Infection of Cowpea Mesophyll Protoplasts with Cowpea Mosaic Virus

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Mesophyll protoplasts were isolated from the primary leaves of cowpeas by a one-step procedure using a mixture of Macerozyme and Cellulase. The protoplasts were inoculated with cowpea mosaic virus and virus multiplication was shown to occur by measuring the virus infectivity at various times after inoculation. Eighty to ninety-six percent of the protoplasts were infected as shown by fluorescent antibody staining. More than $10^8$ progeny virus particles were produced per infected protoplast. A cytopathic structure similar to the structure found in CPMV infected leaf cells occurred in infected protoplasts. Poly-L-ornithine was not essential for CPMV infection of the cowpea mesophyll protoplasts, but it had a stimulating effect. This is the first protoplast system after the tobacco mesophyll protoplast system suitable for virus infection studies. As cowpeas belong to a plant family other than tobacco, the new protoplasts allow comparative infection studies with plant viruses.

INTRODUCTION

The recent development of procedures for the isolation of plant protoplasts and their infection with viruses has greatly enhanced the possibilities for studying plant virus infection at the cellular level. However, only protoplasts from plant species belonging to the Solanaceae, such as petunia, tomato, and tobacco have been used for virus infection (Hibi et al., 1968; Cocking and Pojnar, 1969; Takebe and Otsuki, 1969; Otsuki and Takebe, 1973; Motoyoshi et al., 1973b; Otsuki et al., 1974). A weak point with these systems is that leaves or other tissues, from which protoplasts suitable for virus infection can be isolated, can be obtained only after a long period of growth. Control of the physiological conditions of the plants during such periods is difficult, yet these conditions are important for the survival of the isolated protoplasts and especially for their efficient infection by virus.

We have found that mesophyll protoplasts suitable for studies of plant virus infection can be isolated from the primary leaves of cowpeas belonging to the family Papilionaceae. In this paper we describe the conditions for their isolation and for their infection by cowpea mosaic virus (CPMV). CPMV is a plant virus with a genome distributed between two separate nucleoprotein particles (see Van Kammen, 1972 for review). Our main purpose in isolating cowpea mesophyll protoplasts was to obtain a cellular system with which the genetic information in each of the nucleoprotein components of CPMV could be studied. However, the use of such protoplasts may find a wider application in the study of plant virus infection.

MATERIALS AND METHODS

Isolation of cowpea mesophyll protoplasts. Vigna unguiculata (L.) Walp var. 'Blackeye Early Ramshorn' seeds were germinated in moistened vermiculite at 25°C in the dark. After 2 days the seedlings were put on pots of Hoagland solution in a chamber (Vötsch, Miniphyt VTPH) under
controlled conditions of light, temperature, and humidity: 28°C, 70% relative humidity, 14 hr lights of about 10,000 lux by 12 white fluorescence tubes and 12 150-W bulbs and 22°C, 70% relative humidity, 10 hr darkness each day. Fully expanded primary leaves of 9- to 11-day-old plants were used for the isolation of protoplasts. Protoplasts from younger or older leaves had a low rate of survival in the subsequent inoculation and incubation procedure and a lower percentage of such protoplasts became infected.

The leaf surfaces were sterilized by immersing the leaves in 70% ethanol for 5 set and then in approximately 0.2 M sodium hypochlorite for 20 min, followed by washing several times in double-distilled water. The lower epidermis was peeled off with a forceps and two g of leaf pieces were put into a 100-ml Erlenmeyer flask with 20 ml of enzyme. The enzyme solution contained 1% Cellulase Onozuka R-10 (Kinki Yakult Manuf. Co.), 0.05% Macerozyme R-10 (Kinki Yakult Manuf. Co.) and 0.6 M D-mannitol, pH 5.5. The leaf tissue was infiltrated with enzyme solution in vacuo for 10 min and then the flask was incubated at 25°C in a shaker bath at a frequency of 90 excursions per min. After 30 min the enzyme solution was decanted to remove broken cells and some spongy cells, and 20 ml of fresh enzyme solution were added. The incubation was then continued for 3 hr. The mixture was filtered through a 150-mesh stainless-steel filter and the protoplasts were collected by centrifugation of the filtrate at 800 rpm for 2 min. The protoplasts were washed three times by resuspending them in 0.6 M mannitol and sedimenting at 700 rpm for 2 min, and finally they were resuspended in 0.6 M mannitol to a concentration of ca. 5 x 10^5/ml. Concurrently a solution of purified CPMV, 10 µg/ml, was made in 0.02 M potassium citrate buffer, pH 5.2, containing 0.6 M mannitol and 1 µg/ml poly-L-ornithine. The CPMV solution was kept at 25°C for 5 min and then it was mixed with an equal volume of the protoplast suspension. The final concentration of protoplasts, virus, and poly-L-ornithine in the inoculum were thus ca. 2.5 x 10^5/ml, 5 µg/ml, and 0.5 µg/ml, respectively. The inoculum was kept at 25°C for 15 min and then the protoplasts were washed by three cycles of centrifugation at 600 rpm for 2 min and resuspending in sterile 0.6 M mannitol, 10 mM CaCl₂ solution to remove the not-adsorbed virus.

