STUDIES ON TOBACCO MOSAIC VIRUS USING HYPERSENSITIVE TOBACCO TISSUE CULTURE

I. LOCAL LESION FORMATION IN CULTURED CELLS

II. EFFECT OF CYCLOHEXIMIDE ON TMV SYNTHESIS

III. CHANGES IN SOLUBLE PROTEINS IN TMV INFECTED CALLUS

Thesis for the Degree of Ph.D.

MICHIGAN STATE UNIVERSITY

ROGER NEIL BEACHY

1973

This is to certify that the

thesis entitled

STUDIES ON TOBACCO MOSAIC VIRUS USING HYPERSENSITIVE TABACCO TISSUE CULTURES

presented by

Roger Neil Beachy

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in *Botany and Plant Pathology*

Harry H. Murakishi
Major professor

Date *May 14, 1973*

ABSTRACT

STUDIES ON TOBACCO MOSAIC VIRUS USING HYPERSENSITIVE TOBACCO TISSUE CULTURES

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Callus cultures derived from Nicotiana tabacum cvs Xanthi-nc, Samsun NN, and Burley NN, and from Nicotiana glutinosa reacted with necrotic local lesions 36-44 hours after inoculation with tobacco mosaic virus (TMV). The necrotic response occurred only in cells cultured in medium containing the Murashige and Skoog mineral and vitamin stocks, coconut water, 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthaleneacetic acid (NAA). More than 90% of the virus synthesis in Xanthi-nc callus, as determined by the incorporation of ^{3}H-uridine and ^{14}C-leucine into TMV purified by sucrose gradient centrifugation, occurred after lesions became visible.

Xanthi-nc callus was used to study the effect of the protein synthesis inhibitor, cycloheximide, on the synthesis of TMV-ribonucleic acid (RNA) and complete virus. Within one hour of treatment cycloheximide inhibited TMV synthesis by 90%, as shown by incorporation of ^{3}H-uridine and ^{14}C-leucine into purified virus. The synthesis of TMV-RNA, as measured by the incorporation of ^{3}H-uridine into viral RNA

separated by polyacrylamide gel electrophoresis, was not affected by 4 hours of cycloheximide treatment. The data suggest that the inhibition of complete virus is caused by the inhibition of virus protein synthesis and/or of virus assembly.

Soluble proteins were extracted from TMV-infected and non-infected Xanthi-nc callus at 24 hour intervals after inoculation, and were separated by polyacrylamide disc gel electrophoresis. Changes in extracted proteins (determined by staining with amido black) were evident from 48 to 144 hours after inoculation. Two bands of TMV coat protein, identified antigenically, were evident 48-144 hours after inoculation. An additional, slower migrating protein was extracted from the infected callus but could not be positively correlated to virus infection. The results suggest that callus cultures may be useful in additional studies to identify viral proteins or changes in host proteins caused by virus infection.

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Thanks to the faculty of the A. E. C. Plant Research Laboratory, especially Drs. H. Kende, and M. Jost, for the use of instruments, without which a great deal of this research could not have been possible. The discussions with other faculty members and graduate students of the Department of Botany and Plant Pathology were most helpful.

A fellow graduate student, L. E. Pelcher, deserves a special word of appreciation for his instruction in techniques and for helpful discussion and brain-storming throughout the course of the research.

Financial support which made this research possible was given by the Michigan Agricultural Experiment Station.

DEDICATION

To my late father, and to my mother, whose influence and guidance contributed greatly to the shaping of personal goals; and above all to my dear wife, Terry, whose love, patience, and understanding gave me the confidence that I needed to reach toward those goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

Studies of the hypersensitive or necrotic response to virus infection have previously been made only in whole leaves or leaf discs: these studies were complicated by problems of metabolite uptake, sample variability, microbial contamination, etc. (Matthews, 1970).

A cell system without these problems would be valuable for studies of the virus-cell interaction which leads to the necrotic or local lesion reaction.

Use of plant tissue cultures is a possible solution to such problems. Tissue cultures are known to take up radioactive precursors and specific metabolic inhibitors readily. In addition, use of relatively homogenous cell populations and aseptic conditions would eliminate some of the problems posed by Matthews (1970). Researchers in several laboratories have attempted to induce the necrotic reaction by inoculating cultured cells from known hypersensitive hosts, but none have been successful (Wu et al, 1960; Hirth and Lebeurier, 1965; Nims et al, 1967; Kassanis, 1967). Venketeswaran (1968) inoculated cultured cells of Nicotiana glutinosa with tobacco mosaic virus (TMV) and observed death of many cells and virus multiplication. A relatively high concentration of inoculum (10 μg TMV per ml) was required, however, to trigger the response, which was a "general" cell killing rather than the localized response seen on inoculated leaves (Holmes, 1929).

Recently Murakishi et al (1970) successfully inoculated callus derived from systemic tobacco with TMV, and showed that this method produced high virus titers and virus crystals. Murakishi's results indicated the potential usefulness of the technique for TMV infection of, and, perhaps, local lesion formation in, callus derived from hypersensitive tobacco cultivars.

The first aim of my study was to culture callus tissue from several hypersensitive tobacco cultivars and to test the callus for the ability to form necrotic lesions in response to TMV infection. Next, I hoped to apply the system to studies in plant virology. After establishing the cultural conditions for lesion formation (Appendix) and characterizing the response (Chapter 1), the system was used in the following studies: (1) to elucidate the TMV growth curve in the hypersensitive callus in relation to symptom development (Chapter 2); (2) to examine the effect of the protein synthesis inhibitor, cycloheximide, on the synthesis of TMV-RNA and complete virus during the period of rapid virus accumulation (Chapter 2); and (3) to evaluate the usefulness of the callus-TMV system for identifying changes in host and viral proteins which may occur as a result of virus infection (Chapter 3).

CHAPTER 1

LOCAL LESION FORMATION IN TOBACCO TISSUE CULTURE

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Local Lesion Formation in Tobacco Tissue Culture

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Michigan Agricultural Experiment Station Journal Paper 5114. The advice of L. E. Pelcher and J. X. Hartmann is gratefully acknowledged.

ABSTRACT

Callus cultures derived from *Nicotiana glutinosa* and *N. tabacum*, 'NN Samsun', 'NN Burley', and 'Xanthi-nc' responded with reddish-brown local lesions when infected with tobacco mosaic virus (TMV). Cultured cells of NN Burley inoculated with 0.083 to 83 µg TMV/ml gave a virus dilution curve similar to that obtained with intact leaves of Xanthi-nc tobacco plants. Lesion formation on tissue cultures occurred only when cultures were grown on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid, naphthaleneacetic acid, and coconut water. Phytopathology 61:877-878.

Tobacco mosaic virus (TMV) infection of tobacco leaves usually results in systemic mosaic symptoms or in localized necrotic lesions. The latter "hypersensitive" reaction is governed by the *"NN"* gene, which is present in *Nicotiana glutinosa* L. (2) and *N. tabacum* L., 'NN Samsun' (3), 'NN Burley' (9), and 'Xanthi-nc' (8). Earlier work in this laboratory (5, 6) showed that TMV infection of tissue culture from a systemic host resulted in a high virus titer and a high percentage of cells with inclusions, but no macroscopic symptoms. In subsequent studies with tissue cultures from 13 species and cultivars of *Nicotiana*, local lesions were produced only in cell cultures from *N. tabacum* 'NN Samsun' (1). We now find that cell cultures from each of the hypersensitive hosts mentioned develop local lesions when the culture medium is suitably modified.

Interveinal leaf segments (1-1.5 cm^2) of the hypersensitive hosts were surface-sterilized and washed in sterile, distilled water. Tissue explants were placed on a basic Murashige and Skoog (MS) medium (7) modified by the addition of 240 µg of 2,4-dichlorophenoxyacetic acid (2,4-D), 40 µg of naphthaleneacetic acid (NAA), and 50 ml of coconut water/liter, and solidified with 1% agar. Callus formed from the explants was transferred to a similarly modified liquid MS medium and grown in flasks on a rotary shaker (120 rpm).

Cell suspensions (150-250 mg fresh wt) in the log phase of growth were aseptically inoculated by dispersing a TMV solution throughout the tissues with a Vortex mixer (Scientific Industries, New York) as previously described (6). The cells were then spread over the surface of modified MS agar and incubated at 22-24 C under 90 ft-c of fluorescent light. Reddish-brown necrotic lesions were macroscopically visible on cell clumps as early as 39 hr after inoculation (1), and were 1-2 mm in diam after 6 days (Fig. 1). Cultures inoculated with heat- or ultraviolet-inactivated TMV, with phosphate buffer, or with southern bean mosaic virus (which does not infect tobacco) never developed lesions. To determine whether virus multiplication had occurred in the necrotic areas, lesions were excised and assayed for infectious virus. Excised lesions and surrounding symptomless tissues were ground separately 1:50 (w/v) in 0.1 M phosphate buffer, pH 7.3, and the homogenates were rubbed on opposite half-leaves of Xanthi-nc tobacco plants. Homogenates of excised lesions and of symptomless tissues produced an average of 433 and 21 lesions/half-leaf, respectively. Moreover, microscopic examination revealed that 25% of the cells within a 0.5-mm radius of the lesions contained the characteristic hexagonal crystals of TMV. Symptomless areas more distant from the lesions were free of inclusions.

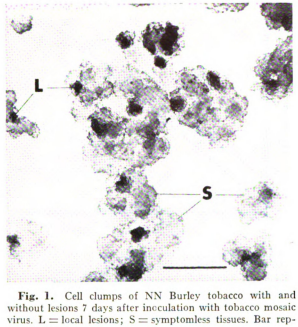

Fig. 1. Cell clumps of NN Burley tobacco with and without lesions 7 days after inoculation with tobacco mosaic virus. L = local lesions; S = symptomless tissues. Bar represents 1 cm.

To determine the TMV dilution curve of cultured cells from hypersensitive tobacco, NN Burley cell suspensions were inoculated with 0.083-83 µg TMV/ml (12 replicates/dilution). The log of the number of lesions produced per g fresh wt of cells increased linearly with the log of the virus concentration between 0.083 and 10 µg virus/ml. At higher virus concentrations, the slope of the curve leveled off, suggesting near saturation of the infectible sites. To determine the TMV dilution curve on intact plants, 24 half-leaves of greenhouse-grown Xanthi-nc tobacco were inoculated in a Latin square randomization scheme with 0.083 to 83 µg TMV/ml. Dilution curves of the tissue culture and leaf systems were similar in the range of virus concentrations tested (Fig. 2).

Tissue cultures of Xanthi-nc, NN Samsun, and *N. glutinosa* also produced lesions when inoculated with

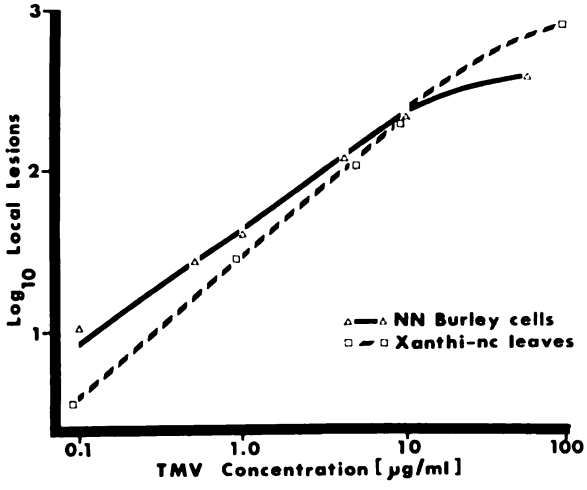

Fig. 2. Comparison of the dilution curves of tobacco mosaic virus (TMV) inoculated on NN Burley tissue culture (Log_{10} number of local lesions per g cells inoculated) and Xanthi-nc leaves (Log_{10} number of local lesions per half-leaf).

