Infection of Tobacco Mesophyll Protoplasts with Tobacco Mosaic Virus

By R. H. A. COUTTS AND E. C. COCKING
Department of Botany, University of Nottingham, University Park, Nottingham NG7 2RD
AND B. KASSANIS
Rothamsted Experimental Station, Harpenden, Hertfordshire, England

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SUMMARY

Isolated protoplasts from healthy tobacco mesophyll tissue were infected with a Rothamsted culture of the common strain of tobacco mosaic virus. The average yield of virus/infected protoplast was estimated at \(1.4 \text{ to } 5.8 \times 10^6\) particles, which was almost as much as found in intact plants. Virus titre was assessed by infectivity assay, electron microscopy, and serological techniques.

INTRODUCTION

Plants become infected by viruses only after the cell wall is injured by mechanical inoculation or penetrated by a vector, and cells of callus tissues are often even more difficult to infect than plants (Kassanis, 1967). Cells with walls removed by enzymatic digestion are much more easily infected (Cocking, 1970). Recently, Takebe and co-workers showed how to use enzymes to isolate mesophyll cells from tobacco leaves and to remove their cell walls without affecting their susceptibility to infection with tobacco mosaic virus (TMV) (Takebe, Otsuki & Aoki, 1968, 1971; Takebe & Otsuki, 1969). By adding 0.1 to 2 \(\mu\)g/ml of TMV to a protoplast suspension, they synchronously infected 30 to 70% of the protoplasts, provided poly-L-ornithine was also added. Apparently this polypeptide facilitates pinocytosis and entry of virus particles into the protoplasts. Infectivity tests by the Japanese workers showed that more than \(10^6\) TMV particles were produced/infected protoplast. Earlier work (Takebe & Otsuki, 1969) reported maximum infectivity in the first day after infection followed by a 50-fold reduction of infectivity in the next 24 h. On the contrary, Takebe et al. (1971) reported that \(10^6\) virus particles/protoplast were produced at the end of the third day without any decrease in infectivity. Carborundum used in their infectivity tests (assays) (personal communication) probably increased errors and might account for the discrepancy.

We have repeated Takebe’s experiments using different infectivity tests to estimate the concentration of virus and omitting carborundum. The virus concentration was also estimated serologically and by counting in the electron microscope the number of virus particles in drops in comparison with a known concentration of polystyrene latex particles.

METHODS

Preparation of protoplasts. Sixty- to seventy-day-old Nicotiana tabacum, varieties Samsun and Xanthi nc, were grown in John Innes compost in 7 in pots at 22 to 25 °C, under warm white fluorescent-tube illumination, 1000 to 1100 lx at the middle leaf surface, for 16 h/day.
The relative humidity was kept at 70 to 75%. Plants were left unwatered for 24 h before use, to cause a slight loss in turgor which facilitates epidermal peeling. Leaves not quite fully expanded (20 to 25 cm in length) gave protoplasts that were most stable and susceptible to virus infection. The leaves were surface-sterilized by immersing in 70% (v/v) alcohol for 30 to 60 s, followed by 2.0% (v/v) sodium hypochlorite (prepared by dilution from B.D.H. sodium hypochlorite solution) for 30 min. They were then washed in three changes of sterile distilled water to remove the surface-sterilants, that often damage protoplasts (Power, 1971). All subsequent work was done in a sterile-air cabinet. Protoplasts of palisade parenchyma cells were prepared as described by Takebe et al. (1968) with slight modifications to the concentrations of enzymes used for cell and protoplast isolation (Coutts, 1973).

Infection of protoplasts with TMV particles. Infection was made with 2 μg/ml of a purified preparation of the Rothamsted culture of the common strain of TMV in 0.02 M-potassium citrate buffer (pH 5.2) containing mannitol at 13 g/100 ml and poly-L-ornithine (mol. wt. 120000, Sigma Chemical Company) at 2 μg/ml. After standing at 25 °C for 10 min, the TMV solution at 2 μg/ml was added to an equal volume of protoplast suspension (1 to 4 x 10^6/ml) and the whole incubated at 25 °C for 1 h, with occasional swirling. Similar methods were used to infect with potato virus X (25 μg/ml) and tobacco necrosis virus (23 μg/ml). After incubation, the protoplasts were washed free of excess virus by centrifuging at slow-speed (100 g for 1 min) in mannitol (13 g/100 ml) and calcium chloride (0.1 mM).

