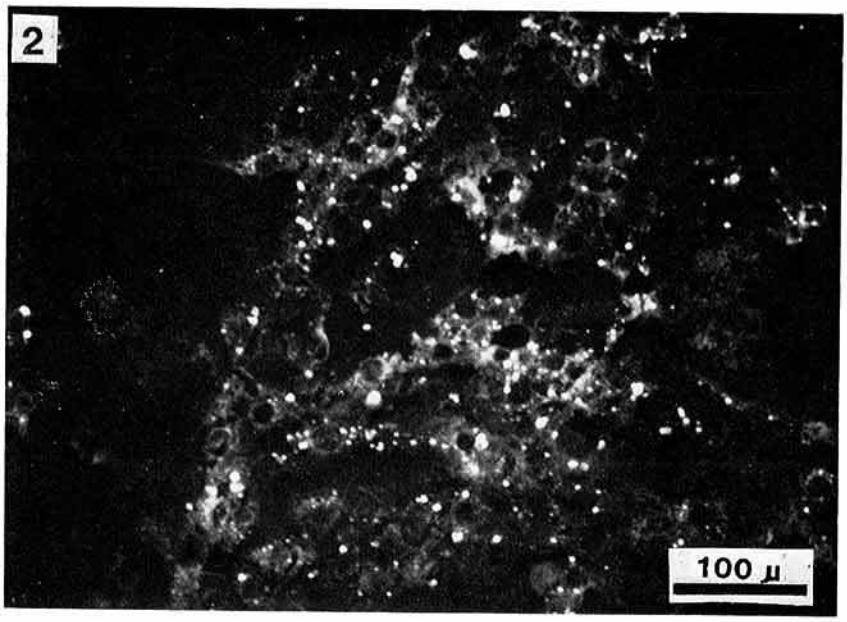

Plate 2. Cells of the NN-7 cell line stained with fluorescent antibody 42 hr after inoculation with RDV
Non-infected cells, which do not fluoresce, are not clearly seen in the picture.

then fixed with cold acetone for 5 min, and stained with fluorescent antibodies at 37°C for 40–60 min. Excess stain was removed by dipping in 2 successive beakers of PBS followed by 30–60 min of soaking in PBS. The stained specimens, mounted in PBS containing 50% glycerol (pH 7.3), were examined under a Nikon fluorescence microscope (Plate 2).

5) Fluorescent focus count method

The fluorescent cells were counted under a 20X objective lens. The method of counting infections was "focus count method". In this method employed in the experiments, an infected cell and any adjoining infected cells and neighboring infected cells were counted as one infection focus. The focus counts were carried out at a magnification of ×200 fold under a Nikon fluorescence microscope. Diametral and whole area counts of coverslips were done by the focus count technique. The diametral factor (whole area counts/diametral counts) was about 10 as shown in Table 3. Numbers of foci per diametral count between 4 and 150 were considered satisfactory. When the diametral count was less than 4, foci over the whole area of the coverslip were counted and taken instead.[10]

Table 3. Diametral factor in focus count method

Coverslip No.	Average of two diametral counts	No. of foci on whole coverslip	Diametral factor
1	19.5	201	10.30
2	18.0	178	9.88
3	56.0	641	11.45
4	58.0	605	10.43
5	150.0	1383	9.22
6	152.5	1647	10.80
7	4.0	45	11.25
8	3.5	38	10.86
9	73.5	762	10.37
10	77.0	763	9.90
			10.45±0.45

6) Relation of virus concentration to counts of foci of infected cells

Infectivity assays of serially diluted inoculum were performed on cell monolayers.

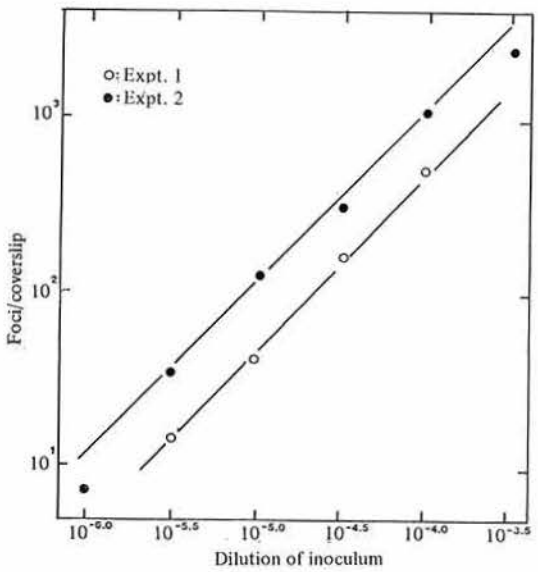

Fig. 1. The relationship between relative concentration of rice dwarf virus and corresponding focus counts of infective cells by the fluorescent focus counting technique

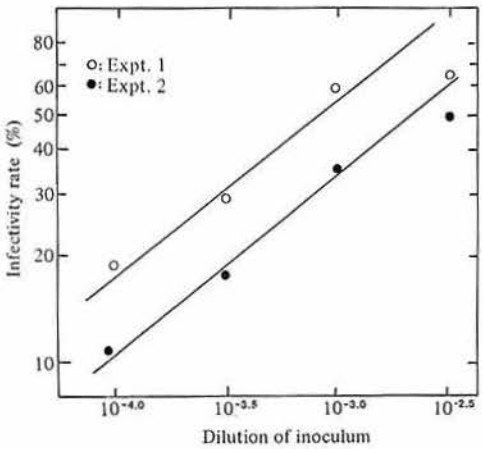

Fig. 2. The relationship between relative concentration of rice dwarf virus and corresponding infectivity rate (%) by insect vector injection method

The number of foci was counted in diametral microscopic fields of coverslip monolayers using the standard assay technique.[3,7,10] Under our experimental condition, relationship between RDV concentration and number of foci of infected cells was linear in the range of $10^{-6.0}$ to $10^{-4.0}$ relative virus concentration based on fresh weight of infected leaves as shown in Fig. 1. Therefore, a dilution end point of RDV was between $10^{-6.5}$ and $10^{-6.0}$. On the other hand, that[9] (Fig. 2) of the virus by the insect vector injection method was $10^{-4.5}$ to $10^{-4.0}$. Thus, the vector cell monolayer method detected up to about 1/100 virus concentration, compared with results by vector injection.

Conclusion

Six NC and four NN cell lines were established from embryonic fragments of their leafhopper vectors, and these have been passed through 85–147 and 81–121 subcultures respectively, employing Liu and Black's medium modified slightly. All cell lines were usually epithelial cell monolayers attached bottom surface of plastic flask, and have also been very susceptible for RDV infection. These cell lines were frozen at −80°C in a deep freezer for a night, and then stored at −196°C in a stocker containing liquid nitrogen.

Infectivity assays of RDV were made accurately on vector cell monolayers by using fluorescent focus count method. By the method, experimental results were shown quantitative, experimental period was shortly within 48 hr, the virus was detected up to $10^{-6.0}$ relative concentration, so that the method was 100 fold sensitive compared with vector injection technique. In addition, the technique will be applied to many plant viruses which are transmitted by insect vectors, and moreover propagated in the insect bodies.

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