TMV. In previous studies [S. Venketeswaran, *personal communication;* and (4)], local lesions were not formed in cultured cells of *N. glutinosa* nor in hybrids of *N. glutinosa* and *N. tabacum*. Our positive results with tissues from *N. glutinosa* and the NN hybrids suggested that either the culture medium or the inoculation technique we used was important for lesion formation. In earlier experiments with nonsupplemented MS medium and with White's medium (10) supplemented with 2,4-D, NAA, and coconut water, no lesions were produced on inoculated cells. Clearly, both the supplements and the substances present in the basic MS medium are crucial for lesion formation. In addition, the method of cell culture following inoculation is important. In repeated experiments, NN Burley cells were inoculated with 5 µg TMV/ml and were incubated on liquid or agar media. Incubation in the liquid medium resulted in 6 ± 6 lesions/g, whereas incubation on the agar medium resulted in 125 ± 14 lesions/g.

In more recent tests, the number of lesions produced in cultured cells increased fourfold over that of previous experiments. Maintaining this higher efficiency of inoculation has not been consistent, however. Parameters such as the growth medium, the cell culture techniques, and the physiologic state of the cells may be critical for optimizing the efficiency of inoculation. Experiments are in progress to more precisely determine the extent that these factors are involved in lesion formation. The plant tissue culture-virus system we describe should be a useful tool for studies of the virus-induced hypersensitive reaction.

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CHAPTER 2

EFFECT OF CYCLOHEXIMIDE ON TOBACCO MOSAIC VIRUS SYNTHESIS IN CALLUS FROM HYPERSENSITIVE TOBACCO

EFFECT OF CYCLOHEXIMIDE ON TOBACCO MOSAIC VIRUS SYNTHESIS IN CALLUS FROM HYPERSENSITIVE TOBACCO

SUMMARY

Local lesions were formed on callus derived from Nicotiana tabacum cv Xanthi-nc 36-44 hours following inoculation with tobacco mosaic virus (TMV). The necrotic lesions, however, had little or no inhibitory effect on virus synthesis. The greatest accumulation of ^{3}H-uridine and ^{14}C-leucine labeled virus occurred 48-84 hours after inoculation. When infected callus tissue was treated with cycloheximide (1 µg per ml) during the period of rapid virus accumulation, the synthesis of host protein and complete virus was reduced by 90% within 1 hour. The synthesis of TMV-RNA was not affected even after 4 hours of treatment. The results suggest that cycloheximide prevented the formation of complete virus by inhibiting the synthesis of viral protein and/or by preventing the assembly of virus particles.

INTRODUCTION

Cycloheximide (CHX) is a potent inhibitor of protein synthesis (McKeehan and Hardesty, 1969) in plant tissue cultures (Nudel and Bamberger, 1971). Reports indicate that it may stimulate or inhibit tobacco mosaic virus (TMV) synthesis in inoculated leaves of Nicotiana tabacum. Ouchi et al (1969) reported that 1 µg CHX per ml stimulated virus synthesis in infected leaf discs, whereas higher concentrations

reduced synthesis. Takebe et al (1971) and Zaitlin et al (1968), using infected leaf protoplasts and leaf discs, respectively, reported near complete inhibition of TMV synthesis by CHX at 10-15 µg per ml. We re-examined the effect of CHX on the synthesis of TMV in tobacco tissue cultures and also studied the effects of CHX on the synthesis of TMV-RNA.

Tissue cultures have certain advantages over leaf discs or protoplasts used in previous work. Callus tissue can be cultured in a metabolically active state under aseptic conditions; thus, problems caused by tissue senescence or by microbial contaminants (Matthews, 1970) are avoided. We used the TMV and Xanthi-nc callus cultures previously described by Beachy and Murakishi (1971 a). The degree of virus infection in such cultures is indicated by the number of necrotic lesions produced after inoculation. The conditions for maximum efficiency of infection were determined in a preliminary study. In addition, the virus growth curve was characterized because this had not previously been done for a hypersensitive callus culture.

MATERIALS AND METHODS

Callus cultures

The culture medium was a combination of the media described by White (W) (1963) and Murashige and Skoog (MS) (1962). The W and MS media were prepared separately, each were brought to pH 5.9 and combined in a ratio of 3 MS:2 W. The combined medium contained 50 ml of coconut water 30 µg of kinetin, 250µg of 2,4-dichlorophenoxyacetic acid, and 40 µg of naphthaleneacetic acid per liter. The medium (MS-W) was sterilized by autoclaving. Solid medium was prepared by adding agar (1% by weight).

Stem pith cores (explants) of Nicotiana tabacum cv Xanthi-nc were removed aseptically with a cork borer, placed on solidified MS-W medium and grown under Gro-lux fluorescent lamps (80 foot candles). Callus growth usually became green pigmented within 2 weeks and was maintained by monthly transfers on solid medium. To prepare cells for inoculation, 1 gram of agar-grown callus was transferred to 20 ml of liquid medium in 125 ml flasks and placed on a rotary shaker (120 rpm). After 6-9 days, 1-2 ml of resulting cell suspension was transferred with a large mouth pipette (3 mm opening) to fresh liquid medium. After 6-9 days of additional growth, the cells had developed into small aggregates, 20-75 cells per aggregate, and were inoculated.

Virus inoculation

A common strain of TMV was purified according to Knight (1962) from leaves of N. tabacum cv Havana-38 inoculated 2-4 weeks earlier. The virus was freed of microorganisms by passage through an ultrafine sintered glass filter.

Suspension cell cultures were inoculated with 150 µg TMV per ml by the vibratory technique of Murakishi et al, (1970; 1971). Cells from several flasks were combined on funnels lined with Miracloth filters, washed with fresh medium and dispensed in tubes containing 3 ml of MS-W medium. After adding the TMV suspension the tubes were vibrated for 10 seconds on a Vortex mixer (Scientific Products Corp., Evanston, Ill.), the cells were collected on Miracloth-lined funnels, and washed with fresh liquid medium. The cell clumps were gently stirred to ensure randomization, transferred to filter pads resting on solid medium, and incubated at 24°C under the Gro-lux lamps (Pelcher et al, 1972).

Incorporation of ^{3}H-uridine and ^{14}C-leucine in Xanthi-nc callus

The incorporation of radioactive metabolites into Xanthi-nc callus was determined by placing cell samples into 2 ml of liquid medium containing ^{3}H-uridine (specific activity 9-27 curies per millimole) or ^{14}C-L-leucine (specific activity 230 millicuries per millimole) from New England Nuclear. Cell samples were harvested, washed with ice cold 0.25 M sucrose containing 10^{-4} M uridine and/or 10^{-3} M L-leucine, and ground in a conical glass homogenizer in 0.1 M Tris-HCl (pH 8.0) containing 0.25 M sucrose, 10^{-4} M uridine, 10^{-3} M L-leucine, and 75 mM 2-mercaptoethanol. The homogenate was centrifuged at 1,000 g for 10 minutes and aliquots of the supernatant were precipitated at 4°C in 10% trichloroacetic acid (TCA), collected on glass fiber filters, and washed with cold 5% TCA, 95% ethanol and ethanol:ether (1:1). The filter-trapped materials were digested at 40°C for 4 hours with 0.5 ml of a solution containing 20% NCS Solubilizer (Amersham/Searle, Des Plaines, Ill.), 3.75% water, and 76.25% toluene. Ten milliliters of toluene-based POPOP-PPO solution was added and samples were counted in a Packard Tri-Carb, or Beckman LS-133 liquid scintillation counter with a counting efficiency of 17% for ^{3}H, and 51% for ^{14}C. Doubly labeled samples were corrected for bleedover using ^{14}C-leucine standards prepared in an identical manner.

To determine the efficiency of the labeling procedure, randomly mixed callus samples were placed in liquid medium containing 2.5 μCi ^{3}H-uridine per ml and 0.1 μCi ^{14}C-leucine per ml. Callus samples were harvested after 1, 3, 6, 9, and 12 hours, and the acid-insoluble radioactivity was determined as described above. Both isotopes were incorporated at a constant rate under these labeling conditions (Figure 6).

Cycloheximide (Sigma Chem. Co., St. Louis, Mo.) was prepared in liquid MS-W medium and sterilized by Millipore filtration.

Extraction of labeled TMV from infected callus

Infected cells were incubated in ^{3}H-uridine (12.5-25 μCi per ml) and/or ^{14}C-leucine (0.5-1.0 μCi per ml) in liquid medium, harvested by washing with 0.25 M sucrose containing 10^{-4} M uridine and 10^{-3} M leucine, weighed, and frozen. Thawed cells were ground in a glass homogenizer in 0.1 M Tris-HCl, pH 8.0, containing 10^{-4} M uridine, 10^{-3} leucine, and 75 mM 2-mercaptoethanol and centrifuged at 1,000 g for 10 minutes. The supernatant fluid was centrifuged at 12,000 g for 15 minutes. The final supernatant was layered on 1 ml of 30% sucrose in the Tris buffer and centrifuged in the SW 39L rotor in the Spinco Model L ultracentrifuge at 35,000 rpm for 90 minutes. The final pellet was resuspended in 1 ml of water and given a low-speed centrifugation, and the resultant supernatant was layered on 10-40% linear sucrose gradients prepared in 0.01 M potassium phosphate buffer (pH 7.3). After centrifugation at 23,000 rpm for 90 minutes in the SW 25.1 rotor, gradients were fractionated with an ISCO Model D fractionator coupled to a Model UA-2 UV (254 nm) analyzer. One milliliter fractions were collected and co-precipitated with 0.5 mg bovine serum albumin by 10% TCA. Precipitates were trapped on glass fiber filters and radioactivity was determined as described above.

Radioactivity profiles of sucrose gradients from extracts of TMV-infected and non-infected cells labeled with ^{3}H-uridine were used to determine incorporation into TMV (Figure 3). The peak of radioactivity representing ^{3}H-labeled TMV was found in fractions 9-11. TMV purified by the differential centrifugation procedure (Knight, 1962) sedimented to the same level under these conditions.

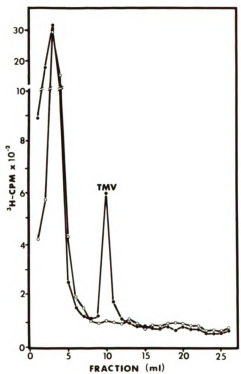

Figure 3. Radioactivity profiles of sucrose gradients. TMV-infected and non-infected Xanthi-nc callus were incubated in 12.5 μCi ^{3}H-uridine per ml of medium and extracted for TMV as described in the text. The extracts were centrifuged in 10-40% sucrose gradients, and radioactivity in 1 ml fractions was determined. Non-infected, 0--0, TMV-infected, ●--●.

RNA extraction and polyacrylamide gel electrophoresis

Inoculated callus was incubated in 20 µCi ^{3}H-uridine per ml, harvested by washing with the cold sucrose solution, weighed, and RNA extracted at 4°C by the sodium-dodecyl-sulfate (SDS)-phenol procedure described by Pelcher et al (1972). The RNA was taken up in the electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA and brought to pH 7.8 with glacial acetic acid) made 5% with respect to sucrose. The RNA concentration of the solution was determined assuming an $E^{0.1\%}_{260nm} = 30$.

