Infection of Cowpea Mesophyll Protoplasts with Clover Yellow Mosaic Virus

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SUMMARY

Several factors were investigated in relation to infection and multiplication of clover yellow mosaic virus (CYMV) in cowpea mesophyll protoplasts. The extent of virus multiplication was determined by fluorescent antibody staining and infectivity assay. The conditions that gave the most infection (38%) were: pre-incubation of the virus with 0.4 μg/ml poly-L-ornithine, a virus concentration of 4 μg/ml, an inoculum containing citrate buffer at pH 6.0, and contact between inoculum and protoplasts for at least 15 min. Time-course studies revealed synchronous one-step growth of virus with rapid accumulation of virus antigen from 12 to 48 h after inoculation.

INTRODUCTION

In recent years, plant protoplasts have been successfully used for synchronous infection and multiplication of many plant viruses (Takebe, 1975). However, there are only a few reports of plant virus infection using protoplasts isolated from plant species outside the Solanaceae (Hibi, Rezelman & van Kammen, 1975; Renaudin et al. 1975; Okuno, Furusawa & Hiruki, 1977). With the cowpea, a species belonging to the Papilionaceae, mesophyll protoplasts have been used in studies on cowpea mosaic virus (CPMV; Beier & Bruening, 1975; Hibi et al. 1975), tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV; Koike, Hibi & Yora, 1976). Because clover yellow mosaic virus (CYMV) infection of intact cowpea leaves results in a very high virus concentration (D. V. Rao, unpublished data), this host has been chosen for protoplast isolation and virus multiplication studies. The present paper reports the various factors that affect infection and multiplication of CYMV in cowpea mesophyll protoplasts.

METHODS

Virus. The purified vetch isolate of CYMV (Hiruki, Shukla & Rao, 1976) was used throughout the investigation. The virus was extracted, using a Waring Blendor, from the infected pea tissue in 0.1 M-phosphate buffer, pH 7.0, containing 0.5% ascorbic acid. The sap was clarified by low-speed centrifugation and the virus precipitated by 8% polyethylene glycol (mol. wt. 6000). The resulting precipitate was dissolved in 0.025 M-phosphate buffer at pH 7.0, containing 0.001 M-ethylene diaminetetraacetate. Low-speed centrifugation was performed and the supernatant fluid which contained the virus was subjected to two cycles of differential centrifugation. The final virus pellets were dissolved in 0.025 M-phosphate buffer, pH 7.0.

Isolation of protoplasts. Cowpea seeds were incubated on a moist filter paper in a Petri dish at 28 °C for 2 days. The germinated seeds were planted in moist vermiculite of fine
grade and were grown in a controlled environment cabinet (Model E30, Percival Co., Boone, Iowa, U.S.A.) at an approximate relative humidity of 80%. A 14-h light period at approx. 11800 lux provided by 6 fluorescent lamps (F24712/CW/HO, Sylvania Ltd, Montreal, Canada) and two 40 W incandescent bulbs, at a temperature of 28 ± 2 °C was alternated with a 10-h dark period at 23 ± 2 °C.

The plants received half strength Hoagland's solution throughout the growth period. The one-step procedure of Hibi et al. (1975) was used for isolation of protoplasts. Protoplasts were counted using a haemocytometer.

**Inoculation.** Unless otherwise stated, protoplasts were inoculated by the direct method. The purified preparation of CYMV was diluted to 10 μg/ml in 0.01 M-potassium citrate buffer, pH 5.8, prepared in 0.6 M-mannitol. The concentration of poly-L-ornithine (mol. wt. 150000; Pilot Chemicals Co., Boston, Mass., U.S.A.) was adjusted to 0.8 μg/ml in citrate-buffered mannitol. Two ml of poly-L-ornithine, mixed with an equal amount of virus solution and kept at 25 °C for 30 min, was used to suspend freshly sedimented protoplasts, the concentration of which was adjusted to 2.5 x 10^6/ml. Final concentrations were thus 0.4 μg/ml poly-L-ornithine, 5 μg/ml virus, 2.5 x 10^6/ml protoplasts and 0.01 M-potassium citrate buffer, pH 5.8. The samples were shaken at 25 °C for 15 min at 90 excursions/min.

Where the indirect method was used, freshly sedimented protoplasts were suspended in 0.01 M-citrate buffered mannitol, pH 5.8, and the protoplast concentration was adjusted to 5 x 10^6/ml. The virus-poly-L-ornithine mixture, incubated as in the direct method, had a concentration of 0.8 μg/ml poly-L-ornithine and 10 μg/ml virus. This mixture was added to the protoplast suspension at a 1:1 volume ratio, so that final concentrations were the same as those in the direct method.

In both the direct and the indirect method, the protoplasts were washed after inoculation at least three times in 0.6 M-mannitol containing 10 mM-CaCl_2 to remove unabsorbed virus.

