1988

Inoculation of apple protoplast with tobacco mosaic virus (TMV) /

Xiao Hua Li
University of Massachusetts Amherst

INOCULATION OF APPLE PROTOPLAST WITH TOBACCO MOSAIC VIRUS (TMV)

A Thesis Presented

by

Xiao-hua Li

Submitted to the Graduate School of the

University of Massachusetts in partial fulfillment

of the requirements for the degree of

Master of Science

May 1988

Department of Plant Pathology

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Xiao-hua Li

Approved as to style and content by

George N. Agrios

Dr. George N. Agrios, Chairman of Committee

Mark S Mount

Dr. Mark S. Mount, Member

Mark S Mount

Mark S. Mount, Department Head,

Department of Plant Pathology

ACKNOWLEDGEMENTS

Sincere thanks to Dr. George Agrios for providing advice and encouragement throughout the course of this research. Thanks also to Marcy Walker for her help in this project.

Thanks are extended to Patrick Shiel and to all my other friends in the Department of Plant Pathology for their support.

Additional thanks to my husband for his understanding, patience and love, and to my parents for their concern and continuing support.

ABSTRACT

Apple protoplasts obtained from callus were inoculated with 10-100 ug/ml tobacco mosaic virus (TMV) in the presence of 5-20 ug/ml poly-L-ornithine (PLO). Several combinations of TMV and PLO concentrations resulted in infection of protoplasts with TMV. The best protoplast infection results were obtained when 5 X 10^5 protoplasts/ml were inoculated with final TMV concentrations ranging from 20 to 50 ug/ml while the PLO concentration was kept at 10 ug/ml. The protoplasts and the inoculum were mixed for 10 minutes. The protoplasts were then washed to remove excess TMV and incubated for 42-72 hours at 25°C. Inoculation results were monitored primarily by ELISA. An increase of TMV antigen in apple protoplasts could be detected as early as 6 hours after inoculation and reached a maximum concentration 24-36 hours after inoculation. A 10-fold increase in ELISA readings (absorbance at 405 nm) was obtained under the above conditions at 24-36 hours compared to the readings at the completion of inoculation. The infection was further confirmed by bioassaying infected protoplasts on the TMV local lesion host Nicotiana glutinosa, and by FITC staining of infected protoplasts. Infection of apple protoplasts with TMV was also obtained by electroporation-mediated inoculations.

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INTRODUCTION

The discovery that plant protoplasts could be infected with viruses was made at a time when there was intense interest in the replication process of plant viruses. Because virus particles infect a large proportion of protoplasts simultaneously and the infections proceed synchronously, protoplast-virus systems provide a valuable tool for critical studies of virus replication in plant cells, compared to previously available rather coarse studies of viruses in whole plants or excised tissues. The findings from protoplast systems should help improve our understanding of what happens in tissues or whole plants after virus infection.

Because of the problems encountered in traditional studies of woody plant viruses, the nature of virus infection in woody plants is understood far less than that in herbaceous plants. It is, therefore, especially important to develop a better system for studying viruses of woody plants.

Although successful inoculations of protoplasts with viruses have been made in a number of virus-host

combinations, all of them have been carried out with herbaceous hosts such as tobacco while no similar work has been done on woody plants. To date, no research has been reported on apple protoplast inoculation with viruses. It was the purpose of this research to determine the conditions for inoculation of apple protoplasts with a well characterized and stable virus such as tobacco mosaic virus (TMV), which is known to infect apple. Successful inoculation of apple protoplasts with this stable virus should determine the conditions under which apple protoplasts can be infected with other viruses, including the more labile viruses that cause severe diseases on apple. The information gained from studying the apple protoplast/TMV system may also prove applicable to inoculation of protoplasts of other woody plants with some or all of their viruses.

C H A P T E R II

LITERATURE REVIEW

Tobacco mosaic virus (TMV) is the best characterized among plant viruses. The virus is the type member of the tobamovirus group. It consists of one molecule of linear positive-sense single-stranded RNA. The virus particle is elongated and rigid, about 18nm in diameter and 300nm long, with helical symmetry. Because of the high titer it reaches in its systemic host, TMV is readily transmitted by mechanical inoculation (Matthews, 1981).

TMV causes severe diseases in tobacco and tomato (Holmes, 1946; Zaitlin and Isreal, 1975). It was reported to infect apple in 1964 (Kirkpatrick, 1964) and has been recovered from apple seeds and from young apple seedlings (Gilmer and Wilks, 1967). Because of the high titer it reaches in its hosts and because of its stability, TMV can be easily purified (Steere, 1959; Zaitlin and Isreal, 1975).

Apple protoplasts have been isolated from fruit (Anderson et al., 1979; Mattoo and Lieberman, 1977) by incubation of the tissue overnight with appropriate enzymes, from cotyledon tissue (Yamaki, 1981) after a 3-4

hour incubation with enzymes, from newly emerging leaves of apple (Huang and Millikan, 1983) after a four-hour shaking of a mixture of leaf tissue rinsed with sterilized water and maceration with high concentrations of enzymes (6.0% cellulysin and 1.0% macerase), and from callus and cell suspension cultures (Hurwitz and Agrios, 1984) by incubation of the callus for 4-5 hours in 2% cellulysin and 0.5% macerase enzyme solutions. There are several advantages to obtaining protoplasts from cultured tissue: (1) Conditions for protoplast isolation can be standardized more easily; (2) cultured cells or callus can be obtained in any season; and (3) the cultured tissues are already sterile and therefore can be used directly without further sterilization and injury.

The potential of plant protoplasts for studies of virus infection was first demonstrated by Cocking (1965, 1966). His electron microscope observations suggested that isolated tomato fruit protoplasts had the ability to take up TMV (Cocking, 1966). It was shown later that TMV could multiply in plant protoplasts (Takebe et al., 1968; Hibi et al., 1968; Cocking and Pojnar, 1969).

Generally, two methods have been employed to infect plant protoplasts with plant viruses by polycation-mediated inoculation. The procedures currently used to inoculate protoplasts with virus particles derive from that of Takebe and Otsuki (1969) which involves incubation of protoplasts

with virus particles pretreated with PLO. An operational modification called the "direct method " was used by Motoyoshi et al (1974b) to inoculate tobacco mesophyll protoplasts with CCMV. In this method, pelleted protoplasts are suspended directly in the virus-PLO solution instead of being suspended first in mannitol solution and then exposed to virus.

PLO, which has a strong positive charge, is usually essential for protoplast inoculation with viruses because it helps change the charges on the surface of the generally negatively charged viruses so that they may come into contact with the protoplast membrane, the surface of which is also negatively charged (Takebe, 1977). When PLO is omitted from inocula containing tobacco mosaic or tobacco rattle viruses at 1ug/ml, little or no infection results. However, Zhuravlev et al. (1980) were able to infect about 20% of tobacco protoplasts by treating them with PLO-free inocula containing much greater concentrations (0.1-1 mg/ml) of tobacco mosaic virus in 0.1M sucrose, 0.6 M mannitol, 0.1 M phosphate buffer pH 7. Moreover some viruses, such as brome mosaic and pea enation mosaic viruses, which are positively charged at the pH of the inoculum, do not require poly-L-ornithine to infect protoplasts (Motoyoshi and Hull, 1974; Motoyoshi et al., 1974a, Okuno and Furusawa, 1978). Even in these cases, however, PLO stimulates infection of protoplasts. Some

other polycations with a similar charge, such as poly-L-lysine, poly-L-arginine, or protamine sulfate, can be substituted for PLO with greater or lesser effectiveness (Motoyoshi et al., 1974b).

Both pH and kind of buffer used for inoculation can influence the level of infection of protoplasts by viruses. The best pH and buffer to be used varies with the virus and the species of protoplasts being inoculated (Takebe, 1977). For some viruses (e.g. tobacco rattle virus; Kubo et al., 1974) phosphate buffer gives better results than citrate buffer while infection of turnip protoplasts with cauliflower mosaic virus (CaMV) is more efficient in citrate buffer than in phosphate buffer (Furusawa et al., 1980). For infection of tomato protoplasts with tobacco mosaic virus, tris hydrochloride buffer is better than either of the above (Motoyoshi and Oshima, 1975, 1976).

The buffer used to prepare the virus inoculum also influences the optimum concentration of protoplasts to be inoculated (Mayo, 1978). For different virus-protoplast combinations, pH influence on the efficiency of infection varies (Barnett et al., 1981; Motoyoshi and Hull, 1974; Motoyoshi,et al., 1974a). It was also observed that the optimum pH shifts with different buffers. Generally, potassium citrate buffer at a lower pH (4.2-5.2) allows a greater frequency of infection of protoplasts by viruses (Hibi et al., 1975), whereas phosphate buffer is more

efficient at a higher pH (5.5 -9.0) for infection of protoplasts (Barker and Harrison, 1977; Jarvis and Murakishi., 1980; Lesney and Murakishi, 1981; Kubo et al., 1975).

The effect of ions, such as Ca^{++}, on infection of protoplasts with viruses was noticed by several investigators. As reported by Lesney and Murakishi (1981), combination of PLO and $CaCl_2$ had a synergistic effect in enhancing infection of soybean protoplasts with bean pod mottle virus, especially at lower virus concentrations. When virus was preincubated for 15 minutes with $CaCl_2$ or $MgCl_2$ prior to inoculation, infection of protoplasts was significantly increased over inoculation with virus which had not been preincubated. Similar results were obtained by Jarvis and Murakishi (1980) who reported that when $CaCl_2$ was added, the level of infection of soybean protoplasts with southern bean mosaic and cowpea mosaic viruses increased six-fold in both potassium phosphate buffer and tris-HCl buffer.

There seems to be no general guide to the best inoculation temperature. Inoculation of cowpea protoplasts with alfalfa mosaic virus was more effective at 0°C than at the usual 25°C, presumably because the virus particles were less readily inactivated (Alblas and Bol, 1977). However, with other viruses, inoculations at low temperatures were either as effective as (Motoyoshi et al., 1974b) or less

effective than (Jarvis and Murakishi, 1980) those at room temperature.

Since electroporation results in formation of "holes" or pores in the plasmalemma of protoplasts through which molecules may pass (Watts et al., 1987), electroporation has been developed as a modern technique for introduction of foreign genetic material into plant protoplasts (Potrykus et al., 1985; Fromm et al., 1986). Infection of protoplasts with virus particles by electroporation was obtained in 1986 when Okada et al. infected tobacco mesophyll protoplasts with TMV and cucumber mosaic virus (CMV). The concentrations of virus needed for protoplast inoculation, however, were very high (500 ug/ml) compared with those needed for protoplast inoculation with RNA (10 ug/ml) (Okada et al., 1986). Subsequently, Nishiquchi et al. (1987) reported that up to 80-90% of the protoplasts became infected with TMV after exposure to a 10 usec pulse at 200V in a 0.5M mannitol solution. These authors felt that a longer electric pulse was necessary to induce TMV particle entry. The successful inoculations with TMV (18 X 300nm) and CMV (30nm) suggested that some of the pores produced under proper conditions are larger than 30nm in diameter. The size of the pores is related to voltage (Zimmermann et al., 1984). From the evidence that RNA of these viruses can enter protoplasts even when it is added some time after the electric pulse, it was also suggested

that pores probably persist for several minutes (Okata et al., 1986). Shiel and Agrios (unpublished) have also found recently that when apple protoplasts were inoculated with apple mosaic virus, the most efficient infection was obtained when the virus was added to protoplasts after rather than during electroporation.

There are several ways to detect presence of virus in protoplasts (Harrison and Mayo, 1983). Fluorescent antibody staining, first applied by Otsuki and Takebe in 1969 to detect virus in protoplasts, has become a standard method for detecting and counting virus-infected protoplasts in an inoculated population. Enzyme-linked immunosorbent assay (ELISA), which was applied by Clark and Adams in 1977 for detecting viruses in plants, can be used to assay virus yield in infected protoplasts (Mayo and Barker, 1983). Finally the local lesion assay is still important for measuring infectivity in extracts of infected protoplasts after a desired incubation period.

C H A P T E R III

MATERIALS AND METHODS

The Virus

Source and maintenance of tobacco mosaic virus (TMV)

White Burley tobacco plants, used as the propagation host for tobacco mosaic virus, were grown in a greenhouse at 75-80° F. White Burley plants with 2-4 leaves were transplanted into 6-inch diameter plastic pots containing Pro-mix. The plants were fertilized weekly. When the plants had 5-7 leaves that were approximately 70 to 90% fully expanded, the leaves were dusted with celite and inoculated with a purified TMV preparation or with sap from infected tissue prepared in 0.5M phosphate buffer at pH 7. Leaves that developed good mosaic symptoms were harvested and kept in a -20° C freezer until use.

Purification of TMV

TMV was purified from systemically infected leaves primarily by alternating cycles of low-speed and high-speed centrifugation.

Frozen infected White Burley leaves (200 to 500 g) were ground in 3% K_2HPO_4 with a rolling bar device. The sap was

clarified by straining through cheesecloth and centrifuging at 8,000 g for 15 minutes. The supernatant was saved and centrifuged at 44,000 g for one hour. The pellet was resuspended in 0.03M phosphate buffer, pH 7.0. The low and high speed centrifugation procedures were repeated 3 to 4 times until the virus suspension appeared clear and whitish. The virus pellet from the last high speed centrifugation was resuspended in 2-5 ml distilled water and kept in the refrigerator. Virus infectivity was bioassayed on its local lesion host Nicotiana glutinosa. The amount of virus in purified preparations was determined by measuring its absorbance at 260nm with a spectrophotometer.

