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Study of Tobacco Mosaic Virus Replication in the Protoplasts of Saccharomyces Cervesisiae Using Assay Host Phaseolus Vulgaris Cv. Pinto and Fluorescence Microscopy

Gurdeep Kaur
Loyola University Chicago

STUDY OF TOBACCO MOSAIC VIRUS REPLICATION IN THE PROTOPLASTS OF *SACCHAROMYCES CERVESISIAE* USING ASSAY HOST *PHASEOLUS VULGARIS* CV. PINTO AND FLUORESCENCE MICROSCOPY

by

Gurdeep Kaur

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VITA

The author, Gurdeep Kaur, daughter of Sohan Singh, was born on July 8, 1952, in Ludhiana, Punjab.

Her secondary education was obtained in the Government Girls Higher Secondary School, Moge, India, where she graduated in 1969.

In July, 1970, she entered Khalsa College for Women, Ludhiana, India, and in July, 1973, received the degree of Bachelor of Science with a major in Biology and Chemistry.

In July, 1973, she entered Malwa Central College of Education, and in July, 1974, received the degree of Bachelor of Education.

In January, 1976, she entered the graduate program in Biology. In January, 1979, she was awarded the Master of Science degree in Biology.

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INTRODUCTION

Protoplasts are the cellular units lacking cell wall. Klercker in 1892 was the first to isolate protoplasts from the onion epidermis by microdissection and plasmolysis of the onion epidermis in sucrose solution; only small quantities of protoplasts were obtained. The shortcomings of the mechanical method was later avoided by using the enzymatic method to isolate protoplasts.

Weibull (1953), Giaja (1919), and Cocking (1960) successfully isolated protoplasts from bacteria, fungi and higher plants respectively, using cell wall degrading enzymes.

Viruses are nucleoprotein highly specific to their hosts. Animal virus will infect animal cell and plant virus will infect plant cell. It has been established that animal virus can infect the animal cell through the process of pinocytosis, such phenomenon is absent in case of plants because plant cell walls act as a barrier against the entrance of the virus.

Lederberg (1956) suggested that protoplasts might be useful material for studying the biosynthesis of plant viruses. In 1968 Takebe and Otsuki successfully isolated protoplasts from Tobacco leaf infected with Tobacco Mossaic Virus. Using fluorescent antibody staining technique Otsuki and Takebe (1969) located TMV antigen in infected tobacco protoplasts. Coutts, Cocking, and Kassanis (1972)

successfully isolated Saccharomyces cerevisiae (yeast) protoplasts and infected them with Tobacco Mosaic Virus. The yeast protoplasts provide a very desirable system for studying the infection process of Tobacco Mosaic Virus.

The purpose of this research is to isolate protoplasts of Saccharomyces cerevisiae, infect them with a common strain of Tobacco Mosaic Virus (TMV) and study the replication process using Fluorescent antibody staining technique and conventional assay procedure using Phaseolus vulgaris cultivar (cv.) Pinto as assay host under environment conditions such as effect of the temperature and concentration of the virus.

REVIEW OF LITERATURE

The following review of literature pertains mainly to methods for isolating and preparing the protoplasts and the procedures of various investigators to infect protoplasts with the virus. Emphasis is given to bring all the literature related to the isolation and preparation of Saccharomyces protoplasts and the infection of protoplasts with Tobacco Mosaic Virus.

Early History of Protoplasts Preparation

In 1892 Klercker was the first to isolate protoplasts from the onion epidermis. He accomplished this by microdissection and plasmolysis of the onion epidermis in sucrose solution, but only small quantities of protoplasts were obtained by this method. Later on, the method was improved by using cell wall degrading enzyme. In 1953 Weibull reported that in the presence of sucrose, the enzyme lysozyme digested the cell wall of certain bacteria releasing the protoplasts intact.

Yeast Protoplasts

In 1956 Giaja treated yeast cell with enzyme obtained from the gut juice of snail (Helix pomatia) and demonstrated the preparation of yeast cell protoplasts in the presence of high concentration of sugar. Giaja further reported that to prepare protoplasts, young

cells of yeast must be used, and the yield of protoplasts was influenced by the nature of sugar. For example, glucose caused yeast cell to lyse rapidly during digestion unless the temperature was reduced to about 4°C in the later stages. This was avoided by the use of rhamnose, a sugar which was not metabolized.

Eddy and Williams (1957) discovered that the protoplasts isolated from Saccharomyces carlsbergensis plasmolysed in sugar solutions more concentrated than 0.5M and tend to swell and eventually burst at lower concentrations. They also reported in 1959 that isolated protoplasts are capable of regenerating a normal yeast cell wall. They modified the method for isolating yeast protoplasts by using mannitol instead of rhamnose as an osmotic stabilizer.

In 1962 Garcia Mendoza and Villanueva isolated a species of streptomyces from the soil and found that an enzyme, Strepzyme, produced by this species of streptomyces was able to lyse the cell wall of a number of yeasts (Saccharomyces fragilis, S. rossei, S. cerevisiae, and Candida utilis) and mold spores. They also observed that it was much easier to obtain protoplasts from the young growing culture of yeasts. In 1964 Garcia Mendoza and Villanueva further reported that there is no difference in the mode of action of lytic enzyme strepzyme and the gut juice of snail (Helix pomatia).

Millbank and Macrae (1964) reported that cell wall of Saccharomyces was composed mainly of glucan, mannan, and protein, and in order for the cell wall to be digested, both mannan and glucan of the cell wall must be at least partially degraded. Degradation of

either glucan or mannan alone did not lead to protoplasts formation. Tanaka and Phaff (1965) made comparative studies of the composition of cell wall from different yeasts and preferred microbial enzyme instead of the snail enzyme in digesting yeast cell walls because of the complex composition and difficulty to obtain in large quantity.

Gascon and Villanueva (1965) found that preparation of protoplasts through treatment with snail or microbial enzyme does not affect the biochemical and biological activities of the microbial cell. He concluded that stabilizing media containing the solute magnesium sulfate at a concentration of 0.8 M to 1.0 M is the best of all the osmotic stabilizers tested, yielding a stable protoplasts suspension.

Regeneration of Yeast Protoplasts

In 1966 Svoboda and Necas reported that yeast protoplasts prepared by snail enzyme showed a high rate of regeneration of cell wall when embedded in nutrient media containing gelatin.

Protoplasts from Higher Plants

Cocking (1960) isolated protoplasts from the root tips of tomato seedling (Lycopersicum esculentum) by treatment with fungal cellulase obtained from Myrothecium verrucaria, using 0.6M sucrose as an osmotic stabilizer.

Ruesink and Thimann (1966) prepared protoplasts from higher plant tissue using concentrated Myrothecium cellulase. The addition

of polyuronidases or of ethylene diamine tetra acetate (EDTA) did not increase the yield of protoplasts.

In 1968 Takebe et al., reported the successful use of the commercially available Trichoderma viride cellulase for the formation of protoplasts from isolated leaf mesophyll cells of tobacco plants.

Mechanism of Virus Infection

The mechanism of plant virus infection as reported by Frankel Conrat et al. (1958), takes place in two steps; 1) separation of protein coat, and 2) infection of host cell by the separated nucleic acid.

The lesions produced by the nucleic acid appeared in a more synchronized fashion and reached the final maximal value appreciably earlier (about 24 hrs) than did those by the intact TMV. A few tests performed in different hosts have shown similar differences in rate between nucleic acid and virus infection as in Xanthi variety of tobacco and pinto beans. The 5 hour lag period corresponds to the average time required for release of an active nucleic acid from the intact virus.

Susceptibility of Phaseolus vulgaris to Tobacco Mosaic Virus

Irving Rappaport and Jia-Hsi Wu (1963) reported that the bean plants Phaseolus vulgaris cv. Pinto, although normally immune to the mild strain of TMV U_2 can be made susceptible to infection by

subjecting primary leaves previously inoculated with virus to a sublethal heat treatment 6 hours before inoculation. There was a lag period of 4 hours in response to heat after inoculation followed by a rapid rise in number of lesions between 4 and 6 hours. It was found that infectious unit invades the cell and then progresses through a few replication steps before infection becomes latent.

Isolation of TMV Infected Cells and the Study of Virus Replication

Takebe et al. (1968), isolated mesophyll cells from TMV infected tobacco plants and found that isolated cells supported the replication of virus during further incubation. Motoyoshi and Oshima (1968) demonstrated that isolated tobacco leaf cells could be infected with TMV by mechanical stirring. Cocking and Pojnar (1969) presented the electron microscopic evidence for the infection of isolated tomato fruit tissue protoplasts by Tobacco Mosaic Virus (TMV). After the uptake of TMV into pinocytic vesicles, the protein coat of the virus disappear and on regeneration of cell wall by isolated protoplasts, the virus appeared in the cytoplasm in an aggregate characteristic of TMV infection.

Infection of Isolated Protoplasts with TMV

In 1969 Takebe and Otsuki succeeded in isolating tobacco leaf protoplasts and infecting them with TMV. Infection occurred in the presence of Poly-L-Ornithine and virus multiplied to the concentration of 10^6 virus particules per infected protoplast during 24 hours

of incubation. Burgess, Motoyoshi and Fleming (1973) suggested that the function of Poly-L-Ornithine and other treatment used to obtain virus infection of protoplasts is to injure the cell membrane and allow a non physiological entry of higher molecular weight materials. Pinocytosis appears to be unnecessary for uptake of virus particles under conditions of membrane stress.

Study of TMV Replication by Fluorescent Antibody Staining Technique

Otsuki and Takebe (1969) developed a fluorescence antibody staining technique to locate TMV antigen in infected tobacco protoplasts, a method to determine the number of TMV infected protoplasts.

Protoplasts Infected with TMV-RNA

Aoki and Takebe (1969) infected tobacco protoplasts by brief contact with infectious TMV-RNA. Upon incubation, a synchronous multiplication of TMV occurred and virus titer reached a maximum after 22 hours of incubation period. Fluorescent antibody staining technique showed 3-7% of the protoplasts infected.

Tobacco Protoplasts Infected with Cucumber Mosaic Virus (CMV) and TMV

Honda and Matsui (1971) infected tobacco protoplasts with Cucumber Mosaic Virus (CMV) and Tobacco Mosaic Virus (TMV) and found that in addition to TMV particles within cytoplasm, large and small amounts of rod-shaped particles were observed within the matrix of nuclei and plastids respectively. In the nuclei, particles were

arranged at random whereas, in plastids, they were arranged parallel to each other. The particles could be detected only within nuclei and plastids of TMV infected leaf cells grown in daylight.

Otsuki, Takebe, Honda, and Matsui (1972) examined thin sections of tobacco protoplasts infected with TMV during 45 hours of incubation and observed one end of TMV rods adsorbed to the plasmalemma; the virus entered protoplasts by the process of pinocytosis.

Yeast Protoplasts Infected with TMV

Coutts, Cocking, and Kassanis (1972) showed that yeast cells can become infected with viruses normally found in different fungal species. They thought that yeast protoplasts might provide a suitable system for demonstrating the infection of yeast cells with virus such as TMV. In 1977 Patel isolated Saccharomyces cerevisiae protoplasts and studied the infection of protoplasts with TMV by Fluorescent antibody staining technique.