**Incubation.** Protoplasts were incubated in 10 ml aliquots of standard incubation medium in 125 ml Erlenmeyer flasks according to the method of Hibi et al. (1975) except that the concentration of cephaloridine was reduced to 500 μg/ml. After the incubation period, 3 ml samples were removed for a fluorescent antibody test and the protoplasts in the remaining 7 ml were collected by centrifugation and re-suspended in 0.6 M-mannitol. The protoplasts were then washed twice, re-pelleted and kept at −20 °C for use in the infectivity assay.

**Infectivity assay.** The samples containing frozen protoplasts were thawed at room temperature, suspended in 0.25 ml of 0.005 M-phosphate buffer, pH 7.0, and ground individually in glass tissue grinders (Bellco Biological Glassware, Vineland, U.S.A.). The homogenates were centrifuged at 17000 g for 15 min and the supernatants were adjusted to a final volume of 0.25 ml with the same buffer. Inoculation of Chenopodium amaranticolor plants was made by the half-leaf method using eight half-leaves for each treatment.

**Preparation of immunoglobulin.** The immunoglobulin fraction of the antiserum was precipitated with ammonium sulphate according to Campbell et al. (1970) except that phosphate-buffered saline (PBS) was used instead of borate-buffered saline. After the third precipitation, the precipitate was dissolved in and dialysed against PBS until it was free from ammonium sulphate as checked by the silver nitrate test.

**FITC-conjugation.** After adjusting the protein concentration of the preparation to 1%, conjugation with fluorescein isothiocyanate (FITC; ICN Pharmaceuticals Inc., Ohio, U.S.A.) was performed according to Otsuki & Takebe (1969). The conjugated preparation had a staining titre of 1/32 and the molar ratio of fluorescein to protein, determined from the absorbance at 495 nm and 280 nm, was 1.7. The preparation was stored at −20 °C in small glass tubes in 1 ml lots until use.
To minimize non-specific staining, an acetone powder preparation of healthy pea leaves was mixed with the conjugated globulin at a rate of 50 mg/2 ml for 2 h at room temperature. The mixture was then centrifuged at 2000 g for 15 min to obtain the supernatant.

Conjugated normal rabbit globulin, prepared as described for anti-CYMV globulin, served as a control.

Staining. The staining method of Otsuki & Takebe (1969) was modified to minimize the undesirable effects of direct dehydration such as distortion and crumbling of protoplasts. Protoplasts were fixed in 3% glutaraldehyde for 1 h and washed twice with PBS. A drop of thick suspension was placed on a glass slide previously smeared with Meyer's albumin and dried quickly in a stream of warm air. The slide was then immersed in 95% ethanol for 10 min and washed for 30 min in PBS, after which a drop of fluorescent antibody was applied to the fixed sample and incubated for 2 h at 37 °C in a water bath. The slide was then washed with two changes of PBS for 90 min, mounted in phosphate buffered glycerol and examined with a Leitz Ortholux I microscope fitted with a 200 W ultra-high pressure mercury lamp. The exciter filters UG r (1 mm), BG 3 (1 mm) and a red suppression filter BG 38 (4 mm) were used with an eyepiece barrier filter K 490. Observations were recorded with an automatic camera using a Kodak Tri-X film.

Except for time-course studies, protoplasts were harvested 48 h after inoculation for fluorescent antibody staining. Usually, 300 protoplasts were counted to assess the percentage of protoplasts showing fluorescence in each experiment.

Scanning electron microscopy. The protoplasts were fixed in 3% paraformaldehyde-glutaraldehyde in 0·2 M-Millonig's buffer, pH 7·4, for 3 h, then post-fixed in 2% osmium tetroxide, and passed through a graded series of ethanol, followed by a concentration series of amyl acetate. They were then dried by the critical point method using liquid carbon dioxide as a transitional fluid in a Denton vacuum drier (Model DCP-I). The dried protoplasts were examined with a Cambridge Stereoscan S4 electron microscope operated at 20 kV and 15 to 30° tilt.

Transmission electron microscopy. After negative staining with 2% phosphotungstate, pH 7·0, protoplast extracts were examined for the presence of intact virus particles using a Philips Model 200 transmission electron microscope at 80 kV.

RESULTS

Preparation of protoplasts

The one-step procedure, applied to the primary leaf tissues of cowpea, yielded approximately 1 to 3 × 10^6 protoplasts/g (fresh weight) of leaf tissue. From 80 to 83% of the protoplasts were apparently intact when counted immediately after isolation and washing. Fig. 1 and 2 show the appearance of the protoplasts as observed with the light and the scanning microscopes respectively. The majority of the epidermal protoplasts were removed by low-speed centrifugation, although sometimes 1 to 3% of a total protoplast population was found to be of epidermal origin.