The 2.4% polyacrylamide gels were prepared as described by Bishop et al (1967). Polymerized gels were allowed to stand in the electrophoresis buffer containing 0.2% SDS (w/v) at 4°C for 72 hours prior to use. The gels were pre-run for 30 minutes at 5 mA per gel, and then loaded with 15 µg of the RNA in volumes of 15-25 µl. Following electrophoresis for 90 minutes at 5 mA per gel (10 V per cm), the gels were scanned at 260 nm in a Gilford 2400 spectrophotometer with attached gel scanner. The gels were frozen in dry ice, sliced into 1 mm sections and the radioactivity was determined as previously described (Pelcher et al, (1972). Specific activities (CPM per µg RNA) were determined by calculating the µg of RNA from optical density profiles of gels, and by establishing the CPM in RNA from the corresponding radioactivity profiles of the gels.

RESULTS

Efficiency of virus infection

The efficiency of TMV infection of Xanthi-nc callus is expressed by the numbers of lesions produced (Beachy and Murakishi, 1971 a). The greatest number of lesions was produced on callus subcultured 2 times

in liquid medium prior to inoculation while fewer lesions were obtained from callus continuously subcultured. Rapidly growing cultures consisting mainly of single cells produced fewer local lesions than did slowly growing cultures composed of small (0.5-2 mm in diameter) cell aggregates with a "grainy" texture. Cultures composed of grainy cell aggregates were produced by carefully regulating the amount of cells transferred in the second liquid subculture. New explants were taken when the efficiency of infection began to drop.

TMV synthesis in Xanthi-nc callus

The TMV growth curve in Xanthi-nc callus was determined by measuring the incorporation of ^{3}H-uridine (12.5 μCi per ml) and ^{14}C-leucine (0.5 μCi per ml) into virus over 12 hour intervals until 144 hours after inoculation. TMV was purified from 1.5 grams of freshly harvested cells. The results are expressed in counts per minute (cpm) in TMV for each 12 hour interval. The rates of virus synthesis and total yields of TMV from inoculated Xanthi-nc callus varied slightly with each experiment but in general the growth curves were similar to the curve given in Figure 4. Virus synthesis was first detected 12-24 hours after inoculation, and the rate of synthesis increased until 60-72, or 72-84 hours. The high rate of synthesis was followed by a sharp decline (12-24 hours in duration) and then by a second peak of virus synthesis. The second burst of synthesis lasted for 12 or 24 hours and was followed by a drop in the rate of synthesis and the maintenance of the low rate through the remainder of the experiment.

Local lesions were observed 36-44 hours after inoculation in all experiments and continued to enlarge until 84-96 hours (Figure 4). More than 90% of the extracted virus was produced after lesions appeared.

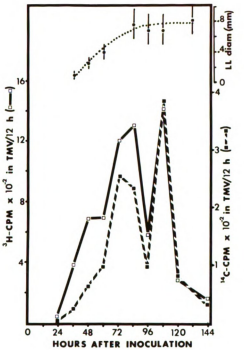

Figure 4. TMV growth curve in Xanthi-nc callus. Cells were incubated for 12 hours in 12.5 μCi ^{3}H-uridine and 0.5 μCi ^{14}C-leucine per ml, and extracted for TMV as described in the text. After sucrose gradient purification, radioactivity in TMV was determined as described. The upper portion of the figure indicates the diameter (mm) of the local lesions (with standard deviation) over the experimental period.

To determine whether or not lesions affected virus extraction, callus containing lesions and callus without lesions were homogenized in the presence of purified ^{3}H-uridine-TMV. Analysis of sucrose gradients of the two extracts showed that extractability of virus was not affected by lesions in the callus.

Effect of cycloheximide on incorporation of ^{14}C-leucine and ^{3}H-uridine

The sensitivity of Xanthi-nc callus to cycloheximide (CHX) was determined by placing randomly mixed cell samples in liquid medium containing ^{14}C-leucine (0.1 μCi per ml) and various concentrations of CHX for 3 hours. Radioactivity of acid insoluble fractions was determined. CHX at 1 μg per ml gave near maximum inhibition of ^{14}C-leucine incorporation (Figure 5) without cell damage. This concentration was used in all later experiments unless otherwise indicated.

The effect of CHX on the incorporation of ^{14}C-leucine and ^{3}H-uridine into the acid-insoluble fraction as a function of time was determined (Figure 6). CHX gave 50% inhibition of ^{3}H-uridine incorporation in 12 hours and about 90% inhibition of ^{14}C-leucine within 1 hour. There was no inhibition of the incorporation of either isotope into the acid soluble fraction.

Under these conditions, CHX remained an effective inhibitor of protein synthesis for 16-20 hours. Thereafter, the rate of ^{14}C-leucine incorporation approached that of the non-treated controls. The recovery of Tetrahymena and rat cells from CHX inhibition was observed previously (Frankel, 1970; Yeh and Shils, 1969).

Effect of cycloheximide on complete virus synthesis

Forty-eight hours after inoculation, Xanthi-nc callus was treated with 20 μCi ^{3}H-uridine per ml in the presence or absence of CHX.

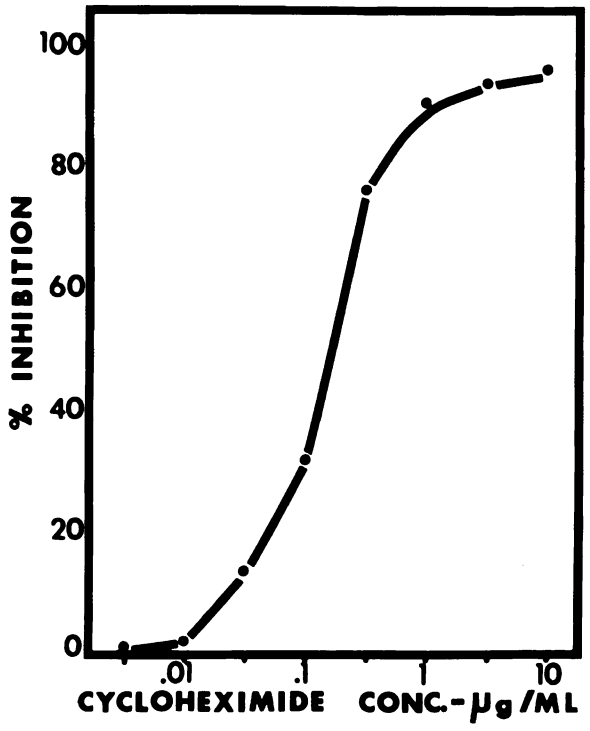

Figure 5. Effect of increasing concentrations of cycloheximide on protein synthesis in Xanthi-nc callus. Cells were incubated with ^{14}C-leucine (0.1 µCi per ml) for 3 hours, washed with cold 0.25 M sucrose containing 10^{-3} M leucine, and homogenized in 0.1 M Tris-HCl, pH 8.0, with 0.25 M sucrose, 10^{-3} M leucine, and 75 mM 2-mercaptoethanol. The homogenate was centrifuged and aliquots were precipitated with 10% trichloroacetic acid (TCA); the precipitate was collected on glass filters and washed with 5% TCA, ethanol, and ether. Radioactivity was determined by liquid scintillation. Percent inhibition was determined by comparing CPM in CHX treated and non-treated samples. Non-treated cells incorporated 1500-2000 CPM per 0.06 gram of cells in 4 experiments.

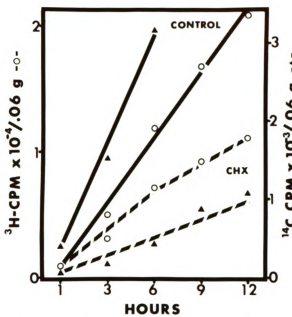

Figure 6. Effect of cycloheximide (CHX, 1 µg per ml) on the incorporation of ^{14}C-leucine (0.1 µCi per ml) (▲) and ^{3}H-uridine (1.0 µCi per ml) (O) into TCA insoluble fractions of Xanthi-nc callus. Radioactive materials and CHX were added simultaneously. See Figure 5 for details.

After 2, 4, and 6 hours, 3.0 gram cell samples were harvested and analyzed for ^{3}H incorporation into completed virus. Cycloheximide reduced the rate of ^{3}H-uridine incorporation into TMV throughout the 6 hour period, and complete virus formation was inhibited by approximately 90% at the end of this time (Figure 7).

Ouchi et al (1969) reported that the inhibition of TMV synthesis in leaf discs by CHX at 1 µg per ml was bipartite in nature; treatment at 0-12 hours after inoculation resulted in decreased virus synthesis, and treatment beyond 12 hours caused a 2-fold increase in virus titer. To determine whether or not CHX inhibition of TMV synthesis in our system was dependent upon the time of treatment, inoculated tissue cultures were incubated in 0.4 µCi ^{14}C-leucine per ml and 12.5 µCi ^{3}H-uridine per ml in the presence or absence of CHX for 12 hour intervals at 4 time periods after inoculation. When CHX was applied at 24-36, 36-48, 48-60, and 60-72 hours after inoculation, the incorporation of both isotopes into TMV was inhibited approximately 90% (Figure 8). To determine whether or not TMV extractibility was affected by the CHX treatment, callus cultures were placed in a solution of CHX or in growth medium alone, harvested, and homogenized with purified ^{3}H-uridine-TMV. After purification by sucrose gradient centrifugation, equal amounts of ^{3}H-TMV were recovered from tissues given both treatments. When inoculated callus was treated with CHX at 5 µg per ml (0-30 minutes after inoculation), virus synthesis and local lesion formation were inhibited for at least 84 hours.

Effect of cycloheximide on TMV-RNA synthesis

The results suggested that synthesis of complete virus was inhibited by CHX (1 µg per ml) to a degree similar to the inhibition

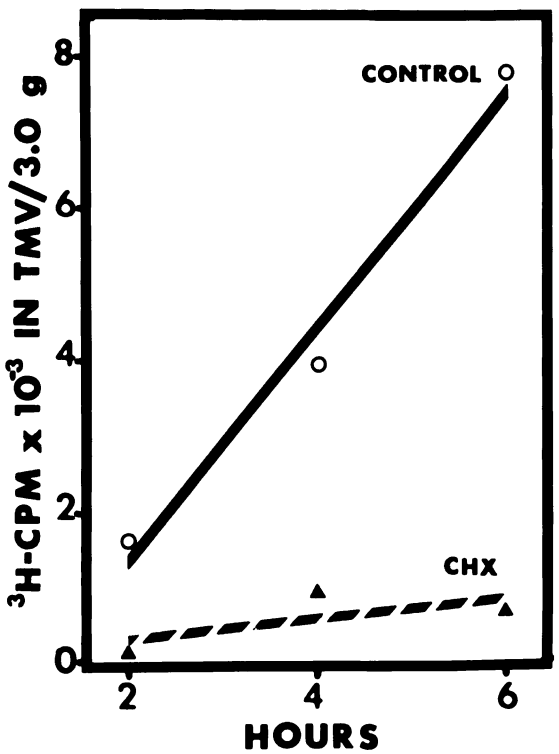

Figure 7. Effect of cycloheximide (CHX, 1 µg per ml) on the incorporation of ^{3}H-uridine (20 µCi per ml) into TMV in Xanthi-nc callus. At 48 hours after inoculation, cells were treated simultaneously with ^{3}H and CHX. TMV was extracted from 3.0 grams of cells homogenized in Tris buffer, followed by differential centrifugation, and 10-40% sucrose gradient centrifugation. Gradients were monitored at 254 nm and radioactivity in 1 ml fractions was determined. Incorporation into TMV was determined by comparing optical density and radioactivity profiles.