MATERIALS AND METHODS

Cultivation of Saccharomyces cerevisiae

A common strain of Saccharomyces cerevisiae cultivar ellipsoideus, No. 566 obtained from the American Type Culture Collection was maintained on agar slants at 4°C. The yeast was grown for cell isolation in 125 ml Erlenmeyer flasks containing 30 ml of the pre-culture liquid media (Appendix A) at 25°C for 24 hours. Inoculum from the 24 hour culture was transferred into another 125 ml Erlenmeyer flask containing 30 ml of the shake culture liquid medium (Appendix A) and was grown for 18 hours at 25°C with constant agitation. Yeast cells from the 18 hour culture were collected by centrifugation in an International Clinical Centrifuge (Model CL-424-M-4, International Equipment Company) at approximately 1000 rpm for 5 minutes. Cells were washed twice with sterile glass distilled water and then used for the isolation of protoplasts. The composition of the agar medium, pre-culture liquid medium, and shake culture liquid medium used by Darling et al. (1969) are given in Appendix A.

Isolation of Protoplasts

One gram wet weight of yeast cells (Fig. 1) were suspended in an osmotic stabilizer (sorbitol-citric acid pH 5.0) and incubated for 30 minutes at 30°C with occasional shaking. Yeast cells were collected by centrifugation and treated with 2 ml of enzyme B-Glucuronidase/Aryl sulfatase from the gut juice of snail (Helix pomatia) obtained from Sigma Chemical Company Activity - B-Glucuronidase 6.5 IU/ml, Aryl sulfatase

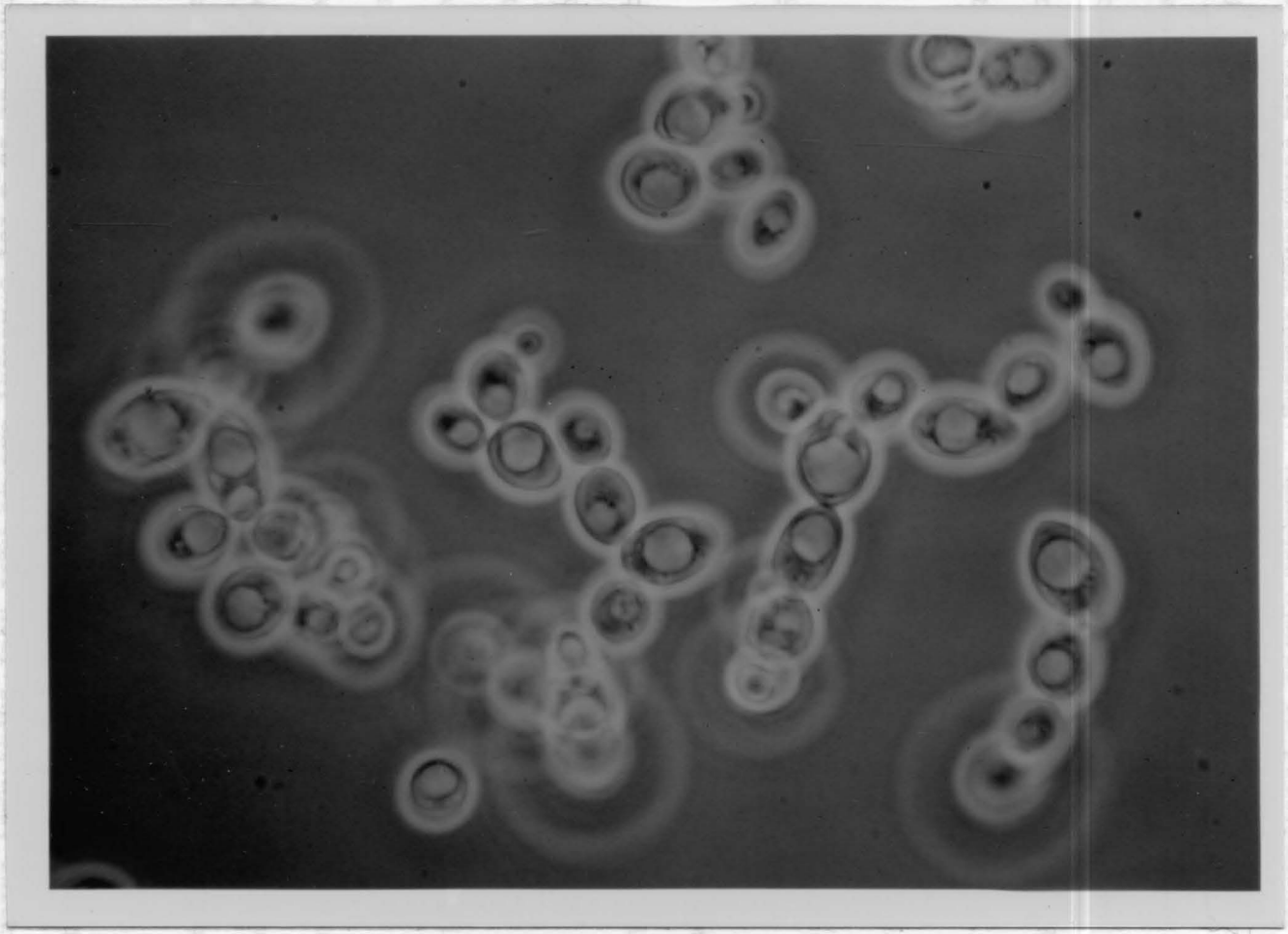

FIGURE 1: Saccharomyces cerevisiae (yeast normal cells before enzyme treatment.

4.4 IU/ml at 37°C. The mixture of yeast cells and enzyme was incubated at 38°C with occasional shaking. Maximum number of protoplasts were obtained within 3 to 4 hours. The enzyme and the cell wall debris were removed by centrifuging at approximately 1000 rpm for 5 minutes and the protoplasts were finally suspended in fresh osmotic stabilizer. Isolated protoplasts were examined under the phase contrast microscope (Fig. 2) and the number of protoplasts were determined using the formula

$$\% \text{ of protoplasts} = \frac{\text{No. of protoplasts x 100}}{\text{No. of protoplasts \& yeast cells}}$$

Inoculation of Protoplasts with TMV

The protoplasts suspension in osmotic stabilizer was mixed with an equal volume of the common strain of diluted purified TMV (dilution of the TMV was made in osmotic stabilizer, 1 ml of TMV in 50 ml of osmotic stabilizer 1:50). Inoculum used on the six half leaves produced an average of 100 lesions per half leaf. 0.25 ml of the protoplasts virus mixture was taken in sterile test tubes immediately after mixing TMV with protoplasts. The protoplasts were isolated by centrifugation and washed three times with osmotic stabilizer. This sample represents the zero time incubation. This zero time sample was further divided into two parts; one part used for fluorescent antibody staining and the second part for bio-assay. The remaining protoplast-virus mixture was incubated at 28°C for 5 hours, and at half hour intervals, infected protoplasts were isolated from the incubation medium by centrifugation and washed three times with osmotic stabilizer. These protoplasts were divided into three equal parts. One part was suspended in 0.25 ml of

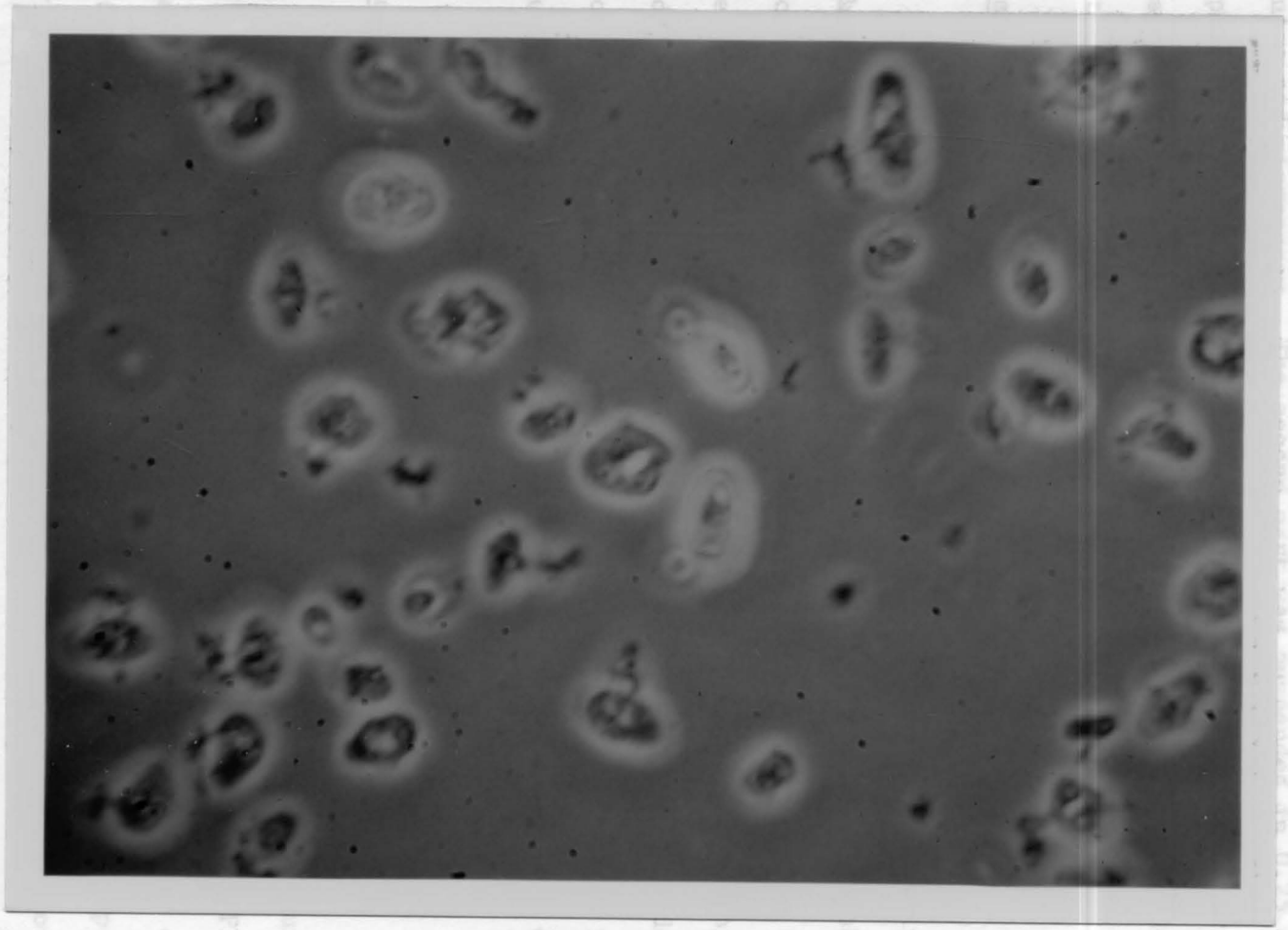

FIGURE 2: Saccharomyces cerevisiae (yeast) protoplasts obtained after 4 hours treatment with enzyme B-Glucuronidase/Aryl Sulfatase at 38°C.

osmotic stabilizer and slides were prepared for fluorescent antibody staining and microscopy. The second part was suspended in 0.25 ml of phosphate buffer solution (Appendix A) and was kept in a freezer for bio-assay. The third part was fixed in 1 ml of 5% glutaraldehyde for electronmicroscopic study.

