

Infection and Synthesis Rate of Southern Bean Mosaic Virus in Soybean Callus Cells under Selected Cultural Conditions

Fang-Sheng Wu and Harry H. Murakishi

Graduate Student and Professor, respectively, Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824.

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ABSTRACT

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Soybean (*Glycine max* 'Harosoy 63') callus cells grown in either Eriksson's or Linsmaier and Skoog's media were inoculated with southern bean mosaic virus (SBMV) and then incubated in either liquid or agar media. The composition and type (liquid or agar) of the medium significantly affected the growth curve of the virus. Assays in *Phaseolus vulgaris* 'Pinto' leaves indicated that soybean callus grown in liquid and incubated in liquid medium after inoculation gave the most rapid virus growth rate compared

with that of Prince bean callus treated identically or with infected soybean plants grown in the greenhouse. Vortexing of the cell-suspension/virus-inoculum mixture as is done for tobacco mosaic virus—tobacco callus infection was not necessary with the soybean callus-SBMV system. Infection was achieved by adding virus inoculum to a suspension culture of callus cells, washing the cells with fresh medium and incubating the cells in liquid media on a rotary shaker at 120 rpm.

Additional key words: virus-tissue culture interactions.

The prospect of using pipettable suspensions of virus-infected cells or protoplasts has elicited considerable excitement among plant virologists (16). Callus cultures (1, 9, 10, 11, 12, 13), separated cells (16), and protoplasts (15) have been used by different investigators to study virus replication and virus-host interactions. Early studies of virus infection of callus cultures resulted in low efficiency of infection and slow growth rate of virus (4, 6). The low efficiency of infection was attributed to the inability of virus to penetrate the cellulose wall (4). Infection efficiency was improved by vortexing friable, suspension cultures of tobacco callus in the presence of virus and incubating the vortexed callus on agar medium (11). When callus was not vortexed or when inoculated cells were incubated in liquid medium the infection efficiency was reduced significantly (10).

Plant virus vs. plant tissue culture relationships have been studied so far using tobacco tissue and tobacco mosaic virus (TMV) almost exclusively (1, 4, 5, 6, 9, 10, 11, 13). Other viruses with different morphology, transmission characteristics, and host range have not been studied because a suitable experimental system was not available. Strains of southern bean mosaic virus (SBMV) are world-wide in distribution and affect primarily bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.), and cowpea (*Vigna sinensis* L.) (14). The SBMV is an isometric virus with a diameter of 25 nm (8, 14). It may be seed-borne and is transmitted by the bean leaf beetle (*Ceratoma trifurcata*) as well as by leaf rubbing (14). The yield of SBMV from intact plant tissue is relatively high; the purification procedure is simple, and a

dependable bioassay host is also available (2). Callus from its host plants can be easily induced and maintained in chemically defined media. These favorable experimental conditions encouraged us to study SBMV-callus interactions. This report describes several parameters that affect the multiplication rate and better synchronization of SBMV synthesis so that it could be used for the study of SBMV replication in cells at macromolecular levels.

MATERIALS AND METHODS

Tissue culture and media.—Soybean (*Glycine max* 'Harosoy 63') or bean (*Phaseolus vulgaris* L. 'Prince') seeds were surface-sterilized with 70% ethanol for 3 min and 20% Clorox for 20 min, rinsed in 0.01 N HCl, and washed several times with sterilized distilled water. The seeds then were transferred to sterile 50-ml test tubes in which filter paper was folded and placed at the bottom of the tubes to support the seeds. The seeds were kept moist by the addition of 9 ml of Linsmaier and Skoog (7) medium diluted 1:4 with water. The seeds were allowed to germinate under the same light and temperature conditions used for growing tissue cultures. The hypocotyl was cut into 1-cm segments and placed on designated media solidified with 1% agar. Then the induced calli were maintained either on liquid media (liquid-grown) or on media solidified with 1% agar (agar-grown). Gro-Lux fluorescent lamps [861 lux (80 ft-c)] and a temperature of 24 C \pm 1.0 C were used under all growth conditions. Unless otherwise specified, calli grown in liquid media were incubated in Erlenmeyer flasks and placed on a rotary shaker at 120 rpm.