Incubation of protoplasts for virus multiplication. The washed inoculated protoplasts were transferred aseptically into incubation medium. The incubation medium was the same as that used by Aoki and Takebe (1969) for tobacco mesophyll protoplasts except that the concentration of mannitol was 0.6 M instead of 0.8 M, 6-benzyladenine was omitted, and the concentration of Cephaloridin was 1 mg/ml instead of 300 µg/ml. The concentration of protoplasts was made ca. 2.5 x 10^5/ml. Incubation was stationary in 10-ml portions in 100-ml Erlenmeyer flasks at 25°C under continuous illumination (ca. 2200 lux).

Infectiuity assay of protoplast extracts.
After appropriate periods of incubation the protoplasts from a 10-ml portion were collected by centrifugation, washed once with 0.6 \( M \) mannitol containing 10 \( mM \) \( CaCl_2 \), and finally suspended in 0.25 ml of 0.1 \( M \) phosphate buffer, pH 7.0. The suspension of protoplasts was homogenized in a minipotter homogenizer and the homogenate was centrifuged at 17,000 \( g \) for 15 min. The supernatant was aspirated with a pipet and its volume adjusted to 0.25 ml with 0.1 \( M \) phosphate buffer. The infectivity of the extract was then tested in a local lesion assay on six half leaves of *Phaseolus vulgaris* L. var. 'Pinto.' If necessary, the extracts were diluted with 0.1 \( M \) phosphate buffer, pH 7.0, so that lesion counts would not exceed 100 per half leaf. In each assay the other halves of the leaves were inoculated with 0.5 \( \mu g \) CPMV/ml as a control.

**Fluorescent antibody staining of infected protoplasts.** Fluorescein isothiocyanate-conjugated antibodies to CPMV were prepared according to the method for the preparation of anti-TMV fluorescent antibodies (Otsuki and Takebe, 1969). The preparation had a staining titer of 1:8 and the ratio of the absorbancies at 495 nm and 280 nm was \((A_{495}/A_{280}) = 1.9\). Samples of infected protoplasts were stained with fluorescent antibodies according to the staining procedure described for TMV-infected protoplasts (Otsuki and Takebe, 1969) except that 96% ethanol was used for fixation. The stained specimens were examined with a Wild fluorescence microscope in a dark field with BV filters. Yellow-green fluorescence was observed in the infected protoplasts, but not in healthy protoplasts nor in infected protoplasts which had previously been treated with unconjugated CPMV-antibodies.

**Electron microscopy.** Samples of infected and healthy protoplasts were collected by centrifugation after 24 hr of incubation. The protoplasts were prepared for electron microscopy by the method described above. The pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 hr, washed three times with 0.1 M phosphate buffer, and postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 1 hr. After being dehydrated in a concentrated alcohol series, the pellets were embedded in Epon. Ultra-thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. They were examined with a Jeol 100CX transmission electron microscope.
and stained for electron microscopy by the method described previously (Hibi and Yora, 1972), except that Spurr medium was used for embedding instead of Epon 812. The sections of protoplast pellets were examined under the Hitachi HU-12 electron microscope.