Incubation of infected protoplasts. The washed protoplasts were transferred aseptically into an incubation medium (Takebe & Otsuki, 1969) at a concentration of 1 to 4 x 10^5 protoplasts/ml, and incubated in 5 ml portions in L-shaped incubation tubes as used by Takebe et al. (1968). The 5 ml samples of infected protoplasts were incubated without shaking under fluorescent light (3500 to 3900 lx). Incubation temperature varied between 25 and 29 °C in different experiments. At set times protoplast samples were harvested by centrifuging at slow-speed (100 g for 1 min) in mannitol (13 g/100 ml) and calcium chloride (0.1 mM), and usually stored at 4 °C. In Expts. 2 and 3 the samples were frozen at −20 °C.

Extraction of virus from infected protoplasts. Preparations of infected protoplasts were suspended in 4 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.02%, sodium azide, and homogenized by hand in a ground-glass homogenizer in an ice bath. The homogenate was centrifuged at 8000 g for 10 min and the supernatant fluid made up to 5 ml for assessing virus content. The samples were usually stored at 4 °C but in Expts. 2 and 3 they were frozen at −20 °C.

Infectivity tests. Infectivity was assessed in tobacco plants, N. tabacum cv Xanthi nc, trimmed to five fully expanded leaves so that, by inoculating half-leaves, ten replications of ten inocula could be compared. Inocula were arranged systematically on half-leaves so that each appeared only once on a plant and once in each half-leaf position. The samples were at first inoculated undiluted, but in later tests were diluted up to 1/100, depending on the amount of virus suspected to be present. Four control dilutions of known concentrations, usually 2.5, 0.6, 0.15 and 0.04 μg/ml, were also inoculated. All virus solutions were made in 0.1 M phosphate buffer (pH 7.0) and inoculated without carborundum. The infectivities of the samples were determined by comparison with the log graph of the number of lesions obtained from the control dilutions and recorded as log virus concentration.

Serological tests. The virus concentration was estimated serologically by mixing different dilutions of the samples in narrow tubes with the antisera diluted 1/100 (antiseraum titre 1/1600). The dilution end-point of the samples was compared with the end-point of a dilution series of known concentrations of purified virus, 1.0 or 1.5 μg/ml was the minimum at which
TMV infection of tobacco protoplasts

TMV reacted, after the tubes were kept for 8 h in the water bath at 40 °C. The serological precipitin test was the least accurate method of estimating virus concentration because the dilution factor was 2, so that errors of up to 100 % were expected.

Electron microscopy. The samples were dialysed against water and mixed with a known concentration of polystyrene latex particles. Droplets of the mixture were sprayed on to a collodion-coated grid using Nixon & Fisher's (1958) method, modified by staining with phosphotungstate. Six droplets of each sample were photographed and enlarged. The number of particles was counted but for TMV the number of whole particles was estimated by dividing the total length of the particles in the 6 drops by 300 nm, the normal length of a TMV particle. The number of TMV particles/unit volume of the solution was estimated from their number in the droplets relative to that of latex particles (concentration of latex particles $3.3 \times 10^{11}$/ml) and the virus concentration in μg/ml was found by dividing the number of particles by $0.15 \times 10^{11}$.

RESULTS

Samples of the protoplasts were usually taken 0, 10, 18, 24, 48 and 72 h after inoculation with virus of a final concentration of 1 μg/ml. As the infectivity tests were made without carborundum, the 0 h samples never produced lesions, but when carborundum was added there were usually 5 lesions/half-leaf. Lesions were few with the 10 and 15 h samples except when the protoplasts were incubated at 28 °C. Later the infectivity increased rapidly to a maximum at the 48 h sample (Fig. 1). The first sample to react serologically (undiluted) was the 18 h sample from an experiment at 28 °C. In different experiments the titre increased to 1/32 or 1/128 by 48 or 72 h. The serological titre remained unchanged or occasionally doubled between 48 and 72 h, but in most experiments the infectivity fell slightly (Fig. 1, Table 1). Virus concentration estimated by electron microscopy was very close but always slightly greater than that obtained by infectivity tests. By contrast, the virus concentration estimated serologically was much higher than that from infectivity tests, but the former was known to be less sensitive. The greatest difference between infectivity and serology was in experiments 2 and 3 (Table 1). In these experiments, in which samples were frozen before and after extracting the virus, the extracted virus was much aggregated and this probably explains the higher serological titres. There was no aggregation when samples were not frozen.