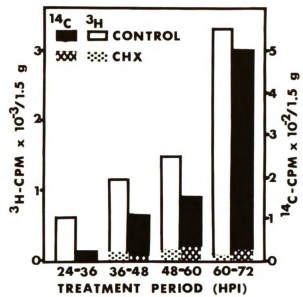

Figure 8. Effect of cycloheximide (CHX, 1 µg per ml) on incorporation of ^{14}C-leucine (1 µCi per ml) and ^{3}H-uridine (20 µCi per ml) into TMV. Each sample was treated for 12 hours during 24-72 hours post inoculation (HPI) and radioactivity in TMV was determined as described for Figure 5.

of cellular protein synthesis. To determine the effect of CHX on the synthesis of TMV-RNA, inoculated cells (at 48 hours after inoculation) were incubated in 20 μCi ^{3}H-uridine per ml with and without CHX for 2, 4, and 6 hours.

The specific activity (CPM per μg RNA) of the 25 S and 18 S ribosomal RNA (Loening and Ingle, 1967) increased throughout the 6 hour period in the untreated cells (Figure 9). Cycloheximide, however, reduced the rate of incorporation of ^{3}H-uridine into 25 S and 18 S ribosomal RNA and caused a 90% reduction in their specific activities by 2 hours of treatment. There was no inhibition of ^{3}H-uridine into 4-5 S RNA. Similar effects of CHX on the synthesis of cellular RNA were reported in Neurospora (Viau and Davis, 1970) and in rat liver (Muramatsu et al, 1970). The synthesis of TMV-RNA, expressed as specific activity, was not affected by CHX treatment. The specific activity of TMV-RNA from the CHX-treated and non-treated cells was approximately 2.5 times that of ribosomal RNA from non-treated cells by the fourth hour of labeling.

DISCUSSION

Leaves of tobacco cultivars such as Samsun NN and Xanthi-nc contain the N gene (Holmes, 1939), and react by producing necrotic local lesions after inoculation with TMV. Callus cultures from these cultivars also produce lesions following TMV infection (Beachy and Murakishi, 1971 a). The necrotic response to virus infection has not been demonstrated, however, in isolated cells (Jackson and Zaitlin, personal communication) nor in protoplasts (Otsuki et al, 1972) from Samsun NN and Xanthi-nc. Otsuki et al (1972) suggested that the necrotic reaction

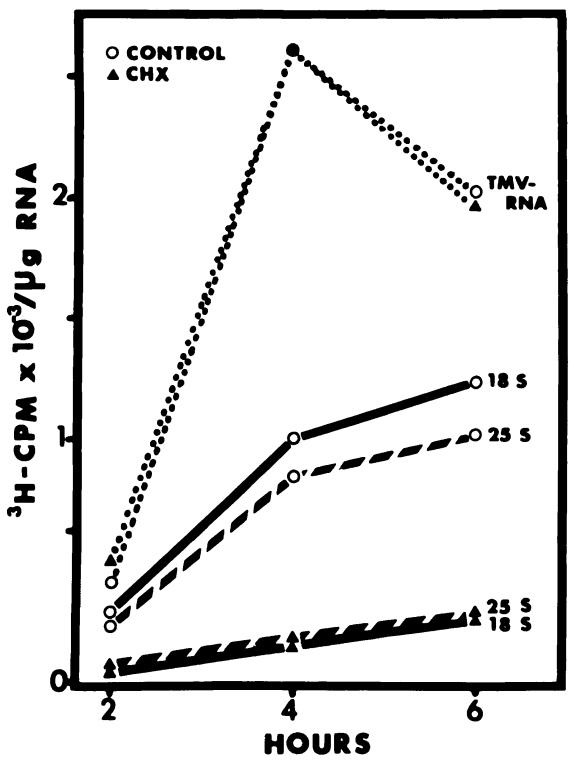

Figure 9. Effect of cycloheximide (CHX, 1 µg per ml) on the incorporation of ^{3}H-uridine in RNA of virus infected Xanthi-nc callus. RNA was extracted from 3.0 grams of cells in SDS-phenol and separated by electrophoresis on 2.4% polyacrylamide gels. Gels were scanned at 260 nm, and radioactivity in 1 mm slices was determined. CPM in each RNA species was determined by comparing optical density and radioactive profiles.

depends upon the interactions between infected and neighboring cells. Our results support their suggestion since the reaction occurred only in callus cultures composed of cell aggregates. The interactions could occur by plasmodesmatal connections between cells in tobacco callus aggregates (Hartmann, 1971).

Tobacco mosaic virus synthesis in Xanthi-nc callus occurred in 2 major bursts during 144 hours and was similar to TMV synthesis in callus from a systemic tobacco (Pelcher et al, 1972). The necrotic reaction appeared to have little or no inhibitory effect on virus synthesis since more than 90% of the virus was synthesized after lesions were evident. In leaf systems it is well established that virus synthesis occurs in advance of the spreading necrotic lesion (Osawa and Yamaguchi, 1970; Hayashi and Matsui, 1965). Recently Otsuki et al (1972) compared the synthesis of TMV in leaf discs of Samsun tobacco (a systemic host) and Samsun NN, and reported that the rate of virus synthesis slowed after lesions became apparent. Their data showed about a 10-fold difference in extractible virus (as measured by infectivity assays) by 96 hours after inoculating the two cultivars. The possibility that the lower infectivity could have resulted from decreased virus extractibility from the local lesion host was apparently not explored. From all of these data it appears that the effects of the necrotic response on TMV synthesis in the initially infected and immediately adjacent cells cannot be specified at this time.

Cycloheximide inhibits protein synthesis by preventing peptide elongation (McKeehan and Hardesty, 1969) and is effective in tobacco callus cultures (Figure 5; Nudel and Bamberger, 1971). We found that tobacco callus recovered from the inhibition caused by low concentrations of CHX (0.01-1.0 μg per ml) but not from higher concentrations.

A similar recovery phenomenon was observed with Tetrahymena (Frankel, 1970) and rat tissues (Yeh and Shils, 1969) when low concentrations of CHX were used. The data clearly emphasize the importance of monitoring the length of time that CHX remains effective.

The inhibition of complete virus synthesis by cycloheximide appeared to be similar to the inhibition of host cytoplasmic protein synthesis (compare Figures 6 and 7) in Xanthi-nc callus. The data compare favorably with those for tobacco leaf discs (Zaitlin et al, 1968). Synthesis of TMV-RNA, however, was not affected by 4 hours of CHX treatment (Figure 9). The results agree with those obtained for several animal viruses (Nobel and Levintow, 1970; Friedman and Grimley, 1969). Harrison and Crockatt (1971) and McCarthy et al (1972) reported that the synthesis of tobacco rattle and tobacco necrosis viral RNAs, determined by measuring the infectivity associated with phenol extracts of leaf tissues, continued in the presence of CHX. To the best of my knowledge, my results represent the first such report for TMV. Failure of the specific activity of TMV-RNA to increase during the fourth to sixth hour of labeling (in CHX treated and non-treated cells) is not clear. During the same time period, complete virus continued to accumulate in untreated cells (Figure 7).

Since TMV coat protein is present in extracts from infected Xanthi-nc callus (Beachy and Murakishi, 1971 b) and in TMV-infected Samsun NN leaves (van Loon and van Kammen, 1970), it is possible that TMV-RNA synthesized in the absence of viral protein synthesis (CHX sensitive) would have been encapsulated in viral protein already present in the cells. This was not the case, since the incorporation of ^{14}C-leucine and ^{3}H-uridine into complete virus was equally inhibited by CHX treatment (Figure 8). The possibility remains, however, that the

techniques used were not sufficiently sensitive to detect whether or not existing proteins were used.

Inhibition of complete virus formation could be caused by the inhibition of viral coat or other protein synthesis, or to the inhibition of virion assembly, as suggested by the failure of viral protein present in the cells to encapsulate viral RNA. Further studies utilizing appropriate plant tissue-TMV-inhibitor systems may be useful in elucidating the nature of TMV assembly in vivo, and the nature of virus protein pools.

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CHAPTER 3

PROTEINS FROM CULTURED CELLS OF XANTHI-NC TOBACCO INOCULATED WITH TOBACCO MOSAIC VIRUS

*PROTEINS FROM CULTURED CELLS OF XANTHI-NC TOBACCO INOCULATED WITH TOBACCO MOSAIC VIRUS

SUMMARY

Cell suspensions of Nicotiana tabacum Var. Xanthi-nc tissue culture were inoculated with 100 µg tobacco mosaic virus (TMV) per ml, washed with fresh medium to remove excess inoculum, and incubated on agar medium under diffuse light. Local lesions were observed on inoculated cell cultures after 40 hours. At 24-hour intervals inoculated and noninoculated control cells were harvested, and cell extracts were prepared by homogenization and centrifugation at low (15,000 g) and high (144,000 g) speeds. The supernatant from the high speed centrifugation was subjected to electrophoresis on 7% and 10% polyacrylamide disc gels, stained for protein in amido black, and scanned in a Gilford densitometer. On a fresh weight basis, more proteins were extracted from lesion-bearing inoculated tissue than from control tissues. Two new protein bands, characterized as TMV protein, were detected from extracts of infected cells. Intensities of several normal host protein bands diminished, whereas others increased following infection. Data indicate that protein changes can be detected in virus-infected cells from tissue cultures more easily than in virus-infected leaves.

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INTRODUCTION

Tobacco mosaic virus (TMV) is a rod-shaped virus composed of a single strand of ribonucleic acid (RNA) encapsulated in coat protein (Hart, 1955; Fraenkel-Conrat, 1955). TMV-RNA contains approximately 6340 nucleotides with a total molecular weight of 2.05×10^6 daltons (Takahashi, 1967). Assuming the triplet code for translation of messenger RNA (Crick, 1962), there are enough nucleotides to code for 2133 amino acids. The coat protein contains 158 amino acids (Fraenkel-Conrat, 1965) coded for by 474 nucleotides. This represents only 7-8% of the total genetic material, and, clearly, additional viral proteins could be made from the TMV-RNA template.

Suggestions of the possible nature of the TMV gene products come from studies of other single-stranded RNA viruses of similar size. The small RNA coliphages ($1.1\text{-}1.3 \times 10^6$ daltons) are known to have several gene products including a replicase enzyme, maturation protein, and coat protein (Kozak and Nathans, 1972). Poliovirus RNA (1.9×10^6 daltons) is known to code for at least 14 proteins including 4 which assemble into the coat protein (Baltimore, 1969). These viruses are amenable to study because of their ability to shut off host RNA and protein synthesis, facilitating the detection of virus products. This does not occur in TMV infection, however, and studies of virus gene products are more difficult. Several methods of study have been used.

One method has been the use of cell free systems as described by Nirenberg and Matthaei (1961). Repeated attempts to use E. coli cell-free systems primed with TMV-RNA as messenger initially produced interest, but it now appears that only fragments of the genetic message are being randomly translated (Reichmann and Clark, 1968; Matthews, 1970).

In vitro systems from plant cells have also not been successful (Reichmann and Clark, 1968; Takahashi, 1967), probably because of the large amounts of nucleases released upon homogenization.

Kiho (1968) reported the isolation of TMV-specific polyribosomes from infected tobacco leaves. It may be possible to identify proteins other than coat protein by using this procedure for isolating the elongating peptides.

Another method used has been the attempted isolation and characterization of specific proteins. From studies which elucidated the nature of the replication of RNA-containing bacterial and animal viruses (Kozak and Nathans, 1972; Baltimore, 1969), an RNA-dependent-RNA-polymerase was implicated in the replication of TMV-RNA. Though efforts to demonstrate the presence of the enzyme in infected tissues have been relatively successful (Ralph and Wojick, 1969; Bradley and Zaitlin, 1971), only a single report on characterization of the enzyme has been presented (Brishammar, 1970). Recent work by Zaitlin *et al* (personal communication) indicated that they had partially purified a host mediated RNA-dependent-RNA-polymerase which is stimulated by TMV infection. The role of virus infection on enzyme activity and specificity remains to be determined.