Further purification of the virus was carried out using sucrose density gradient centrifugation (SDGC). Half ml of a 2 mg/ml TMV preparation was placed on top of a sucrose density gradient in a cellulose nitrate centrifuge tube (14 X 95mm, Ultra-Clear). The sucrose density gradient was prepared by successive layering of sucrose solutions of 292, 250, 200, 135 and 0 mg/ml dissolved in 1.4, 3.5, 3.2, 2.6 and 1.6 ml of distilled water from bottom to top. Density gradient centrifugation was carried out in an SW 40 Ti rotor at 100,000 g for 2 hours at 4°C. The amount of TMV in each fraction was determined by measuring its absorbance at 260nm with a spectrophotometer. The peak fractions were collected and dialyzed against distilled water overnight

and then centrifuged at 100,000 g in a 75 Ti rotor for one and a half hours. The virus, which had sedimented in a pellet, was resuspended in 0.5 to 1 ml distilled water.

Assay of purified virus

The concentration of purified TMV in suspension was determined by measuring its absorbance at 260nm with a spectrophotometer. The degree of purification was determined from the ratio of its absorbance at 260 and 280nm (A260/280 ratio). The infectivity of purified TMV was measured by bioassay on its local lesion host Nicotiana glutinosa. TMV was diluted in 0.05 M phosphate buffer, pH 7.0, and inoculated on celite-dusted Nicotiana glutinosa leaves. The lesions were counted 3 days after inoculation. Purified virus particles were also checked under an electron microscope.

Anti-TMV Antibody

Production of TMV antiserum

Antiserum to TMV was produced in rabbit by administering four weekly intramuscular injections followed by four weekly intravenous injections. Each intramuscular injection consisted of 3-5 mg of purified TMV in 1-2 ml distilled water emulsified with an equal volume of Freund's incomplete adjuvant. Each intravenous injection consisted

of 0.5-2.5 mg purified TMV in 0.3-1.25 ml distilled water without adjuvant. The rabbit was bled 10 days after the final injection, the blood was allowed to clot, and the serum was collected after low-speed centrifugation (30 min at 5,000 g) of the clotted blood. The TMV antiserum, to which 0.02% NaN_3 was added as a preservative, was kept in the freezer until use.

"Purification" of TMV antibody

For purification of TMV antibody, 9 ml distilled water was added to 1 ml TMV antiserum. Then, 10 ml saturated ammonium sulfate solution was added slowly and drop-wise while stirring (Campbell et al., 1970). The mixture was left at room temperature for 30-60 min. After centrifugation at 5,200 g for 5 min, the precipitate was collected by dissolving it in 2 ml 1/2-strength PBS (phosphate buffered saline), and was dialyzed three times against 500 ml half strength PBS (including once overnight). The antibody was further purified with the aid of a chromatographic column filled with DEAE-sephacel. Ten one-ml fractions were harvested from each column. The concentration of -globulin proteins (part of which comprised the TMV antibody) in each fraction was determined by measuring its absorbance at 280nm.

The "purified" TMV antibody, to which 0.02% NaN_3 was added, was kept in the refrigerator (4°C) until use.

Assay of "purified" antibodies

The specific antibody titer was determined by the standard microprecipitin procedure (Ball, 1974). The sensitivity and the optimum concentration of the antibody needed for detecting and measuring the virus in apple tissues was determined by the ELISA test.

The Protoplasts

Source and isolation of protoplasts

Apple calli obtained from cultured apple twigs were used as a source of protoplasts. Twigs of the current year's growth with a diameter of 0.4-0.8 cm were obtained from healthy apple trees growing in the orchard. The bark was peeled off the twigs and the twigs were cut into about 1 inch-long pieces that were placed in a beaker containing 20% bleach with 0.5% Tween-20 in which they were surface sterilized for 5 min. The twigs were then transferred to 70% ethanol for 10 seconds. Sterilized twigs were split in the middle lengthwise and the halves were placed cut face up onto a modified Murashige and Skoog (MS) medium prepared in 60 X 15 mm sterile Petri dishes as described by Hurwitz and Agrios (1984). Callus was produced along the phloem of the cut surfaces of the twigs. The calli used for protoplast isolation were harvested within 7 to 15 days

after twigs were placed onto media. The twigs were then transferred onto fresh media. Fresh calli were obtained every 7 to 16 days after the twigs were transferred onto fresh media. Good calli could be obtained continuously from twigs which were cultured for about four months. Only young, fresh, whitish, and soft-looking calli were used for protoplast isolation.

The method of isolation was a modification of the procedure described by Hurwitz and Agrios (1984). Enzymes consisting of 0.05 g macerase (Calbiochem) and 0.2 g cellulysin (Calbiochem) were dissolved in 8.5 ml 0.7 M mannitol containing 0.1% $CaCl_2$ at pH 5.2 to 5.4. The concentrations of enzymes in this solution were 0.6% for macerase and 2.4% for cellulysin. The enzyme solution was sterilized by passing it through sterile filter having pores of 0.22 um diameter. About 1 g of young callus was placed into the enzyme solution and digested for about 2 hours on a Gyrotory Water Bath Shaker at 80 rpm. The protoplasts were separated from the cell wall debris by filtering the solution, after completion of cell wall digestion, through a 52 um pore sterile filter. The protoplasts were then washed with 0.7 M mannitol containing 0.1% $CaCl_2$.

Apple protoplasts were also isolated from young emerging leaves of greenhouse grown juvenile MM 106 apple plants using either the same method as described above or a

modification of the method described by Huang and Millikan (1983). With the latter method, newly emerging leaves of MM 106 apple are excised and sterilized by a 3-minute exposure to 5% bleach and rinsing 3 times with sterile distilled water. About one g of these ground leaves were transferred to 10 ml of a sterilized enzyme solution consisting of 6.0% cellulysin and 1.0% macerase in 0.7M mannitol containing 0.1% $CaCl_2$. The mixture was then placed on a Gyrotory Water Bath Shaker and rotated at 80 to 100 rpm for 4 to 24 hours at room temperature. The tissue was examined for protoplasts under a microscope at 30 min to 6 hour intervals.

Viability test and detection of cell wall regeneration

The viability of apple protoplasts was ascertained by the fluorescein diacetate (FDA) staining method (Larkin, P. J., 1976). Cell wall formation by protoplasts was detected by the Calcofluor White staining method (Nagata and Takebe, 1970).

Inoculation of Apple Protoplasts with TMV

The PLO-mediated inoculation and the electroporation-mediated inoculation procedures were used to infect apple protoplasts with TMV.

PLO-mediated inoculation

Apple protoplasts obtained from callus were inoculated with TMV in the presence of PLO. Certain concentrations of TMV (2-200 ug/ml) and PLO (2-40 ug/ml) were mixed in potassium citrate-buffered mannitol at room temperature for 10 minutes. At that time, an equal volume of one million/ml freshly isolated protoplasts, which were prepared in 0.7M mannitol solution containing 0.1% $CaCl_2$, were added to the virus inoculum mixture. This procedure produced final concentrations of 1-100 ug TMV, 1-20 ug PLO, and 0.5 million protoplasts per ml. The mixture of protoplasts and TMV/PLO inoculum were kept at room temperature with occasional gentle shaking for 10 minutes and then the virus inoculum was removed by centrifugation of the protoplast-TMV/PLO mixture at 800 rpm. The pellet of inoculated protoplasts was resuspended and washed with 0.7 M mannitol 1 to 5 times to remove excess virus. Finally, the inoculated, washed protoplasts were resuspended in fresh mannitol to give a concentration of one million protoplasts per ml and these were then subdivided into several equal samples. One sample, collected and frozen immediately after the protoplasts were washed, was the 0 time control. The other samples were incubated at 25° C for variable periods of time at the end of which they were frozen until all the samples were collected and were then tested for virus increase.

Electroporation-mediated inoculation

Protoplasts were prepared and maintained in the same way as described in PLO-mediated inoculation. TMV was diluted in 0.7 M mannitol buffered with potassium-PBS at pH 7.0 to produce a TMV preparation with a concentration of 100 ug/ml. Equal volumes of the TMV preparation and of freshly isolated protoplasts were mixed giving a final TMV concentration of 50 ug/ml and 0.5 X 10^6 protoplasts/ml. Half an ml of the protoplast-TMV mixture was transferred into a spectrophotometer cuvette supplied with two parallel electrodes set 4 mm apart (Watts et al, 1987; Nishinguchi et al, 1987). Electroporation was carried out by discharging a capacitor (10-100 uF) through the cuvette with the electrodes. The power supply gave a continuous 400 V voltage. The preparation containing the virus and the protoplasts was held at 0^oC for at least 10 min after electroporation. The protoplasts were then collected by low speed centrifugation (80g) for 3 min and resuspended in 1 ml 0.7 M mannitol. Inoculated protoplasts were washed and divided into sub-samples in the same way as in PLO-mediated inoculation. In each treatment, 0.5-1.0 ml of protoplast suspension was used to get sufficient protoplasts for quantification of infection.

Techniques for Assaying TMV in Infected Protoplasts

Three tests have been used to detect infection of apple protoplassts with TMV.

ELISA

The increase of virus antigen in protoplasts was determined primarily with indirect ELISA. Indirect ELISA conditions were similar to those in the double sandwich ELISA procedure described by Clark and Adams (1977) except that the initial coating of wells was with antigen in 200 ul of coating buffer. Virus coating buffer equal in volume to the protoplast suspension was added to 0.5-1.0 ml of infected protoplast sample. In order to release any virus they contained, the protoplasts were broken by sonicating them in coating buffer at 100 watts for 10 min in a sonifier cell disrupter (W158). For each treatment an average ELISA value was obtained from at least 4 replicate wells. The TMV antibody preparation was cross absorbed with about 1% of acetone-dried tobacco leaf tissue in PBS-tween for 2 hours. Tobacco tissue was removed by centrifugation at 5,000 rpm for 15 min. For ELISA, the TMV antibody was used at a concentration of 1.5 ug/ml and the anti-rabbit IgG alkaline phosphatase conjugate (Sigma) at 1:1,000.

To assess the sensitivity of indirect ELISA in detecting TMV in the presence of apple protoplasts, an ELISA test was carried out by using 2-fold dilutions of protoplasts representing different concentrations of protoplasts ranging from 1 X 10^6 to 0.016 X 10^6 protoplasts/ml in the presence of a constant TMV concentration of 100 ug/ml. In the same ELISA test, 100 ug/ml TMV was used as a positive control.

FITC staining

The percentage of protoplasts infected with TMV was determined by staining the inoculated protoplasts with fluorescein isothiocyanate (FITC) conjugated with anti-TMV antibody (Nishiguchi et al., 1986). One drop of protoplast suspension was placed on a glass slide coated with a very thin layer of albumin fixative (Fisher), and was air dried. The slide was immersed in 95% ethanol for 10 min at room temperature to fix the protoplasts. After washing in PBS for three 1-min periods, the slide was air dried again. One drop of 1:10-diluted virus-specific antibody solution was added to the protoplasts and incubated for 2 hours. After immersing the slide in PBS for three more 1-min periods, the protoplast-containing area was covered with a 1 or 2 drops of 1:50 dilution of FITC-conjugated goat anti-rabbit antibody (Calbiochem-Behring Corp) in PBS. The slides were kept in dark at 37°C for 3 hrs and were then subjected to

three 1-min washes in PBS. The proportion of infected protoplasts was determined by observing the treated protoplasts with a Leitz Dialux 20 epi-fluorescence microscope.

Alternatively, FITC staining of protoplasts was carried out in suspension in a centrifuge tube. Protoplasts were spinned to remove mannitol solutions and were fixed by adding 0.5 ml 95% ethanol into the pelleted protoplasts. Protopalsts were suspended in ethanol by gently tapping the tube and were kept at room temperature for 3 minutes. Protoplasts were spined again to remove the ethanol, washed once and then kept as a pellet. Four to five drops of 1:10 TMV-antibody were added to the protoplast pellet. The mixture was incubated at 37°C for one hour. Protoplasts were then stained with 1:50 FITC-conjugate as described above. After each treatment (fixation, antibody, conjugate), the protoplasts were sedimented by centrifugation at 80 rpm and were washed 1-2 times with PBS. Finally, the protoplasts were suspended in 2 to 3 drops of PBS buffer. One drop of the protoplast suspension was then placed on a microscope slide and checked under an epi-fluorescence microscope.

Bioassay on local lesion host

The infectivity of TMV produced in infected protoplasts was determined and measured by bioassaying inoculated

protoplasts on leaves of the TMV local lesion host Nicotiana glutinosa. The protoplasts were broken to release the virus by using a 3-ml syringe in which the protoplasts were passed back and forth through a small 26G 1/2" needle at least 10 times. The leaves were observed for local lesions daily and the local lesions were finally counted 7 to 10 days after leaf inoculation.

To assess the infectivity of TMV in the presence of apple protoplasts, desired concentrations of TMV were tested on N. glutinosa leaves after the virus was mixed with different concentrations of protoplasts. Two sets of protoplasts were prepared for this test. For each set, protoplasts were diluted in mannitol solution to obtain concentrations ranging from 1 X 10 to 20 protoplasts/ml. Then, 20 or 100 ug/ml TMV were mixed with the two sets of protoplast preparations, respectively. Each sample was bioassayed by rubbing it on 7 half leaves of N. glutinosa. The number of local lesions induced by each sample was obtained by averaging the lesions developed on the 7 half leaves.

C H A P T E R IV

RESULTS

Purification of TMV

In preliminary purification trials, a clear TMV pellet could be obtained after 6 or 7 cycles of low and high speed centrifugation. However, when the pellet was resuspended in phosphate buffer after two cycles of centrifugation and was then kept frozen for one day, only one more centrifugation cycle was needed to obtain the same result. The pellet could thereby be obtained totally free of green material after a three-cycle centrifugation.