RESULTS

Growth curve. The increase of CPMV in cowpea mesophyll protoplasts was determined by measuring the infectivity of protoplast extracts at various times after inoculation (Fig. 2). A small amount of virus infectivity was found at the beginning of the incubation period and could be attributed to virus particles adsorbed to and taken up into protoplasts upon inoculation. The infectivity decreased to near zero during the next few hours. After 12 hr of incubation the infectivity in the protoplasts began to increase and continued to increase very rapidly until 24 hr. The rate of multiplication then decreased and the amount of virus reached a maximum at 48 hr of incubation. The dose of virus particles per protoplast in the inoculum (5 µg CPMV/ml, ca. 2.5 x 10⁶ protoplasts/ml) was about 10⁶. The average number of virus particles adsorbed per protoplast was estimated to be about 10⁵ from the infectivity of the protoplasts at time zero. Therefore, it appears that approximately 0.1% of the virus particles in the inoculum was adsorbed to protoplasts. The average yield of progeny virus per infected protoplast was estimated to be about 3.6 x 10⁶ from the infectivity of the extracts of protoplasts after 48-hr incubation, and the number of infected protoplasts as determined by fluorescent antibody staining. These calculations indicate that the virus particles increased at least 1000-fold over the initial number during the 48 hr of incubation. Such calculations are rather approximate as they do not take into account that both middle and bottom component particles of CPMV are necessary for infectivity. Calculation of the number of virus particles was, for the sake of simplicity, based on an average molecular weight of 5.5 x 10⁸ daltons for an infectious CPMV particle (Geelen, 1973).

Accumulation of virus antigen. At various times after inoculation of the protoplasts with CPMV, samples of the protoplasts were stained with fluorescent CPMV antibodies and examined under the fluorescence microscope. Figure 3 shows the increase of the percentage of infected protoplasts detectable by fluorescent antibody staining at different times of incubation. The first fluorescence due to CPMV antigen was visible at 12 hr of incubation as weakly fluorescent specks mostly in the center of the protoplasts. The appearance of CPMV specific fluorescence coincided with the increase of virus infectivity in the

**Fig. 2.** The increase of CPMV in cowpea mesophyll protoplasts inoculated with CPMV. The protoplasts were incubated in 10-ml portions at a cell density of 2.5 x 10⁶/ml. The increase of CPMV was determined by measuring the infectivity of an extract of protoplasts from a 10-ml portion at various times after inoculation. Each point represents the total number of lesions produced on six half-leaves of *Phaseolus vulgaris*, var. 'Pinto.'
protoplasts (cf. Fig. 2). The percentage of infected protoplasts increased very rapidly from 13% to 91% between 12 and 24 hr of incubation and then more slowly to reach a maximum at 36 hr of incubation; at this time 96% of the protoplasts were found to fluoresce. Between 15 and 30 hr of incubation the virus antigen was observed as one or two big amorphous fluorescent masses in the cytoplasm of many protoplasts (see Fig. 4, arrow for example) distinctly different from the crystalloid structures which are seen for example in TMV-infected protoplasts. At 36 hr and later, when the amount of virus had reached a maximum, the fluorescence was even brighter and was found distributed throughout the cytoplasm as a network between the chloroplasts, which remained dark (Fig. 4).

Fig. 3. The increase of the percentage of CPMV-infected protoplasts, inoculated with CPMV. Samples of protoplasts at various times after inoculation were stained with fluorescent CPMV antibodies and the percentage of infected protoplasts was determined under the fluorescence microscope.

Fig. 4. Fluorescence micrograph of cowpea mesophyll protoplasts infected by CPMV and stained with fluorescent antibody at 48 hr of incubation. Viral antigen is seen as giant amorphous masses (arrow) and also dispersed in the cytoplasm. The scale represents 25 µm.
If the inoculated protoplasts were cultured in the dark the percentage of protoplasts with specific fluorescence after 24-hr incubation was reduced to 32% of that in the control cultured in the light.

The production of CPMV antigen was completely suppressed by adding cycloheximide (10 µg/ml) to the incubation medium. With chloramphenicol (200 µg/ml) in the incubation medium the number of fluorescent protoplasts after 24-hr incubation was reduced to 36% of the control. Actinomycin D (10 µg/ml) and 2-thiouracil (30 µg/ml) did not markedly reduce the percentage of infected protoplasts by comparison with the appropriate controls.

