The Effect of Low-Temperature Pre-Incubation Treatment of Tobacco and Soybean Callus Cultures on Rates of Tobacco- and Southern Bean Mosaic Virus Synthesis

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Journal Series Article No. 7636, Michigan Agricultural Experiment Station, East Lansing.
Accepted for publication 4 August 1976.

ABSTRACT

WHITE, J. L., F. S. WU, and H. H. MURAKISHI. 1977. The effect of low-temperature pre-incubation treatment of tobacco and soybean callus cultures on rates of tobacco- and southern bean mosaic virus synthesis. Phytopathology 67: 60-63.

Tobacco callus cultures inoculated with tobacco mosaic virus (TMV) and soybean callus cultures inoculated with southern bean mosaic virus (SBMV) held for 4 days at 10 C (nonpermissive temperature) and subsequently incubated at 24 C (permissive temperature) revealed a higher rate of virus synthesis than those continuously maintained at 24 C. In infected tobacco callus, TMV infectivity dropped slightly 5 hours after a shift to the permissive temperature, then rose logarithmically for 8 to 24 hours after which a linear increase occurred until 60 hours. In infected soybean callus, SBMV infectivity dropped slightly within 8 hours after a change to the permissive temperature, followed by a logarithmic increase in titer to 20 hours, after which the titer increased less rapidly. The total amount of recoverable TMV or SBMV infectivity at the conclusion of the experiments (120 hours) was equal in temperature-treated or nontemperature-treated callus. The similarities in increase of titer in temperature-treated callus, when compared to synthesis of virus in other plant, bacterial, and animal systems, suggest synthesis is occurring in a synchronous manner or approaching synchrony.

Callus culture provides a readily available aseptic source of metabolically active cells for studies of virus replication (10, 12) and necrotic local lesion formation under closely controlled conditions (2). Generally, in tissue systems relatively few cells are infected at inoculation and virus replication does not proceed synchronously. A very important advance was made when protoplasts from leaf mesophyll were successfully isolated and infected, which made possible demonstration of synchronous replication of TMV and other plant pathogenic viruses (13,14). However, because protoplasts lack a cell wall, they are fragile and require very careful handling. Also, the necrotic reaction associated with the hypersensitive tobacco (7) was absent in protoplasts infected with TMV (11).

To alleviate asynchrony in the plant system, Dawson and coworkers (3, 5) used a procedure in which mechanically inoculated lower leaves of intact tobacco plants (*Nicotiana tabacum* L.) were kept at a permissive temperature which allowed virus replication while the young, upper leaves were maintained at nonpermissive temperatures of 5 or 12 C. Ten days later, the upper leaves were exposed to permissive temperatures and infectivity increased rapidly in these leaves for 48 hours after an initial lag. The rate of synthesis in the low-temperature-treated upper leaves was similar to that of synchronously infected protoplasts. We investigated the effect of incubation of virus-inoculated tobacco and soybean callus tissues at an initial low temperature before incubation at a temperature which allows virus synthesis in a manner similar to that of Dawson et al. (5).

MATERIALS AND METHODS

The green-pigmented cell cultures that were used were derived from the stem pith of tobacco (*N. tabacum* L. 'Havana 38') for experiments with TMV or soybean (*Glycine max* L. 'Harosoy 63') for experiments with SBMV. The tobacco cells were maintained and inoculated as previously described (10). Half-gram batches of tobacco cells were suspended in 3 ml of liquid media (10) and inoculated with the U-1 strain of TMV at a concentration of 150 μg per milliliter of cell suspension. Inoculated cells were pooled, stirred gently to ensure randomization, and washed with 25 ml of fresh medium. One-gram samples of cells were transferred to 7-cm disks of Whatman No. 4 filter paper in petri dishes containing 20 ml of medium solidified with 1% agar. The cells were incubated at either 10 or 24 C under 968 lx of fluorescent light. At designated intervals, cells were harvested, weighed, and frozen at −20 C until all samples could be assayed together. Frozen cells were thawed, and ground in a conical tissue grinder with 0.1 M phosphate buffer, pH 7.3. Furthermore, the homogenates usually were diluted 1:100 or 1:1000 for assay. Tobacco mosaic virus infectivity was assayed by the half-leaf method on Xanthinc tobacco plants with six replications for each determination.