Isolation and Purification of Tobacco Mosaic Virus

The method used for the purification of TMV was basically of Venekamp and Mosch (1964). About 3 week old TMV infected Turkish tobacco plants were harvested and the midribs were removed from the leaves. Three hundred grams of leaves were macerated in a Waring blender with 10% polyethylene glycol (PEG) and 4% sodium chloride (NaCl) (calculated on weight/volume basis). The macerated leaves were pressed through cheesecloth to remove fibrous materials. The filterate was then ready for chromatography.

Preparation of Column and Purification of TMV

A glass column 24 inches long, 2 inches in diameter, equipped with sintered glass filter was packed with cellulose using 10% PEG and 4% NaCl as solvent. The cellulose was allowed to settle in the column and the excess of 10-4 solution was drained (Fig. 3). Plant sap from the TMV infected tobacco leaves in 10-4 solution was passed through the column and the plant impurities were removed with 5% PEG and 2% NaCl (5-2 solution). When the effluent was clear, the virus from the column was eluted with 5% PEG (5-0 solution). The virus was precipitated from

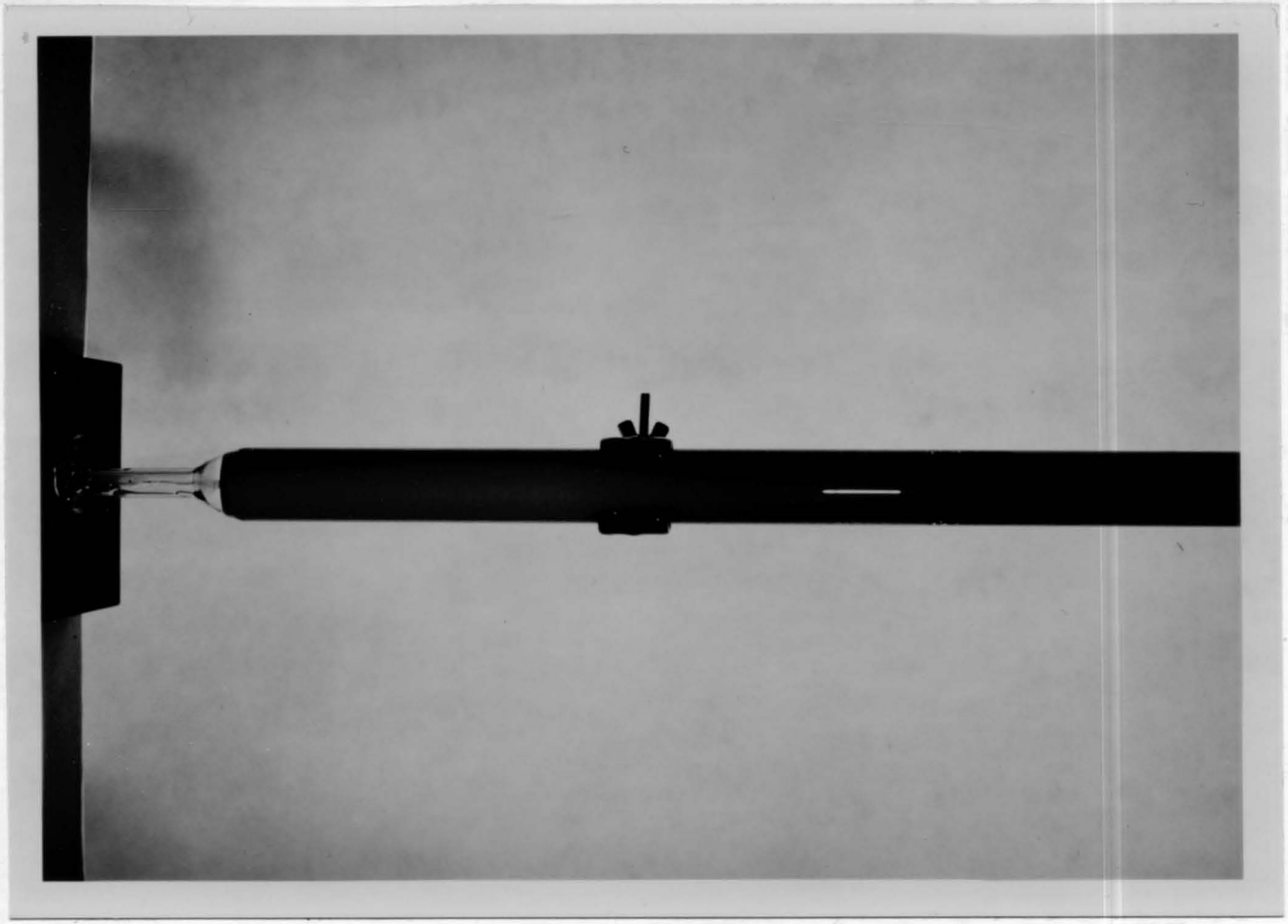

FIGURE 3. Microcrystalline cellulose column to purify TMV by chromatography.

the 5-0 solution (containing TMV) by the addition of 2% NaCl. This solution was then centrifuged at 8000 rpm for 10 minutes in a refrigerated low speed centrifuge (Sorvall RC-2B). The supernatent was discarded and the pellet was suspended in 0.1 M phosphate buffer pH 7.0. Virus was further purified by using an ultracentrifuge (Sorvall Oil Turbine Drive #2) for 2 hours at 171,000 R.C.F. The pellet which was the concentrated pure virus was resuspended in a phosphate buffer pH 7.0 and the supernatent was discarded.

ASSAY OF INFECTIVITY

Preparation of Fluorescent Antibody Stain

Fluorescent antibody stain was prepared by following the procedure described by Clark and Shepard (1963). One volume (1 ml) of the serum containing specific antibodies (titer 1064 determined by ATCC) was adjusted to 1% protein with 10 ml of 0.025 M Na_2CO_3 - $NaHCO_3$ buffer, pH 9.8 and placed in dialysis tubing to be dialyzed against 100 ml of 0.001% Fluorescein Isothiocyanate (FITC) (obtained from Sigma Chemical Company) in the same buffer. The dialysis was carried out in a graduated cylinder at 4°C for 24 hours with constant stirring by a magnetic stirrer. After 24 hours the contents of the dialysis tubing was dialyzed against phosphate buffered saline pH 7.3, 0.67 M at 4°C until no fluorescein was detected in the dialysate with ultraviolet light. The stain prepared above was then kept in a freezer until needed.

Fluorescent Antibody Staining of Infected Yeast Protoplasts

TMV infected protoplasts of Saccharomyces cerevisiae were stained with 1:8 dilution of the fluorescent antibodies stain prepared above following the method of Takebe and Otsuki (1969). Smears of the infected protoplasts were made on clean slides coated with bovine albumin. Smears were air dried with a warm stream of air for 30 minutes. Fixed smears were washed four times, 30 minutes each with PBS solution, stirred constantly with a magnetic stirrer, and

then stained with conjugated antibodies at 36°C for 2 hours in a moist chamber. Stained slides were washed 4 times, 30 minutes each with PBS, stirring constantly. After washing, slides were air dried and observed under the fluorescent microscope for stained protoplasts (Fig. 4).

Assessment of the Rate of Virus Replication in Yeast Protoplasts

Preparation of Assay Host

Phaseolus vulgaris cv. Pinto was used as an assay host. Plants were grown in vermicula in 16 x 20 x 30 cm plastic trays in the greenhouse at 20 to 28°C, relative humidity approximately 50 to 60%, and supplied with continuous artificial light (2,000 lux). Primary leaves of 10 day old plants were subjected to hot water treatment at 45°C for 1 minute. The midribs were removed and the half leaves were distributed at random on 1% agar in 11 x 7 x 1¼ inch aluminum trays. Surface moisture from the leaves were removed with paper strips and 350 mesh carborandum was dusted evenly on the half leaves. Leaves were then ready for inoculation.

Inoculation of Half Leaves

Method for inoculation of leaves was the same used by Lamborn, Cochran and Chidester (1971). The protoplasts infected with TMV were suspended in 0.1 M phosphate buffer (pH 7.0) and treated with ultrasonic waves to release the virus from the protoplasts. A drop of this solution was placed at the tip of a half leaf using a Pasteur

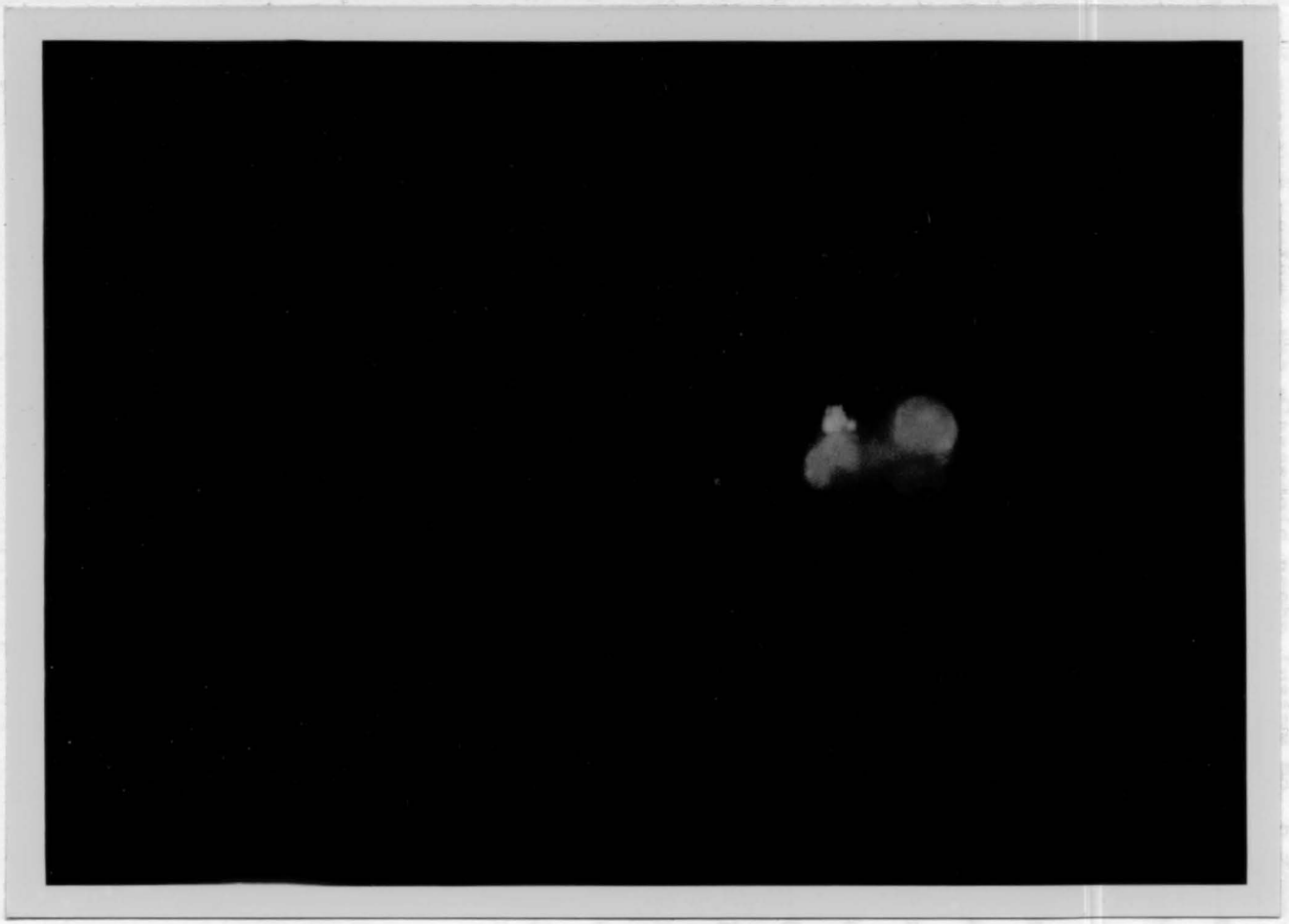

FIGURE 4: Saccharomyces cerevisiae protoplasts infected with Tobacco Mosaic Virus, photographed after staining with fluorescent antibodies, whole cell fluorescing.

