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Study of Tobacco Mosaic Virus Replication In Protoplasts of *Saccharomyces Cerevisiae*

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ABSTRACT

The objective of this research was to study the effect of temperature, time and concentration of Tobacco Mosaic Virus (TMV inoculum) on its replication in isolated *Saccharomyces cerevisiae* (yeast) protoplasts. The protoplasts were prepared by treating the yeast cells with enzyme B — Glucuronidase/Aryl Sulfatase. Suspensions of protoplasts in osmotic stabilizer were infected with a common strain of TMV and incubated at 28°C for five hours. TMV-infected protoplast samples were taken at half-hour intervals, washed three times with osmotic stabilizer, suspended in 0.1 M phosphate buffer pH 7.0 and then TMV replication was assessed by inoculating ten day old primary leaves of *Phaseolus vulgaris* cv. Pinto, and by staining the infected protoplasts with fluorescent antibodies.

Both the assay methods used to determine the extent of TMV replication indicated the maximum increase at four hours of incubation. TMV-RNA replication may have taken place earlier but the whole virus was detected two and one half hours after inoculation. TMV replication in yeast protoplasts seemed to be temperature dependent. Maximum infectivity was obtained when protoplasts infected with TMV were incubated at 28°C. Protoplasts inoculated with 10X virus dilution showed double the infectivity as compared to 100X and 1,000X dilutions.

INTRODUCTION

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

Lederberg (1956) suggested that protoplasts are useful for studying the biosynthesis of plant viruses. Takebe, Otsuki, and Aoki (1968) successfully isolated protoplasts from tobacco leaf infected with Tobacco Mosaic Virus (TMV). Using the 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 TMV.

The purpose of this research is to isolate protoplasts of *Saccharomyces cerevisiae*, infect them with a common strain of TMV and study the replication process of TMV using fluorescent antibody staining technique and conventional assay procedure using *Phaseolus vulgaris* cultivar Pinto as assay host under different conditions such as temperature, duration of incubation, and concentration of TMV inoculum.

MATERIALS AND METHODS

Cultivation of *Saccharomyces cerevisiae*

A common strain of *Saccharomyces cerevisiae* cultivar ellipsoideus 556, 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 medium 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 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 at approximately 1000 rpm for 5 minutes. Cells were washed twice with glass distilled water and then used for the isolation of protoplasts. The composition of the agar medium, preculture liquid medium, and shake culture liquid medium is given in Table 1 (Darling *et al.*, 1969).

Isolation of Protoplasts

One gram wet weight of yeast cells was suspended in an osmotic stabilizer (sorbitol-citric acid pH 5.0) and incubated for 30 minutes at 30°C with occasional agitation. 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). The mixture of yeast cells and enzyme was incubated at 38°C with occasional agitation. A 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 then suspended in fresh osmotic stabilizer.

Inoculation of Protoplasts with TMV

The protoplast suspension in osmotic stabilizer was mixed with an equal volume of the common strain of diluted purified TMV (1:50 in osmotic stabilizer). TMV was purified by using Venecamp and Mosch method (1963). This mixture, used as inoculum, produced an average of 100 lesions per half leaf. 0.5 ml of the mixture was taken in sterile test tubes immediately after mixing TMV with protoplasts and this sample represented the zero time incubation. The protoplasts were washed three times with osmotic stabilizer, and divided into two parts. One part was suspended in 0.25 ml of osmotic stabilizer and slides were prepared for fluorescent antibody staining and microscopy. The other part was suspended in 0.25 ml of phosphate buffer and was kept in a freezer for bioassay. The remaining protoplast-virus mixture was incubated at 28°C. Samples were taken from this mixture at half-hour intervals for the first 5 hours and at longer intervals thereafter. Infected protoplasts were washed three times with osmotic stabilizer and were treated and studied in the same manner as the protoplasts from the zero time sample.

The data were analyzed statistically using log transformation, analysis of variance technique, and t-test for independent sample means.

Assay of Infectivity:
Fluorescent Antibody Staining of Infected Yeast Protoplasts

TMV infected protoplasts of *Sacchraomyces cerevisiae* were stained with 1:8 dilution of the fluorescent antibody stain (Clark and Shepard, 1963) following the method of Otsuki and Takebe (1969) and observed under the fluorescent microscope for stained protoplasts (Fig. 1).