Method of inoculation

The relative efficiency of the direct and indirect methods was tested. The results, representing the percentages of infected protoplasts from three experiments for the direct/indirect methods, are as follows: 28/24, 20/19, 29/31. These results indicate no significant difference between the two methods. Although either of them could be used in inoculations, the direct method was preferred in subsequent experiments for its simplicity.
Figs. 1 and 2. A light micrograph and a scanning electron micrograph, respectively, of cowpea mesophyll protoplasts sampled after 12 h incubation. Protoplasts were suspended in 0.6 M mannitol.

Figs. 3 and 4. Fluorescence micrographs of cowpea mesophyll protoplasts, sampled 24 h and 36 h, respectively, after inoculation with clover yellow mosaic virus, and stained with fluorescent antibody to virus.

Fig. 5. Particles of clover yellow mosaic virus obtained from a 100-fold dilution of an extract of cowpea mesophyll protoplasts sampled 60 h after inoculation with the virus.

Concentration of poly-L-ornithine

When protoplasts were inoculated in the absence of poly-L-ornithine, only 4 to 9% of them were infected. Hence, experiments were carried out to investigate the effect of poly-L-ornithine on the percentage of infection. The percentage of infected protoplasts was highest at 0.6 μg/ml. At this level, however, only 45% of the protoplasts survived. A lower poly-L-ornithine concentration (0.4 μg/ml) gave a significantly higher percentage survival of protoplasts (Fig. 6).
Infection of protoplasts with CYMV

Fig. 6. Effect of different concentrations of poly-L-ornithine on clover yellow mosaic virus infection (○—○) and survival of cowpea mesophyll protoplasts (●—●). Protoplasts were sampled 48 h after inoculation.

Table 1. Effect of pre-incubation of clover yellow mosaic virus with poly-L-ornithine on the frequency of infection in cowpea mesophyll protoplasts

<table>
<thead>
<tr>
<th>Time of pre-incubation (min)</th>
<th>Percentage of protoplasts showing fluorescence*</th>
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<tr>
<td>0</td>
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<td>10</td>
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<td>28</td>
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<td>60</td>
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* Protoplasts were inoculated at a concentration of 5 μg/ml virus and 0.4 μg/ml poly-L-ornithine at pH 5.8. Percentage of protoplasts that stained with fluorescent antibody to the virus was determined by sampling 48 h after inoculation.

Pre-incubation of virus with poly-L-ornithine

The percentage of infected protoplasts was more than doubled when the virus was pre-incubated with poly-L-ornithine for 10 to 60 min (Table 1).

Duration of contact of virus with protoplasts

Immediately after adding a virus-poly-L-ornithine mixture to protoplasts, the mixtures were incubated for different periods of time in a shaker bath. A brief contact of virus with protoplasts for 15 min gave rise to reasonably good infection, which did not change appreciably with increasing incubation periods up to 60 min (Table 2).
Table 2. Effect of duration of contact of clover yellow mosaic virus with cowpea mesophyll protoplasts on the frequency of infection

<table>
<thead>
<tr>
<th>Duration of inoculation (min)</th>
<th>Percentage of protoplasts showing fluorescence*</th>
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<tbody>
<tr>
<td>0</td>
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<td>15</td>
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<td>30</td>
<td>23</td>
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<td>45</td>
<td>23</td>
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<td>60</td>
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* Protoplasts were inoculated with 4 μg/ml and 0.4 μg/ml of virus and poly-L-ornithine respectively (pH 6.0). Protoplasts were sampled 48 h after inoculation and stained with fluorescent antibody.

Fig. 7. Effect of the concentration of clover yellow mosaic virus on infection of cowpea mesophyll protoplasts. Protoplasts were inoculated at a poly-L-ornithine concentration of 0.4 μg/ml, and pH 5.8, and were sampled 48 h after inoculation.

Fig. 8. Effect of pH of inoculum on infection of cowpea mesophyll protoplasts with clover yellow mosaic virus. Protoplasts were inoculated with 5 μg/ml virus and 0.4 μg/ml poly-L-ornithine, and were sampled 48 h after inoculation.

Concentration of virus in inoculum

The largest proportion of infected protoplasts was obtained at an inoculum concentration of 4 μg/ml, beyond which there was no increase (Fig. 7).

PH of inoculation medium

Potassium citrate buffers with pH values from 4.9 to 6.3 were used to determine the influence of pH on the percentage of infection. The highest percentage was obtained at pH 6.0 (Fig. 8).
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Time-course study

The time-course of CYMV multiplication was studied by assaying the infectivity of protoplast extracts with increasing incubation periods up to 60 h (Fig. 9). Immediately after inoculation, the protoplasts retained a small amount of infectivity, which decreased to about 40% after 6 h. The infectivity increased rapidly and continuously from 12 h to 48 h after inoculation. Thereafter, virus multiplication ceased. At least a 100-fold increase in virus infectivity was obtained between 0 and 60 h after inoculation as determined by assaying a series of dilutions of the protoplast extracts. Intact virus particles were observed with the electron microscope when the extracts were examined 60 h after inoculation (Fig. 5).