pipette. The half leaf was then placed on a 5 x 2¼ x 1 inch sponge pad and pressed against the ultrasonic probe (using No. 6 power level, Branson Sonifier Cell Disrupter) with even pressure starting at the tip of the leaf and proceeding towards the base (Fig. 5). Each treatment was repeated on six half leaves. The inoculated half leaves were placed between two strips of paper and stored in air tight plastic containers. The containers were kept in an incubator at 28°C for 18 hours. After 18 hours the leaves were placed on 1% agar in trays. The trays were covered with glass plates and kept at room temperature under constant fluorescent light intensity of 3,000 lux for 3-4 days when the lesions appear on the leaves (Fig. 6). Lesions were counted with a Wild binocular microscope using 500x magnification. Lesions were also observed for color and morphology in order to find out whether the lesions were caused by intact TMV or TMV-RNA alone.

FIGURE 5: Ultrasonic inoculation of a half leaf of Phaseolus vulgaris cv. Pinto, with yeast protoplasts infected with Tobacco Mosaic Virus. Leaves were pushed upon and drawn under the metal probe with even pressure.

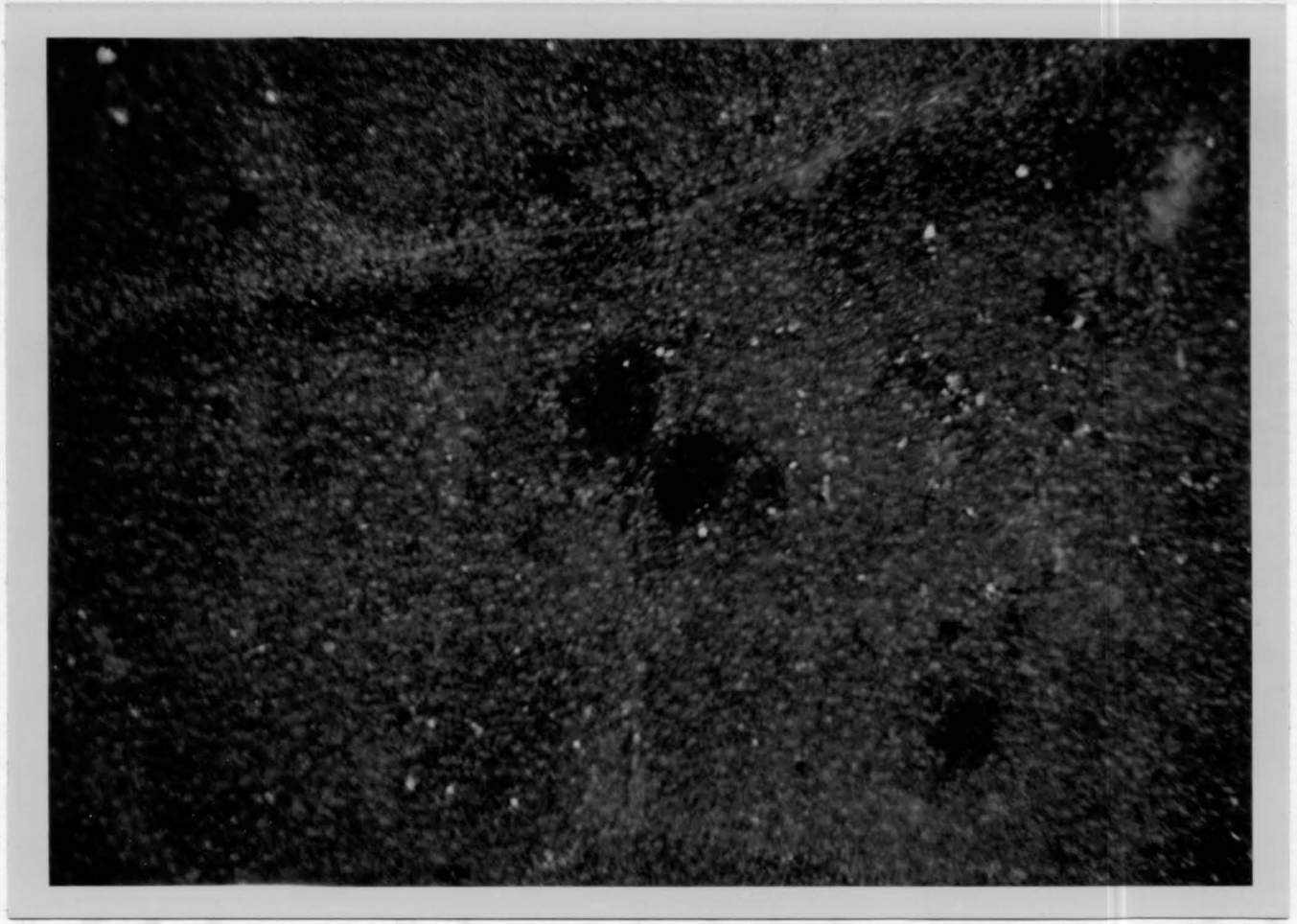

FIGURE 6: Local lesions produced by Tobacco Mosaic Virus infected Saccharomyces cerevisiae (yeast) protoplasts in Phaseolus vulgaris cv. Pinto, 3 to 5 days after inoculation at 20 to 25°C.

RESULTS AND DISCUSSION

The data obtained on the extent of TMV replication in yeast protoplasts under several environmental conditions are presented in this chapter. For the sake of convenience to the author, results and discussion are presented together.

Early Replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae (Yeast) Protoplasts

The isolated protoplasts were inoculated with Tobacco Mosaic Virus (TMV) and were incubated at 28°C for 5 hours. Samples of the inoculated protoplasts were taken at every half hour interval for 5 hours and were tested on six half leaves of Phaseolus vulgaris cv. Pinto beans, and stained with the fluorescent antibodies stain. The results are shown in Tables 1 and 2, and in Fig. 8. The 0.5 hours showed the presence of virus particles. The samples taken at 1.0, 1.5 and 2.0 hours showed the decrease in the number of virus particles and then increase around 3.0 hours, reaching the maximum at 4.0 hours. After 4.0 hours there was a drop again

The lesions produced by 0.5 hour sample might possibly be due to the adsorption of virus particles on the surface of the yeast protoplasts. The progressive decrease in the number of lesions at 1.0, 1.5, 2.0, 2.5 hours after incubation might be due to the fact that as virus particles enter the protoplasts through the process of pinocytosis, the protein coat of the virus is removed and the virus

Table 1: Assessment of the early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris cv. Pinto.

INCUBATION TIME(HOURS)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
	1	19		
	2	20		
	3	31		
0.5	4	9	95	15.8
	5	5		
	6	11		
	1	2		
	2	12		
	3	14		
1.0	4	14	57	9.5
	5	7		
	6	8		
	1	7		
	2	5		
	3	10		
1.5	4	12	47	7.8
	5	8		
	6	5		
	1	5		
	2	6		
	3	9		
2.0	4	5	36	6.0
	5	3		
	6	8		
	1	7		
	2	5		
	3	9		
2.5	4	10	39	6.5
	5	4		
	6	4		
	1	3		
	2	3		
	3	4		
3.0	4	17	33	5.5
	5	6		
	6	0		

Table 1 Continued

INCUBATION TIME(HOURS)	NO OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
	1	9		
	2	10		
	3	10		
3.5	4	10	54	9.0
	5	8		
	6	7		
	1	14		
	2	14		
	3	18		
4.0	4	19	107	17.8
	5	14		
	6	28		
	1	6		
	2	9		
	3	6		
4.5	4	5	39	7.8
	5	13		
	6	0		
	1	6		
	2	7		
	3	8		
5.0	4	18	52	8.6
	5	7		
	6	6		

Table 2: Assessment of the early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris cv. Pinto Number of protoplasts/ml not calculated.

INCUBATION TIME(HOURS)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO. OF LESIONS/ HALF LEAF
0.5	1	25	72	12.0
	2	10		
	3	15		
	4	11		
	5	11		
	6	0		
1.0	1	12	53	8.8
	2	18		
	3	12		
	4	11		
	5	0		
	6	0		
1.5	1	4	30	5.0
	2	5		
	3	5		
	4	9		
	5	3		
	6	4		
2.0	1	4	24	4.0
	2	7		
	3	3		
	4	4		
	5	4		
	6	2		
2.5	1	2	17	2.8
	2	4		
	3	3		
	4	3		
	5	3		
	6	2		
3.0	1	3	19	3.1
	2	2		
	3	8		
	4	3		
	5	2		
	6	1		

continued

INCUBATION TIME(HOURS)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO. OF LESIONS/ HALF LEAF
3.5	1	7	**54**	**9.0**
	2	5		
	3	7		
	4	6		
	5	2		
	6	4		
4.0	1	5	34	5.6
	2	10		
	3	7		
	4	4		
	5	3		
	6	5		
4.5	1	26	43	7.1
	2	3		
	3	5		
	4	4		
	5	5		
	6	o		
5.0	1	11	39	6.5
	2	9		
	3	8		
	4	4		
	5	1		
	6	6		