Bioassay

Phaseolus vulgaris cultivar Pinto was used as an assay host. Plants were grown in the greenhouse and 10 day old leaves were inoculated with TMV infected protoplasts according to the method described by Lamborn, Cochran and Chidester (1971).

Lesions were counted with a Wild binocular microscope using 500X magnification. Color and morphology of the lesions were observed in order to find out whether the lesions were caused by intact TMV or TMV-RNA alone. Light brown and spreading type lesions are mostly caused by viral RNA, whereas, TMV produces dark brown and compact lesions.

RESULTS AND DISCUSSION

Effect of Time of Incubation On the Replication of TMV in *Saccharomyces cerevisiae* Photoplasts

The lesions produced by 0.5 hour sample may 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, and 2.5 hours of 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 enters the eclipse phase, during which time the virus particles are not detected. After the removal of the protein coat, the viral nucleic acid in the protoplasts probably acts 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. Increase in infectivity at 3.0 hours after inoculation is probably due to the appearance of new copies of the viral RNA which 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 the RNA is enclosed in the protein coat. The number of infectious particles again increased at 20.5 hours and 47.0 hours, followed by a drop at 120.0 hours. The infectivity was more with the 47.0 hour sample as compared to 4.0 hour samples (Fig. 2). This decrease and increase in infectious particles might be due to the quick synthesis and degradation of the viral RNA before it was enclosed in the coat protein which was synthesized 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 remained 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 in coat protein.

TMV infected protoplasts stained with fluorescent antibodies stain followed the same pattern of replication as indicated by the bio-assay.

Effect of Temperature on the 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 at 0 time and one-half hour after inoculation showed the infectivity due to the adsorbed virus. The decrease in infectivity up to 2.0 hours incubation probably indicates the period during which viral synthesis occurs 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, 28°C, and 45°C showed that the maximum infectivity occurred at 4.5, 7.0, and 8.0 hours respectively, thus indicating that 28°C is the optimum temperature for TMV replication in yeast protoplasts (Fig. 3).

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

Virus suspensions containing 500 lesion forming units was diluted 10X, 100X, and 1000X. Yeast protoplasts were inoculated with these virus dilutions, and incubated at 28°C for 5.0 hours. Samples of the inoculated protoplasts were taken every half hour and tested on half leaves of *Phaseolus vulgaris* cultivar Pinto, the results of which are shown in Fig. 4. The 0 time and half-hour incubation periods for all three dilutions showed the presence of adsorbed virus particles as indicated by the lesions produced on the assay host. The lesser the inoculum dilution, the more the virus adsorbed on the surface of the protoplasts. However, the adsorption was about the same at the dilution of 100X and 1000X. Infection, using 100X and 1000X dilutions, started decreasing at 1.0 hour 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 again at 4.0, 4.5, and 5.0 hours of incubation. The infectivity in the protoplasts inoculated with 10X dilution increased after 4.0 hours of incubation.

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Table 1. Composition of slant culture medium, preculture medium, shake culture medium and protoplast medium for the maintenance of yeast and yeast protoplasts.

	Grams/Liter			
	Shake Culture Medium	Protoplast Medium	Slant Culture Medium	Preculture Medium
Potassium dihydrogen orthophosphate	0.20	0.20	2.00	2.00
Magnesium sulfate	0.25	0.25	1.00	1.00
Glucose	0.10	1.00	20.00	20.00
Yeast extract	3.00	3.00	3.00	3.00
Sodium lactate	10.00	1.00	—	—
Ammonium hydrogen phosphate	3.50	3.50	—	—
Sorbitol	—	127.52	—	—
Ammonium sulfate	—	—	1.00	1.00
Difco peptone	—	—	3.50	3.50
Difco special agar	—	—	30.00	—