The polyacrylamide gel electrophoresis technique has been used in recent studies which attempted to characterize virus proteins, or virus-induced host proteins, in infected cells. Using disc gel electrophoresis, which separates native proteins on the basis of size and charge (Davis, 1964), van Loon and van Kammen (1970) identified the viral coat protein in cell extracts from systemically infected leaves, but other viral proteins were not detected by this procedure. They also reported

the appearance of 4 new proteins in extracts from leaves bearing local lesions, and it was suggested that they were produced by the host in response to virus infection.

Other workers used sodium-dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis, which denatures proteins and provides for their separation on the basis of size (Maizel, 1966; Weber and Osborn, 1969), and radioactive labeling to detect virus-directed or stimulated proteins in tobacco leaves systemically infected with TMV. Zaitlin and Hariharasubramanian (1971, 1972) and Singer (1971, 1972) fed TMV-infected leaves with ^{3}H-amino acid and non-infected leaves with ^{14}C-amino acid. The 2 samples were combined, homogenized, and subjected to co-electrophoresis. The ratio of the isotopes (CPM ^{3}H÷CPM ^{14}C) throughout the gel remained constant in the proteins not affected by virus infection, but stimulated or repressed protein synthesis resulted in a change of the isotope ratio. Zaitlin and Hariharasubramanian (1971, 1972) reported finding the coat protein and several larger molecular weight proteins; they suggested that one of these might represent the replicase. Singer (1971), however, reported finding only the coat protein.

Plant tissue cultures capable of being infected with virus have been developed in this laboratory and it was hoped that the tobacco callus system would provide a useful tool for the identification of viral proteins. Since local lesions were produced on callus taken from hypersensitive tobacco cultivars following TMV infection (Beachy and Murakishi, 1971), I wished to re-examine the occurrence of the 4 new proteins reported by van Loon and van Kammen (1970). Therefore, extracts of TMV-infected and non-infected Xanthi-nc callus were made at 24 hour intervals until 144 hours after inoculation and compared for soluble proteins by disc gel electrophoresis.

MATERIALS AND METHODS

Callus cultures and TMV inoculation

Nicotiana tabacum Xanthi-nc callus was prepared from aseptically removed cores of stem pith grown on the solid Murashige and Skoog - White (MS-W) medium (Appendix). Pigmented callus was maintained on solidified medium, and prepared for inoculation as previously described (Chapter 2, Materials and Methods). Callus tissue grown in liquid medium was inoculated with 100-120 µg TMV per ml by the vibratory technique of Murakishi *et al* (1970), and incubated on solidified medium under Gro-lux lamps.

A mannitol-containing medium (MS-W-mannitol) was prepared in the MS-W formula (Appendix) by substituting equimolar amounts of mannitol for dextrose and glucose in the separately prepared media. When this medium was to be used, callus was produced in the normal MS-W medium, then inoculated and washed with MS-W-mannitol, and incubated on solidified mannitol medium.

Extraction buffer and electrophoresis solutions

The extraction buffer, modified from that described by Staples and Stahmann (1964) as reported by van Loon and van Kammen (1968), contained 0.1 M Tris-HCl, pH 8.0 (room temperature), 0.5 M sucrose, 1% ascorbic acid, and 75 mM 2-mercaptoethanol. The original buffer contained 1% cysteine-HCl, but it was found that substituting 2-mercaptoethanol at this concentration prevented browning during cell homogenization. The buffer was not kept more than 48 hours.

The electrophoresis solutions were prepared as described by Davis (1964), except for the small-pore gel buffer (solution B); the solution

was brought to pH 6.9 with phosphoric acid (van Loon and van Kammen, 1968) instead of pH 6.7 with hydrochloric acid.

Soluble protein extraction and electrophoresis

Cell samples were harvested at 24 hour intervals, weighed and cooled to 4°. The cells were ground in a conical glass homogenizer in buffer 1:5 (v:w). The homogenate was immediately squeezed through 4 layers of cheesecloth (delay at this step resulted in extensive browning of the homogenate), centrifuged at 10,000 g for 30 minutes, and the supernatant was subjected to 144,000 g for 150 minutes in the SW 39L rotor (Spinco Model L, 35,000 rpm). The final supernatant was removed and made 5% with respect to sucrose. Bromophenol-blue was added to serve as a marker. The procedure was designed to extract the soluble cell proteins, and to remove bound proteins by centrifugation. By this method, intact TMV would not be disrupted, and would be removed by centrifugation.

Seven per cent and 10% polyacrylamide disc gels were prepared according to Davis (1964) in glass tubes 6 mm (inside diameter) x 8 cm which were acid-washed, and rinsed with Photoflo (Eastman Kodak Co.). The gels were placed in the electrophoresis apparatus and 500 ml of reservoir buffer (Davis, 1964) was added to the upper and lower reservoirs. After placing 0.1 ml of the protein extract on the gel surface, electrophoresis was performed at 4° with a Spinco Duostat constant current/constant voltage power source at 2 mA per gel (constant current) for 20 minutes, and 3 mA per gel until the marker dye reached the end of the gel (75-90 minutes). Gels were removed from the tubes, stained overnight in a solution of 0.5% amido black (van Loon and van Kammen, 1968) in 7% acetic acid, and electrophoretically destained at 6 mA per

gel (constant voltage) with 7% acetic acid in both reservoirs. The gels were scanned at 660 nm in a Gilford 2400 spectrophotometer with attached gel transporter. Electropherograms were traced directly from the originals.

Immunodiffusion

For immunodiffusion experiments 7% gels containing the extracted proteins were sliced lengthwise, and placed flat side down in a petri plate. The antiserum solution, consisting of rabbit anti-TMV serum 1:4 in a solution of 0.9 M NaCl, and 0.9% agar at 37-40°, was poured around the sliced gels, allowed to harden, and left at room temperature for several days.

RESULTS AND DISCUSSION

Burley NN and Xanthi-nc callus cultures were tested; both produced local lesions after infection with TMV. In repeated experiments the Xanthi-nc callus, a cell line with green pigments, consistently produced more local lesions following inoculation, and was less likely to become brown after homogenization, than was Burley NN callus. Browning of the cell extract is caused by the oxidation of phenolic compounds (Loomis and Battalie, 1966) which are abundant in tobacco callus (Fritig and Hirth, 1971). Electrophoresis and subsequent staining of such extracts resulted in poor protein separation and diffuse staining throughout the gels. This confirms the observations of van Loon and van Kammen (1968). The Xanthi-nc callus was used in all further experiments.

In early experiments, extracts of healthy and TMV infected callus were separated on 7% and 10% gels to make more complete comparisons of protein components (van Loon and van Kammen, 1968). Essentially the

same results were obtained from both gels, but protein bands were separated better on the 10% gels (Figure 10). There appeared to be better resolution of both the fast and slow migrating proteins on 10% gels, which were used routinely for all other experiments.

In the first two experiments in which the cells were grown on the usual MS-W medium, no distinct changes in soluble proteins were detected from 24-144 hours after inoculation. Since it had been previously shown that Xanthi-nc callus grew rapidly on this medium (more than a 3-fold increase in fresh weight by 144 hours, Figure 19, Appendix), it was probable that changes which occurred following virus infection were "diluted" by the new cell growth. Virus is localized in and around the lesion area of Xanthi-nc callus (Beachy and Murakishi, 1971) and the growth of uninfected cells would reduce the proportion of virus-infected cells. To reduce cell growth, mannitol was substituted for sucrose and dextrose to make the MS-W-mannitol medium described in Materials and Methods. Mannitol is an efficient osmoticum but is not metabolized by tobacco cells (Trip et al, 1964). There was less than a 10% fresh weight increase in Xanthi-nc callus after 110 hours of incubation on mannitol medium. Callus kept under these conditions became greener under Grolux illumination than did other callus, and the cells appeared to remain normal when observed by light microscopy.

Xanthi-nc callus was inoculated with TMV, incubated on the mannitol medium, and extracted for proteins at 24 hour intervals until 144 hours after infection for disc gel electrophoresis. There were 3 experiments of this type, and the same general observations were made in each experiment. The results of one experiment in which the greatest differences were observed are described. Since the gel lengths varied

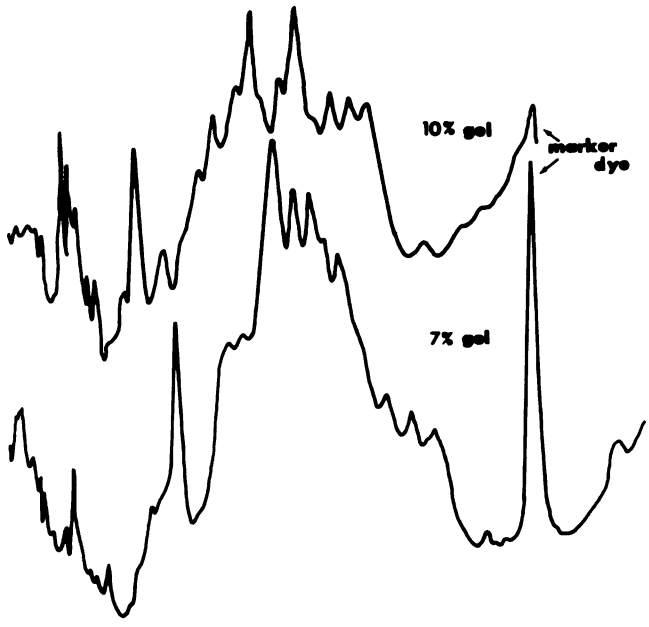


Figure 10. Soluble proteins from Xanthi-nc callus separated on 7% and 10% polyacrylamide disc gels. Gels were stained with amido black and scanned in a Gilford gel scanner. Migration is from the left.

slightly, and the rate of migration was not constant between gels, an R_f value was calculated by setting the migration of the marker dye equal to 1.00.

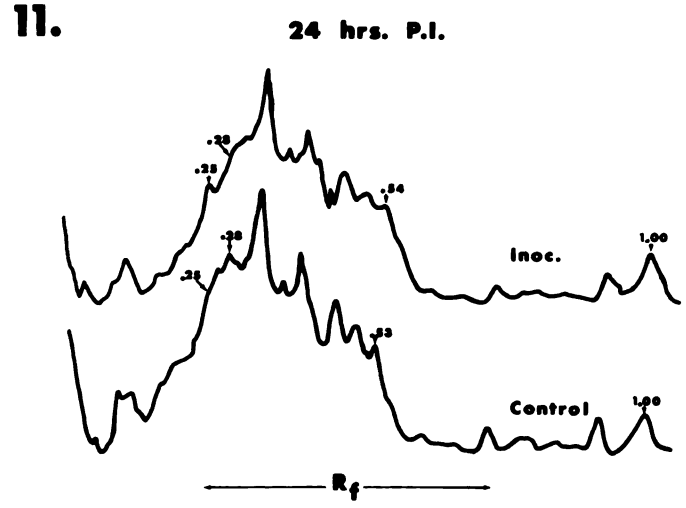
Electropherograms from cells extracted 24 hours after inoculation are shown in Figure 11. The slight differences in proteins between the samples, such as those at R_f 0.25 and 0.28, were probably within the range of variability, and will not be considered in the discussion below.