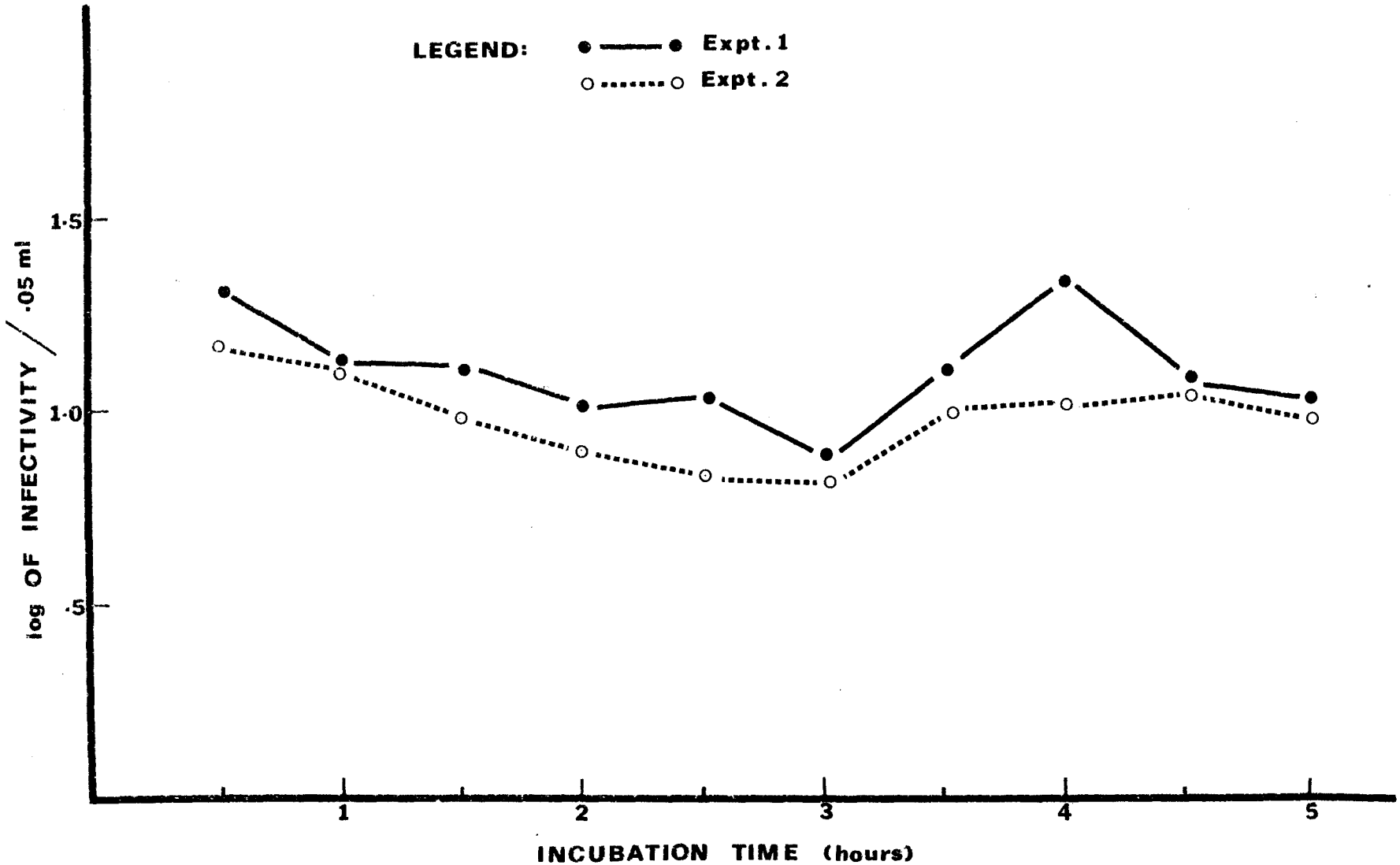

FIG. 8: Assessment of the early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris cv. Pinto

is entering the phase called eclipse phase. At eclipse phase, virus particles are not detected. After the removal of the protein coat, the viral nucleic acid in the protoplasts probably act as mRNA and combines with the host ribosomes to form polysomes which are responsible for the synthesis of polymerases and other enzymes required for the multiplication of the nucleic acid of the virus. New copies of the viral RNA started to appear around 3.0 hours after inoculation and reached maximum value at 4.0 hours. The decrease in the number of virus particles after 4.0 hours could possibly be due to the destruction of viral RNA by RNAase present in the host cell, before RNA was enclosed in the protein coat, and also partly due to the susceptibility of the assay host. The susceptibility of the leaves differ from plant to plant, even if they were grown under the same environmental conditions. Even the leaves of the same plant differs in their susceptibility.

Two experiments in Fig. 8 show a similar pattern of virus replication, although the number of lesions produced are different. In one of the experiments the samples were taken beyond 5.0 hours after inoculation; at 20.5, 47.0 and 120.0 hours. The results are shown in Table 3 and Fig. 9. There was an increase in the number of infectious particles at 20.5 hours and at 47.0 hours, and a drop at 120.0 hours. The infectivity was more at 47.0 hours as compared to 4.0 hours. This decrease and increase in infectious particles might possibly be due to the quick synthesis and degradation of the viral RNA before it was enclosed in the coat protein which was synthesized

Table 3: Assessment of the early replication of Tobacco Mosaic Virus in *Saccaromyces cerevisiae* protoplasts by local lesion assay on six half leaves of *Phaseolus vulgaris* cv. Pinto.

INCUBATION TIME(HOURS)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
0.5	1	60	646	107.6
	2	113		
	3	150		
	4	120		
	5	107		
	6	96		
1.0	1	28	148	37.0
	2	32		
	3	38		
	4	50		
	5	*		
	6	*		
1.5	1	18	153	30.6
	2	42		
	3	27		
	4	23		
	5	43		
	6	*		
2.0	1	30	151	25.1
	2	21		
	3	38		
	4	40		
	5	17		
	6	5		
2.5	1	49	144	28.8
	2	20		
	3	18		
	4	23		
	5	34		
	6	*		
3.0	1	21	129	25.8
	2	35		
	3	18		
	4	23		
	5	32		
	6	*		

*Leaves died before lesion counts were made.

continued

INCUBATION TIME(HOURS)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO. OF LESIONS/ HALF LEAF
3.5	1	24	182	30.3
	2	70		
	3	37		
	4	18		
	5	18		
	6	50		
4.0	1	44	218	36.3
	2	59		
	3	40		
	4	45		
	5	30		
	6	0		
4.5	1	11	288	48.0
	2	8		
	3	18		
	4	46		
	5	40		
	6	160		
5.0	1	40	96	16.0
	2	80		
	3	6		
	4	9		
	5	17		
	6	6		
20.5	1	190	467	77.8
	2	46		
	3	35		
	4	165		
	5	6		
	6	25		
47.0	1	60	625	104.1
	2	180		
	3	40		
	4	120		
	5	65		
	6	160		
120.0	1	26	130	21.6
	2	16		
	3	33		
	4	6		
	5	34		
	6	15		

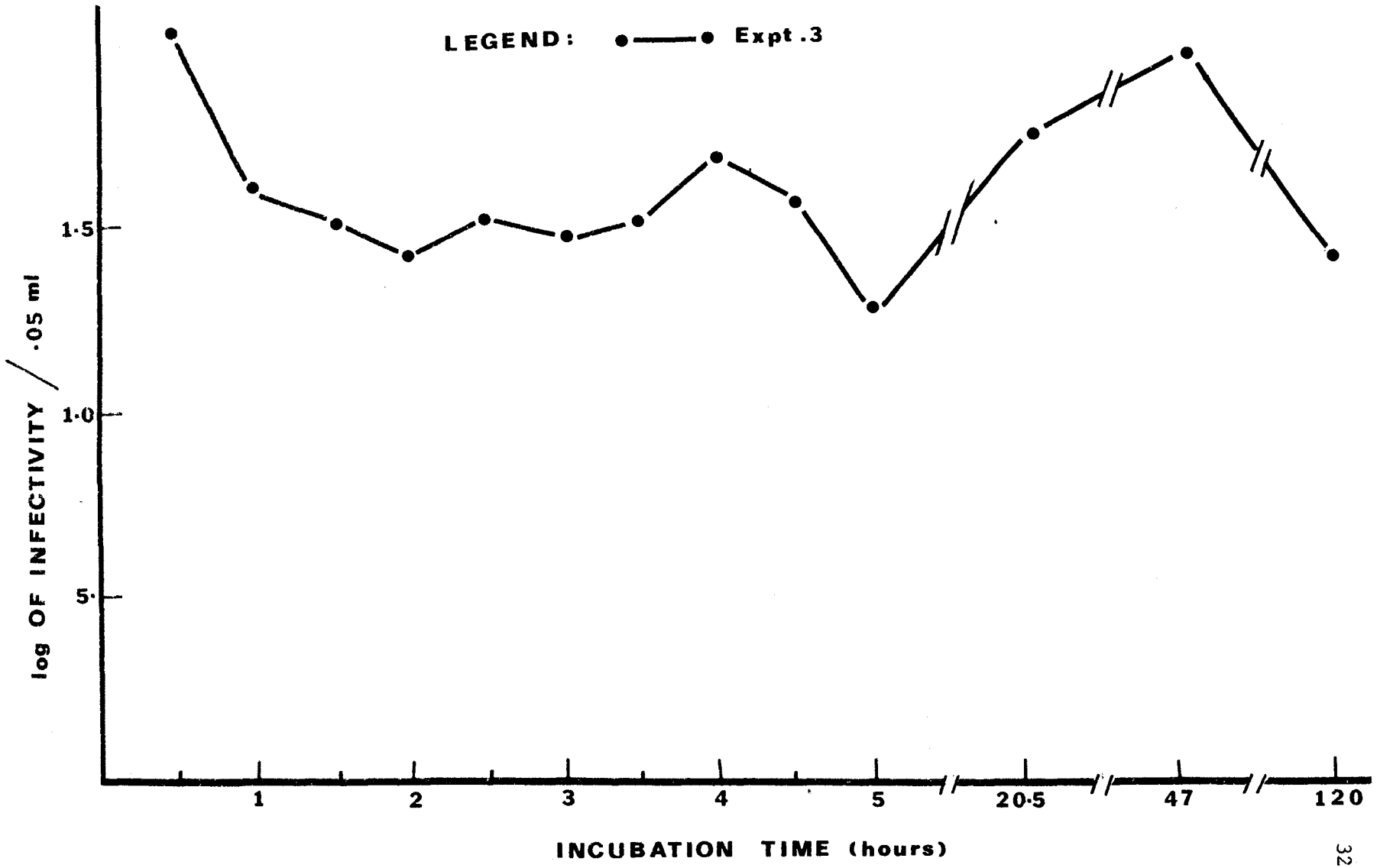

FIG. 9: Assessment of the early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris cv. Pinto

at later stages of the viral replicative cycle. It is well known that viral RNA is degraded by the ribonucleases while RNA enclosed in the coat protein is not affected by the nucleases.

It is clear from this data that virus synthesis continued at least up to 120.0 hours. If virus synthesis was complete, that is, RNA synthesized was enclosed in coat protein, the infectivity would have been maintained at the level as indicated at 47.0 hours of incubation. The fact that infectivity decreased indicated the degradation of RNA which was recently synthesized and was not enclosed on coat protein.

TMV infected protoplasts stained with fluorescent antibodies stain followed the same pattern of replication as indicated by an assay host. Results are shown in Table 4.

Effect of Concentration of Inoculum on the Replication of TMV in Saccharomyces cerevisiae Protoplasts

Virus suspension producing 500 L.F.U./ml was diluted (2 times, 100 times and 10,000 times). Yeast protoplasts were inoculated with these dilutions of virus and incubated at 28°C for 5.0 hours. Samples of the inoculated protoplasts were taken every half hour for 5 hours and tested on six half leaves of Phaseolus vulgaris cv. Pinto. The results are shown in Table 5 and Fig. 10. The half hour incubation periods for all three dilutions showed the presence of the adsorbed virus particles as indicated by the lesions produced on the assay host. The lesser the dilution, the more the virus was adsorbed on

TABLE 4 Study of Tobacco Mosaic Virus replication in protoplasts of *Saccharomyces cerevisiae* by Fluorescent antibody staining technique.