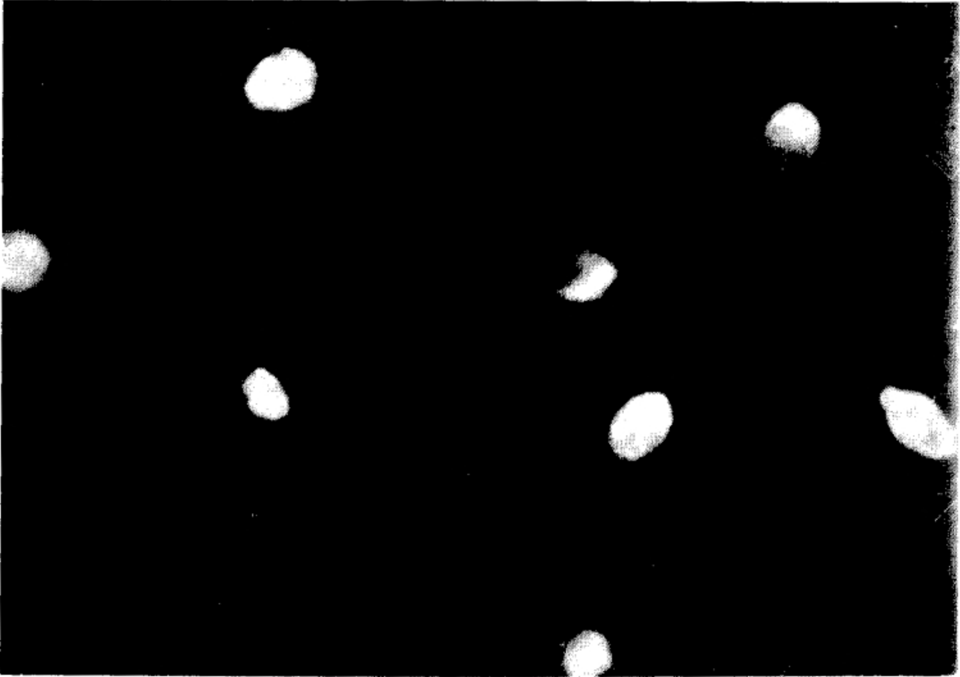

Fig. 1. Saccharomyces cerevisiae protoplasts four hours after inoculation with tobacco mosaic virus at 35°C under 100 ft. c. light intensity. Photographed after staining with fluorescent antibodies. Magnification 1000X.

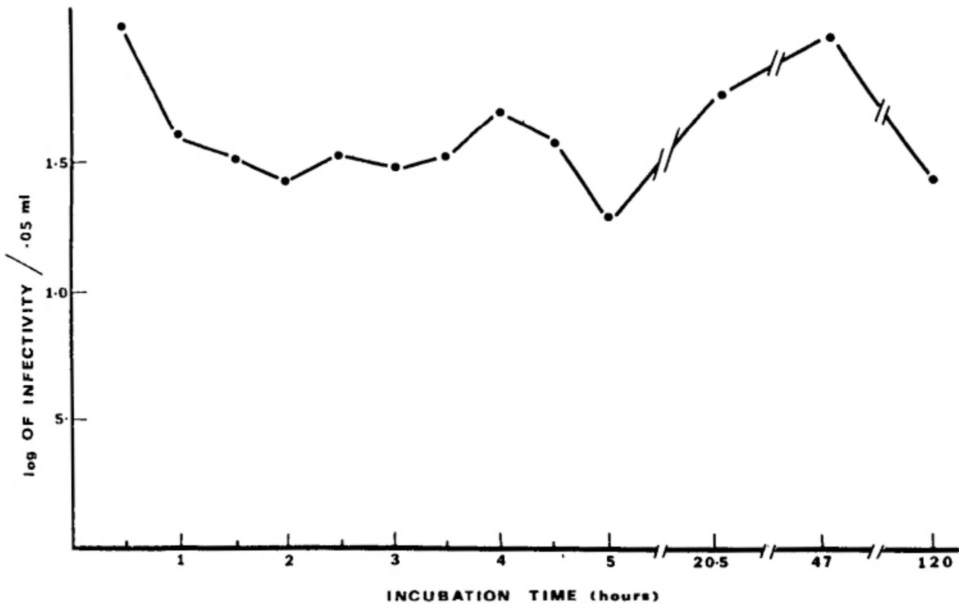

Figure 2. Effect of time of incubation on the replication of tobacco mosaic virus in *Saccharomyces cerevisiae* protoplasts assessed by local lesion assay on six half leaves of *Phaseolus vulgaris* cv. pinto.