Spectrophotometer readings after the initial purification gave an A 260/280 absorbance ratio of the TMV preparation of 1.17-1.18. The same pattern of absorbance was obtained after sucrose density gradient centrifugation, but the ratio at A 260/280 usually increased slightly to 1.20-1.21. Electron microscope photographs (Fig.1) showed that about 98 percent of the virus particles were intact. After further purification, over 90 percent of the virus particles were aggregated. Virus infectivity tests showed that, within TMV concentrations ranging from 0.1 ug/ml to

Fig.1 Electron microscope photograph of TMV particles
(Magnification: 120,000 X)

10 ug/ml, local lesion numbers increased linearly with 10-fold increases of virus concentration. No lesions formed on leaves inoculated with less than 0.01 ug/ml TMV (Fig.2).

"Purification" of Anti-TMV Antibody

The antiserum obtained from the rabbit injected with TMV had a microprecipitin titer of 512. When the antiserum was purified via a chromatographic column and the 3 or 4 fractions containing antibody were collected and combined, the purified antibody had an A280 reading of 1.092, which represents an antibody (protein) concentration of 0.78 mg/ml. In ELISA tests, in which a 1:1,000 dilution of sap from healthy tobacco was used as a negative control, purified virus could be detected in as low a concentration as 0.1 ug/ml when rabbit anti-TMV -globulin and goat-antirabbit IgG conjugated with alkaline phosphatase were used at 1.5 ug/ml and 1 ug/ml, respectively.

Isolation of Apple Protoplasts

In the majority of the experiments, frequent, i.e., biweekly transfer of twigs to new media was found advantageous for producing young and vigorous callus. Over 70 percent of such twigs developed callus within one week after transfer and over 90 percent formed callus within 10

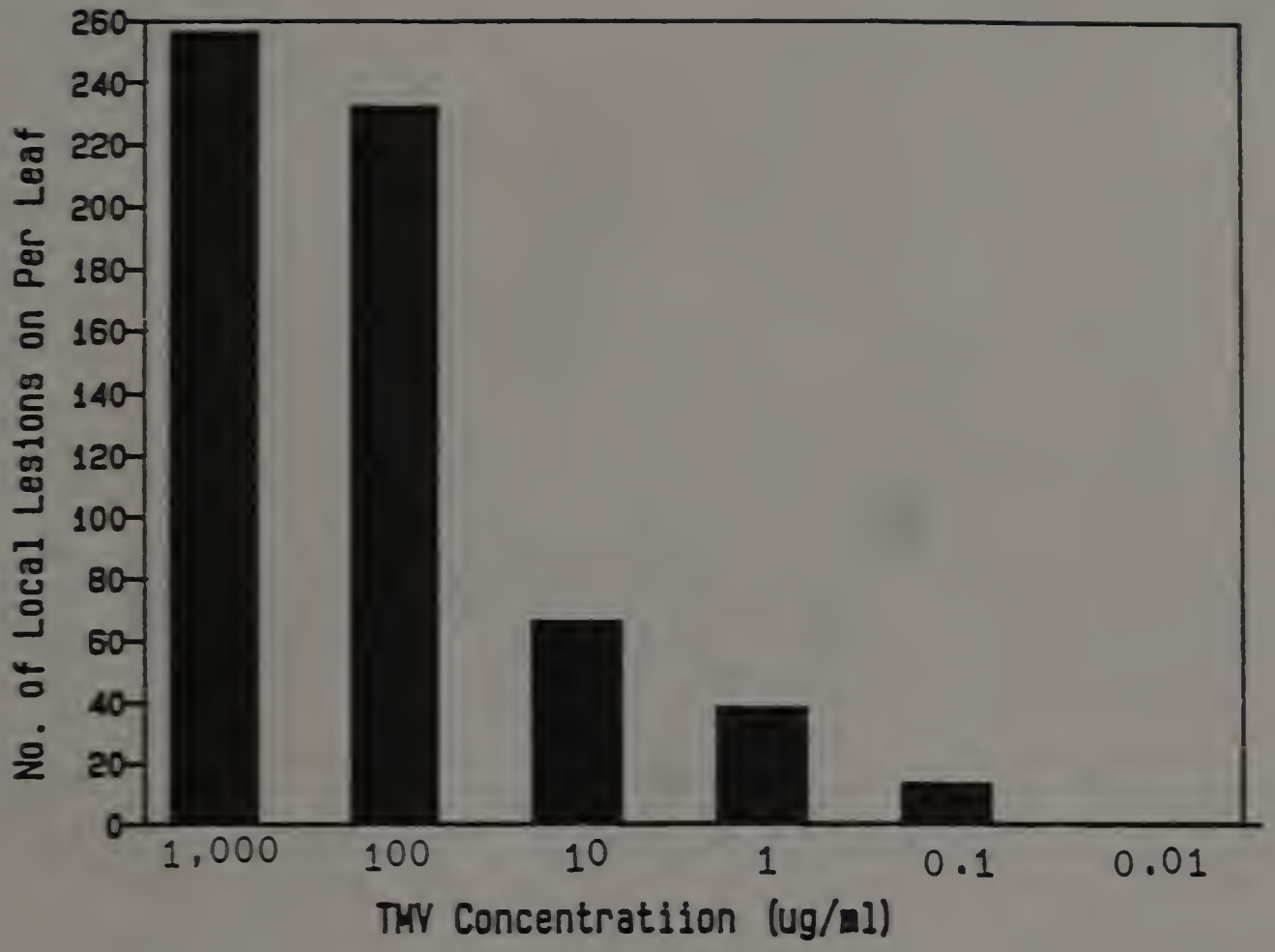

Fig.2 Infectivity of tobacco mosaic virus (TMV) used to inoculate apple protoplasts as determined by the number of local lesions produced on Nicotiana glutinosa leaves inoculated with 10-fold dilutions of purified TMV

to 15 days. The callus produced by such twigs was young and soft and separated quickly into single cells when it was placed in the enzyme solution.

Several factors influenced the yield and quality of protoplasts. Such factors included the concentration of enzymes used, the quality of callus employed, and the age of the callus.

When cellulysin was used at 2% and marcerase at 0.5%, generally about one million protoplasts could be obtained from 1 g of 1-month-old fresh callus. When the same age callus was treated with 2.4% cellulysin and 0.6% macerase, the number of protoplasts obtained from 1 g callus was often 2 to 3 times greater. Statistical analysis of the data showed that there is a highly significant difference in the number of protoplasts released by the two enzyme concentrations. The difference was more apparent when younger callus was treated (Table 1).

The quality of callus, as judged by its ability to release protoplasts, plays a most important role in isolation of protoplasts. It varies with the duration of twig culture. The influence of duration of Mutsu twig culture on protoplast production is shown in Table 2. Twigs that had been in culture for four months or more (since their initial plating on nutrient media) produced considerably less high-quality, protoplast-releasing callus, even though the callus was harvested every two

Table 1. Effect of two concentrations of cell wall degrading enzymes on the number of protoplasts released from 1 g of Mutsu apple callus of two different ages

	Kind and concentration of enzyme			
	0.5% macerase 2% cellulysin		0.56% macerase 2.4% cellulysin	
Experiment No.	Callus age (days) 12-16	28-32	Callus age (days) 12-16	28-32
1	3.0[a]	1.0	7.0	2.2
2	5.5	1.1	5.0	2.5
3	3.2	2.0	8.6	2.0
4	4.0	1.8	13.3	3.1
5	2.0	0.7	4.3	3.0
6	2.0	0.9	8.2	4.0
7	-	1.6	6.4	1.8
Average	3.3	1.3	7.5	2.7

[a] Millions of protoplasts per g callus

Table 2. Effect of duration of twig culture on nutrient media on the number of protoplasts released from new calli scraped off from the twigs every two weeks

Experiment	Months twigs were kept in culture					
No.	0.5	1	2	3	4	over 4
1	4.8[a]	4.3	13.3	7.0	3.0	1.9
2	4.0	5.6	10.0	6.3	2.1	1.5
3	3.2	6.5	5.7	6.4	1.5	0.3
4	5.0	4.6	5.0	-	-	0
5	4.6	4.1	4.3	-	-	0
Average	4.32	5.02	7.66	6.66	2.20	0.75

[a] Millions of protoplasts per g of callus

weeks and looked young and fluffy. The best protoplast-releasing callus was obtained from twigs that had been cultured for about two to three months but which were transferred after every time the callus was scraped off, i.e., every two weeks.

The age of callus is calculated from the day the twig was transferred to fresh medium to the day the callus is harvested and used for protoplast isolation. When appropriate concentrations of enzymes were used, protoplast yields were much greater when the callus was about 2 weeks old than when it was one month old or older. The differences can be seen in the data given in Tables 1 and 3. All the calli in these tables were obtained from twigs which had been kept in culture for 2-3 months.

The apple variety Macoun showed an appreciably greater potential for high yields of protoplasts than the varieties Golden Delicious, Lord Lambourne or Mustu. One gram of young Macoun callus produced from 5 - 13.3 X 10^6, while Golden Delicious produced from 2.1 - 8.1 X 10^6, Lord Lambourne from 1.7 - 7.6 X 10^6, and Mutsu from 3.5 - 5.7 X 10^6 protoplasts (Table 4). When other conditions were the same, Macoun callus usually produced 1.4 to 1.7 times as many protoplasts as other varieties (Table 4). In a separate experiment, 1 g Macoun callus produced 14.6 X 10^6 protoplasts.

Placing the callus and enzyme mixture in a refrigerator

Table 3. Effect of callus age on protoplast yield

Apple variety	Mutsu			Golden Delicious		
	Days callus grown on nutrient media			Days callus grown on nutrient media		
Experiment No.	7-16	28-33	over 35	7-16	28-33	over 35
1	7.0[a]	2.5	0.9	9.3	3.5	0.3
2	5.0	2.2	1.2	6.3	3.6	0.0
3	8.6	2.0	0.7	3.5	1.7	0.4
4	13.3	3.1	0.9	4.8	1.1	-
5	4.3	3.0	4.9	13.5	1.1	-
6	8.2	1.8	-	-	1.7	-
7	6.4	4.0	-	-	2.1	-
Avr.	7.5	2.7	1.7	7.5	2.1	0.2

[a] Millions of protoplasts per g callus

Table 4. Effect of apple variety on number of protoplasts rleased from 1 g of twig callus approximately 2 weeks old

Experiment No.	Apple varieties: Lord Lambourne	Mutsu	Golden Delicious	Macoun
1	7.6[a]	-	8.1	13.3
2	-	5.7	-	8.3
3	-	3.5	3.2	5.5
4	1.7	-	2.1	5.0

[a] Millions of protoplasts per g callus

overnight resulted in a much shorter cell wall digestion time once the callus-enzyme mixture was removed from the refrigerator and placed on a shaker at room temperature. Following overnight refrigeration, the time for completion of cell wall digestion was shortened from 2 -4 hours to 1-2 hours. The yield and the quality of protoplasts were often improved by such a treatment; for example, in such preparations fewer protoplasts had cell wall fragments on them. These results were somewhat erratic however when using older callus.

High quality protoplasts were generally present when the preparation was clean, exhibited a greater proportion of small, round and full protoplasts, and could survive the inoculation and the required incubation period which often extended for 72 to 96 hours.

The quality and survival of protoplasts were determined from both general microscope observations without any staining and from microscope observation of protoplast preparations treated with fluorescein diacetate (FDA). Under the conditions of our experiments, especially when high quality callus was used, 87 to 95% of protoplasts were viable immediately after enzyme digestion while 73 to 83% of protoplasts survived incubation periods of as long as 96 hours, as determined by FDA staining (Fig.3). Most of these protoplasts were round and full. Clean and high quality protoplast preparations were usually obtained from 7 to 20