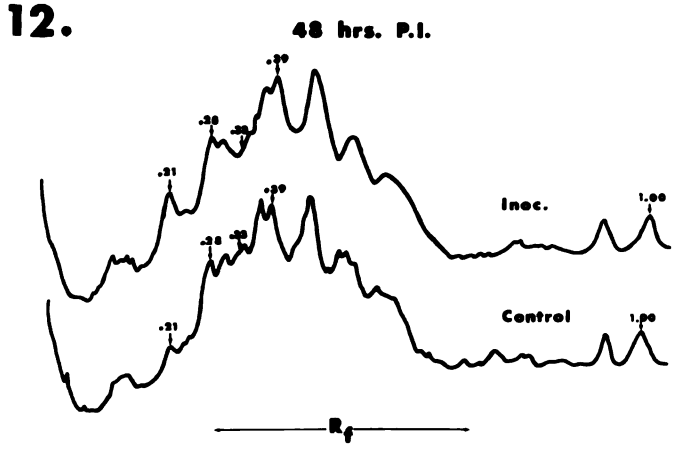
At 48 hours after inoculation, several changes in the stained proteins were observed (Figure 12). There was an increase in the 0.21 band in the inoculated sample and the 0.33 cells. The migration of other proteins were similar in both gels (for example, components 0.28 and 0.39).

By 72 hours after inoculation a large peak, R_f=0.21, was prominent in the inoculated cells only (Figure 13). In addition, the 0.28 component was present in larger proportions in the inoculated cell extract. It appeared that the 0.21 and 0.28 peaks at 72 hours were similar to those with the same migration observed at 48 hours.

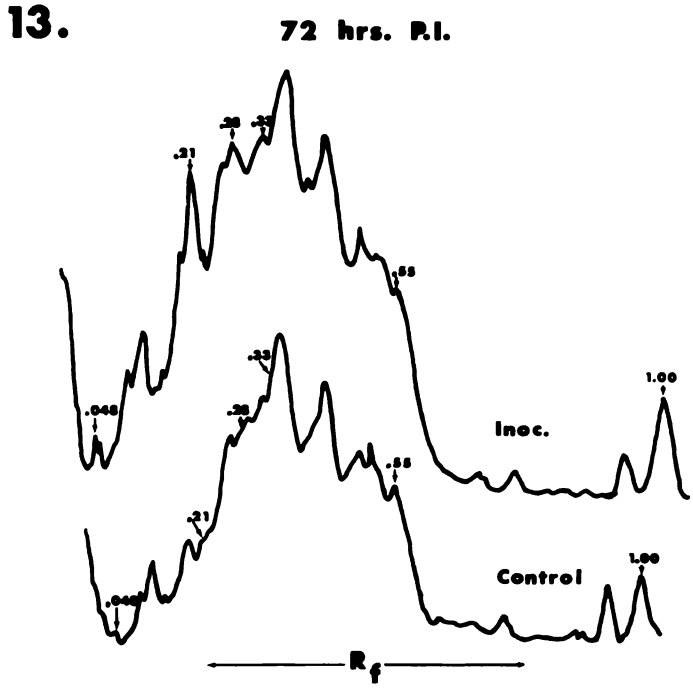
Ninety-six hours after inoculation the 0.20 component remained prominent, and there were major changes at R_f 0.26-0.41 (Figure 14). The apparent increases in components 0.26 and 0.35 are substantiated by the similarity of the 0.41 and 0.54 bands in both cell extracts. This indicated that relative protein migration was consistent between the gels.

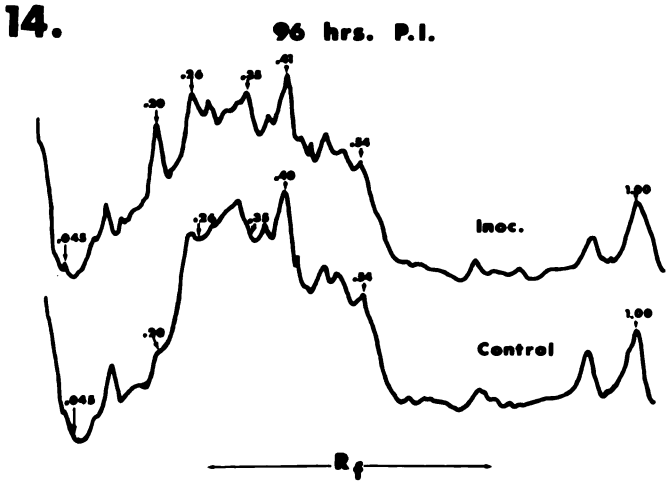
The extracts made at 120 and 144 hours after inoculation (Figures 15, 16) showed the same kinds of differences between inoculated and control extracts as were seen at 96 hours. In addition, the component 0.043-0.05 was present in the inoculated sample as a distinct peak, but



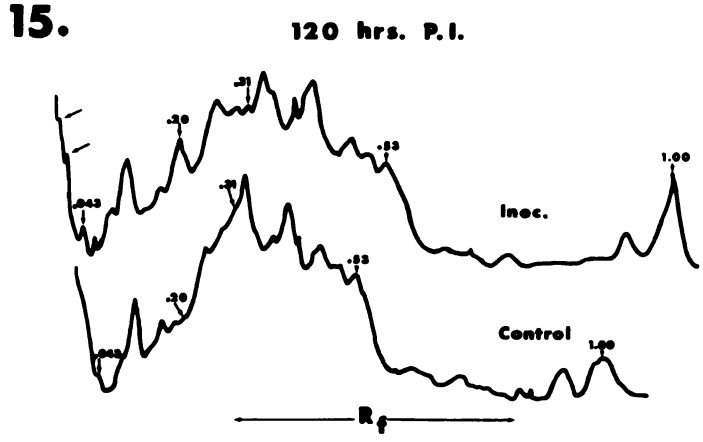


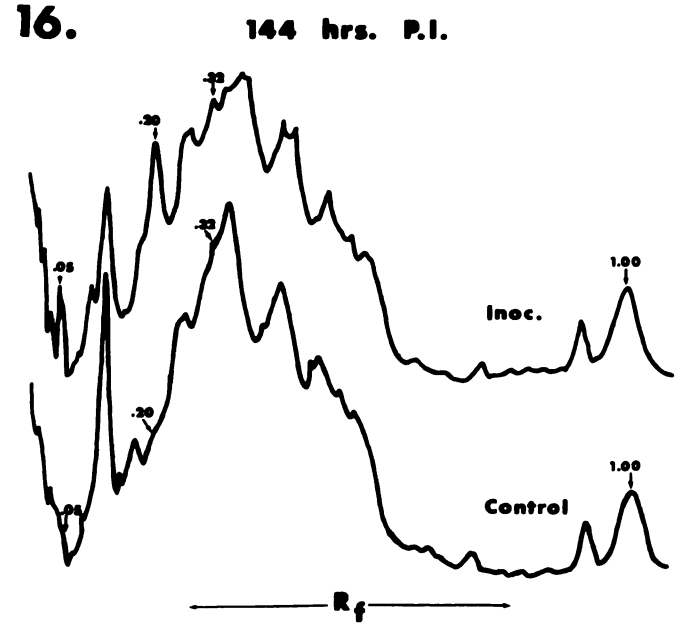
Figures 11, 12. Soluble proteins from infected and non-infected Xanthinc callus. Proteins were separated on 10% polyacrylamide disc gels and stained with amido black. The gels were monitored at 660 nm with a Gilford gel scanner. Migration is from the left. R_f was calculated from the migration on the marker dye. Extracts were taken at 24 and 48 hours post inoculation (hrs. P. I.).





Figures 13, 14. Soluble proteins from infected and non-infected Xanthinc callus. See Figure 11 for details. Extracts were taken at 72 hours (Figure 13) and 96 hours (Figure 14) post inoculation (hrs. P. I.).





Figures 15, 16. Soluble proteins from infected and non-infected Xanthinc callus. See Figure 11 for details. Extracts were taken at 120 hours (Figure 15) and 144 hours (Figure 16) post inoculation (hrs. P. I.).

remained as a shoulder in the control cells. This component was also extracted at 72 and 96 hours in lesser proportions.

In summary, the 0.20-0.21 component increased by 48 hours after inoculation and was found through 144 hours. Other components with R_f values of 0.20-0.41 varied from day to day. An exception was the 0.26-0.28 component which was extracted at 48-144 hours, but not always in sufficient proportions to be definitely correlated with virus infection. Another component, 0.043-0.05, was observed in only the inoculated cell extracts. Initially observed at 72 hours, the peak was also present at 96 and 120 hours and became intense by 144 hours. Since this band was most noticeable at 72 and 144 hours when the 0.20-0.21 component was also most intense, the two bands may be interrelated. It is possible that a quantitative aspect is also being observed, however, since the amount of protein applied to the gels at 72 and 144 hours was greater than the other times (as indicated by the height of the tracings above the baseline).

The changes in protein bands other than virus antigen bands may represent changes in normal host proteins due to the infection. Curtis (1971) reported changes in isozymes of peroxidase in bean leaf extracts following the formation of tumors induced by Agrobacterium tumefaciens. The changes that he observed on 7% gels occurred at approximately R_f 0.4-0.55. The changes which were observed in stained proteins from tobacco callus after virus infection were at R_f 0.3-0.4 on 10% gels; on 7% gels, their relative migration would have been 0.36 -0.48. The results suggest that changes in extractible proteins other than coat protein may represent increases or decreases in normal host proteins, perhaps isozymic changes.

van Loon and van Kammen (1970) were unable to demonstrate TMV antigen in cell extracts from infected Samsun NN, or N. glutinosa leaves, local lesion hosts of TMV. To determine whether or not soluble TMV-antigen was present in Xanthi-nc callus proteins were extracted from infected and non-infected cells at 96 hours after inoculation and subjected to electrophoresis on 7% gels. The gels were exposed to TMV antiserum as described in Materials and Methods. After 2-3 days of incubation, an antigen-antibody precipitin reaction was observed at the surface of the gel containing the extract from the infected cells (Figure 17).

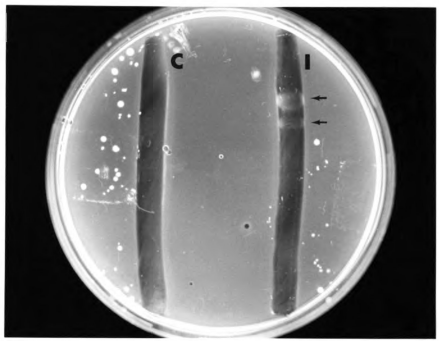


Figure 17. Identification of TMV protein by antigenic reaction. Soluble proteins from infected and non-infected Xanthi-nc callus were separated on 7% polyacrylamide disc gels. Gels were sliced, and immersed in TMV antibody as described in the text. Migration was from the top. C = non-inoculated cells; I = inoculated cells. The arrows mark the 2 bands of antigen-antibody precipitation.

The non-infected cell extracts did not show this reaction. In other gels run at the same time and stained for proteins, two bands were observed in the infected cell extracts at positions identical to the two precipitin lines, and corresponded closely to the 0.20-0.21 and 0.26-0.28 components reported above. van Loon and van Kammen (1970) reported that TMV coat protein in extracts from infected leaves of a systemic tobacco host had an R_f value of 0.22 in 10% gels. On these bases the 0.20-0.21 component from Xanthi-nc callus was thought to represent coat protein. The second precipitin band indicated that the 0.26-0.28 peak was also TMV protein, and may represent aggregated coat protein, perhaps X-protein (Takahashi and Ishii, 1953). Whether this band is an artifact of the isolation procedure, or is a true reflection of coat protein in the cells is not known. An interesting speculation is that the second band is a result of the hypersensitive response, and is somehow related to inhibition of virus synthesis in the host.

van Loon and van Kammen (1970) reported finding 4 host proteins in larger amounts in leaves of Xanthi-nc 7 days after infection, with R_f values of 0.55-0.84 on 10% gels. Similar protein changes were not observed in the callus-TMV system, and several reasons are suggested: (1) synthesis of the proteins did not occur in the callus; or (2) the concentration of the proteins was too low to be detected in the callus system. van Loon and van Kammen (1970) suggested that the new proteins were related to the acquired resistance phenomenon described by Ross (1961a, 1961b), but no further work on characterization of the proteins has yet been forthcoming. If their suggestions are correct, the failure of the infected callus to produce the proteins implies the absence of acquired resistance in TMV-infected Xanthi-nc callus.

