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A Simplified Method of Obtaining Tobacco Protoplasts for Infection with Tobacco Mosaic Virus

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SUMMARY

Incubating tobacco leaf tissue, from which the lower epidermis was peeled, overnight with 0·3 to 0·4 % Macerozyme and 0·6 to 1·2 % cellulase, depending on leaf condition, produced a good yield of protoplasts that were susceptible to infection by TMV. Fluorescent antibody staining showed that 20 to 80 % of protoplasts became infected, and infectivity tests indicated that an average of about 4×10^6 virus particles/infected protoplast, or 100 to 500 μg virus/10^6 protoplasts were produced by 2 days after inoculation. The production and infection of protoplasts depended less on the season when the plants were grown than with the 'two-step' method. Also, plentiful stable protoplasts were obtained without adding potassium dextran sulphate to the macerating medium. Calcium is required in the incubating medium for virus infection but can be partially replaced by Mg, which is not essential in the presence of Ca. Virus attained the greatest concentration when the protoplasts were inoculated as soon as they were washed free from the enzymes.

INTRODUCTION

Tobacco protoplasts have been infected with tobacco mosaic virus (TMV) (Takebe & Otsuki, 1969; Coutts, Cocking & Kassanis, 1972) and three other viruses (Otsuki & Takebe, 1972, 1973; Motoyoshi *et al.* 1973), using a 'two-step' method. In this method, pieces of stripped tobacco leaf are incubated first with Macerozyme, which attacks the pectin of the middle lamella and separates the mesophyll cells; these are then incubated with cellulase to remove their cell walls and release the protoplasts. Macerozyme first separates spongy parenchyma cells, but these are discarded so that protoplasts from palisade parenchyma cells can be collected separately as they are believed to be more suitable for virus infection. A practised person can complete both incubations in 5 to 6 h but the necessary shaking damages some protoplasts. We therefore tried to simplify the method by incubating overnight in mixed enzymes as described by Power & Cocking (1970), but found the protoplasts could not be infected with virus. This paper describes how we modified the method to produce susceptible protoplasts and comments on the role of some of the chemicals used.

METHODS

Plants. We used middle leaves (20 to 25 cm long) from 7- to 9-week-old tobacco plants (*Nicotiana tabacum* L. White Burley type cv. Judy's Pride), grown as described by Motoyoshi *et al.* (1973).

Preparation of protoplasts. The leaves were washed in distilled water and then allowed to wilt for about 45 min. The lower epidermis was peeled off by inserting fine jeweller's forceps into the main veins and pulling the epidermis away. Leaf pieces of 3 to 6 cm^2 were

cut and placed, peeled side down, in Petri dishes (14·5 cm diam.) each containing 30 ml of 0·3 to 0·4 % Macerozyme (Yakult Biochemicals Co Ltd., Nishinomiya, Japan), 0·6 to 1·2 % cellulase 'Onozuka' SS (All Japan Biochemicals Co Ltd, Nishinomiya, Japan) and 13·2 % (w/v) D-mannitol. We describe later how the concentration of enzymes was varied depending on the state of the plants. The dishes were placed overnight in a dark incubator at 25 °C. After 17 h, the dishes were gently swirled and the released protoplasts passed through butter muslin to separate them from debris. Haemocytometer counts showed that one Petri dish, well covered with leaf pieces, produced 5 to 10×10^6 protoplasts. The protoplasts were removed from the enzyme solution by centrifuging at 35 ***g*** for 3 min, washed three times with 15 ml of 13·2 % (w/v) mannitol and finally suspended in infection medium. The protoplasts were pelleted by centrifuging at 35 ***g*** for 3 min after the first two washes and at 100 ***g*** for 2 min after the third wash.

Infection and incubation of protoplasts. The infection medium consisted of 0·02 M-potassium citrate buffer, pH 5·2, containing 13·2 % (w/v) mannitol, 4 μg/ml poly-L-ornithine (approx. mol. wt. 120000) and 2 μg/ml of purified TMV. The medium was incubated in a conical flask at 25 °C for 10 min and 5 ml added to each centrifuge tube containing the 100 ***g*** protoplast pellet and 5 ml of a 13·2 % (w/v) solution of mannitol. The mixture was returned to the conical flask and incubated at 25 °C for 10 min while gently rocked (15 oscillations/min). Inoculated protoplasts were then centrifuged from the infection buffer at 35 ***g*** for 3 min and washed three times with 13·2 % (w/v) mannitol solution containing 0·1 mM-$CaCl_2$. The washed protoplasts were suspended in modified Aoki & Takebe (1969) medium containing 13·2 % (w/v) mannitol, 0·2 mM-KH_2PO_4, 1 mM-KNO_3, 0·1 mM-$MgSO_4$, 10 mM-$CaCl_2$, 1 mM-KI, 0·01 μM-$CuSO_4$ and gentamycin* at 10 μg/ml, and adjusted to pH 5·4. Sterility was not maintained when preparing and incubating the protoplasts, so there was often yeast contamination after 48 h incubation but this did not seem to affect virus yield. When testing how different factors influenced virus multiplication one protoplast suspension was used for all treatments and subdivided only just before incubation, using $2{\cdot}5 \times 10^6$ protoplasts/treatment. The protoplasts were incubated in light at 28 °C because they produced no virus in darkness.