INCUBATION TIME (HOURS)	NO. OF PROTOPLASTS COUNTED	NO. OF PROTOPLASTS FLUORESCING
0.5	25	7
1.0	25	8
1.5	25	7
2.0	25	1
2.5	25	0
3.0	25	3
3.5	25	3
4.0	25	16
4.5	25	3
5.0	25	2

Table 5: Effect of concentration of inoculum and time on early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesions assay on six half leaves of Phaseolus vulgaris cv. Pinto.

INCUBATION TIME(HOURS)	VIRUS DILUTION (500 LFU/ml)	NO. OF HALF LEAVES INOCULATED	NO.OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
0.5	2 times	1	4	327	54.5
		2	3		
		3	60		
		4	120		
		5	70		
		6	70		
	100 times	1	68	203	33.8
		2	65		
		3	12		
		4	25		
		5	29		
		6	4		
	10,000 times	1	55	288	48.0
		2	49		
		3	25		
		4	8		
		5	124		
		6	27		
1.0	2 times	1	15	271	45.2
		2	8		
		3	139		
		4	62		
		5	38		
		6	9		
	100 times	1	25	333	55.5
		2	25		
		3	63		
		4	32		
		5	132		
		6	56		
	10,000 times	1	40	213	35.5
		2	81		
		3	17		
		4	9		
		5	20		
		6	46		

Table 5 (continued)

INCUBATION TIME (HOURS)	VIRUS DILUTION (500 LFU/ml)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
3.5	2 times	1	4	169	28.1
		2	21		
		3	40		
		4	70		
		5	21		
		6	13		
	100 times	1	2	485	80.8
		2	55		
		3	180		
		4	60		
		5	110		
		6	78		
	10,000 times	1	2	493	82.1
		2	80		
		3	203		
		4	45		
		5	63		
		6	100		
4.0	2 times	1	15	946	157.6
		2	500		
		3	175		
		4	70		
		5	36		
		6	150		
	100 times	1	10	193	32.1
		2	12		
		3	60		
		4	11		
		5	8		
		6	92		
	10,000 times	1	25	122	20.0
		2	5		
		3	17		
		4	43		
		5	26		
		6	6		

Table 5 (continued)

INCUBATION TIME(HOURS)	VIRUS DILUTION (500 LFU/ml)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG.NO OF LESIONS/ HALF LEAF
2.5	2 times	1	12	146	24.3
		2	50		
		3	11		
		4	62		
		5	7		
		6	4		
	100 times	1	2	239	39.8
		2	20		
		3	4		
		4	125		
		5	73		
		6	15		
	10,000 times	1	5	49	8.1
		2	29		
		3	0		
		4	4		
		5	8		
		6	3		
3.0	2 times	1	20	157	26.1
		2	17		
		3	31		
		4	14		
		5	28		
		6	47		
	100 times	1	12	139	23.1
		2	4		
		3	23		
		4	35		
		5	23		
		6	42		
	10,000 times	1	2	78	13.0
		2	10		
		3	9		
		4	25		
		5	3		
		6	29		

Table 5 (continued)

INCUBATION TIME(HOURS)	VIRUS DILUTION (500 LFU/ml)	NO. OF HALF LEAVES INOCULATED	NO.OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS HALF LEAF
1.5	2 times	1	16	151	25.1
		2	19		
		3	48		
		4	10		
		5	30		
		6	28		
	100 times	1	16	113	18.8
		2	13		
		3	13		
		4	12		
		5	35		
		6	24		
	10,000 times	1	73	128	21.3
		2	5		
		3	13		
		4	2		
		5	24		
		6	11		
2.0	2 times	1	29	99	16.5
		2	23		
		3	11		
		4	4		
		5	27		
		6	5		
	100 times	1	19	65	10.0
		2	4		
		3	9		
		4	16		
		5	9		
		6	8		
	10,000 times	1	59	106	17.6
		2	13		
		3	5		
		4	4		
		5	21		
		6	4		

Table 5 (continued)

INCUBATION TIME(HOURS)	VIRUS DILUTTON (500 LFU/ml)	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
4.5	2 times	1	6	84	14.0
		2	7		
		3	10		
		4	45		
		5	8		
		6	8		
	100 times	1	2	70	11.6
		2	30		
		3	11		
		4	4		
		5	7		
		6	16		
	10,000 times	1	17	63	10.5
		2	16		
		3	13		
		4	6		
		5	6		
		6	5		
5.0	2 times	1	40	202	33.6
		2	52		
		3	50		
		4	35		
		5	25		
		6	0		
	100 times	1	21	104	17.3
		2	21		
		3	12		
		4	8		
		5	12		
		6	30		
	10,000 times	1	8	76	12.6
		2	5		
		3	11		
		4	35		
		5	12		
		6	5		

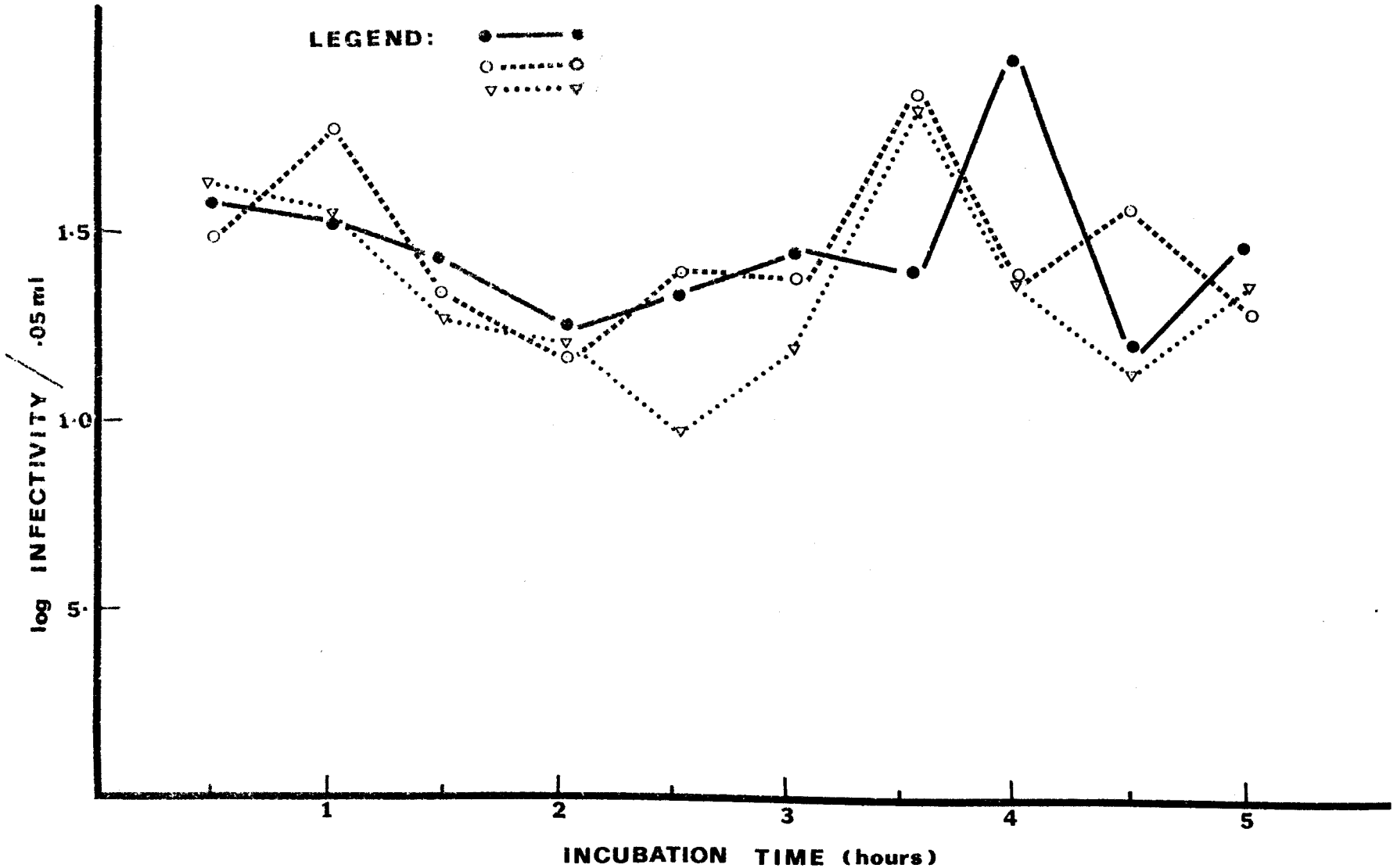

FIG. 10: Effect of conc. of virus and time on the early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris cv. Pinto

the surface of the protoplasts. However, the adsorption was about the same at the concentration of 100 times and 10,000 times. Infection started decreasing at 1.0 hours and continued until 3.0 hours, which probably indicates the eclipse phase of the viral replicative cycle. Infectivity increased after 3.5 hours of incubation period and decreased at 4.0, 4.5, and 5.0 hours of incubation in the protoplasts samples inoculated with virus diluted at 100 times and 10,000 times virus particles/ml. The infectivity peaks at 3.5 hours might possibly be due to the quick synthesis of viral RNA just after the eclipse phase. The decrease in infectivity probably is due to the degradation of newly synthesized RNA with the ribonucleases already present in the protoplasts. This degradation of viral RNA takes place only if the viral RNA was not enclosed in the coat protein.

The infectivity in the protoplasts inoculated with 2 times dilution increased at 4.0 hours after inoculation. This increase was almost double the increase in protoplasts inoculated with 10 times and 10,000 times dilution. The infectivity then decreased at 4.5 hours at the same level as those protoplasts inoculated with a 100 times and 10,000 times dilution. The one-half hour delay in the development of the peak infectivity in the protoplasts inoculated with 2 times dilution probably was due to the delay in taking the samples for bio-assay, but the infectivity almost doubled in this sample of protoplasts as compared to the protoplasts inoculated with lower concentration is unexplainable.

Effect of Temperature on Early Replication of TMV in Saccharomyces cerevisiae (Yeast) Protoplasts

Saccharomyces cerevisiae protoplasts were inoculated with TMV and incubated at 15°C, 28°C and 45°C. Samples were taken at half hour intervals from all three temperatures for infectivity tests. Samples taken one-half hour after inoculation showed the infectivity due to the adsorbed virus. The decrease in infectivity up to 2.0 hours incubation is probably due to the synthesis of virus in the protoplasts. The trend in increase or decrease in infectivity is the same in the three incubating temperatures under study. Samples taken from 15°C and 28°C showed the maximum infectivity at 4.5 hours, whereas samples from 45°C showed maximum infectivity at 4.5 hours after incubation. The rate of increase in infectivity was faster in samples incubated at 15°C and 28°C, but was slow at 45°C. However, the maximum synthesis of infective particles as indicated by the infectivity assay occurred at 45°C after 7.0 hours of incubation.