The greater estimate of virus concentration by electron microscopy than by infectivity may reflect an error inherent in this method, in which both normal and broken particles were measured. However, in three out of four experiments in Table 1 the virus concentration estimated by infectivity was a little less at 72 than at 48 h after inoculation. As no virus was found in the incubation medium the specific infectivity of the virus probably deteriorated during this interval, and this is supported by the continued increase in virus concentration measured by electron microscopy. This loss of specific infectivity cannot be explained by the many broken particles in the 72 h samples because as many were found in the 48 h sample. We found that poly-L-ornithine did not affect the infectivity of TMV but there was a reduction in the infectivity when TMV at 25 μg/ml was mixed with the incubation medium. After 4 days in the incubation medium the virus was dialysed against water and its infectivity was compared with that of virus similarly treated but kept all the time in water; virus in the medium had only about 0.25 of the infectivity of that in water. Before extracting the virus from the protoplasts they were centrifuged and suspended in 0.1 M phosphate buffer (pH 7.0) and therefore the virus should not have come into direct contact with the incubation medium, but medium might have infiltrated the cells.
Fig. 1. Time course of appearance of TMV in infected protoplasts (Expt. 4). Infectivity was measured by serology (●—●), electron microscopy (□—□) and local lesion assay (○—○).

Table 1. Concentration of TMV in protoplasts*

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Temperature of incubation (°C)</th>
<th>Incubation time (h)</th>
<th>No. of protoplasts/ ml</th>
<th>Virus concentration (µg/ml)</th>
<th>Average concentration (µg/ml)</th>
<th>Virus particles/protoplast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>48</td>
<td>10^3</td>
<td>Infectivity 61</td>
<td>96</td>
<td>78</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>48</td>
<td>9.8 × 10^4</td>
<td>9</td>
<td>128</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td></td>
<td>9.8 × 10^4</td>
<td></td>
<td>25</td>
<td>(25)</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>48</td>
<td>1.1 × 10^5</td>
<td>24</td>
<td>128</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td></td>
<td>1.1 × 10^5</td>
<td></td>
<td>13</td>
<td>(13)</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>48</td>
<td>1.3 × 10^5</td>
<td>24</td>
<td>48</td>
<td>28</td>
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<tr>
<td></td>
<td>72</td>
<td></td>
<td>1.3 × 10^5</td>
<td></td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>48</td>
<td>4 × 10^5</td>
<td>28</td>
<td>96</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td></td>
<td>4 × 10^5</td>
<td></td>
<td>25</td>
<td>96</td>
</tr>
<tr>
<td>6</td>
<td>23 to 27</td>
<td>48</td>
<td>3.3 × 10^5</td>
<td>22</td>
<td>30</td>
<td>32</td>
</tr>
</tbody>
</table>

* The virus concentration was estimated by infectivity, serology and by counting the particles in the electron microscope. In Expts. 2 and 3 the virus was extremely aggregated, which could account for the high serological titres; the serological titres in these cases were not considered when estimating virus particles/protoplast. In Expt. 5 the results were means of two replications. Expt. 6 was made during power cuts, hence the wide range of temperature. Expts. 1 to 5 were made using Nicotiana tabacum cv. Samsun and 6 cv Xanthi.

Assuming the mol. wt. of TMV to be 4 × 10^7, there would be 1.5 × 10^10 particles/µg of virus. On this basis the number of virus particles/protoplast was 1.2 × 10^5 in Expt. 1, but in the other five experiments in Table 1 it varied between 1.4 × 10^6 and 5.8 × 10^6, assuming every protoplast was infected. However, Takebe et al. (1971) found that only half of the protoplasts become infected so the number of particles/infected protoplast should perhaps
be doubled. Fluorescent-antibody studies on TMV infected protoplasts showed that at the 48 h incubation stage 50% to 80% of the protoplasts were infected (R. H. A. Coutts, unpublished observations).