The R3 medium is composed of Linsmaier and Skoog minerals (7), and 30 g sucrose supplemented with 0.5 mg

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-dichlorophenoxyacetic acid (2,4-D) per liter of medium. The pH was adjusted to 5.8 with 0.2 N NaOH solution.

Medium E152 from Eriksson (3) had been found suitable for growing SBMV-infected bean callus (12). This medium contained 0.10 mg 2,4-D, 0.5 mg naphthaleneacetic acid (NAA), and 0.02 mg kinetin per liter of medium. The E112 medium was Eriksson's medium containing 0.10 mg 2,4-D, 0.10 mg NAA, and 0.02 mg kinetin per liter of medium. The E152 medium provided better callus growth on agar medium than in liquid medium, but medium E112 produced the opposite effect.

Virus preparations.—The bean strain of SBMV was propagated in leaves of Prince bean. Plants grown in the greenhouse were harvested 3 to 4 wk after inoculation and stored at -25°C until used. The virus was purified by homogenizing infected leaves in 0.5 M potassium phosphate buffer, pH 7.5. The resultant slurry was passed through four layers of cheesecloth and *n*-butanol was then added to the sap to a final concentration of 10%. After stirring for 30 min, the mixture was centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor. Then $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to a final concentration of 60% with constant stirring for 20 min and stored at 4°C for 1 hr. The precipitates were collected by centrifugation and resuspended in distilled water. The suspension was subjected to four or five cycles of differential centrifugation (12,000 *g* for 15 min and 97,000 *g* for 1 hr). The final pellet was resuspended in 0.01 M phosphate buffer, pH 7.2, and was freed of microorganisms by filtration through a Millipore filter with an average pore size of 0.45 μm .

Inoculation of callus.—Callus grown either on agar or in liquid medium was collected in 1-g batches and placed in 50-ml test tubes containing 3 ml of liquid medium and SBMV at a concentration of 180 $\mu\text{g}/\text{ml}$. Unless specified, the mixture of callus and virus was vortexed immediately with a Model S8220 Vortex mixer (Scientific Products, Evanston, IL 60201) for 20 sec at approximately 800 rpm. In experiments in which vortexing was omitted the callus-virus mixture was agitated on a rotary shaker at 120 rpm (amplitude of 2.5 cm as is normally done when growing callus cells. After vortexing or shaking, the cells were collected on Miracloth-lined funnels and washed three times with fresh medium. To randomize the cell clumps and enhance the removal of nonadsorbed virus, the cells were stirred gently with a spatula during the washing. After being washed, cells were transferred to liquid or agar medium. Incubation was under the same light and temperature conditions as those used for maintaining callus cell growth. The liquid medium was renewed periodically by adding fresh medium and the callus grown on agar medium was transferred periodically to fresh medium. This process of regular subculture maintained actively growing cells. At various periods after incubation, the cells were collected, weighed, and stored at -25°C until they were used for infectivity assays.

Bioassay.—Frozen callus cells were homogenized with 3 ml of cold 0.5 M phosphate buffer (pH 7.5) per gram of callus in a TenBroeck tissue grinder. The homogenate was diluted to 10^{-1} with 0.01 M phosphate buffer pH 7.1.

Before the assay, plants were placed in the dark for four days. This procedure increased the number and size of lesions over those that developed on nondark-treated plants. Fully expanded primary leaves of Pinto bean dusted with 0.22 μm (600-mesh) Carborundum were used for inoculation with a glass spatula according to a randomized block design. Each point of the virus growth curves (Fig. 1 and 2) represents the mean number of local lesions per half-leaf based on a total of 24 half-leaves (eight replications of each of three bioassays). The variation of mean values for each determination among individual experiments was less than 16%.

RESULTS AND DISCUSSION

Effects of liquid or agar medium.—Callus cells grown either in liquid or on agar were inoculated with virus and then incubated either in liquid or agar media. The four combinations of liquid and agar media resulted in different multiplication rates of virus in cells (Fig. 1-2). When the R3 medium was used (Fig. 1), the liquid-grown/liquid-incubation condition gave the most rapid synthesis rate of virus in cells. Logarithmic increase of virus titer began 2 days after inoculation and reached a near maximum at 5 days. The agar-grown/agar-incubation condition had a 4-day lag period with a slow increase in virus multiplication rate during the first eight days after inoculation. The liquid-grown/agar-incubation condition did not show significant increase in virus titer until 2 to 4 days after inoculation. The virus titer increased slowly thereafter until it reached a near maximum 8 days after inoculation. The agar-grown/liquid-incubation condition had a virus synthesis rate similar to liquid-grown/agar-incubation conditions but there was a 1-day delay in the initial increase of infectivity. Under all four conditions, the infectivities were approximately equal at 60 days after inoculation and the high level of infectivity was still maintained at 120 days.