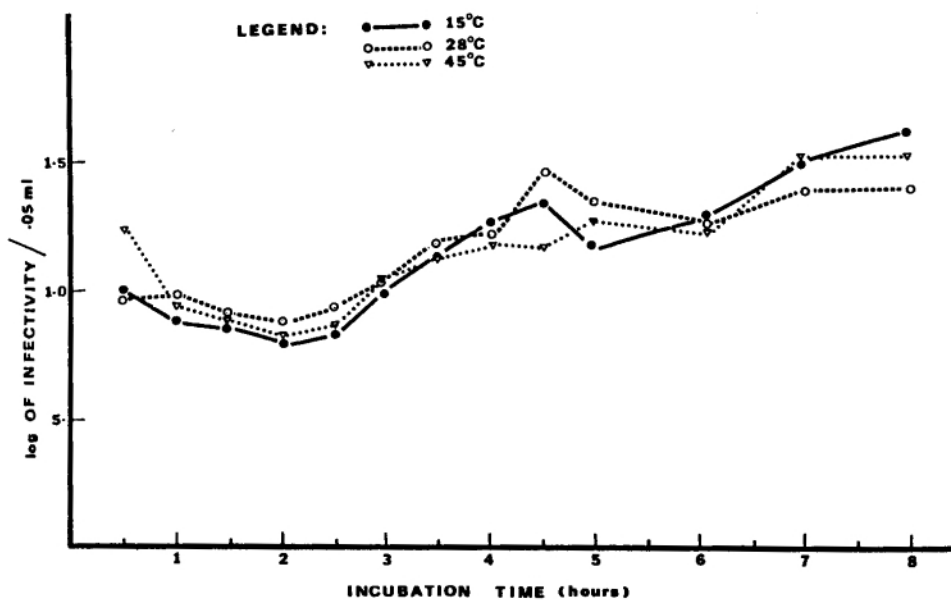

Figure 3. Effect of temperature on the replication of tobacco mosaic virus in Saccharomyces cerevisiae protoplasts assessed by local lesion assay on six half leaves of Phaseolus vulgaris cv. pinto.

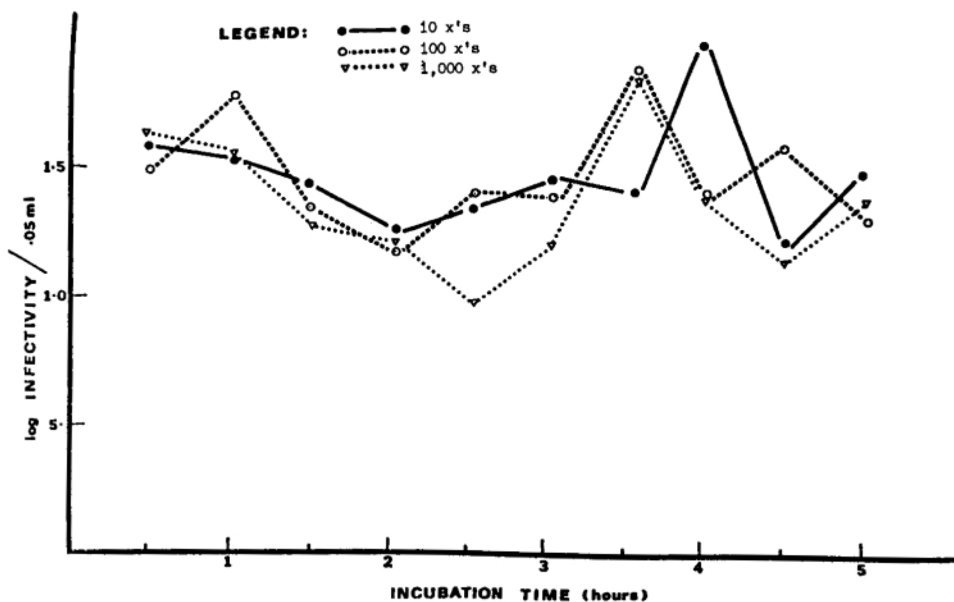

Figure 4. Effect of concentration of virus inoculum on the replication of tobacco mosaic virus in Saccharomyces cerevisiae protoplasts assessed by local lesion assay on six half leaves of Phaseolus vulgaris cv. pinto.