Infection of protoplasts with CYMV was monitored by using the fluorescent antibody staining technique. The first fluorescence due to CYMV antigen was detected as early as 12 h after inoculation as small, faint, yellowish-green specks in the cytoplasm. The number of fluorescing protoplasts, however, was only 3% at this time. Small fluorescent masses were
Table 3. Time-course study of cowpea mesophyll protoplasts by the fluorescent antibody staining technique after inoculation with clover yellow mosaic virus

<table>
<thead>
<tr>
<th>Time after inoculation (h)</th>
<th>Percentage of protoplasts stained with fluorescent antibody*</th>
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<tbody>
<tr>
<td>0</td>
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<td>12</td>
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<td>48</td>
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* Protoplasts were inoculated with 4 μg/ml virus and 0.4 μg/ml poly-L-ornithine respectively at pH 6.0.

observed after 24 h of incubation (Fig. 3) and after 36 h most of the cytoplasm was occupied by large diffuse masses of fluorescent material. These were apparently formed by coalescence of the smaller masses observed earlier. Fig. 4 shows such a mass occupying most of the protoplast. Chloroplasts were observed as unstained dark bodies. No fluorescence was observed when protoplasts were similarly treated with conjugated normal globulin. Percentages of protoplasts showing fluorescence increased rapidly (from 3 to 31%) from 12 to 48 h incubation, and more slowly thereafter (Table 3).

DISCUSSION

In this investigation, up to 38% of cowpea mesophyll protoplasts were infected by CYMV (Fig. 8). The time-course study, involving the infectivity assay and fluorescent antibody staining, indicated synchronous infection resulting in single step growth of CYMV, with a growth curve similar to those reported for other viruses (Takebe & Otsuki, 1969; Hibi & Yora, 1972; Hibi et al. 1975). The amount of virus accumulated in the protoplasts was apparently large as revealed by the extensive fluorescence observed in the infected protoplasts and by electron microscopy of protoplast extracts at dilutions of 100-fold (Fig. 5).

CYMV requires poly-L-ornithine to infect a large percentage of protoplasts. Poly-L-ornithine has been shown to be essential for infecting tobacco protoplasts with several viruses (Aoki & Takebe, 1969; Takebe & Otsuki, 1969; Otsuki & Takebe, 1973; Motoyoshi et al. 1973; Kubo, Harrison, Robinson & Mayo, 1975; Huber et al. 1977), Brassica protoplasts with TYMV (Renaudin et al. 1975) and cowpea protoplasts with TMV (Koike et al. 1976). All these viruses are negatively charged at the pH ranges used for inoculation; the negatively charged CPMV and CMV, on the other hand, do not require poly-L-ornithine to infect a high percentage of cowpea protoplasts (Hibi et al. 1975; Koike et al. 1976). Thus, the requirement of poly-L-ornithine seems not to be a universal feature of negatively charged viruses.

The evidence, presented for CYMV with regard to pre-incubation of virus with poly-L-ornithine, shows that virus infection was enhanced by the presence of the polycation (Table 1). However, it would seem that the requirement for poly-L-ornithine is less marked with CYMV than with TMV (Takebe & Otsuki, 1969). The actual mechanism of action of poly-L-ornithine is not conclusively known.

The results of inoculation with CYMV under the inoculation conditions used in this investigation are strikingly similar to those with PVX, the type member of the potexvirus group (Fenner, 1976) and for which the optimal conditions for infection have been worked
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out (Otsuki et al. 1974). The most important similarity is in response to pH of the inoculation medium. CYMV required an optimal pH of 6.0 for maximum infection, PVX also required a very similar but broader optimal pH (around 5.8). The reason for such a requirement has not been fully explained although, for certain virus-protoplast combinations at least, the electrical charge on the virus particles seems to be very important. The isoelectric point of CYMV is reportedly 5.3 (Purcifull & Shepherd, 1964) and, in this investigation, infection was low at pH 5.4 (Fig. 8). Since the net charge on the virus particles is zero at the isoeionic point, one would expect very little virus adsorption to the surface of the protoplast membrane if this depends on the charge of virus particles. It will therefore be interesting to determine whether, as the pH value increases, there are increases in the net negative charge of the virus particles to an optimum at pH 6.0 which for CYMV causes maximum adsorption to the protoplast surface. Finally, it should be pointed out that the kind of buffer in the inoculum may be important, because inocula prepared in phosphate buffer at pH 6.0 gave more infection with tobacco rattle virus than those prepared in citrate buffer (Kubo et al. 1976).

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REFERENCES


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