The results of this study suggest that Xanthi-nc callus inoculated with TMV and incubated on mannitol-containing medium would be a good system in which to study viral proteins or virus-induced host-proteins: (1) local lesions (indicative of virus infection) are produced under these conditions; (2) electrophoresis of extracts of cells grown on mannitol-containing medium demonstrated that changes in cell proteins could be observed by this technique; (3) since the weight of cells does not increase under these conditions, it is possible to study the changes which occur only in those cells present at the time of inoculation; (4) soluble protein extracts of tobacco callus resolved into more, distinct bands upon electrophoresis than extracts from infected tobacco leaves (see van Loon and van Kammen, 1968, 1970). In other preliminary experiments it was determined that mannitol treatment did not greatly affect the incorporation of ^{14}C-amino acid into an acid insoluble fraction of the cell homogenate. The system would, therefore, be amenable to the type of double labeling experiments such as those of Singer (1971, 1972) and Zaitlin and Hariharasubramanian (1970, 1972). This procedure would more accurately detect changes in extracted proteins due to virus infection.

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SUMMARY AND RECOMMENDATIONS

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Cultured cells of 4 hypersensitive tobacco cultivars produced necrotic local lesions following infection with TMV. Specific cultural conditions are required for high lesion numbers: (1) the callus culture must be composed of cell aggregates with a "grainy" texture at the time of inoculation; and (2) the growth medium must contain Murashige and Skoog mineral and vitamin stocks, coconut water, 2,4-D, and NAA. An important advance towards characterization of the necrotic response in cultured cells would be the elimination of coconut water which remains largely undefined. If the growth, virus infectibility, and lesion expression of the cultured cells could be controlled by altering specified components in a defined medium, the steps involved in these processes could be closely examined. Whether or not lesions can be produced in a defined medium, clearly warrants further study.

More than 90% of the TMV synthesized in Xanthi-nc callus occurred after necrotic lesions were formed. Microscopic studies of TMV-infected leaves of hypersensitive bean indicated that materials deposited between the cells blocked the plasmodesmatal connections, and thereby prevented virus spread (Wu and Dimitman, 1970). This occurs, however, several cells distant from the point of infection, and 5-7 days after necrosis is first observed. All of the data raise questions regarding the effect, if any, of the necrotic response on the synthesis and/or processing of viral protein(s), and of viral RNA. A comparison of the kinetics of viral RNA and protein synthesis in callus from Xanthi-nc

and Xanthi (a systemic host) before, during, and after lesion development on the hypersensitive callus may elucidate the effects of the necrotic reaction, and the nature of the disease resistance conferred by the necrotic (N) gene (Holmes, 1939).

Within 1 hour of applying cycloheximide (CHX) to infected Xanthi-nc callus, the formation of complete TMV was prevented, but the synthesis of TMV-RNA was unaffected even after 4 hours of treatment. Further studies may answer questions related to the nature of TMV synthesis and assembly such as the following: (1) How long does the synthesis of TMV-RNA continue in the absence of protein synthesis (in the presence of CHX)? Answering this question will help to characterize the lifetime of the enzyme (replicase) responsible for the synthesis of viral RNA. (2) Does CHX prevent the formation of complete virus by blocking the movement of TMV-RNA from its "site of synthesis" to its "site of assembly"? (3) Are pre-existing pools of coat protein available for encapsulating TMV-RNA synthesized in the presence of CHX, or (4) are proteins, other than virus coat protein, required for virus assembly? Questions 2, 3, and 4 were raised when TMV-RNA synthesized in the presence of CHX was not incorporated into complete virus, even though a pool of coat protein had been identified in Xanthi-nc callus. Further studies are needed to clarify the nature of TMV synthesis and assembly.

Changes in soluble proteins following TMV infection of Xanthi-nc callus were detected by polyacrylamide disc gel electrophoresis. The TMV coat protein was detected by the antibody-antigen reaction. An additional protein component was detected (by staining) which may be related to virus infection. The staining technique, however, is not accurate for detecting small changes in protein synthesis. Tissue culture cells take up radioactive amino acids readily, and double

labeling experiments similar to those reported by Zaitlin and Hariharasubramanian (1970) and Singer (1971) would more accurately detect the effects of virus infection on host cell protein synthesis, and may lead to the identification of additional viral proteins other than coat protein.

APPENDIX

APPENDIX

The three chapters of the thesis were prepared primarily for publication. Additional observations and results of experiments not recorded in the thesis proper are present in the Appendix. It gives information related to tissue culture cell lines used, the value of the growth medium, and the inoculation procedures used. It is hoped that the information given here will serve as a background for further work on the hypersensitive callus - TMV system.

*Local lesion and viral crystal formation in tissue culture cells of different species of Nicotiana

Callus derived from cultivars of Nicotiana tabacum L. and other Nicotiana species was studied to determine which cell lines would produce large amounts of virus and would therefore be amenable to further studies of virus infection. Most of the cultures were developed by H. H. Murakishi during 1965-1968 and were maintained on agar-solidified White's medium (1954). Nicotiana tabacum cvs Samsun NN (SNN), Burley NN (BNN), and N. glutinosa were developed by the author. Two cultivars of N. tabacum, an albino mutant, and an habituated cell line, were obtained from S. Venketeswaran (University of Houston).

Agar-grown callus (300-400 mg) were placed into 40 ml test tubes containing 3 ml liquid medium and 36 µg TMV, and the tubes were vibrated with a Vortex mixer for 15 seconds. Contents of each tube were poured into a funnel lined with coarse filter paper and allowed to drain for 5 minutes. After washing with 15 ml fresh medium, the cells were dispersed on solid medium and placed under Gro-lux lamps. A small sample of cells was harvested at 0-time and frozen. After 7 days the inoculated cells were harvested, frozen, and later homogenized with 0.01 M potassium phosphate buffer, pH 7.3, and appropriately diluted to give an easily countable number of local lesions on the assay host. Homogenates of the 0-time and 7-day harvests were applied with a glass spatula to opposite half-leaves of N. tabacum Xanthi-nc previously kept in the dark for 24 hours, and dusted with carborundum. Virus increase

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*This material, presented at the Sixty-Second Annual Meeting of the American Phytopathological Society, Hot Springs, Arkansas, October 4-8, 1970, was published as an abstract (Phytopathology 60, 1283, 1970).

was determined by comparing the number of lesions produced by the two homogenates.

The data are given as the number of local lesions produced by the homogenates of 4 samples, each rubbed on 6 half leaves of Xanthi-nc (total of 24 half leaves). The cell lines H-38 (Havana-38), White Burley, Maryland Mammoth, "Habituated", and N. sylvestris produced large amounts of virus (Figure 18). Only the H-38 callus produced high numbers of TMV crystalline inclusions, with 79% of 500 cells examined containing crystals. Only 3-5% of the cells of other cultivars contained inclusions.

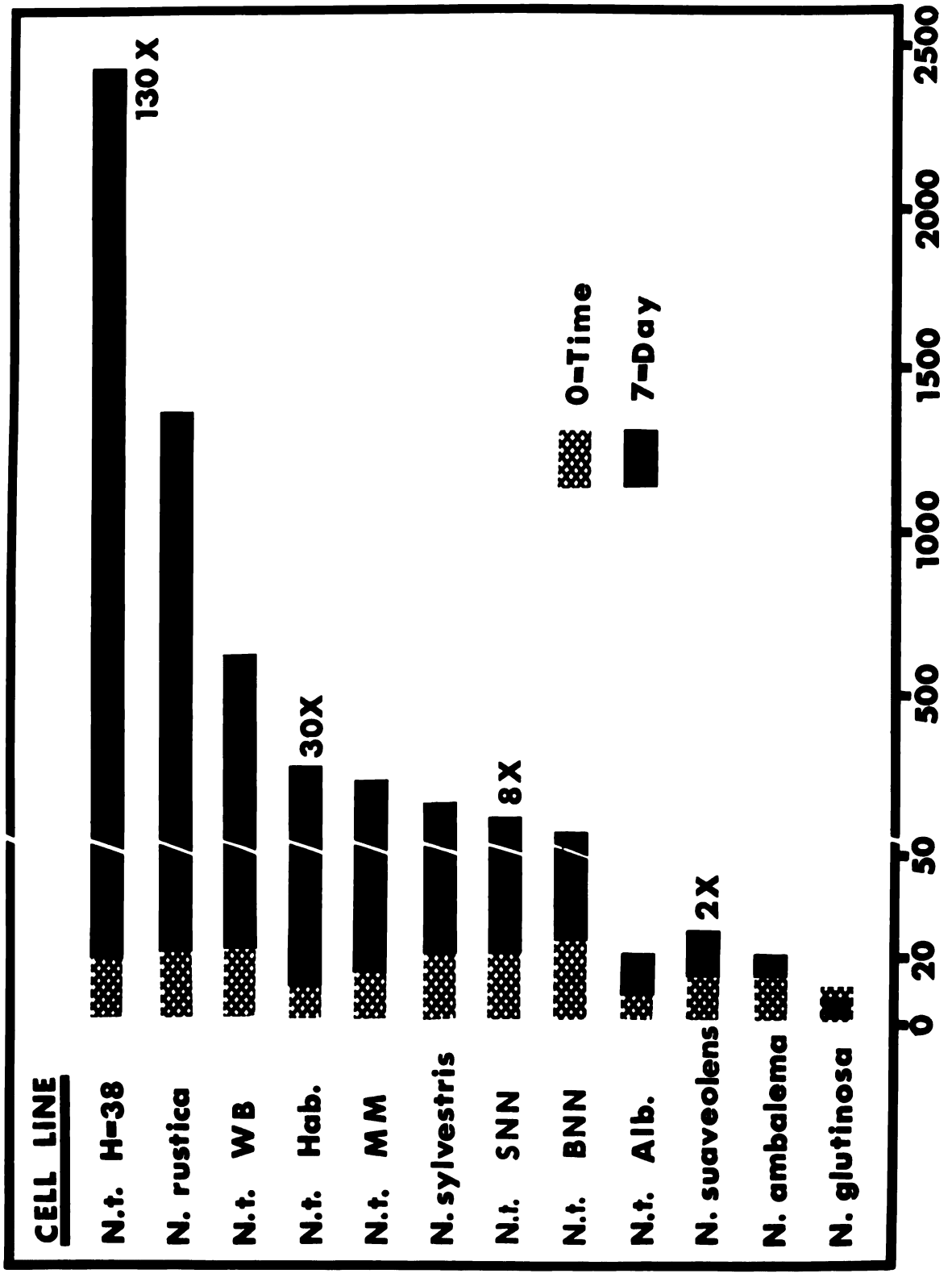
The lack of virus development in N. ambalema was expected because this cultivar was reported to be a "tolerant" host of TMV. Callus from N. tabacum cvs Samsun NN, and Burley NN, and N. glutinosa produced necrotic spots after TMV inoculation; crystalline inclusions were found only around the local lesions.

The data indicate that the H-38 callus produced more virus in 7 days than did the other cultures, under the conditions of these experiments. It is clear, however, that several cell lines produce virus following TMV infection, and could be used in further virus studies.

