Efficient Mechanical Inoculation of Turnip Yellow Mosaic Virus Using Small Volumes of Inoculum

(Accepted 23 March 1979)

SUMMARY

When purified turnip yellow mosaic virus was inoculated mechanically on to Chinese cabbage leaves, using known numbers of virus particles in 0.1 to 1.0 μl volumes of inoculum, as few as 10 to 30 particles were required to produce a single local lesion. A simple procedure is described for obtaining local lesions from the virus in single infected protoplasts.

Mechanical inoculation of leaves with plant viruses has been widely regarded as a very inefficient process (Matthews, 1970; Gibbs & Harrison, 1976; Luria et al. 1978). Numbers of particles required to be applied to produce a single local lesion have frequently been reported to be in the range of $10^4$ to $10^6$. However, when known numbers of TMV particles were inoculated on to tobacco leaves in small volumes (2.5 μl), single lesions were obtained with as few as 450 virus particles (Walker & Pirone, 1972). Walker and Pirone pointed out that the large number of particles required in the conventional assay procedures is due mainly to the large volume of inoculum applied to the leaves. They also suggested that inoculation of a larger number of plants with even smaller volumes of inoculum might reduce estimates of the number of TMV particles required to below 450. In the work reported here we have confirmed and extended their results using turnip yellow mosaic virus (TYMV) in Chinese cabbage (Brassica pekinensis), and have developed a simple method for examining the virus content of single protoplasts.

A severe (white) strain of TYMV, which usually gave clearly visible local lesions, was purified from systemically infected leaves by differential centrifugation, suspended in 1×SSC (0.15 M-NaCl, 0.015 M-citrate buffer, pH 7.0) and stored at 4 °C mixed with an equal vol. of glycerol. Aliquots of this preparation were diluted with 0.1 M-phosphate buffer (pH 6.2) and used as inoculum. The concentration of unfractionated TYMV was calculated using the factor, $A_{260} = 7.6$ for 1 mg/ml. An approximate allowance for the presence of empty protein shells and non-infectious nucleoproteins which occur in small amounts in these preparations was made by subtracting one fifth of the estimated concentration. The number of TYMV nucleoprotein particles per ml was then calculated using a mol. wt. of $5.4 \times 10^6$ (Matthews, 1977).

Plants were grown in pots in a glasshouse maintained at 21 ± 3°C. Plants at the 4 to 6 leaf stage were dusted with carborundum powder before the application of inoculum. One μl of a suspension containing a known number of TYMV particles was dispensed with a 1 μl graduated Hamilton syringe on to one leaf of each plant, the inoculum being then spread evenly over part of the leaf surface with a round-ended glass rod. When 0.1 μl of inoculum was used, inoculations were made under conditions of high humidity to slow the rate of evaporation. The 0.1 μl drop of inoculum was first transferred to the end of the glass rod and then the rod was rubbed on to the surface of the leaf. In each experiment equal numbers of control plants were sham inoculated with phosphate buffer. Groups of control plants were positioned between groups of infected plants; no infection of control plants occurred in any experiment. The data for one experiment are given in Table 1. Inoculation of a plant with approx. 10 TYMV particles was sufficient to produce one local...
Table 1. Numbers of TYMV particles required to produce local lesions in mechanically inoculated Chinese cabbage leaves

<table>
<thead>
<tr>
<th>Approximate number of particles in the applied inoculum</th>
<th>Numbers of lesions on each of ten inoculated leaves*</th>
<th>Average numbers of particles per lesion</th>
<th>Number of plants systemically infected (out of 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied in 1 μl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6,6,4,3,2, 2,1,0,0,0, 2,2,1,1,1, 1,0,0,0,0, 2,1,1,1,1, 0,0,0,0,0,</td>
<td>50 35 7</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (30 plants)</td>
<td>No local lesions</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Applied in 0.1 μl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>3,2,2,1,1, 0,0,0,0,0, 1,1,0,0,0, 0,0,0,0,0, 1,1,0,0,0, 0,0,0,0,0,</td>
<td>100 50 5</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (30 plants)</td>
<td>No local lesions</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

* One μl of inoculum covered about 10 cm² and 0.1 μl about 1 cm² of the inoculated leaf surface. All the local lesions that appeared were in the inoculated areas. All plants that became systemically infected developed at least one local lesion in the inoculated area.

lesion and consequential systemic infection in a proportion of the inoculated plants. This number is comparable to the number of particles of some animal viruses required to infect a cell in culture (Fenner et al. 1974).

In other experiments, not detailed here, using volumes and virus particle numbers similar to those of Walker & Pirone (1972), we obtained efficiencies of infection very similar to those for TMV in tobacco. Thus, these high efficiencies may apply generally for plant viruses with monopartite genomes.