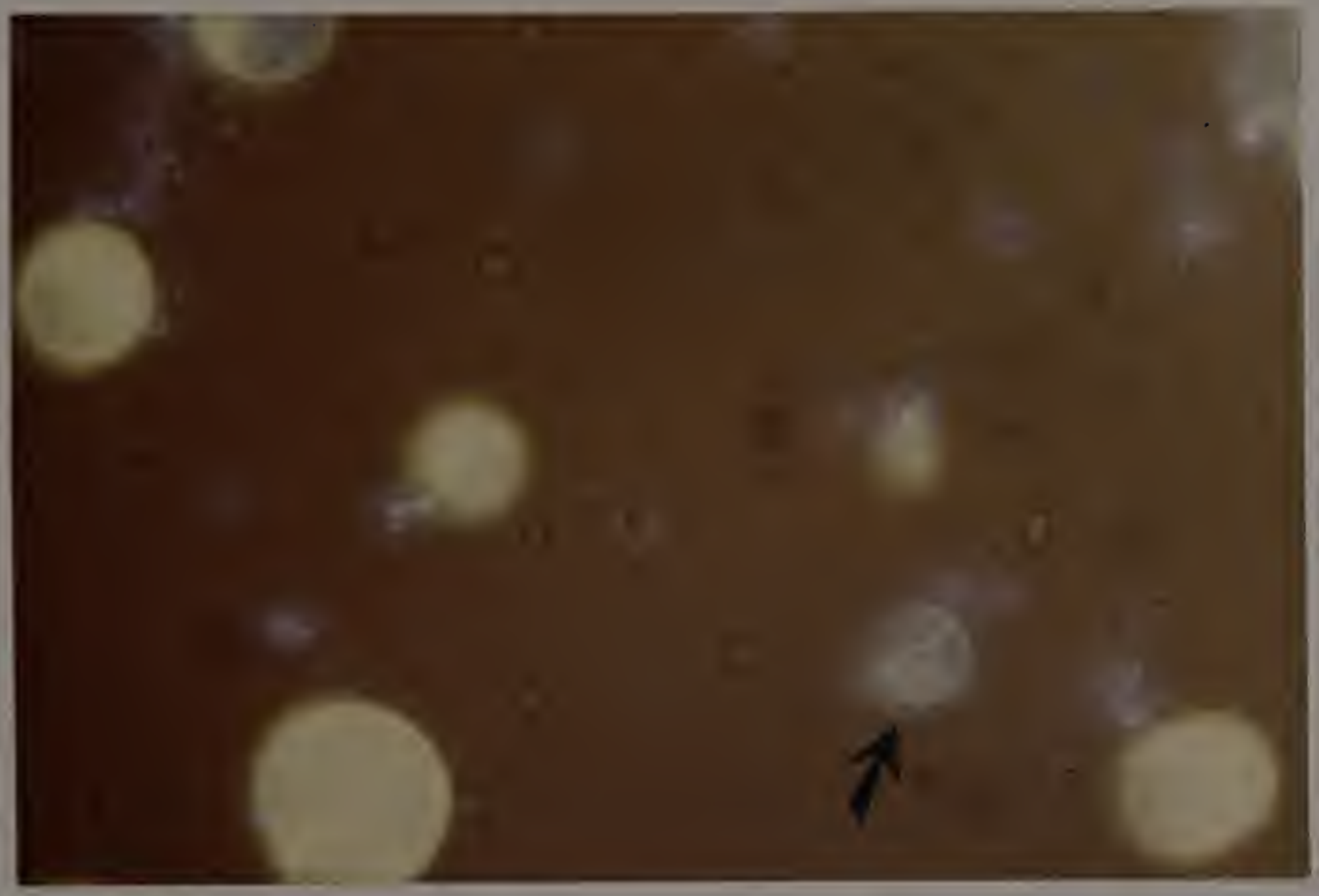

Fig.3 Fluorescent diacetate (FDA) staining of apple protoplasts. Living protoplasts exhibit bright fluorescence while non-living ones (arrow) do not fluoresce

day old callus. Calcofluor White staining showed that under our experimental conditions, more than 85% of the apple protoplasts were totally free of cell walls immediately after enzyme digestion and 66% were free of cell wall material 24 hours after isolation (Fig.4).

No differences in yield and quality of released protoplasts were obtained from calli kept in an incubator set at 25°C and 16 hour light period and in calli kept under room temperature and natural light conditions.

Protoplasts could not be obtained from apple leaves under any of the conditions described previously. Although the majority of the leaf cells were separated in the enzyme solutions within 4 hours from the beginining of the digestion, no protoplasts were released from such cells even after 24 hours of digestion.

Effect of Protoplast Concentration on ELISA and on Bioassy of TMV

ELISA

When compared to the ELISA values of purified TMV alone, ELISA values of equal concentrations of TMV decreased significantly if apple protoplasts were added to the TMV (Fig.5). The ELISA readings decreased almost linearly within the concentration of protoplasts tested. The

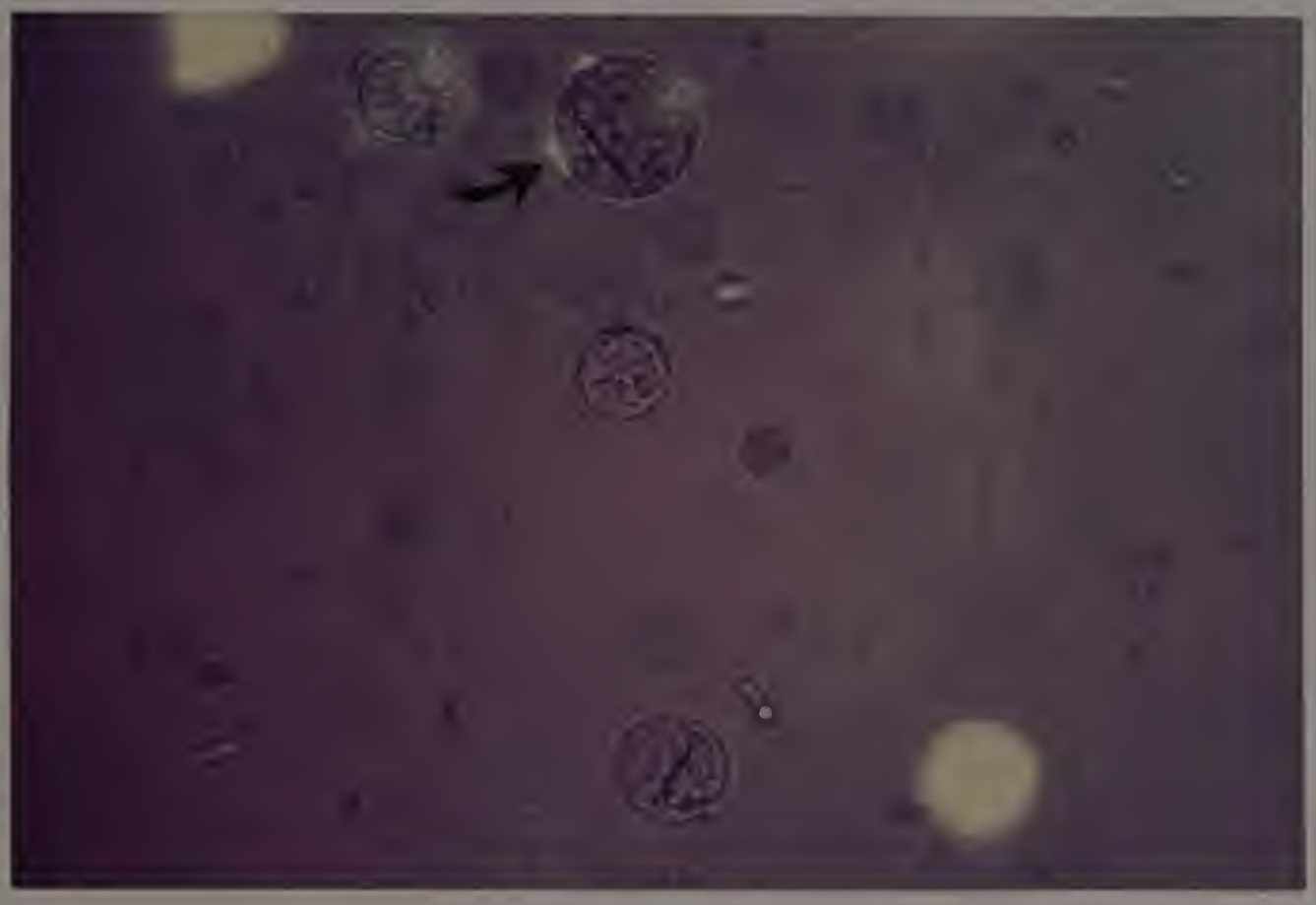

Fig.4 Calcofluor White staining of protoplasts. Undigested or regenerated cell wall material on protoplasts (arrows) shows a bright white fluorescence

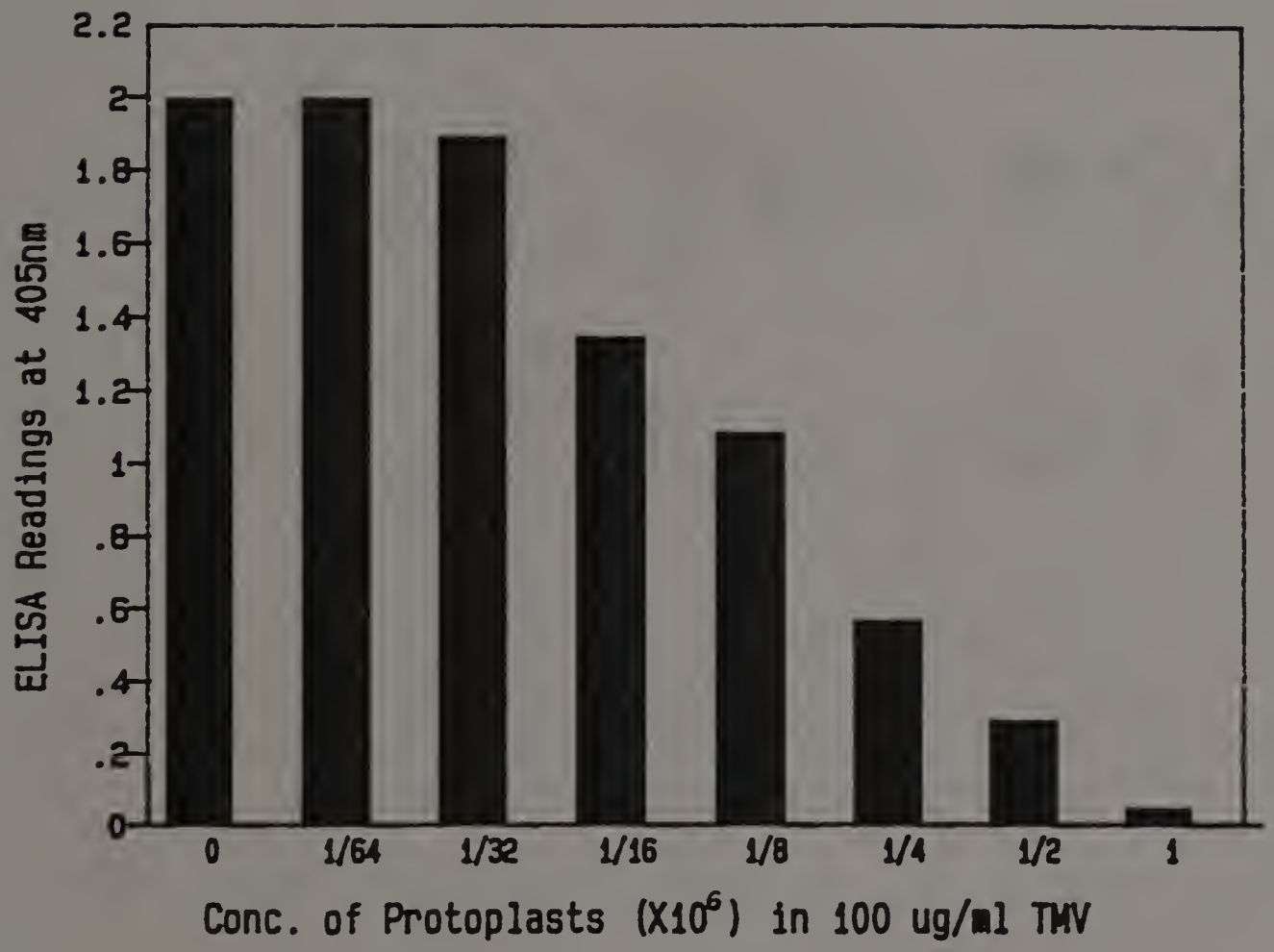

Fig.5 Influence of protoplast concentration on the ELISA readings of a constant dilution (100um/ml) of purified TMV. The higher the concentration of protoplasts in the TMV preparation, the lower the ELISA readings

protoplast concentrations were made by a 2-fold dilution of protoplasts on a constant TMV and PLO concentrations and ranged from 1/64 million to 1 million/ml protoplasts in 100 ug/ml TMV and 20 ug/ml PLO. While the ELISA reading of the lowest concentration of protoplasts (1/64 million/ml) in 100 ug/ml TMV and that obtained from 100 ug/ml TMV alone were both above the reading limit (2.0) of the ELISA scanner, ELISA readings decreased from 1.9 in the presence of 1/32 (0.031) million/ml protoplasts to less than 0.1 in the presence of 1 million/ml protoplasts.