Cultural procedures to increase the efficiency of virus infection

The study reported above indicated that callus from N. glutinosa, and N. tabacum cvs Samsun NN, and Burley NN responded to TMV infection by producing the necrotic response, similar to the reaction known to occur on the leaves of these hosts (Holmes, 1929; Holmes, 1938; Valleau, 1952). Nicotiana tabacum cv Xanthi-nc (Takahashi, 1956) callus responded in a similar manner.

Figure 18. Increase of TMV in callus tissue from cultivars of Nicotiana tabacum, and other Nicotiana species. Virus increase was calculated by comparing the infectivity associated with homogenates of 0-time and 7-day harvests.


CELL LINE
N.t. H-38
N. rustica
N.t. WB
N.t. Hab.
N.t. MM
N. sylvestris
N.t. SNN
N.t. BNN
N.t. Alb.
N. suaveolens
N. ambalema
N. glutinosa
130 X
30X
8X
2X
0-Time
7-Day
0
20
50
500
1000
1500
2000
2500
NUMBER OF LOCAL LESIONS

In initial experiments production of the necrotic reaction (indicative of TMV infection) was not consistent, and non-inoculated controls occasionally produced a "spot" similar to the virus-induced reaction. In order to begin to evaluate the cultural conditions required for successful lesion formation, observations on growth of the cells, and of lesion formation on the hypersensitive cultivars were made and are summarized below.

N. glutinosa: callus grew readily after explanting the stem pith, and became slightly green pigmented; leaf lamina explants did not produce callus. The callus was not easily cultured in liquid medium. The ability to produce local lesion symptoms was not as great (based on local lesions per gram of inoculated cells) as with other cultivars, and the capacity to produce lesions appeared to decrease when kept in culture.

N. tabacum Burley NN: callus cultures were taken from leaf lamina and stem pith; the leaf callus grew more readily and was used throughout. The callus did not become green pigmented but was an off-white to tan color. It could be subcultured repeatedly in liquid medium by means of transferring with a wide mouth (3 mm opening) pipette. Because of the lack of green pigmentation, necrotic lesions were not as readily identified as on the pigmented cell lines.

N. tabacum Samsun NN and Xanthi-nc: leaf lamina and stem pith calli were developed, and both became green pigmented. The stem pith grew more rapidly and was therefore more easily maintained. Both cultivars produced local lesions when inoculated with TMV. Xanthi-nc was arbitrarily chosen for further experimentation.

Maintaining Xanthi-nc callus in optimal condition for virus infection and local lesion formation was difficult in the early experiments.

Continuous subculture in liquid medium produced cultures made up of large (more than 200 cells per aggregate), slowly growing aggregates, and fewer local lesions were produced after inoculation. The most reproducible results were obtained when callus was prepared for inoculation by the following procedure: callus was grown on agar-solidified medium for 3-5 weeks. Callus (1-2 grams) was then transferred to 20 ml of MS-W medium in 125 ml flasks, vibrated on the Vortex Mixer to break up the callus clumps, and placed on the rotary shaker (120 rpm). After 6-9 days (or when large numbers of single cells and small cell aggregates were produced) 1-3 ml of the cell suspension was transferred to fresh liquid medium. The cultures were ready for inoculation after 6-9 days, when callus aggregates became approximately 0.5-2 mm in diameter. The greatest numbers of lesions were obtained when the cell aggregates had a "grainy" texture at the time of inoculation. Friable cell cultures made up of single cells and small cell aggregates, resulting from the transfer of too many cells in the second liquid subculture, produced fewer local lesions after virus infection.

An alternative inoculation procedure

The usual inoculation procedure was the vibratory technique described by Murakishi et al (1970, 1971), but a second method was developed to reduce the handling of cells during inoculation. TMV suspension was added directly to the flasks of cells, and the flasks were returned to the shaker for a 5 minute adsorption period. The cells were collected on Miracloth-lined funnels, allowed to drain for 5 minutes, and subsequently washed with fresh medium. In repeated experiments an equal number of local lesions per gram of inoculated cells occurred regardless of the method of inoculation. However, the inoculated cells

appeared to be less damaged by the procedure described here than by the vibratory technique. A similar method of inoculation was previously used by Kassanis et al (1958) and Bergmann and Melcher (1957) but only limited success was recorded. Hartmann (1971) showed that the cell walls of vibrated tobacco callus cells appeared ragged and broken, and he suggested that this permitted virus attachment to the plasmalemma (for subsequent uptake by pinocytosis? Otsuki et al, 1972). It would appear that under certain cultural conditions the pathway for virus infection is available, and additional injury by vibration is not required.

The medium

The reports from this laboratory (Murakishi et al, 1970, 1971) on the successful inoculation of systemic tobacco callus referred to the use of modified Murashige and Skoog (MS) medium (1962). The medium was prepared by combining 3 liters of MS medium, pH 5.85, and 2 liters of White's (1954) W-9 (Table 1) medium, pH 5.85. When solidified medium was required the pH was brought to 6.2, and 1% (by weight) of Difco Bacto-Agar was added. The medium was made sterile by autoclaving for 20 minutes at 20 p.s.i., and is referred to as MS-W medium.

Using the 2 media outlined in Table 1, various combinations of media and growth regulators were tested for their ability to produce callus capable of local lesion production (other experiments indicated that virus infection did not occur in the absence of lesion development). It was hoped that the study would elucidate the value of the medium in the process of virus infection, and perhaps lead to the development of a more defined growth medium.

Burley NN callus was placed in the test medium and transferred 1-4 times. Throughout the transfers, cells were removed and inoculated

TABLE 1. Components of the basic media.

Medium	Mineral stock	Vitamin stock	Growth regulators, mg/l			Coconut water ml/l
			2,4-D	NAA	CAP	
MS	MS	MS	---	---	---	---
W-9	White	White	0.6	0.1	2.5	120

MS = medium described by Murashige and Skoog (1962)
W-9 = modified White's medium (1954)
2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = naphthaleneacetic acid; CAP = calcium pantothenate. Coconut water was taken from ripe coconuts, and deproteinized by autoclaving, and freezing and thawing twice. The precipitate was removed by filtering through Whatman 2r filter paper.

with 5 µg TMV/ml. The results are given as local lesions produced per gram of cells inoculated. Local lesions were produced in high numbers only in the media containing the MS mineral and vitamin stocks, coconut water, 2,4-D, and NAA (Table 2).

Callus grown in the MS medium containing kinetin, 2,4-D, and NAA (Table 2) and inoculated with TMV, produced 44 local lesions per gram of cells. This suggested that with appropriate manipulation a completely defined medium (without coconut water) could be devised that would be suitable for virus infection. A defined medium would make possible a more exact study of the factors involved in successful virus infection, and expression of the necrotic response.

From the results in Table 2 it was not possible to determine whether or not the inability of cells to produce local lesions after infection was a permanent or a temporary loss. Therefore callus grown in W(i) and W(ii) media (Table 2) were transferred to the MS-W medium for 2-4 successive transfers, and inoculated with TMV. In both cases the normal numbers of local lesions were produced (150-250 local lesions per gram of inoculated cells). Since it was previously determined that virus infection did not occur in the absence of local lesions, it was probable that the non-permissive media prevented virus infection. Do these media affect lesion development if applied to the cells only after inoculation?

To determine if the incubation medium following TMV inoculation was critical to lesion production, cells were grown in the MS-W medium, and inoculated in WDG or W-9 media and incubated on solidified medium of the same type. The same numbers of lesions were produced under these conditions as were produced when the cells were incubated on the MS-W medium. The results support the suggestion that growth of the

TABLE 2. Effect of culture media on the number of local lesions formed on Burley NN callus inoculated with TMV. Burley NN callus was serially transferred 2.4 times in the designated medium and inoculated with 5 µg TMV per ml. The number of local lesions was determined after 6 days.

Basic Medium[a]	Conc. of growth regulators: Kinetin µg/l	Coconut water ml/l	2,4-D mg/l	NAA µg/l	No. of expts.	No. of samples	Avg. no. of LL[b]
W(i)	30	120	6.0	100	5	9	1
W	--	120	6.0	100	5	9	6
W(ii)	30	120	0.6	100	4	8	6
W	--	120	0.6	100	4	8	1
W	--	50	0.6	100	2	6	1
W min., MS vit.	50	100	0.24	---	3	6	3
MS	50	---	---	---	2	6	0
MS	--	---	---	---	2	6	0
MS	50	25	---	---	2	8	2
MS	50	50	---	---	2	8	0
MS	50	100	---	---	2	8	0
MS	50	---	.1-.5	---	4	15	0
MS	50	---	0.25	40	1	3	9
MS	50	---	0.25	400	1	3	44
MS min., W vit.	--	50	---	---	3	6	0
MS	50	25	0.25	---	3	9	143
[c] MS:W-9 (3:2)	30	50	0.25	40	4	12	250
[c] MS:W-9 (3:2)	--	50	0.25	40	3	9	200
MS	30	50	0.25	40	2	9	199
MS	30	100	0.10	80	2	9	220

[a] MS = medium described by Murashige and Skoog (1962). W = medium described by White (1954). Min. = mineral stock. Vit. = vitamin stock.

[b] Average number of local lesions per gram of inoculated cells.

[c] MS and W-9 media (Table 1) were prepared separately and combined in a ratio of 3MS:2W.

cells in WDG and W-9 media prevented virus infection rather than specifically inhibiting local lesion production.

Growth of Xanthi-nc callus on MS-W medium

Through the course of the experiments with Xanthi-nc callus a fresh weight growth curve of the cells was determined to ensure that the cells remained actively growing following their inoculation. In these experiments weighed samples of inoculated and non-inoculated callus were incubated on 15-20 ml of solid MS-W medium. At intervals until 144 hours, samples were removed from the agar and re-weighed. The results of 5 experiments are presented as fresh weight increase of the cells derived by the formula:

$$\% \text{ fresh weight increase} = \frac{\text{harvested cell wt.}}{\text{inoculated cell wt.}} \times 100.$$

The cells continued to grow through 144 hours under these conditions (Figure 19). There were no differences in the growth of inoculated and non-inoculated callus.

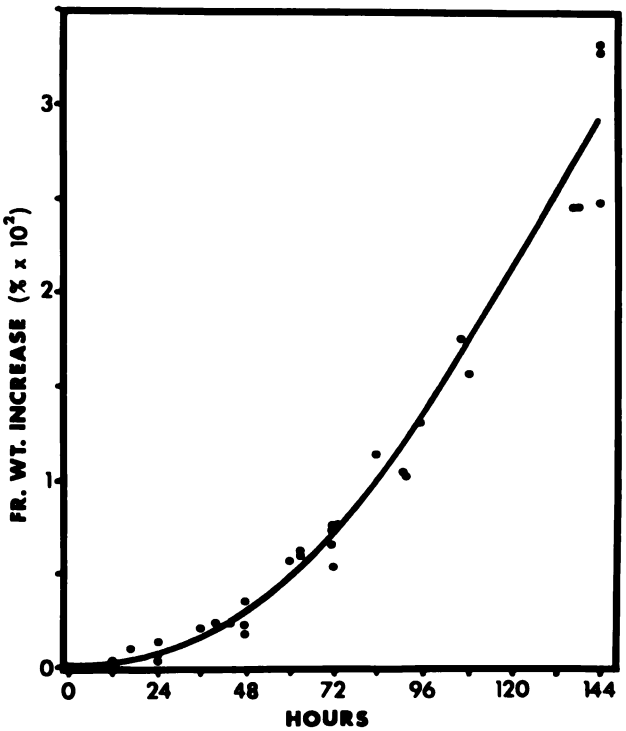


Figure 19. Growth of Xanthi-nc callus on solidified Murashige and Skoog-White (MS-W) medium.

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