Besides mechanical inoculation of leaves two other procedures have been used to determine efficiencies of infection with plant viruses: inoculation of protoplast suspensions, and microinjection of cells. Direct comparison of results obtained by the three procedures may not be meaningful. In principle we can distinguish three quantities in these inoculation procedures – (i) the total number of virus particles applied per local lesion obtained. (Under the limiting conditions used it is reasonable to assume that each local lesion arises from the successful infection of a single cell.) (ii) The number of virus particles that successfully adsorb to the plasma membrane of a viable cell for each successful infection. (iii) The number of virus particles that actually enter a cell for each successful infection.

With mechanical inoculation we can measure only the total virus applied (or adsorbed to the leaf surface, including cell walls, dead cells etc.). When protoplasts are inoculated, the average amount of virus adsorbed to each live cell can be measured. This is usually about 0.1 to 1% of the virus supplied in the inoculum solution. With microinjection the actual virus that is placed within a cell is measured.

Efficiency of infection of protoplasts can be expressed as the average number of virus particles adsorbed per protoplast to give infection in half the protoplasts (ID₅₀). Figures of
about 400 have been obtained for TMV in tobacco protoplasts (calculated from data given by Takebe, 1977); 380 for cowpea chlorotic mottle virus (Motoyoshi et al. 1973); 30 for long rods and 85 for short rods of tobacco rattle virus (Kubo et al. 1976); and 85 for cowpea mosaic virus (calculated from the data given by Hibi et al. 1975). This list includes viruses with monopartite, bipartite and tripartite genomes but the numbers cannot be directly compared since the assays were made in protoplasts from different hosts under a variety of conditions. The most appropriate comparison between the protoplast and whole leaf systems is on the basis of total virus applied for successful infection. In our experiments the lowest number of particles applied per local lesion produced, including leaves with no lesions, was about 20 to 50 (Table 1). The ID$_{50}$ noted above were in the range of about 50 to 500. The numbers of particles supplied in the inoculum for each successful protoplast infection were of the order of 100 to 1000 times the ID$_{50}$ quoted above. Whether we use these figures or the ID$_{50}$, the statement by Takebe (1977) that the efficiency of the infection process in protoplasts is strikingly higher than in intact leaves is not correct.

Halliwell & Gazaway (1975) obtained an ID$_{50}$ of 310 particles injected (in 1 picolitre) for TMV in single tobacco cells. This technique cannot be compared directly with mechanical inoculation and protoplast infection since for these two techniques we have no estimate of how many particles pass into cells that become infected. Nevertheless microinjection does not appear to be more efficient than mechanical inoculation carried out with small volumes.

In addition microinjection is a technically difficult procedure.

The very substantial increase in efficiency of the mechanical inoculation process reported here is probably due to the fact that for a given number of virus particles in the applied inoculum, the smaller the volume, the higher the concentration of the virus applied. A second effect may be operating. The lifetime of many of the potentially infectible sites made by mechanical abrasion of the leaf surface may be short (of the order of one minute; Matthews, 1970). On average, virus particles in a very small volume (and independently of concentration) will have a greater probability of finding such an infectible site within a short time than particles in a larger vol.

Halliwell & Gazaway (1975) obtained 4 to 9 local lesions/half leaf in bioassays of TMV from single infected tobacco cells. Data such as those in Table 1 suggested that it should also be possible to obtain a useful number of local lesions from the TYMV contained in a single cell, since fully infected mesophyll cells have been estimated to contain approx. 2 x 10$^6$ TYMV particles (Ushiyama & Matthews, 1970). Protoplasts were prepared from infected Chinese cabbage leaves by the method of Matthews & Sarkar (1976). The following simple dilution procedure was used to obtain single protoplasts. The protoplast preparation was diluted in 0.4 M-mannitol to give an average density of one protoplast per 50 μl sample which was then dispensed into the compartments of a flat-bottomed serological titre plate. The compartments were inspected using an inverted microscope and samples chosen which contained only one protoplast in good condition. Infection with TYMV was determined by the characteristic rounding and clumping of the chloroplasts induced by TYMV (Renaudin et al. 1975). Selected infected protoplasts were lysed by adding an equal volume of 0.01 M-phosphate buffer, pH 6.2. This was used as inoculum directly, or after further dilution in the buffer. Tests not detailed here indicated that sonication of the extract of a single protoplast did not increase local lesion number and that addition of a healthy protoplast lysate to purified virus did not decrease lesion number.

For reasons not yet understood extracts from single protoplasts have given only about one fifth to one tenth the number of local lesions that would be expected for a virus content of 10$^6$ particles/cell. In various tests no local lesions were obtained when leaves were inoculated with 1 μl of protoplast extract calculated to contain 1000 particles. However, when the 100 μl lysates of single infected protoplasts were inoculated to half leaves at the
rate of 8 µl/half leaf useful numbers of local lesions were obtained. For example, in one such experiment 27 local lesions were obtained from 12 half leaves. The opposite half leaves gave a total of 160 lesions from 10^6 particles of purified virus in 100 µl.

The possibility of obtaining numerous local lesions from the virus production of a single cell may allow the development of new experimental approaches to such questions as the nature of the cross protection phenomenon between related virus strains in the intact plant and the distribution of virus and virus strains in the apical region of a systemically infected host.

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REFERENCES


(Received 7 February 1979)