Bioassay on local lesion host

When protoplasts were present in the TMV inoculum, development of local lesions on N glutinosa was influenced significantly. When N. glutinosa leaves were inoculated with 10 and 50 ug/ml TMV alone, the average number of lesions was 51.1 and 128.1 per half leaf (average of 7 half leaves), respectively. The number of local lesions decreased drastically when even small numbers of protoplasts were added to the TMV inoculum. As shown in Fig.6, the number of local lesions per half leaf was reduced by 50 to 86% whem 10 protoplasts were mixed with one ml of TMV inoculum containing 50 and 10 ug/ml TMV, respectively, and by 50 to 60 percent when 100 protoplasts were added to each ml of TMV inoculum. Higher concentration of protoplasts in the inoculum reduced the infectivity of

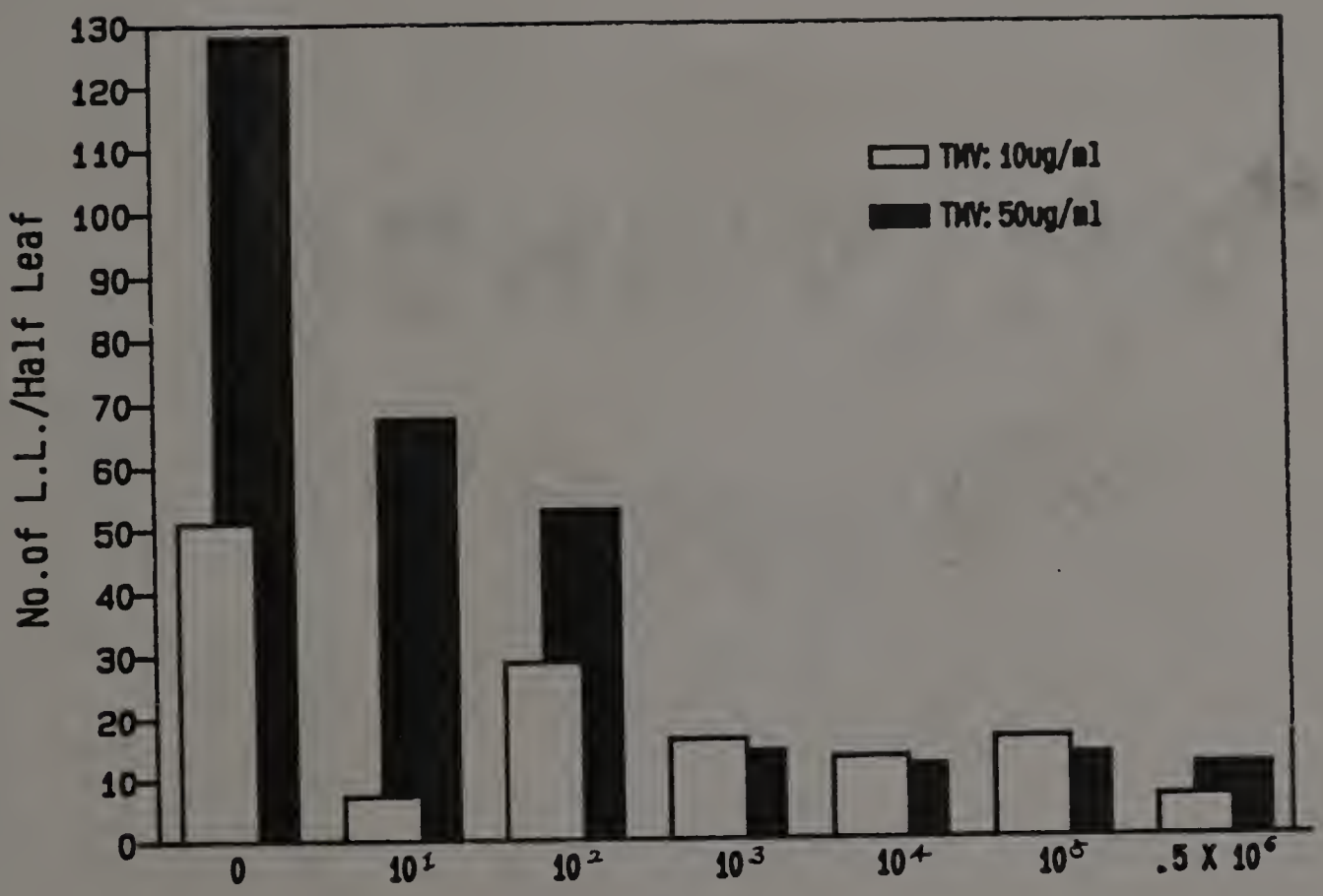

Fig.6 Influence of protoplast concentration in two constant TMV dilutions (10 and 50 ug/ml) on the number of local lesions produced by TMV on N. glutinosa

even the higher (50 ug/ml) TMV concentration by about 90 percent.

PLO-Mediated Inoculation of Apple Protoplasts with TMV

Evidence for infection of apple protoplasts with TMV

Inoculated protoplasts incubated for various periods of time were tested by ELISA. When the ELISA values were at least twice as high as the value obtained from inoculated protoplasts harvested at 0 time, such ELISA values were considered positive, that is, they were taken to indicate that the protoplasts had become infected with TMV and had allowed TMV to replicate within the protoplasts. Over forty experiments were carried out in which positive results were obtained, as shown by ELISA. Some typical results of TMV infection of apple protoplasts are shown in Figures 8 through 12 and Tables 5 and 6.

Protoplasts from experiments in which high ELISA values were obtained, and thereby strongly indicating that the apple protoplasts had become infected with TMV, also induced the formation of a few local lesions on Nicotiana glutinosa leaves inoculated with such TMV-inoculated apple protoplasts (Table 5). The best result obtained by bioassay was 8 lesions per N. glutinosa leaf when the leaves were inoculated with protoplasts harvested 72 hours after inoculation while no local lesions were produced by

Table 5. Comparison of ELISA values (absorbance at 405nm) and numbers of local lesions on Nicotiana glutinosa produced by TMV-infected protoplasts assayed 48-72 hrs after inoculation

Experiment	A 405nm	Lesions/leaf
1	1.043[a]	8.0[b]
2	0.169	3.5
3	0.504	1.5
4	0.163	1.7
5	0.467	0.5
6	0.140	0.5

[a] Average readings of 4-6 wells

[b] Average of lesions on 2 leaves of N. glutinosa

inoculated protoplasts harvested at 0 time. The lesion numbers reported here are the average numbers from two leaves. The ELISA values of the same treatments were 1.043 for the 72 hour incubation after inoculation and 0.097 for the 0 time treatment. In other experiments, either fewer or no lesions were obtained when the ELISA values of inoculations considered "positive" were lower than 0.5.

FITC staining of TMV-inoculated apple protoplasts showed that 2.3-2.8 % of the protoplast were infected with TMV at 36-48 hours after inoculation (Fig.7).

Effect of TMV and PLO concentrations on infection efficiency

The concentration of TMV and PLO in the inoculum proved to significantly affect apple protoplast infection with TMV. In earlier experiments, in which we tried to inoculate apple protoplasts with TMV using virus and PLO concentrations equal to those (1 ug/ml) used to inoculate tobacco protoplasts with TMV, no infection of apple protoplasts with TMV was detected either by ELISA or bioassay.

When the concentration of TMV was increased to 10 and of PLO to 2 ug/ml, occasionally positive results were obtained by ELISA. The ELISA values in these experiments were low, however. For example, the ELISA value at 66 hours after inoculation was 0.16, which is about 5 times greater than

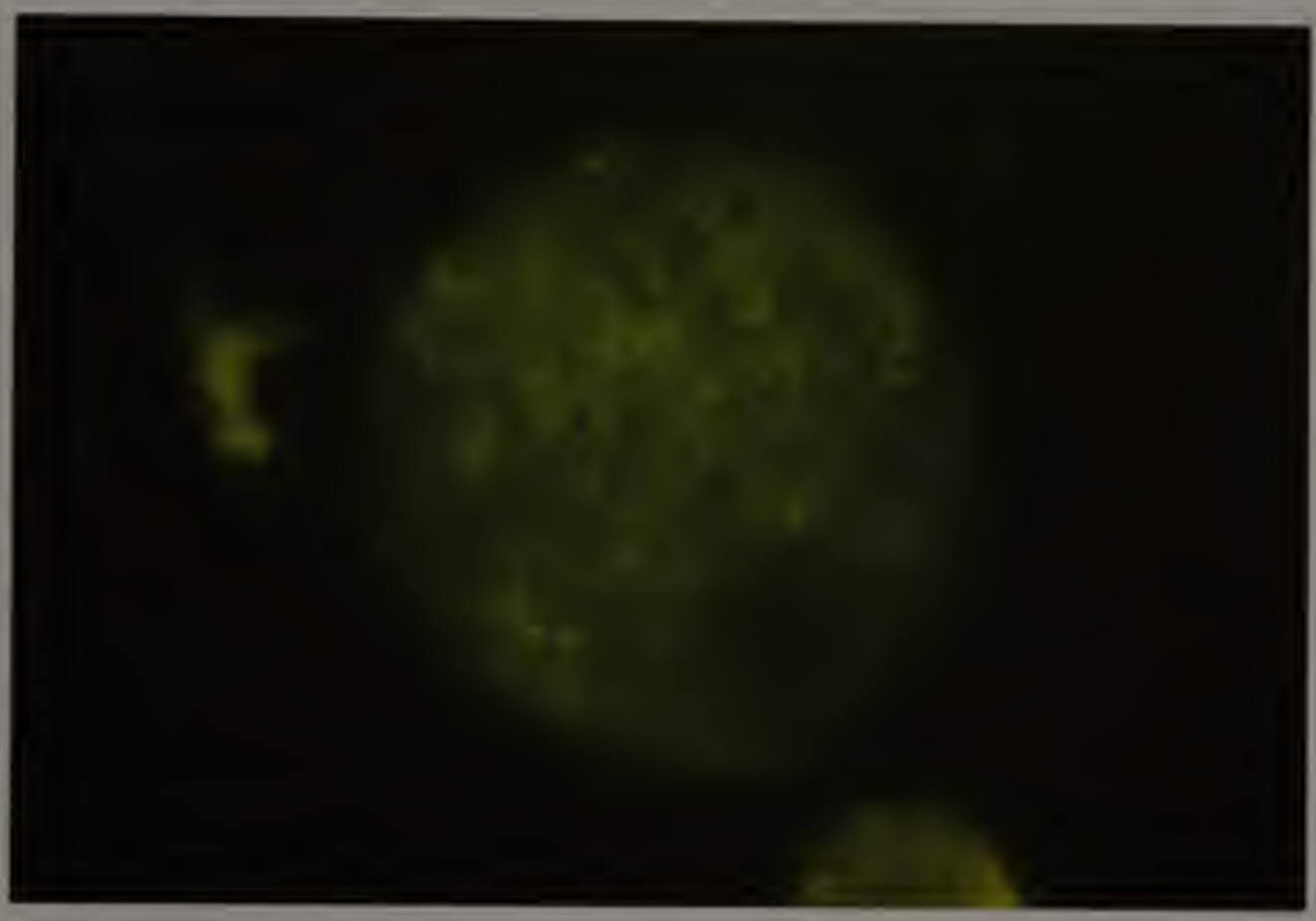

Fig.7 Fluorescein isothiocyanate (FITC) staining of TMV infected protoplasts. Protoplasts or areas of protoplasts containing TMV react with FITC-conjugated TMV antiserum and exhibit a bright flourescence

the readings at 0 time (Fig.8). However, in the majority of the experiments at which such concentrations were used, TMV failed to infect apple protoplasts. Most successful inoculations of apple protoplasts with TMV were obtained from experiments in which TMV concentrations of 25 ug/ml or greater were used as inoculum. Even at these concentrations of TMV, however, no infection of apple protoplasts was obtained if PLO concentration was kept at 2 ug/ml or lower. On the other hand, when PLO concentration was increased to 40 ug/ml, high ELISA readings were obtained even from samples assayed at 0 time. It became clear, therefore, that successful inoculation of apple protoplasts with TMV requires combinations of TMV and PLO concentrations within certain ranges.

In apple protoplast inoculation experiments, in which levels of TMV concentrations ranging from 10 to 50 ug/ml were tested against 3 levels of PLO ranging from 5 to 20 ug/ml, some infection of protoplasts was obtained with all PLO concentrations when TMV was kept at 50 ug/ml, with the highest infection obtained when PLO was at 10 ug/ml. PLO at 20 ug/ml also resulted in good infection of protoplasts with TMV, although somewhat lower than that was obtained with 10 ug/ml PLO (Fig.9). With TMV at 50 and PLO at 10 ug/ml, ELISA values of infected protoplasts were greater than 0.7, which was more than 10 times greater than the ELISA values of samples assayed at 0 time (about 0.05)

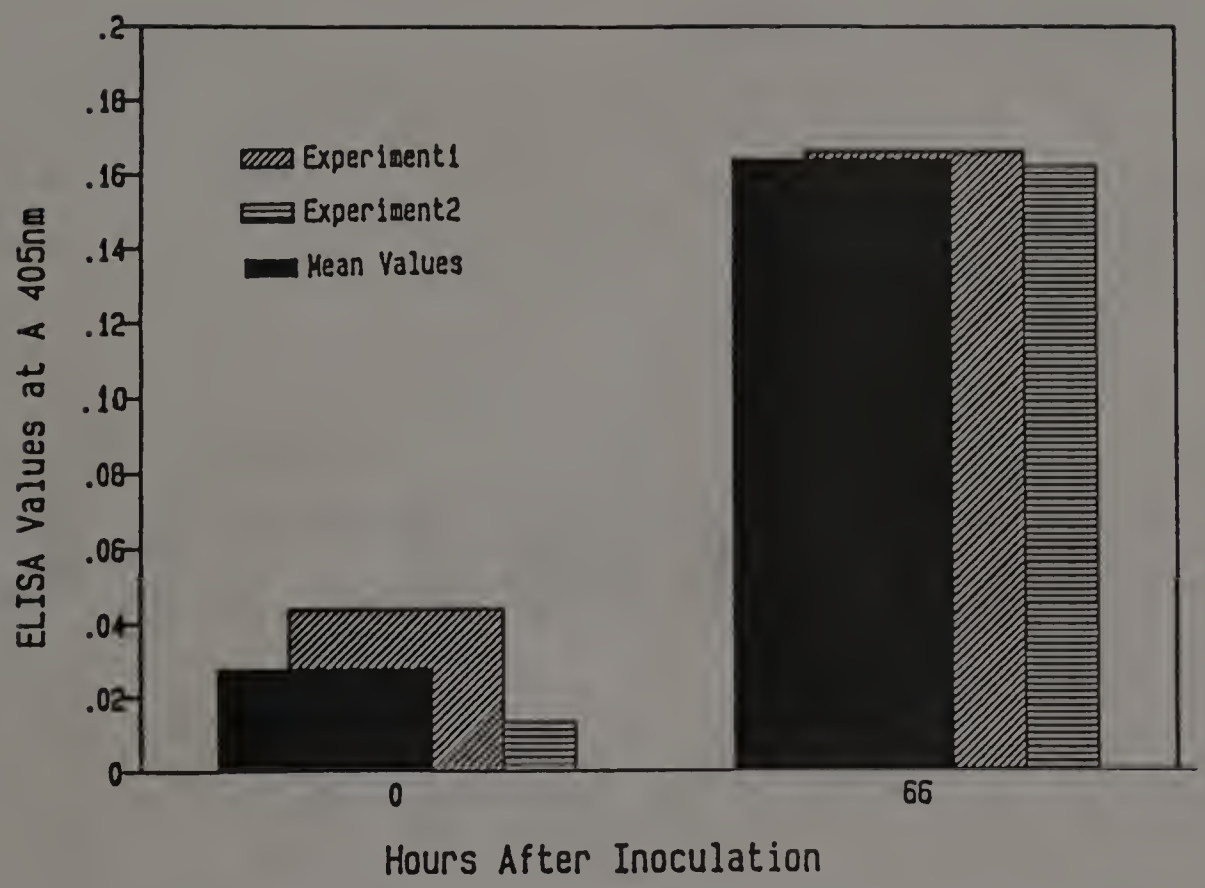

Fig.8 Apple (Mutsu) protoplast infection with TMV at 10ug/ml TMV and 2ug/ml poly-L-ornithine (PLO) as determined by ELISA