Electron microscopy. Ultrathin sections of CPMV-infected protoplasts, collected 24 hr after inoculation, were examined under the electron microscope. Most of the infected protoplasts contained cytopathic structures in the cytoplasm close to the nucleus (Fig. 5); the structures resembled those found in cells of CPMV-infected leaves. They consisted of many vesicles surrounded by electron-dense material, which did not seem to have a clear structure. Such cytopathic structures were not detected in healthy cowpea mesophyll protoplasts. It was very difficult to distinguish CPMV particles clearly in the infected protoplasts, but virus-like particles with a diameter varying from 17 to 22 nm could be recognized frequently in the cytopathic structures (Fig. 5). Apparently CPMV does not form crystalline aggregates in infected protoplasts, nor does it do so in infected leaves. No virus-like particles were found in chloroplasts, mitochondria, or the central vacuole.

![Fig. 5. Electron micrograph of a cowpea mesophyll protoplast infected with CPMV, 24 hr after inoculation. The cytopathic structure (CPS) is found near the nucleus (N), vesicles (Ve), amorphous electron-dense materials (A), and virus-like particles (V) are seen in the cytopathic structure. Furthermore a osmiophilic globule (O), a mitochondrion (M), and part of the central vacuole (CV) can be seen. ×35,000](image-url)
Effects of the inoculation conditions on the frequency of infection. Cowpea mesophyll protoplasts were inoculated with CPMV under various conditions and the percentage of infected protoplasts was determined by staining with fluorescent CPMV antibodies after 36-41 hr of incubation in the culture medium. Figure 6 shows that the percentage of infected protoplasts increased with the concentration of CPMV in the inoculum up to a concentration of 3 μg/ml. At that concentration 90-97% of the protoplasts showed fluorescence. The same frequency of infection was obtained with higher virus concentrations in the inoculum. In the experiment of Fig. 6 the concentration of protoplasts in the inoculating mixture was 1.3 × 10⁶/ml. At a virus concentration of 0.1 μg/ml, at which 52% of the protoplasts became infected, approximately 8.5 × 10⁴ virus particles were thus available per protoplast. Therefore, the multiplicity of infection, being the number of virus particles per protoplast, must be at least 8.5 × 10⁴ to obtain approximately 50% infected protoplasts. The given numbers of particles have only comparative significance.

The effect of the pH of the inoculation medium on the frequency of CPMV infection is shown in Fig. 7. The percentage of infected protoplasts was determined after 36 hr of incubation by staining the protoplasts with fluorescent antibodies.

FIG. 6. The effect of virus concentration in the inoculum on the frequency of CPMV infection. Protoplasts were inoculated at pH 5.2 in the presence of 0.5 μg/ml poly-L-ornithine with various virus concentrations. The percentage of infected protoplasts was determined after 41 hr of incubation by staining the protoplasts with fluorescent antibodies.
which became infected is shown in Fig. 8. A high percentage of infection of cowpea mesophyll protoplasts with CPMV was found when poly-L-ornithine was omitted from the inoculum. This is in marked contrast to tobacco mesophyll protoplasts in which infection by different viruses (Takebe and Otsuki, 1969; Motoyoshi et al., 1973b; Otsuki and Takebe, 1973; Otsuki et al., 1974) required poly-L-ornithine in the inoculum. Preincubation of the cowpea protoplasts and of CPMV with poly-L-ornithine was, however, necessary for a constantly high percentage of infection in every experiment. Poly-L-ornithine apparently stimulated the infection. Concentrations of poly-L-ornithine above 1 µg/ml caused severe damage to the protoplasts as illustrated by the decrease of the percentage living protoplasts. Based on these observations the inoculation procedure described in Materials and Methods was adopted as the standard method.

DISCUSSION

After the report of Takebe et al. (1968) and Takebe and Otsuki (1969) on the isolation of mesophyll protoplasts from tobacco leaves and their infection with TMV similar infections with a number of different viruses have been demonstrated (Otsuki and Takebe, 1973; Motoyoshi et al., 1973b; Otsuki et al., 1974).

In the present paper we show that mesophyll protoplasts from the primary leaves of cowpeas belonging to the plant family of Papilionaceae, are also suitable for virus infection studies. The mesophyll protoplast system from cowpea has a number of advantages over tobacco protoplasts. The cowpea plants can be grown easily under controlled conditions as described under materials and methods. Such controlled conditions of growth are essential if plants with leaves that provide protoplasts, which can be infected with virus, are to be obtained reproducibly. The primary leaves of cowpea are suitable between 9 and 11 days after the seeds have been sown under the conditions given. Tobacco plants, on the other hand, have to be grown for about 60 days before leaves suitable for protoplast isolation are produced, and it is more difficult to keep the growth conditions constant.