The soybean cultures were maintained on R3 media. The R3 medium is composed of Linsmaier and Skoog (9) mineral complement and carbon source supplemented with 0.5 mg of pyridoxine, 0.5 mg nicotinic acid, 0.5 mg thiamine-HCl, 5.0 mg indole-3-acetic acid, 0.3 mg

kinetin, and 0.5 mg 2, 4-dichloro-acetic acid per liter of medium. Cells in 1.0-g batches were suspended in 3 ml of liquid R3 media and inoculated with SBMV at a concentration of 200 μg per milliliter of cell suspension. The tubes were vibrated for 15 seconds at approximately 800 rpm on a Model S8220 vortex mixer (Scientific Products, Evanston, Illinois) and incubated for 30 minutes in a rotary shaker at 120 rpm. Inoculated cells were pooled, stirred gently, and washed with 75 ml of fresh media. Approximately 10 to 20 g of cells were transferred to 50 ml of fresh R3 media in a 250-ml culture flask and incubated with agitation for an additional 4 hours at 24 C. The flasks then were incubated either at 10 or 24 C under 968 lux of light on a rotary shaker. At designated intervals, cells were harvested, weighed, and frozen at −20 C until all samples could be assayed together. Frozen cells were thawed, ground in a Ten Broeck tissue grinder with 0.1 M phosphate buffer, pH 7.5. The homogenates usually were diluted 1:10 for assay. Infectivity of SBMV was assayed by the half-leaf method on primary leaves of bean, *Phaseolus vulgaris* L. 'Pinto', with six replications for each determination.

RESULTS AND DISCUSSION

At 24 C, TMV titer in tobacco tissue culture dropped to a minimum 10 hours after inoculation; this was followed by a logarithimic increase in titer to 60 hours and a linear rise to a maximum at 120 hours (Fig. 1-A); similar results were reported previously (10). However, inoculated cells that were held for 4 days at 10 C before transfer to 24 C demonstrated a different rate of TMV synthesis. After a drop in infectivity at 5 hours, a rapid logarithamic increase in infectivity occurred from 8 to 24 hours, after which a linear increase occurred until 60 hours (Fig. 1-A).

At 24 C, SBMV titer in soybean callus dropped to a minimum 8 hours after inoculation; this was followed by a logarithmic increase in infectivity to 36 hours and a linear rise to a maximum at 120 hours (Fig. 1-B). Inoculated soybean cultures held for 4 days at 10 C and then incubated at 24 C revealed an increased rate of SBMV synthesis. Infectivity dropped 8 hours after the shift to 24 C and then increased logarithmically until 20 hours. After 20 hours, the titer increased less rapidly reaching a maximum at 120 hours. However, the total amount of infectivity of TMV or SBMV recovered was approximately equal in temperature-treated or nontreated infected callus.

The rate of TMV and SBMV synthesis in temperature-treated callus is similar to other systems. Takebe et al. (15) and Otsuki et al. (11) reported a logarithmic increase of TMV infectivity at approximately 6 and 15 hours in protoplasts incubated at 28 and 22 C, respectively. Dawson et al. (5) reported a logarithmic increase in TMV in differentially treated leaves 8 hours after transfer to 25