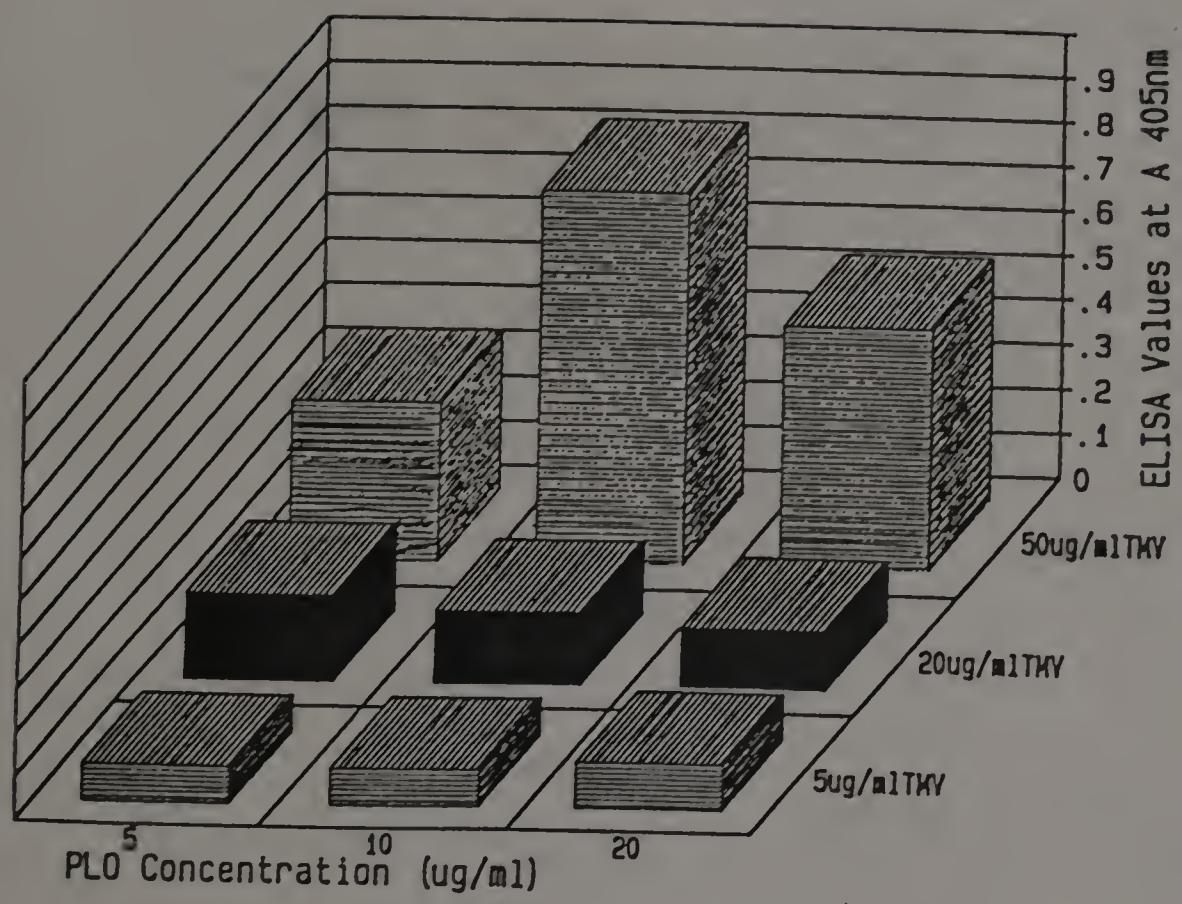

Fig.9 Comparison of the effect of different concentrations of TMV and PLO on infection efficiency of apple protoplasts with TMV as determined by ELISA

(Fig.10). When 50 ug/ml TMV and 20 ug/ml PLO were used, ELISA values of infected protoplasts 72 hours after inoculation were about 0.6, compared to values of about 0.1 obtained from samples assayed at 0 time (Fig.11). Similar results were obtained from experiments with protoplasts of different varieties, one of which, Mutsu, is shown in Fig.12. In other protoplast inoculation experiments, in which TMV was used at 100 ug/ml and PLO at 20 ug/ml, much higher ELISA values (1.3) were obtained from inoculated protoplasts 72 hours after inoculation. At the same time, however, considerably higher ELISA values (0.23) were obtained from inoculated protoplast samples assayed for TMV at 0 time (Fig.13).

Effect of buffer and pH on infection efficiency

In apple protoplast inoculation experiments in which the concentrations of TMV and PLO were kept at 10 and 2 ug/ml, respectively, while the pH was varied from 4.6 to 6.8, no significant differences in the efficiency of protoplast infection could be detected (Table 6).

Similar results were obtained from experiments in which the concentrations of TMV and PLO were kept at 100 ug/ml and 20 ug/ml, respectively.

No infection of apple protoplasts with TMV was obtained from one inoculation experiment in which the TMV and PLO

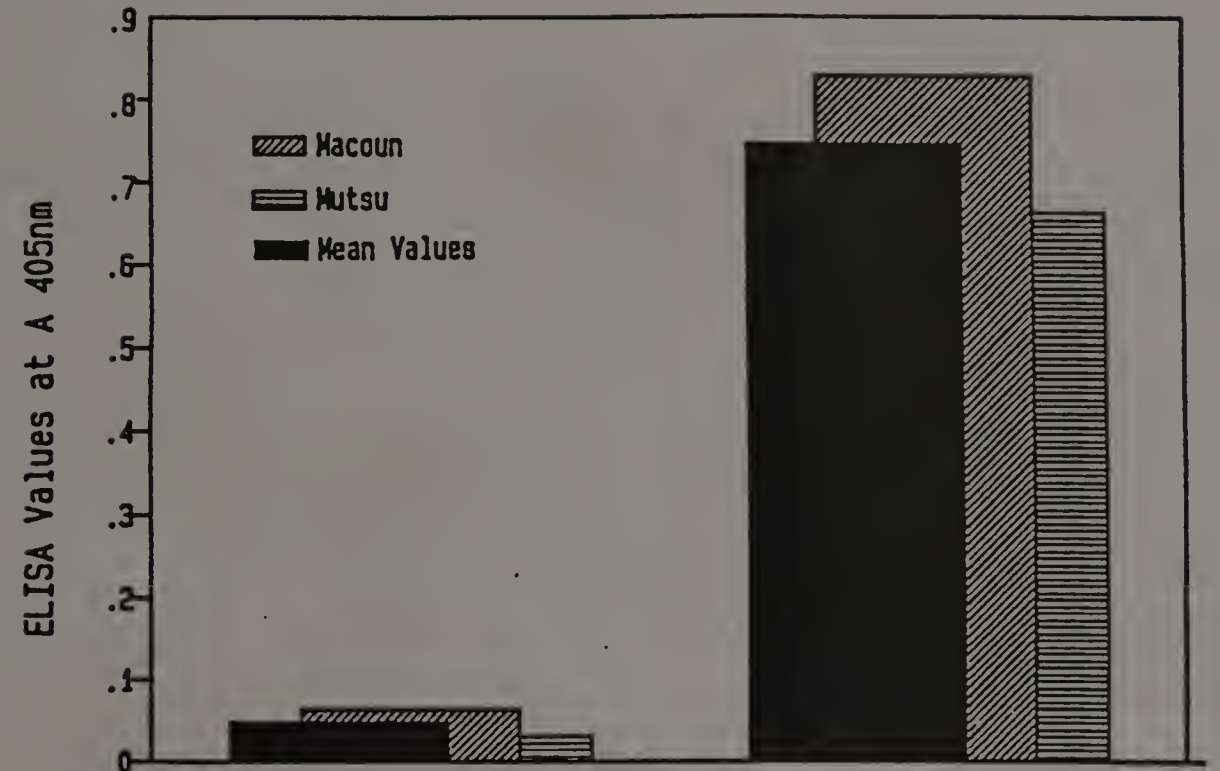

Fig.10 Apple protoplast infection with TMV at 50ug/ml TMV and 10ug/ml PLO as determined by ELISA

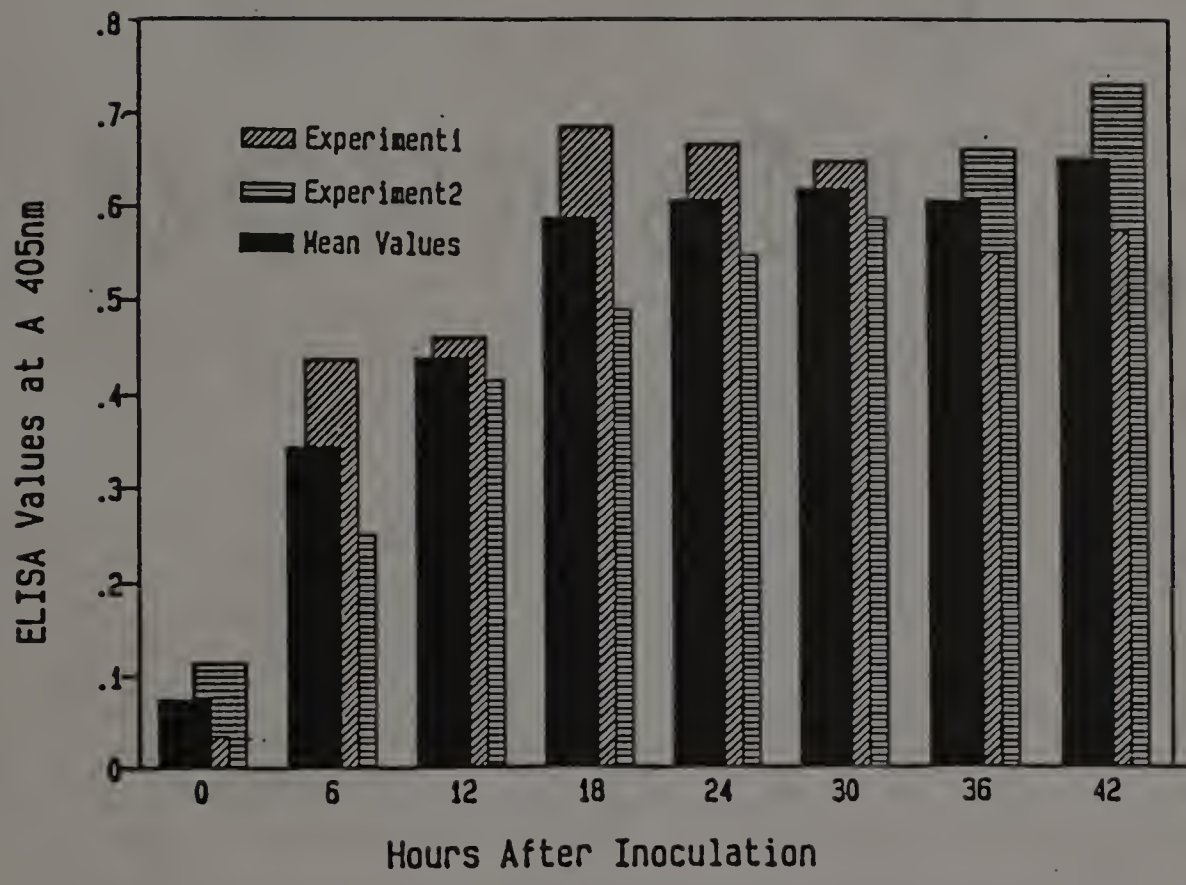

Fig.11 Apple (Macoun) protoplast infection at 50ug/ml TMV and 20ug/ml PLO as determined by ELISA