The method that we used for the isolation of protoplasts from the primary leaves of cowpea was a single-step procedure which involved incubation of the leaf tissue with a solution of Cellulase and Macerozyme. This procedure is simpler and more convenient than the two-step procedure developed by Takebe et al. (1968) for the isolation of tobacco mesophyll protoplasts, and it provides high yields of intact protoplasts. In the procedure of Takebe et al., the cells are first isolated by incubation of the tissue with Macerozyme, and subsequently converted into protoplasts by treatment with Cellulase. The one-step method has also been used successfully to isolate intact protoplasts from various tis-

Fig. 8. The effect of poly-l-ornithine concentration on the frequency of CPMV infection. Protoplasts were inoculated with 5 µg/ml CPMV at pH 5.2 in the presence of various poly-l-ornithine concentrations. The percentage of living protoplasts and the percentage of infected protoplasts stainable with fluorescent antibodies was determined after 36 hr of incubation.
issues of different plants such as petunia leaves (Hibi et al., 1968), petunia petals, carrot roots, turnip leaves, tobacco callus tissue (Hibi et al., unpublished results) and cereal leaves (Evans et al., 1972; Hibi et al., unpublished results). The yields of intact protoplasts from these tissues were equal to or higher than those obtained by the two-step method. However, the one-step method gave lower yields when it was used for the isolation of tobacco mesophyll protoplasts.

The protoplasts isolated from cowpea leaves by the one-step method consist of a mixture of palisade and spongy mesophyll protoplasts and some epidermal protoplasts. During the preincubation some spongy mesophyll protoplasts are liberated and these are discarded. The epidermal protoplasts are removed in the washing procedure after the protoplast isolation; the mesophyll protoplasts are sedimented by centrifugation in 0.6 M mannitol at 700 rpm for 2 min and the epidermal protoplasts remain in the supernatant. Therefore, the protoplast suspension used for infection contains mainly palisade protoplasts and some spongy mesophyll protoplasts. This heterogeneity in the population of cowpea mesophyll protoplasts does not seem to be a disadvantage in infection studies, since most of the protoplasts (80–96%) were infectible with CPMV.

The optimal conditions of inoculation for CPMV infection have been studied in some detail. The optimal pH for infection with CPMV is about 5.2, the lowest pH at which the protoplasts are still stable. The effect of pH on the percentage of protoplasts which became infected with CPMV (Fig. 7) is analogous to that of tobacco mesophyll protoplasts infected with TMV (cited in Otuski et al., 1974), CMV (Otsuki and Takebe, 1973) and CCMV (Motoyoshi et al., 1973b). Virus infection of mesophyll protoplasts from cowpea and tobacco differ strikingly in their dependency on poly-L-ornithine. Cowpea mesophyll protoplasts can be infected with CPMV (Fig. 8), and also with TMV (results not shown) in the absence of poly-L-ornithine, although poly-L-ornithine stimulated the infection by these viruses. On the other hand poly-L-ornithine has been shown to be essential for infection of tobacco mesophyll protoplasts with TMV (Takebe and Otsuki, 1969), CMV (Otsuki and Takebe, 1973), CCMV (Motoyoshi et al., 1973b), and PVX (Otsuki et al., 1974). We have established (Van Kammen et al., to be published) that poly-L-ornithine is also essential for the infection of tobacco mesophyll protoplasts with CPMV. This indicates an interesting difference between the plasmalemma of cowpea mesophyll and tobacco mesophyll protoplasts. It is not yet clear whether this difference affects the virus adsorption or virus entry into the protoplasts, or both. At present, two opposing views are held concerning the role of poly-L-ornithine in the infection of tobacco protoplasts by virus. On the one hand, it is assumed that virus particles are taken up into the protoplasts by phagocytosis (Cocking, 1966; Cocking and Pojnar, 1969; Hibi and Yora, 1972; Otsuki et al., 1972; Honda et al., 1974) and that poly-L-ornithine enhances phagocytic activity (Takebe and Otsuki, 1969; Otsuki et al., 1972). On the other hand, it has been suggested that virus particles enter into the protoplasts nonphysiologically due to local lesions on the plasmalemma caused by poly-L-ornithine (Burgess et al., 1973a; Burgess et al., 1973b). It will be of interest to investigate the virus entry process into the cowpea protoplasts in the absence of poly-L-ornithine, and to investigate the stimulating role of poly-L-ornithine.