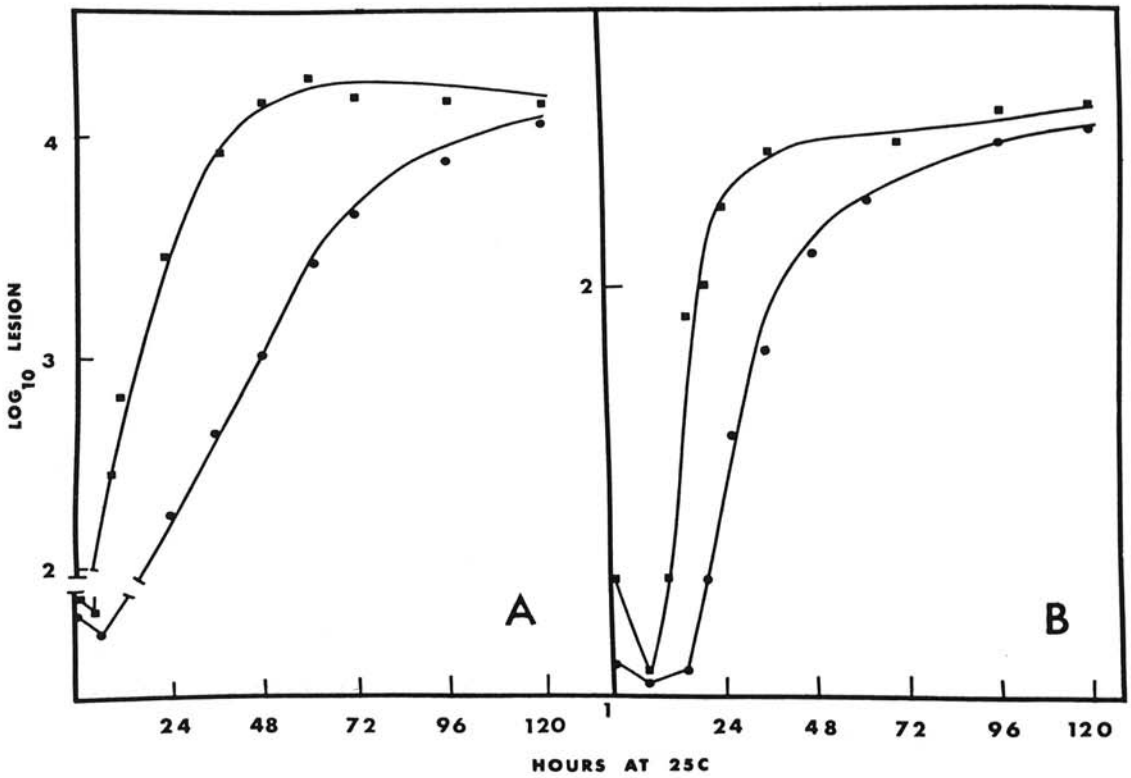

Fig. 1-(A, B). Infectivity of **A)** tobacco callus inoculated with TMV or **B)** soybean callus inoculated with SBMV that either were incubated at 24 C (●——●) or held for 4 days at 10 C and then transferred to 24 C (■——■). Zero time for the temperature-treated curves corresponds to time when the callus was transferred to 24 C. Zero time for the nontemperature-treated curves corresponds to the time when the callus was inoculated.

C from 5 C, whereas in leaves treated at 12 C, the logarithmic increase began at 0 to 6 hours after the temperature shift.

The similarities between the multiplication of TMV in protoplasts and temperature-treated callus is best shown when data from Fig. 1-A are plotted on a linear scale. Infectivity in protoplasts began increasing linearly after 12 hours at 28 C and 24 hours at 22 C, and continued until 72 hours (Fig. 2). With temperature-treated tobacco callus, TMV infectivity began increasing linearly at 12 hours and continued until 60 hours, but the linear increase in infectivity in the nontemperature-treated callus began at 48 hours and continued until 120 hours.

Attempts to determine the percentage of cells infected by the use of fluorescent antibody have been unsuccessful owing to the death of approximately 30% of the cells after the enzymatic treatment necessary to separate the cell aggregates. With nontemperature-treated callus, viral crystalline inclusions were detected first 40 hours after inoculation. With the temperature-treated callus, crystals were observed in approximately 30% of the cells by 48 hours and greater than 60% of the cells 60 hours after inoculation. This increase in the percentage of cells possessing inclusions may reflect a lag between synthesis of virus and inclusion formation. Pelcher et al. (12) reported that peak rate accumulation of complete virus TMV and most rapid viral RNA synthesis preceded maximum crystal inclusion formation by approximately 12 hours in nontemperature-treated tobacco callus. Although the possibility of movement of virus from cell to cell exists, Kassanis et al. (8) have shown that virus spread in inoculated tobacco callus was very slow; therefore, the rapid increase in the rate of virus synthesis observed in the first 48 hours after the shift to the permissive temperature apparently is not a result of extensive virus spread from one cell aggregate to another. The tissues cultures used in this study were composed of small aggregates connected by plasmodesmata. Plasmodesmata could allow spread of virus throughout the aggregate during the incubation at the nonpermissive temperature. Subsequent incubation of inoculated cells at the permissive temperature would allow rapid synthesis of virus in a large proportion of the cells. Recently, Dawson and Schlegel (4) reported that in tobacco leaves mechanically inoculated with TMV and pre-incubated at temperatures nonpermissive for virus synthesis, the rate of TMV synthesis upon shift to a permissive temperature was markedly greater than that in control leaves.

Similarities of the rise of TMV infectivity in the temperature-treated callus, when compared to other synchronous plant, animal (1), and bacterial (6) systems, suggest that TMV synthesis is occurring synchronously or is approaching synchrony. Data on the replication of SBMV in protoplasts are lacking, but the similarities in the rise of virus titer in these cold-temperature-treated callus cultures suggest synchronous or near-synchronous synthesis of virus in a large proportion of the cells after the temperature shift.