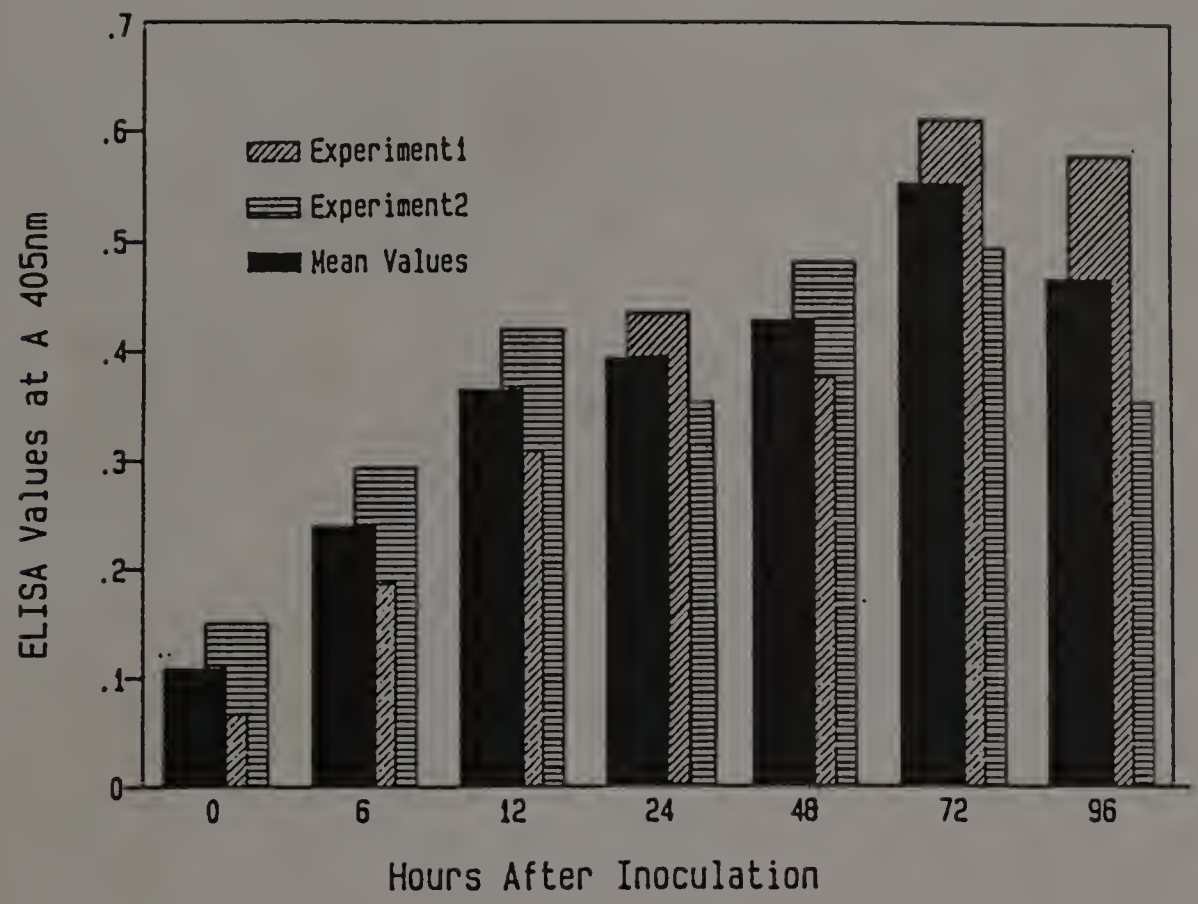

Fig.12 Apple (Mutsu) protoplast infection with TMV at 50ug/ml TMV and 20ug/ml PLO as determined by ELISA

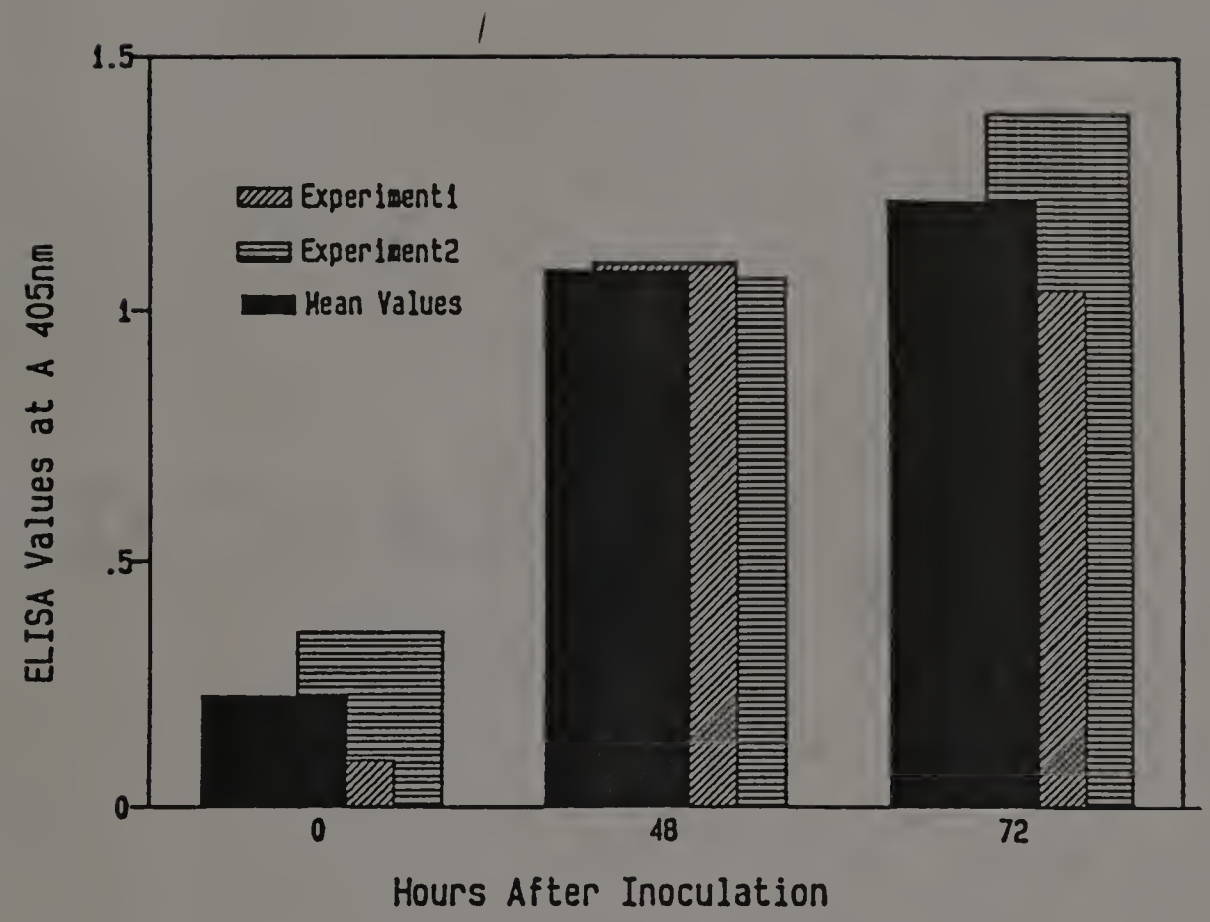

Fig.13 Apple (Mutsu) protoplast infection with TMV at 100ug/ml TMV and 20ug/ml PLO as determined by ELISA

Table 6. Effect of pH of virus inoculum and of apple protoplasts on frequency and efficiency of infection of protoplasts by TMV, as determined by ELISA

pH of TMV/PLO inoculum	pH of Protoplasts	Hours after inoculation	
		0	48-96
5.2	4.6	0.052[a]	0.116
5.2	6.0	0.013	0.162
5.2	6.2	0.095	0.241
5.2	6.2	0.022	0.159
5.2	6.2	0.068	0.127
5.2	6.8	0.044	0.166
5.2	6.8	0.097	0.147
6.2	5.2	0.019	0.155
6.2	5.2	0.017	0.128
6.2	6.2	0.035	0.168

[a] ELISA values at A 405nm

concentrations were 10 ug/ml and 2 ug/ml, respectively, and which was carried out in 0.05M phosphate buffer, pH 7.0.

Effect of apple variety on infection efficiency

Inoculation experiments with TMV were carried out on protoplasts of different apple varieties including Mutsu, Golden Delicious, Macoun and Lord Lambourne. No significant differences in efficiency of protoplast infection with TMV were observed among these varieties. This can be seen in Table 7 which contains the ELISA values of similarly inoculated protoplasts of four apple varieties harvested 48 hours after inoculation. Although ELISA values of individual inoculations sometimes appeared markedly different, the average ELISA values of all inoculations for each apple variety were not significantly different.

Effect of number of washes on ELISA readings of infected protoplasts

To determine the effect of the number of washes on TMV taken up by apple protoplasts, several experiments were carried out in which the protoplasts were washed 1 to 5 times. The supernatant of the inoculum and the wash solution were checked for TMV by ELISA.

Generally, the fewer the washes, the higher the ELISA values were. This was especially apparent at 0 time. The ELISA values obtained from supernatant showed that the

Table 7. Effect of apple varieties on efficiency of infection of apple protoplasts with TMV, as determined by ELISA of inoculated protoplasts 48 hrs after inoculation

Mutsu	Golden Delicious	Macoun	Lord Lambourne
0.167[a]	0.155	0.617	0.227
0.504	0.326	0.169	0.319
0.368	0.343	0.174	0.499
0.326	0.171	0.169	-
0.544	0.201	-	-
-	0.488	-	-
-	0.160	-	-
Aver. 0.382	0.263	0.282	0.348

[a] ELISA values at A 405nm

virus antigen was dissassociated from protoplasts mostly on the first wash. After 3 washes, only a small amount of TMV was present in the wash liquid (Fig. 14).

Time course of apple protoplast infection with TMV

The time course of infection of apple protoplasts with TMV was determined by experiments in which samples of inoculated protoplasts were assayed for TMV by ELISA at 6-hour intervals after inoculation. Figure 15 shows the average ELISA values of six experiments. It is apparent from Figure 15 that an increase in virus antigen as measured by ELISA could be detected in inoculated protoplasts as early as 6 hours after inoculation, and the virus antigen reached maximum concentration 24 to 30 hours after inoculation. Bioassay of infected protoplasts on Nicotiana glutinosa resulted in local lesions only when protoplasts had been incubated for at least 24 hours after inoculation.

Electroporation-Mediated Inoculation of Apple Protoplasts with TMV

Four experiments, in which apple protoplasts were inoculated with TMV via electroporation, resulted in protoplasts exhibiting varying frequency of infection with TMV as determined by ELISA. These experiments were carried

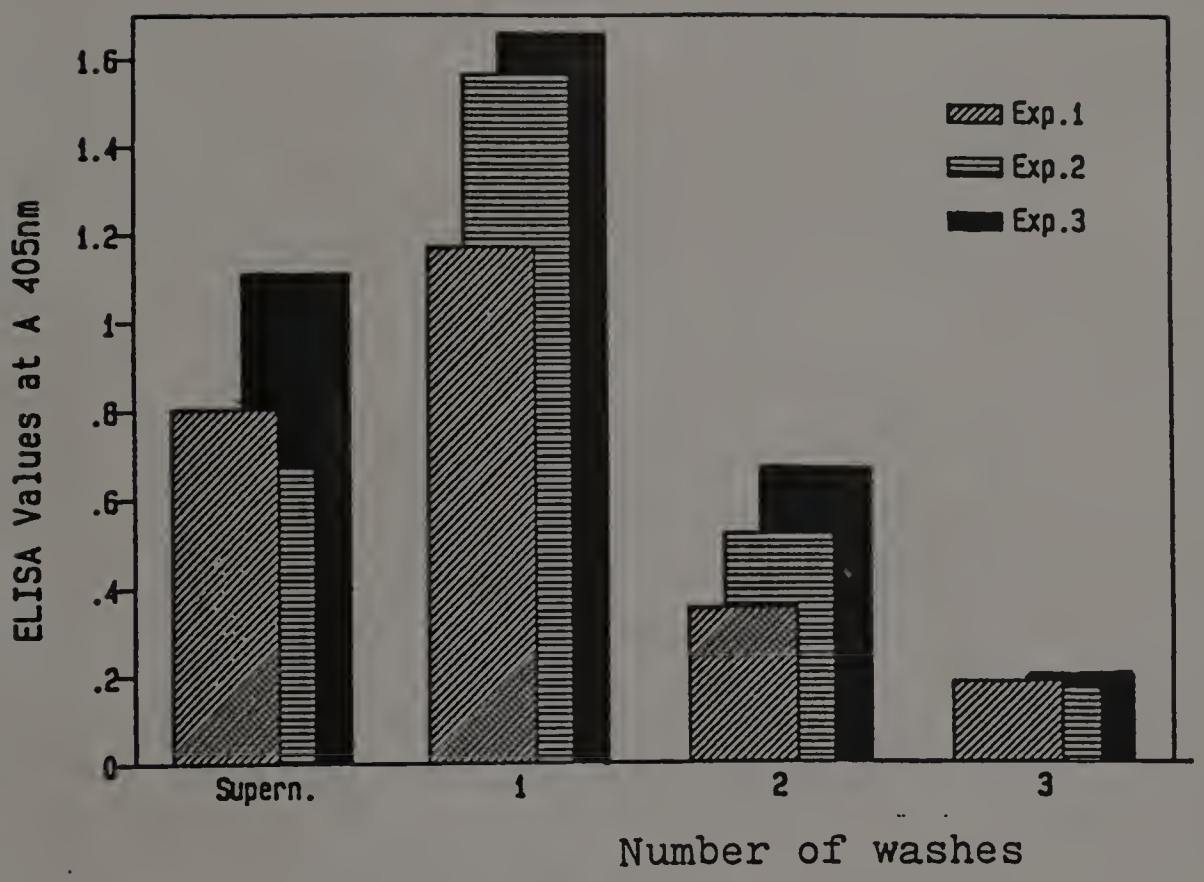

Fig.14 ELISA readings obtained from supernatant of TMV inoculum and from successive washes of inoculated protoplasts