In order to determine whether the effect of liquid or agar medium on the virus multiplication rate was independent of the medium used, Eriksson's medium was compared with R3 medium (Fig. 2). The liquid-grown/liquid-incubation sequence in Eriksson's medium showed the most rapid increase in virus synthesis rate. The agar-grown/liquid-incubation condition showed a similar synthesis rate but had a less steep curve during its exponential phase. The virus synthesis rate increased very slowly under the agar-grown/agar-incubation condition and the total amount of infectivity up to 30 days was less than that of liquid-grown/liquid-incubation and agar-grown/liquid-incubation. After prolonged incubation to 120 days, no infectivity was detected under the agar-grown/agar-incubation conditions.

The liquid-grown/agar-incubation condition in Eriksson's medium did not show a detectable level of infectivity increase in the cells. Evidently, the synthesis of virus in callus cells can be significantly affected not only by the composition but also by the type (agar or liquid) of same medium. The liquid-grown/liquid-incubation condition in both R3 and Eriksson's media gave the best results for virus multiplication. When cultures were shifted from agar to liquid medium or vice versa there was a prolonged lag period before virus synthesis resumed.

These results suggest that actively growing cells in liquid medium were more susceptible to virus infection and that rapid proliferation of cells was conducive to maintaining virus synthesis in cells. This is in contrast to TMV in tobacco tissue culture which required liquid-grown/agar-incubation conditions for high virus yield (10). Under most of the conditions used, SBMV was able to maintain viability in infected soybean callus for 120 days, the longest duration so far tested. This is also different from TMV-infected tobacco callus which lost TMV infectivity after several transfers of callus cells (Murakishi, unpublished).

Effect of vortexing.—Vortexing dissociates tobacco callus into small aggregates and single cells and probably causes minor wounds which help TMV to enter the cells (10). In our experiments with soybean callus the vortexing procedure was not necessary for SBMV infection of soybean callus cells. However, vortexed callus had a steeper exponential increase in the virus growth curve than the nonvortexed callus (data not shown). It is reasonable to assume that the dispersion of cells from callus increased the number of cells that came in contact with virus, and therefore more cells became infected at the time of inoculation. Also it is possible that minor injuries suitable for virus entry and multiplication occurred in otherwise intact cells. Vortexed callus also produced cell debris and broken cells which could not have supported virus multiplication. If an affinity between cell walls and virus exists, the broken cells and cell debris could adsorb considerable amounts of virus from the inoculation medium and reduce the virus

available for infection. The filtrates from vortexed mixture of callus and virus were used as inoculum and added to healthy callus without further vortexing. As a control, healthy callus cultures were vortexed without virus and then were added together with virus inoculum into healthy callus cultures. The two resulting growth curves were similar to other nonvortexed samples. This demonstrated that the cell debris apparently did not absorb a significant amount of virus and that virus attachment sites are probably maximally exposed to virus under these inoculation conditions without vortexing.

Effect of shaking.—For optimum virus multiplication, tobacco protoplasts inoculated with any one of several viruses (15) were usually incubated in liquid without shaking. It was noted in the case of liquid-grown tobacco callus inoculated with TMV and incubated in liquid shake cultures that many virus crystals were found in cells that had adhered to the inner wall of the flask just below the liquid level (Murakishi, unpublished). When liquid-grown soybean callus inoculated with SBMV were transferred to petri dishes for incubation without shaking, the synthesis rate of virus slowed down and the lag period was prolonged. Thus, the liquid-grown/liquid-incubation sequence in R3 medium with continuous shaking provided the best condition for SBMV synthesis (Fig. 1).