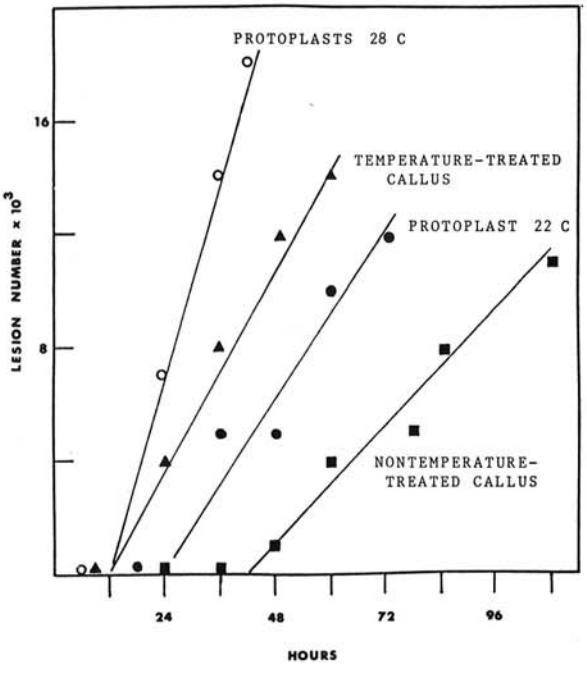

Fig. 2. Linear plots of time-course of TMV multiplication in tobacco protoplast incubated at 28 C or 22 C and temperature-treated and nontemperature-treated tobacco callus. Data for protoplasts were adapted from Takebe et al. (1971, Fig. 2).

LITERATURE CITED

1. BALTIMORE, D., M. GIRARD, and J. E. DARNELL. 1966. Aspects of the synthesis of poliovirus RNA and the formation of virus particles. Virology 29:179-189.
2. BEACHY, R. N., and H. H. MURAKISHI. 1971. Local lesion formation in tobacco tissue culture. Phytopathology 61:877-878.
3. DAWSON, W. O., and D. E. SCHLEGEL. 1973. Differential temperature treatment of plant greatly enhances multiplication rates. Virology 53:476-478.
4. DAWSON, W. O., and D. E. SCHLEGEL. 1976. Enhancement of tobacco mosaic virus spread in mechanically inoculated leaves by pre-incubation at a nonpermissive temperature. Phytopathology 66:625-628.
5. DAWSON, W. O., D. E. SCHLEGEL, and M. C. Y. LUNG. 1975. Synthesis of tobacco mosaic virus in intact tobacco leaves systemically inoculated by differential temperature treatment. Virology 65:565-573.
6. GODSON, G. N. 1968. Site of synthesis of viral ribonucleic acid and phage assembly in MS2-infected Escherichia coli. J. Mol. Biol. 34:149-163.
7. HOLMES, F. O. 1938. Inheritance of resistance to tobacco mosaic disease in tobacco. Phytopathology 28:553-561.
8. KASSANIS, B., T. W. TINSLEY, and F. QUAK. 1958. The inoculation of tobacco callus tissue with tobacco mosaic virus. Ann. Appl. Biol. 46:11-19.
9. LINSMAIER, E. M., and F. SKOOG. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.
10. MURAKISHI, H. H., J. X. HARTMANN, L. E. PELCHER, and R. N. BEACHY. 1971. Growth curve and yield of tobacco mosaic virus in tobacco callus cells. Virology 43:62-68.
11. OTSUKI, Y., T. SHIMOMURA, and I. TAKEBE. 1972. Tobacco mosaic virus replication and expression of the N gene in necrotic responding tobacco varieties. Virology 50:45-50.
12. PELCHER, L. E., H. H. MURAKISHI, and J. X.

HARTMANN. 1972. Kinetics of TMV-RNA synthesis and its correlation with virus accumulation and crystalline viral inclusion formation in tobacco tissue culture. Virology 47:787-796.

13. TAKEBE, I. 1975. The use of protoplasts in plant virology. Annu. Rev. Phytopathol. 13:105-125.

14. TAKEBE, I., and Y. OTSUKI. 1969. Infection of tobacco mesophyll protoplast by TMV. Proc. Nat. Acad. Sci. USA 64:843-848.

15. TAKEBE, I., Y. OTSUKI, and S. AOKI. 1971. Infection of isolated tobacco mesophyll protoplast by tobacco mosaic virus. Colloq. Int. Cent. Nat. Rech. Sci. 193:503-511. (Editor: L. Hirth).