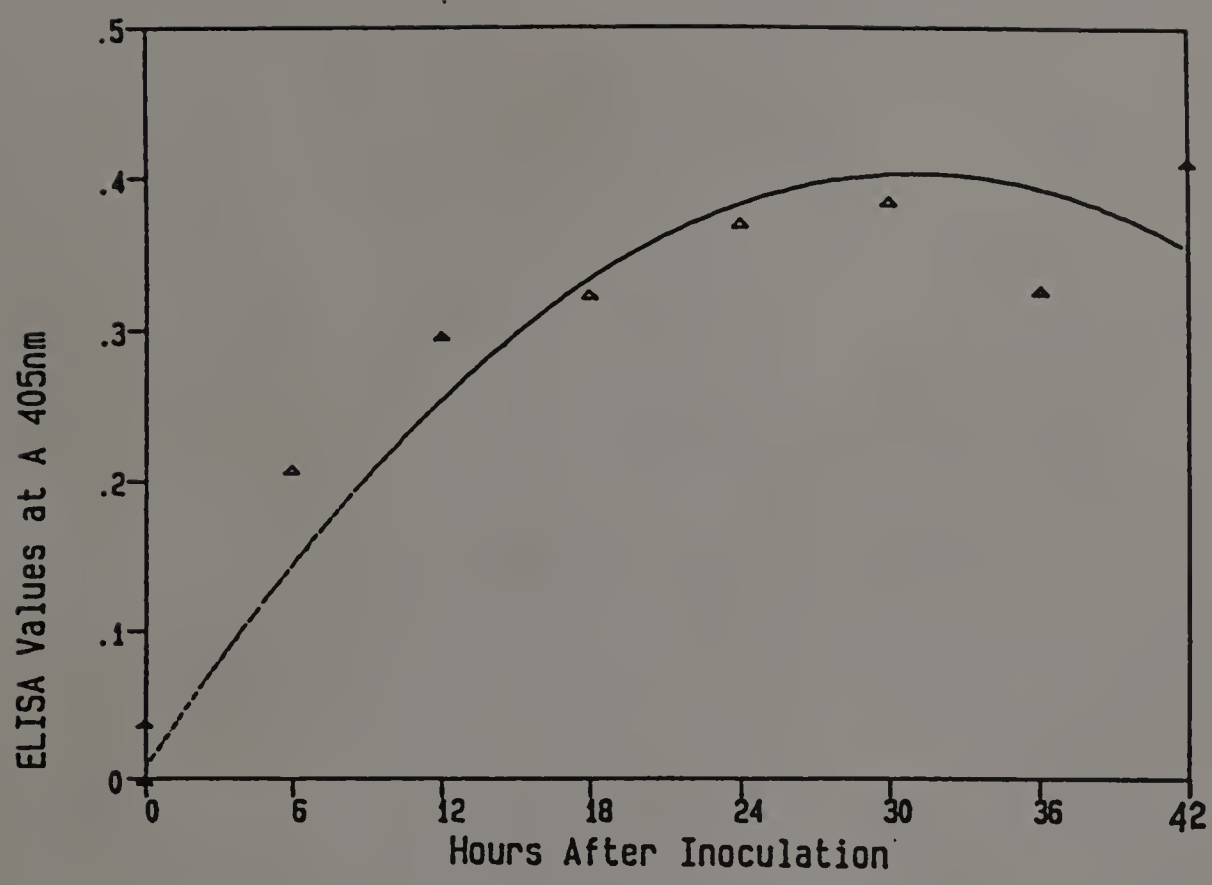

Fig.15 Average ELISA values of 6 experiments of apple protoplasts inoculated with TMV. The curve indicates the change of TMV antigen in infected apple protoplasts at different times after inoculation.

out with 5 X 10^3 protoplasts/ml and 50 ug/ml TMV subjected to electroporation with one discharge of 100 uF capacitance at 400 volts. An average ELISA value of 0.185 was obtained by assaying TMV-inoculated apple protoplasts 60 hrs after inoculation. When electroporation was carried out with three discharges of 100 uF capacitance each, the ELISA value of inoculated apple protoplasts, assayed 48 hrs afte inoculation, was 0.283. The best results were obtained from two experiments in which the protoplasts were electroporated first with a discharge of 2 uF and subsequently with a second discharge of 100 uF capacitance. The ELISA values, obtained by assaying inoculated protoplasts 48 hrs after inoculation in these experiments, were 0.505 and 0.451 which were much higher than the corresponding ELISA values (0.139 and 0, respectively) of protoplast samples assayed immediately after inoculation (Table 8).

Table 8 Infection of apple protoplasts with TMV by means of electroporation-mediated inoculation as determined by ELISA. Discharges of different capacitance influence the efficiency of infection

	Treatments			
Hours after inoculation	100uF(1X)	100uF(3X)	2uF,100uF	2uF,100uF
0	0.069[a]	0.175	0.139	0
48	-	0.283	0.505	0.450
60	0.185	-	-	-

[a] ELISA values at A 405nm

C H A P T E R V

DISCUSSION

Apple Protoplast Isolation

Several authors who worked with apple protoplasts have reported the optimal conditions for protoplast isolation from various tissues, digestion methods, and so on (Anderson et al.,1979; Yamaki, 1981; Huang and Millikan,1983; Hurwitz and Agrios, 1984). This research shows that when using apple calli from twigs, the quality of the callus itself is the most important characteristic determining the ability of the callus to release large numbers of protoplasts. The quality of the callus is controlled mainly by the age of the callus. When other conditions were the same, young callus, produced no later than 15 days after the twigs were placed or transferred onto new media, proved crucial for getting vigorous and clean protoplasts (Table 3).

In occasional experiments it appeared that protoplasts of high yield and quality were produced not by the youngest callus but by callus derived from certain twigs. This suggested that some characteristics of callus are controlled by the conditions of twig growth before the

twigs were cut from apple trees, for example, the position of the twigs, etc,. However, we did not attempt to determine these conditions in the research.

Besides the quality of callus, this research showed that slightly higher concentrations of enzymes than previously used for protoplast digestion (Table 1) and overnight treatment of callus with the enzymes in a refrigerator, improve the number and quality of protoplasts released from callus. Keeping the callus in enzyme solution overnight also shortens the actual digestion time at room temperiture.

Failure to isolate apple protoplasts from newly emerging leaves appears to result from failure of the enzymes to release protoplasts from separated mesophyll cells. This could be due to phenolics (Wallin and Welander, 1985) released from cut up leaves which inhibit cell wall digestion. Butt (1985), who successfully isolated protoplasts from several other mature and juvenile tree species by thoroughly washing chopped leaf tissue prior to addition of digestive enzymes, also suggested a similar mode of inhibition of these enzymes. In our experiments, however, additional washes helped single cells from chopped leaves separate from each other faster, but did not result in release of protoplasts from the cells. It is not known whether the apple leaf cell walls contain compounds not digestible by the enzymes used or whether they contain

enzyme inhibitors that can not be removed by washing from apple leaf cells.

Infection of Apple Protoplasts with TMV Mediated by PLO

This research showed that infection of apple protoplasts with TMV is possible. Three conclusions can be drawn from the results obtained from PLO-mediated inoculations of apple protoplasts with TMV: (1) Apple protoplasts can be infected with TMV by PLO-mediated inoculation; (2) infection of apple protoplasts with TMV requires a much higher concentration of virus than infection of protoplasts of herbaceous plant; this is probably due to the nature of woody plant protoplasts; (3) PLO is required for successful infection of apple protoplasts with TMV and it must also be present at a much higher concentration than that used for inoculation of protoplasts of herbaceous plants with virus. The most important conditions for successful inoculation are the concentrations of TMV and PLO. Other factors, such as pH, varieties of apple, etc, seem to have minimal effects on efficiency of infection.

With the same virus, tobacco mosaic virus, Takebe and Otsuki (1969) infected tobacco mesophyll protoplasts with TMV and PLO concentrations as low as 1 ug/ml concentrations. However, in our TMV-apple protoplast system, a much higher concentration of virus, usually 50 ug/ml, was required for infection. This means that, on the

average, 1,500,000 TMV particles are required to get one apple protoplast infected. This number is 19 times higher than the 80,000 TMV particles required to infect a tobacco protoplast (Takebe, 1977). This indicates that, in the TMV-apple protoplast system, the efficiency of virus infection is lower. It is not known what structural or physiological characteristics of woody plant cells may be responsible for this difference. However, Cocking and Pojnar (1969) showed by electron microscopy that TMV is taken up by and multiplies in protoplasts obtained from tomato fruits. Their system also requires the presence of a very high concentration of virus inoculum for a long period. The efficiency of infection and the rate of virus multiplication in their TMV-tomato protoplast system are apparently much lower than that in Takebe and Otsuki's TMV-tobacco protoplast system. Therefore, specific interaction between viruses and protoplasts from different plant species or different plant tissues may also be important in determining the efficiency of infection.

The time course of TMV increase in apple protoplasts in our experiments was essentially similar to that in tobacco mesophyll protoplasts tested by Takebe and Otsuki (1969). As determined by ELISA, TMV antigen increased rapidly 6 hours after inoculation and reached a maximum concentration 24 to 36 hours after inoculation. No increase of virus antigen was determined at 3 hours after inoculation.

However, no infectious virus was detected by bioassay until 12 to 24 hours after protoplast inoculation. The delayed detection of virus by bioassy implies a delayed replication of infectious TMV RNA compared to the production of virus coat protein antigen in apple protoplasts.

The difference in TMV concentrations required for apple and tobacco protoplast infections and the similarity in the time courses of virus replication in those two hosts may imply that uptaking of virus into rather than multiplication of virus in protoplasts is the most critial step in the infection process in terms of efficiency of apple protoplast infection with TMV.

It is known that poly-L-ornithine is essential for infecting tobacco protoplasts with TMV (Takebe and Otsuki, 1969; Takebe and Nagata, 1973); although some (Zhuravlev et al. 1975) have reported that quite large amounts of the virus are also attached to protoplasts in the absence of poly-L-ornithine, especially at high TMV concentrations. However, PLO proved to be essential for infection of apple protoplasts with TMV even at a very high concentration of TMV.

As pointed out by Takebe (1975) and Kubo et al (1976), the optimal concentrations of virus and PLO may be interdependent since in the inoculum PLO interacts with negatively charged virus particles. Our results agree with this suggestion. In our experiments, since the virus

concentrations used for inoculation were higher than for previously reported combinations, the PLO concentrations required were correspondingly higher. When PLO concentration was low (e.g., 0.4 to 2.0 ug/ml), no infection was obtained at 50 ug/ml TMV. When PLO concentration was high (e.g., 40 ug/ml), the ELISA readings at 0 time also appeared high, presumably due to excessive attachment of TMV to protoplasts.

The fact that ELISA readings of purified TMV preparations are highly influenced by the presence of apple protoplasts makes it hard to evaluate the increase in TMV antigen quantitatively. Since the ELISA readings of constant TMV concentrations decreased dramatically with increase in healthy protoplast concentrations, it is not clear whether the ELISA readings of inoculated protoplasts immediately after inoculation and of inoculated protoplasts 24-36 hours after inoculation reflect precisely the amount of virus antigen in these samples. A similar observation was also made by Lommel et al.(1981); when they used indirect ELISA for quantitative detection of carnation mottle virus (CaMoV) and carnation ringspot virus (CaRSV). By comparing the readings obtained from the viruses in the presence and absence of carnation host proteins, they concluded that the indirect ELISA test would not be useful for accurate quantification of virus in crude plant extracts. They reasoned that in indirect ELISA antigen is

bound directly to the polystyrene surface and therefore, quantitation is directly affected by the binding event. This may also explain the lower ELISA readings of TMV at a higher concentration of protoplasts in our experiments since it is possible that protoplast proteins occupy a greater proportion of the surface of polystyrene wells when they are present in relatively high concentrations in the well in relation to the concentration of the virus. Furthermore, it is possible that the antigenic determinants of the virus were masked by the proteins of the ruptured protoplasts.

However, indirect ELISA is still thought to be a reliable test in our protoplast system since the comparisons are made between inoculated protoplast samples at the beginning of infection (0 time) and inoculated protoplast samples incubated for certain periods of time. In both cases the concentrations of the protoplasts are kept the same. It is, therefore, expected that the increases in ELISA readings of inoculated protoplasts after various periods of incubation indicate proportional increases in TMV antigen in those protoplasts.

Similarly, the interference of healthy protoplasts with local lesion development on N. glutinosa by TMV makes difficult the determination and evaluation of the increase in TMV particles in infected apple protoplasts by bioassay. The nature of this interference is not understood, although

binding of protoplast protein to virus particles and interference with virus entry or multiplication in plant cells is a likely explanation. However, the fact that lesions were obtained only, or in much greater numbers from inoculated protoplasts incubated for one or more days than from protoplasts at 0 time indicates that apple protoplasts were indeed infected with TMV in our experiments.

FITC staining of inoculated protoplasts showed good correspondence with the infection results obtained by ELISA and bioassay. This test confirmed the infection since brightly fluorescing protoplasts were detected only among inoculated protoplasts incubated for one or more days while no fluorescing protoplasts were found among the protoplasts at 0 time. However, the percentage of infected protoplasts as detected by FITC staining was low in comparison to the results obtained by other workers with inoculations of protoplasts of herbaceous plants. The low infectivity of apple protolasts might be due to the properties of woody plant protoplasts.

The observation that the TMV-infected apple protoplasts gave a distinctively bright fluorescence suggests that the large increase in TMV antigen detected by ELISA is contributed by a small number of infected protoplasts.

Staining of protoplasts in suspension was superior to staining them on a slide. Staining protoplasts in suspension resulted in whole, round protoplasts floating

in a clear background with minimum or no nonspecific fluorescence.

ELISA readings obtained from inoculated protoplasts at 0 time washed once were often high, while 0 time readings of inoculated protoplasts washed 3 times were reasonably low. This paralleled the ELISA readings of the washing solutions from inoculated protoplasts. After three washes, the ELISA readings of the supernatant were low.

Infection of Apple Protoplasts with TMV Mediated by Electroporation

Our ELISA observations from electroporation-mediated inoculation experiments proved that electroporation is an alternative way to infect apple protoplasts with TMV. A very low capacitance (2 uF) discharge, given to protoplasts before a high capacitance (100 uF) discharge appeared to protect the protoplasts from damage by electroporation and to increase infection. More work needs to be done to optimize the conditions for inoculation of apple protoplasts with TMV by electroporation.

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