The optimum temperature for TMV replication in yeast protoplasts is 28°C to 30°C. It is well known that optimum temperature is required for the optimum metabolic activity of any living system. This is because the maximum performance of enzymes occur at an optimum temperature. Table 6 and Fig. 11 shows that the temperature required for the maximum replication of the virus in yeast protoplasts is 28°C. However, it is also clear from the figure that the rate of replication of the virus is slow up to 6.0 hours of incubation and

Table 6: Effect of temperature and time on early replication of Tobacco Mosaic virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris cv. Pinto.

INCUBATION TIME(HOURS)	INCUBATION TEMP. °C	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
0.5	15	1	3	46	7.6
		2	17		
		3	17		
		4	1		
		5	4		
		6	4		
	28	1	4	58	9.6
		2	41		
		3	5		
		4	3		
		5	1		
		6	4		
	45	1	7	101	16.8
		2	42		
		3	5		
		4	30		
		5	14		
		6	3		
1.0	15	1	6	23	3.8
		2	5		
		3	3		
		4	2		
		5	3		
		6	4		
	28	1	8	34	5.6
		2	9		
		3	6		
		4	4		
		5	3		
		6	4		
	45	1	5	30	5.0
		2	6		
		3	4		
		4	3		
		5	8		
		6	4		

Table 6 continued

INCUBATION TIME(HOURS)	INCUBATION TEMP. °C	NO. OF HALF LEAVES INOCULATED	NO.OF LESIONS HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
1.5	15	1	6	20	3.3
		2	3		
		3	4		
		4	2		
		5	2		
		6	3		
	28	1	7	29	4.8
		2	12		
		3	3		
		4	2		
		5	3		
		6	2		
	45	1	7	24	4.0
		2	5		
		3	2		
		4	4		
		5	3		
		6	3		
2.0	15	1	1	16	2.6
		2	5		
		3	3		
		4	4		
		5	1		
		6	2		
	28	1	3	24	4.0
		2	9		
		3	5		
		4	3		
		5	2		
		6	2		
	45	1	8	20	3.3
		2	2		
		3	1		
		4	2		
		5	6		
		6	1		

Table 6 continued

INCUBATION TIME(HOURS)	INCUBATION TEMP. °C	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
2.5	15	1	7	19	3.1
		2	3		
		3	5		
		4	2		
		5	1		
		6	1		
	28	1	4	28	4.6
		2	11		
		3	5		
		4	3		
		5	2		
		6	3		
	45	1	6	20	3.3
		2	3		
		3	3		
		4	2		
		5	5		
		6	1		
3.0	15	1	8	36	6.0
		2	7		
		3	2		
		4	11		
		5	5		
		6	3		
	28	1	15	40	6.6
		2	4		
		3	6		
		4	8		
		5	3		
		6	4		
	45	1	6	39	6.5
		2	10		
		3	9		
		4	6		
		5	3		
		6	5		

Table 6 continued

INCUBATION TIME(HOURS)	INCUBATION TEMP. °C	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
3.5	15	1	17	57	9.5
		2	11		
		3	9		
		4	12		
		5	5		
		6	3		
	28	1	12	62	10.3
		2	19		
		3	8		
		4	7		
		5	7		
		6	9		
	45	1	8	54	9.4
		2	7		
		3	17		
		4	11		
		5	4		
		6	7		
4.0	15	1	8	79	13.2
		2	8		
		3	9		
		4	17		
		5	22		
		6	15		
	28	1	4	83	13.8
		2	25		
		3	12		
		4	4		
		5	18		
		6	20		
	45	1	9	72	12.0
		2	4		
		3	7		
		4	24		
		5	21		
		6	7		

Table 6 continued

INCUBATION TIME(HOURS)	INCUBATION TEMP. °C	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
4.5	15	1	20	125	20.8
		2	34		
		3	47		
		4	15		
		5	4		
		6	5		
	28	1	41	173	28.8
		2	7		
		3	53		
		4	22		
		5	10		
		6	40		
	45	1	13	66	11.0
		2	27		
		3	6		
		4	5		
		5	10		
		6	5		
5.0	15	1	5	66	11.0
		2	28		
		3	14		
		4	7		
		5	5		
		6	7		
	28	1	16	105	17.5
		2	27		
		3	14		
		4	22		
		5	22		
		6	4		
	45	1	41	95	15.8
		2	13		
		3	6		
		4	8		
		5	7		
		6	20		

Table 6 continued

INCUBATION TIME(HOURS)	INCUBATION TEMP. °C	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG. NO OF LESIONS/ HALF LEAF
6.0	15	1	23	96	16.0
		2	39		
		3	6		
		4	7		
		5	11		
		6	10		
	28	1	30	96	16.0
		2	26		
		3	6		
		4	3		
		5	10		
		6	21		
	45	1	4	84	14.0
		2	29		
		3	26		
		4	14		
		5	2		
		6	9		
7.0	15	1	21	153	25.5
		2	36		
		3	37		
		4	21		
		5	11		
		6	27		
	28	1	98	154	25.6
		2	4		
		3	15		
		4	14		
		5	14		
		6	9		
	45	1	17	180	30.0
		2	29		
		3	6		
		4	38		
		5	65		
		6	25		

Table 6 continued

INCUBATION TIME(HOURS)	INCUBATION TEMP. °C	NO. OF HALF LEAVES INOCULATED	NO. OF LESIONS/ HALF LEAF	TOTAL NO. OF LESIONS	AVG.NO OF LESIONS/ HALF LEAF
		1	30		
		2	64		
		3	27		
	15	4	14	224	37.3
		5	35		
		6	54		
		1	5		
		2	6		
		3	17		
8.0	28	4	30	114	19.0
		5	26		
		6	30		
		1	12		
		2	35		
		3	36		
	45	4	23	166	27.6
		5	29		
		6	31		

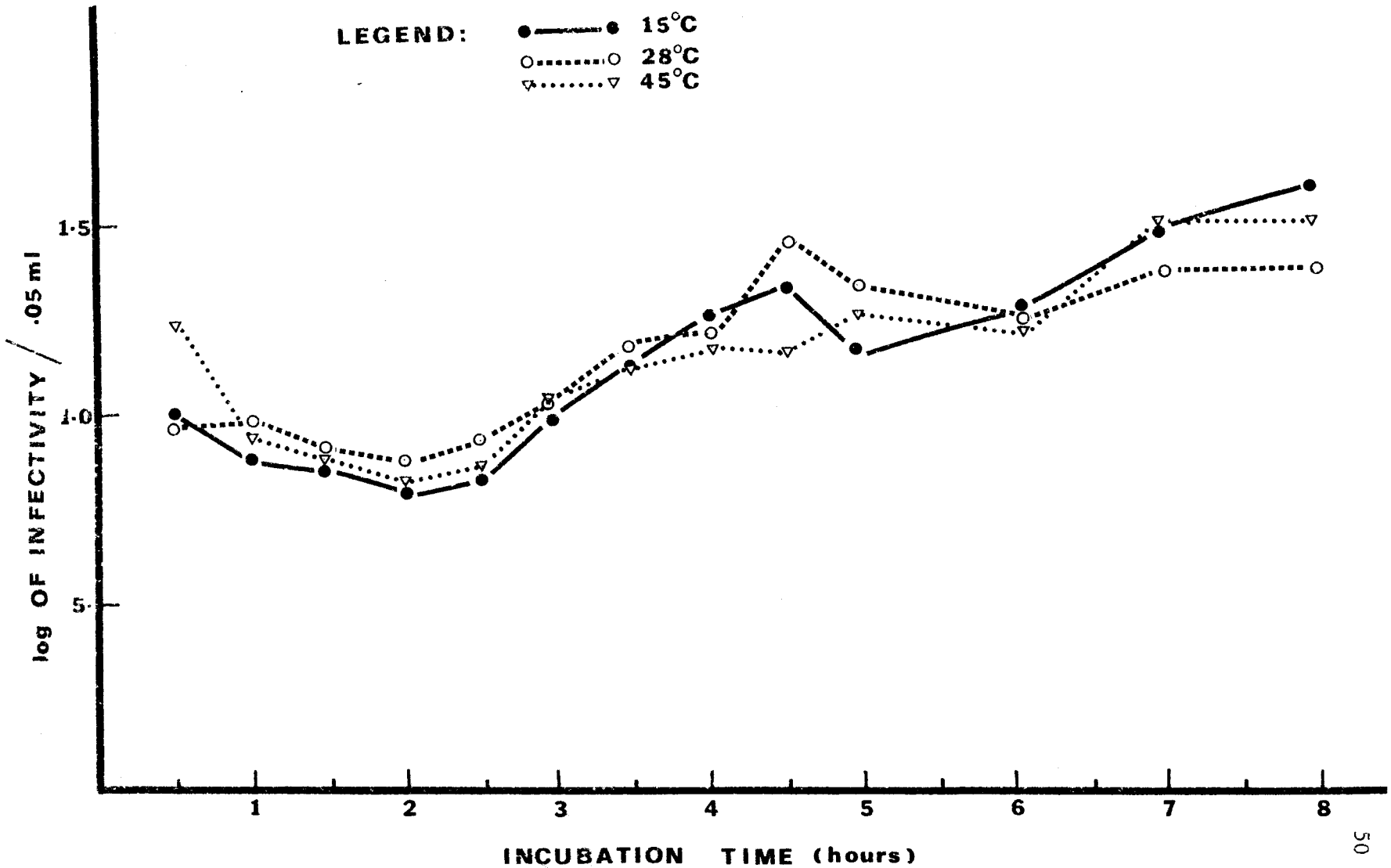

FIG. 11: Effect of temperature and time on early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris cv. Pinto

an increase after 6.0 hours at 45°C which is more than the other two temperatures tested.

The data was analyzed statistically (Table 7) using log transformation (Appendix B), analysis of variance technique (Appendix C), and t-test for independent sample means (Appendix D).

TABLE : 7 Assessment of the early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris CV. Pinto.

SOURCE (FIG 8 (1))	SS	d.f.	MS	F
Time	.867	9	.0963	4.01
Error	1.193	49	.024	

SOURCE (FIG 8 (2))	SS	d.f.	MS	F
Time	.653	9	.0726	2.59
Error	1.362	48	.028	

SOURCE (FIG 9)	SS	d.f.	MS	F
Time	3.15	12	.2625	4.375
Error	3.568	59	.060	

Significant at .01% level.

TABLE : 7 Effect of the concentration of inoculum on early replication of Tobacco Mosaic Virus in Saccharomyces cerevisiae protoplasts by local lesion assay on six half leaves of Phaseolus vulgaris CV. Pinto.

SOURCE (FIG 10)	SS	d.f.	MS	F
Time	6.219	12	.518	4.353
Interaction	3.637	12	.3031	2.547

Effect of temperature and time on early replication of Tobacco Mosaic Virus in protoplasts of Saccharomvces cerevisiae by local lesion assav on six half leaves of Phaseolus vulgaris CV. Pinto.

SOURCE (FIG 11)	SS	d.f.	MS	F
Time	9.211	12	.7676	15.992
Interaction	.965	12	.0804	1.675

SUMMARY AND CONCLUSIONS

Objective

The main objective of this research was to isolate Saccharomyces cerevisiae (yeast) protoplasts and study the effect of time, temperature, and concentration of inoculum on the early events of Tobacco Mosaic Virus (TMV) replication in yeast protoplasts.