It was calculated that about $10^6$ inoculum virus particles are necessary for the infection of one protoplast in the cowpea system. This value is similar to those estimated for the infection of tobacco mesophyll protoplasts by TMV ($10^8$; Takebe and Otsuki, 1969) and by CCMV ($3-5 \times 10^5$ Motoyoshi et al., 1973b). The average number of virus particles adsorbed to one protoplast after washing the protoplasts from the inoculum, was roughly estimated to be $10^4$, which is close to the values obtained in the infection of tobacco mesophyll protoplasts by TMV ($10^5$; Takebe and Otsuki, 1969; $10^4-10^5$: Hibi and Yora,
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1972), and by CCMV (760: Motoyoshi et al., 1973a). In evaluating these numbers one should realize that the CPMV particles referred to are a mixture of middle and bottom component particles, both of which are necessary for infection (Van Kammen, 1968). The average yield of progeny virus per infected cowpea protoplast was about $3-4 \times 10^6$, which is in the same range as the numbers of particles produced in tobacco mesophyll protoplasts by TMV (0.5–1.1 × 10^6; Takebe and Otsuki, 1969; 1.5 × 10^6; Hibi and Yora, 1972), by CCMV (10^6–10^7; Motoyoshi et al., 1973b) and by CPMV (4 × 10^6; Van Kammen et al., to be published).

The viral infectivity in the inoculated cowpea protoplasts began to increase after 12 hr of incubation in the culture medium at the same time that viral antigen was first detectable by fluorescent antibody staining (Figs. 2 and 3). Ninety-one percent of the protoplasts showed specific fluorescence after 24 hr of incubation and between 12- and 24-hr incubation a rapid increase of virus infectivity occurred. The combination of these observations indicates that the CPMV multiplication in the protoplasts proceeds fairly synchronously. The possibility of secondary infection by progeny virus was excluded by control experiments which showed that CPMV is unable to infect protoplasts if inoculated in the culture medium without poly-L-ornithine.

Characteristic cytopathic structures consisting of vesicular membranes embedded in amorphous opaque material (Van der Scheer and Groenewegen, 1971; De Zoeten et al., 1974) have been observed in the cytoplasm of cells of cowpea leaves infected with CPMV. It has been demonstrated that CPMV-RNA replication is associated with the vesicular membranes in the cytopathic structures (Assink et al., 1973; De Zoeten et al., 1974). Similar structures were found to develop in cowpea protoplasts infected with CPMV (Fig. 5). The circularly averaged diameter of negatively stained CPMV particles is about 20 nm and the maximum diameter measured between extreme projections on the periphery is 24 nm (Crowther et al., 1974). Although it was very difficult to distinguish virus particles clearly in the infected protoplast by electron microscopy, many virus-like particles with a diameter of 17–22 nm could be recognized in the cytopathic structures, and no other comparable giant accumulations of virus-like particles could be observed in the cytoplasm. By fluorescent antibody staining the CPMV-antigen appeared to occur in the cytoplasm in one or two giant amorphous masses (Fig. 4). This suggests that the fluorescent amorphous masses correspond to the virus-like particles in the cytopathic structure.

The inhibitor studies show that cycloheximide suppresses CPMV multiplication completely. This might indicate that the 80 S ribosomal system in the cytoplasm is responsible for the synthesis of CPMV-specific proteins.

The effect of chloramphenicol appears to be more complicated. Both infected protoplasts cultured in the light with chloramphenicol and those cultured in the dark without chloramphenicol produced less virus antigen than the control incubated under standard conditions. Possibly the functioning of the chloroplasts, influences the metabolic activities in the cytoplasm.

Such phenomena have not been observed for tobacco mesophyll protoplasts infected by TMV (Takebe and Otsuki, 1969), CMV (Otsuki and Takebe, 1973) and PVX (Otsuki et al., 1974).

Cowpea mesophyll protoplasts can also be infected with CMV (Koike et al., unpublished results), TMV (Hibi et al., unpublished results), and Alfalfa mosaic virus (Bakhuizen and Hibi, unpublished results). In conclusion, the cowpea mesophyll protoplasts provide an additional, more convenient protoplast system for virus infection studies, and it also enables comparative investigations to be done on the infection of different host protoplasts with various plant viruses.

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