Comparison of leaves and calli as virus sources.—Prince bean was used as a propagation host for SBMV throughout the study. The yield of purified virus was about 300 μg per gram of fresh leaf indicating that Prince bean was a good host for the bean strain of SBMV.

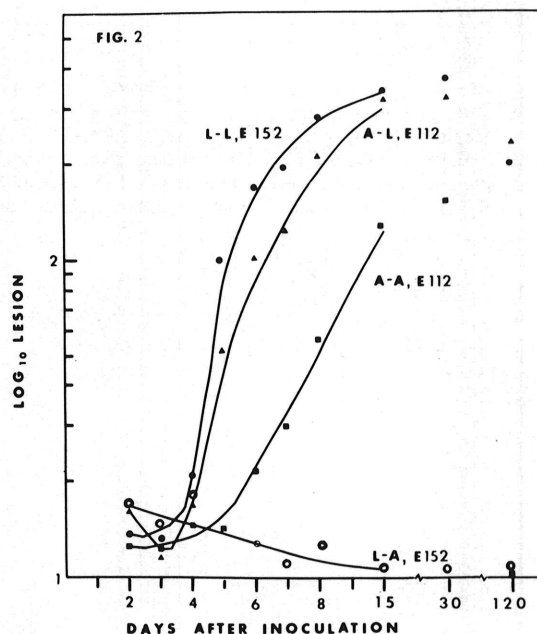
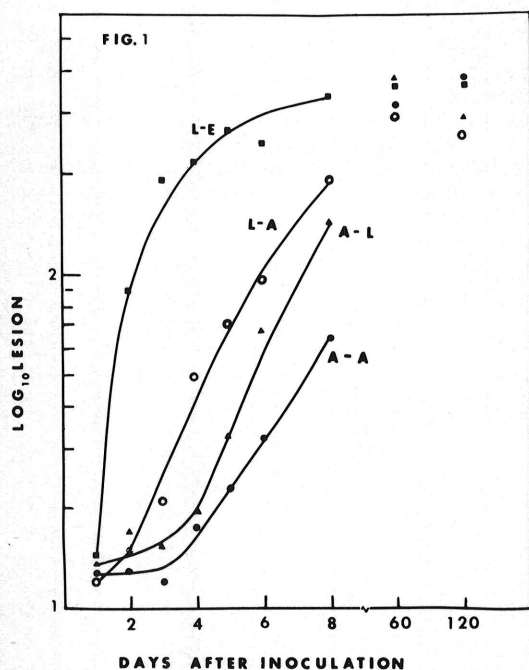


Fig. 1-2. Growth curves of southern bean mosaic virus (SBMV) in soybean callus cells grown in 1) R3 medium or 2) Eriksson medium. The composition of Eriksson medium was either E112 or E152 as in Materials and Methods. Callus cells grown in liquid medium were inoculated with SBMV and then incubated either in liquid medium (L-L) or on medium solidified with 1% agar (L-A). Callus cells grown on agar-solidified medium were inoculated with SBMV and then incubated either in liquid medium (A-L) or on agar-solidified medium (A-A). Infectivity was determined at intervals after inoculation.

The callus derived from the plant was presumed to be a good host for SBMV as well. Consequently, callus cells induced from different media were inoculated with SBMV and virus synthesis was monitored by bioassay. No evidence of infectivity increase in inoculated cells could be detected except when Eriksson's medium was used. A slight increase in infectivity was found in liquid-grown/agar-incubation cells and agar-grown/agar-incubation cells after 8 days of incubation but infectivity could not be detected after 60 days.

Soybean tissue culture was a good host for SBMV but the soybean plant was a poor host for the same virus. Soybean plants were inoculated at the trifoliolate-leaf stage and incubated in the greenhouse. After 8 days and 60 days all leaves except inoculated leaves were ground in 0.1 M phosphate buffer, pH 7.0 (1/3:w/v). The crude juice without dilution then was assayed on the local lesion host as before. No lesions were produced from plants inoculated for 8 days and only 15 lesions (average of 12 half-leaves with four replications) were produced from plants inoculated for 60 days. It is obvious that the soybean callus is more susceptible to SBMV infection and is a much better host for the multiplication of SBMV than the intact soybean plants. In contrast, tobacco callus derived from different species and cultures responded to TMV infection in a manner similar to that of intact tobacco plants (1).

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