Materials Used

A common strain of Saccharomyces cerevisiae cv. ellipsoideus No. 566 obtained from American Type Culture Collection (ATCC). Snail gut enzyme B - Glucuronidase/Aryl Sulfatase (from Sigma Chemical Company). A common strain of Tobacco Mosaic Virus. An assay host Phaseolus vulgaris cv. Pinto. Fluorescent antibodies stain prepared following the method of Clark and Shepard (1963).

Preparation of Protoplasts

Saccharomyces cervisiae protoplasts were prepared by treating the young yeast cells with an enzyme B - Glucuronidase/Aryl Sulfatase. Maximum number of protoplasts were obtained within 3 to 4 hours after the enzyme treatment. Protoplasts were suspended in osmotic stabilizer (sorbital-citric acid pH 5.0). Protoplasts remain viable even after 120 hours.

Infection of Protoplasts with TMV

Suspension of protoplasts in osmotic stabilizer were infected with a common strain of Tobacco Mosaic Virus and incubated at 28°C for 5.0 hours.

Assay of Infectivity

By Conventional Assay Procedure

TMV infected protoplasts were taken at half hour intervals, washed three times with osmotic stabilizer, and, finally, suspended in 0.1 M phosphate buffer pH 7.0. The infectivity was tested on six half leaves of local lesion assay host Phaseolus vulgaris cv. Pinto.

By Fluorescent Antibodies Staining Technique

TMV infected protoplasts were fixed on slides and were stained with Fluorescent antibodies. Stained protoplasts were studied under fluorescent microscope.

TMV replication occurred in yeast protoplasts. The infectivity obtained by half hour samples indicated the adsorption of virus particles on the surface of protoplasts, followed by the eclipse phase which was indicated by low infection until 2.5 hours after infection. Maximum infectivity was obtained at 4.0 hours and then loss of infectivity at 4.5 hours and 5.0 hours after infection. Protoplasts stained with antibodies showed the same pattern of replication as indicated by the assay host.

Effect of Temperature

Isolated yeast protoplasts were infected with TMV and incubated at 15°C, 28°C and 45°C. TMV replication in yeast protoplasts seemed to be temperature dependent. Maximum infectivity was obtained when the protoplasts infected with TMV incubated at 28°C.

Effect of Concentration of Inoculum

Suspension of virus producing 500 lesion forming units/ml was diluted 2 times, 100 times and 10,000 times. Isolated protoplasts were inoculated with virus dilution and incubated at 28°C. Protoplasts inoculated with virus dilution 2 times showed double the infectivity as compared to 100 times and 10,000 times.

Protoplasts provide a suitable system for studying plant virus infection and the virus-host cell relationships.

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APPENDIX A

MEDIUM USED FOR CULTURING YEAST

SLANT CULTURE MEDIUM

	gms./liter
POTASSIUM DIHYDROGEN ORTHOPHOSPHATE	2.0
MAGNESIUM SULPHATE (7 H_2O)	1.0
AMMONIUM SULPHATE	1.0
GLUCOSE	20.0
YEAST EXTRACT	3.0
DIFCO PEPTONE	3.5
DIFCO SPECIAL AGAR	30.0

PRE-CULTURE MEDIUM

	gms./liter
POTASSIUM DIHYDROGEN ORTHOPHOSPHATE	2.0
MAGNESIUM SULPHATE (7 H_2O)	1.0
AMMONIUM SULPHATE	1.0
GLUCOSE	20.0
YEAST EXTRACT	3.0
DIFCO PEPTONE	3.5

APPENDIX A (continued)

FINAL SHAKE CULTURE MEDIUM

	gms./liter
POTASSIUM DIHYDROGEN ORTHOPHOSPHATE	0.2
MAGNESIUM SULPHATE (7 H_2O)	0.25
GLUCOSE	0.1
YEAST EXTRACT	3.0
SODIUM LACTATE	10.0
AMMONIUM HYDROGEN PHOSPHATE	3.5

PROTOPLASTS MEDIUM

	gms./liter
POTASIUM DIHYDROGEN ORTHOPHOSPHATE	0.20
MAGNESIUM SULPHATE (7 H_2O)	0.25
GLUCOSE	1.0
YEAST EXTRACT	3.0
SODIUM CITRATE	1.0
SORBITOL	127.52
AMMONIUM HYDROGEN PHOSPHATE	3.5

APPENDIX B

KLECZKOWSKI TRANSFORMATION

When the mean values of x (raw scores) are greater than 10, the transformation $Z = \log_{10} (x + c)$ (c is a constant, 5-15) is satisfactory, but inapplicable with smaller numbers. In some cases the use of poorly infective is unavoidable. In order to allow statistical analysis of results in such work, a transformation $Z = \log_{10} \frac{1}{2} (x + c + \sqrt{x^2 + 2cx})$ is used, when the mean values are lower than 10.

Z = individual scores transformed by either of the two formula dependent upon the mean score. For example:

x = 2, 12, 14, 14, 7, 8 $\quad \Sigma x = 57 = 9.5\ Mx < 10$

Z = 0.78, 1.22, 1.27, 1.27, 1.06, 1.1 $\quad \Sigma Z = 6.7 = 1.1MZ$

x = 19, 20, 31, 9.5, 11 $\quad x = 95 = 15.8Mx > 10$

Z = 1.38, 1.39, 1.55, 1.14, 1.0, 1.20 $\quad \Sigma Z = 7.68 = 1.28MZ$

APPENDIX C

ANALYSIS OF VARIANCE

Computational procedures for an unweighted means analysis for an unequal number of observations will be described. Suppose that the level of a factor A represents one species of fungi, Saccharomyces cerevisiae, and one level of factor B represents the time periods during which samples of TMV infected protoplasts were taken. The number of lesions produced were counted for each sample for each time period, and are arranged in the table below.

S. cerevisiae

	Time (hours)								
9.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
1.380	0.78	1.06	1.02	1.06	0.85	1.13	1.278	1.02	1.02
1.397	1.22	0.97	0.97	0.97	0.85	1.16	1.278	1.13	1.06
1.556	1.27	1.16	1.13	1.13	0.92	1.16	1.361	1.02	1.1
1.146	1.06	1.22	0.97	1.16	1.34	1.1	1.380	0.97	1.36
1.0	1.1	1.1	0.85	0.92	1.02	1.06	1.278	1.25	1.06
1.204	1.27	0.97	1.1	0.92	0.4	1.16	1.518	0.4	1.02

Next, observations for each sample in the table are counted (n), and scores within each sample are summed (ΣX), squared and summed (ΣX^2) and the sum of the squares is calculated ($SS = X^2 - \frac{(\Sigma X)}{n}$) as shown hereafter:

APPENDIX C (continued)

n	6	6	6	6	6	6	6	6	5	6
X	7.68	6.7	6.48	6.04	6.16	5.38	6.77	8.09	5.39	6.62
X^2	10.04	7.65	7.05	6.13	6.38	5.29	7.65	10.97	5.86	7.39
SS	.209	.174	.051	.052	.056	.463	.008	.046	.051	.083

Thirdly, the harmonic means of the sample frequencies is computed ($\bar{n}_h$). Fourthly, the variance within sample which constitutes error variance is calculated by summing all the sums of squares, in this case it is 1.193. Next, the means of the respective samples are calculated and summarized below. All the following calculations are carried out on the row and column totals of these means.

Time (hours)

	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	Totals
$\bar{X}$	1.28	1.12	1.08	1.01	1.03	.896	1.13	1.35	1.08	1.10	11.062

Compute:

1) $$\frac{(\text{Grand Total})^2}{\text{levels of A x levels of B}} = \frac{(11.062)^2}{(1)\ (10)} = 12.237$$

2) If there is more than one factor to be compared at level A, then

$$\frac{\text{Sum of the means for } A_1 \text{ squared + Sum of the means for } A_2 \text{ squared}}{\text{levels of B}}$$

3) $$\frac{\text{Sum of the means for time}_1 \text{ squared + Sum of the means for time}_2 \text{ squared}}{\text{levels of A}}$$

$$+ (1.28)^2 + (1.12)^2 + (1.08)^2 + (1.01)^2 + (1.03)^2 + (.896)^2 + (1.13)^2 +$$

$$= 12.387$$

Then calculate the sum of squares using the following two quantities:

APPENDIX C (continued)

$$\text{SS for time} = \bar{n}_h\ (12.387 - 12.237) = .867$$

Next, determine the degree of freedom, df, associated with each effect. So then df for time = number of time period - 1 = 10 - 1 = 9 df for within group variation (error) = total number of individual observations = (levels of factor A) x (levels of factor B) = 59 - 10 = 49. In order to determine the mean square value for each effect, divide each sum of square value by its own df. Lastly, divide mean square values (MS) of group by error MS value within group variation to determine F ratio values.

Calculations are summarized in the table below:

Source	SS	df	MS	F
Time	.867	9	.0963	4.01
Within (error) MS	1.193	49		

Using an F distribution statistics table, look up the degree of freedom associated with the effect being considered and the degree of freedom for within variation and determine the critical F value. In this case, for the effect due to time, df are 9 and 49, and the critical F value is 2.72 at the .01 level. Since the observed F value for time 4.01 exceeds the critical F value, the experimenter may conclude that there is a significant difference between the time at which infected protoplasts were taken.

APPENDIX D

T-TEST FOR INDEPENDENT SAMPLE MEANS

The t statistics are used to test the difference between two sample means to determine if one is significantly larger or smaller than the other (two-tailed test). In the present investigation, tests conducted on the overall analysis of variance values were found to be significant in order to determine the locus of the effect. An example is shown below:

	Time 4	Time 5
No. of lesions	1.278	1.02
	1.278	1.06
	1.361	1.10
	1.380	1.36
	1.278	1.06
	1.518	1.02
N	6	6
Mean	1.349	1.103
Variance	.009	.017

$$t = \frac{\text{Mean of time}_1 - \text{Mean of time}_2}{\sqrt{(1/N_1+1/N_2)\times\left[\frac{(N \text{ for time 1}-1)\times(\text{var. time 1})+(N \text{ for time 2})\times(\text{var. time 2})}{(N \text{ for time 1} + N \text{ for time 2}) - 2}\right]}}$$

$$= \frac{1.349 - 1.103}{\sqrt{\frac{2/6\ (5\ (.009) + 5(.017)}{10}}} = 3.739$$

APPENDIX D (continued)

Next determine the df, which are equal to $N_1 + N_2 - 2 = 10$. Check the table for the critical t value = 3.169. Since the calculated value exceeds the tabled value, you can conclude that there is a significant difference between the means of the time.

APPROVAL SHEET

The thesis submitted by Gurdeep Kaur has been read and approved by the following committee:

Dr. Amrik S. Dhaliwal, Director
Professor of Biology, Loyola University

Dr. William C. Cordes
Assistant Professor of Biology, Loyola University

Dr. Mark Goldie
Assistant Professor of Biology, Loyola University

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

12/12/1978
Date

A-S Dhaliwal
Director's Signature