Infected tobacco leaves contain about 2 mg TMV/g of leaf. We estimate that 1 g of tobacco leaf contains approximately $5.4 \times 10^6$ cells. From equivalent numbers of protoplasts we usually obtained between 0.5 and 2 mg of virus (4.2 mg in Expt. 1). This suggests that protoplasts yield from 0.25 to an equal amount of virus to that produced in growing leaves. If the $5.4 \times 10^6$ cells present in 1 g of leaf produce about 2 mg of virus then there would be $6 \times 10^6$ particles/cell, about as many as the most we found in protoplasts excluding Expt. 1 (Table I).

Inoculated protoplasts were incubated at 25 to 29 °C but the final concentration seemed independent of temperature, although the virus multiplied more rapidly at the higher temperatures. Only at 29 °C could the virus be estimated (without the aid of carborundum) in the 10 and 18 h samples (Fig. 1).

**DISCUSSION**

The results show that in protoplasts from mesophyll cells TMV multiplies almost as much as in growing plants. The use of isolated protoplasts in these studies has two major advantages over the use of the plants themselves. First, the protoplasts can be synchronously infected with the virus, and secondly, as a result of the high proportion of naked cells which become infected, it is possible to carry out more meaningful biochemical investigations of virus infection using this protoplast system. As a result, protoplasts will probably become generally used for studying the uptake of viruses and initiation of infection in cells. It may be possible to identify proteins, and other compounds of biochemical interest in infected protoplasts, at earlier stages of infection than in inoculated leaves. A few grams of protoplasts are difficult to prepare compared with the large weight of leaf easily obtained from growing plants, but the extract made from infected isolated protoplasts after incubation is colourless instead of a thick green sap. Nevertheless, such extracts contain sufficient virus to give a serological titre of 1/32 to 1/128, and proportional amounts of other proteins presumably are present in the solution.

Successful protoplast preparation depends on many factors, including the cultural treatment and age of the plant material. We investigated *N. tabacum* varieties Samsun and Xanthi nc and found that the best leaves for protoplast isolation are the ones not quite fully expanded (20 to 25 cm in length), often found in a position 4 to 6 leaves from the stem apex. Fully expanded leaves (25 to 40 cm in length) and young leaves (10 to 20 cm in length) produced unstable protoplasts.

Isolating protoplasts by sequential methods (Takebe et al. 1968) allows separation of different fractions of the mesophyll cells from leaf tissue. The protoplasts from the palisade mesophyll cells support TMV replication better than spongy mesophyll cells or mixed populations of each type (Coutts, 1972). However, we hope to use the mixed enzyme method for the isolation of protoplasts (Power & Cocking, 1970) because, although this method yields a mixed population of both spongy and palisade mesophyll protoplasts, the procedure is much simpler. With *N. tabacum* variety Xanthi nc many more stable protoplasts are obtained by overnight treatment of leaves with a dilute mixture of the enzymes than when using the enzymes sequentially. A few unsuccessful attempts were made to use the protoplast system as described to study the multiplication of potato virus X and also tobacco necrosis virus. This may have resulted from the loss of infectivity of the inoculum or from something more complex. Each virus and type of protoplast may require unique treatment, and to determine whether the infection process has to be modified it may be necessary to study
the conditions for incubation of protoplasts from inoculated leaves. Once the best conditions for virus replication have been determined in protoplasts isolated from these leaves, then the infection of healthy protoplasts with the virus can be studied knowing that, if negative results are obtained, then the incubation conditions are not at fault but rather the conditions for the initiation of infection.

In their first experiment, Takebe & Otsuki (1969) reported a 50-fold decrease in infectivity after the maximum was reached in the first day after infection but later they found no decrease in infectivity during the first 3 days (Takebe et al. 1971). We also found that the 72 h sample was usually a little less infective than the 48 h sample, but in both about half of the virus particles were less than 300 nm long. As the extraction method used preserves the integrity of the particles the loss of infectivity probably occurred during incubation, especially as the incubation medium decreases virus infectivity. Despite this, the system gave a good yield of TMV, but inactivation by the incubation medium may be more important with other viruses.

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REFERENCES


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