Infectivity tests. Samples of 10^6 protoplasts were centrifuged from the incubation medium and the pellets homogenized by hand, using a glass rod fitting closely inside the centrifuge tube. The homogenate was suspended in 2 ml of water and inoculated, without carborundum, to tobacco cv. Xanthi-nc alongside 4 dilutions of known concentrations of TMV (1, 0·2, 0·04, 0·008 μg/ml), using 8 half leaves per treatment. The virus concentrations of the protoplast suspensions were estimated by comparing the log number of lesions obtained with those produced by the known virus concentrations, and assuming that the virus from the protoplasts had the same specific infectivity as the standard virus.

Serological tests. The serological titre of the virus in the protoplast extracts was estimated using the tube precipitation test. The antiserum to TMV had a titre of 1/1600 and was used at 1/100 dilution.

Fluorescent antibody staining. We followed the method of Otsuki & Takebe (1969) except that we used swine anti-rabbit γ-globulin labelled with fluorescein isothiocyanate (Fraburg Ltd, Maidenhead, Berks.). The protoplasts were stuck on slides smeared with Haupt's adhesive, where they were incubated with TMV antiserum (diluted 1/20), washed and then incubated with the anti-rabbit labelled serum (diluted 1/20). After a further wash they were examined with a Leitz Orthoplan fluorescence microscope. Using antisera to tobacco

* Recently we have obtained higher virus yields when gentamicin was replaced with carbenicillin at 100 μg/ml.

necrosis virus and White Burley tobacco protein as controls we obtained respectively no fluorescence and 100% protoplasts fluorescing.

Water, glassware, etc. Successful infection depended on excluding chemical impurities. We used glass-distilled, de-ionized water and treated all glassware with chromic acid before washing it thoroughly with distilled water. All bottles were plastic and were washed with detergent, and all solutions were made freshly each week; stock solutions were used only for the incubation medium.

RESULTS

Production of protoplasts

Using the 'two-step' method, Coutts *et al.* (1972) found that infection rates differed with the season and condition of the plants. Overnight incubation in mixed enzymes gave consistent good yields of protoplasts and virus from hard or soft plants, although plants like those described by Motoyoshi *et al.* (1973) were best.

Power & Cocking (1970) found tobacco leaves released most protoplasts when treated for 4 h with 0·4% pectinase and 4% cellulase. At these concentrations incubating overnight released protoplasts but they remained clumped in groups of 20 or more, even after washing off the enzymes. More than half of the cells looked disorganized instead of having the chloroplasts evenly distributed near the surface. The protoplasts remained clumped during infection, turned brown after 24 h, and produced no virus in 48 h. No infection could be detected even when the protoplast extract was inoculated undiluted. Adding 0·3 or 0·6% potassium dextran sulphate to the enzymes did not improve the result. By contrast, we obtained good protoplasts when the leaf tissues were incubated overnight with 0·3% Macerozyme and 0·6% cellulase and nearly all the protoplasts were released overnight (17.00 to 10.00 next day) but remained *in situ* until the Petri dishes were gently swirled around several times. These protoplasts were always separate from each other and looked in excellent condition. All the experiments reported in this paper were made in the summer and early autumn. However, in the autumn when the plants were given supplementary artificial light, the yield of protoplasts and the virus concentration gradually decreased. The yield of protoplasts was restored and virus yield increased three times when Macerozyme was increased to 0·4% and cellulase to 1·2%. When using these greater concentrations of enzymes the pH tended to decrease but was re-adjusted to 5·4.

Virus concentration

To test infectivity we extracted 10^6 protoplasts in 2 ml water. Protoplast samples incubated for 24 h and 48 h after infection were diluted respectively, 1/10 and 1/100, and inoculated without carborundum. The average number of lesions per half leaf in different experiments varied from 50 to 100 for both samples. When the numbers of lesions obtained in many experiments were converted into concentration, 10^6 protoplasts produced on average 10 and 130 μg of virus, respectively, after 24 and 48 h incubation. Assuming the mol. wt. of TMV to be 4×10^7, there would be $1{\cdot}5 \times 10^{10}$ particles/μg of virus. Therefore, in the 48 h sample there would be on average 2×10^6 virus particles/protoplast if all were infected. This is similar to the concentration obtained by Takebe & Otsuki (1969) and Coutts *et al.* (1972). The serological titre of the 48 h sample ranged from 1/4 to 1/32. Fluorescent antibody staining made in a few experiments showed that between 20% and 80% of the protoplasts became infected, so the number of virus particles/infected protoplast was probably sometimes as high as 10^7. Samples taken 72 h after infection had no more virus than 48 h samples and tests showed that very little virus was lost into the medium.

Early stages of infection

After inoculation the protoplasts were washed three times, so despite using inoculum containing 1 μg/ml, the amount of virus adsorbed on the protoplasts was too small to be detected when the 0 h sample was inoculated to test plants without carborundum. To detect the early stages of virus multiplication, samples taken at 0, 2, 4, 6, 8 and 10 h were inoculated onto leaves that had been dusted with carborundum and produced, respectively, an average of 23, 21, 18, 28, 228 and 395 lesions/half leaf. The results do not show a pronounced decrease in infectivity (eclipse period) 4 h after inoculation, as reported by Takebe & Otsuki (1969), but confirm that after 8 h there is considerable increase in infectivity. In this experiment the final virus concentration was 380 μg/10^6 protoplasts.

In another experiment we attempted to demonstrate the presence of free virus RNA just before the first whole virus appeared as shown by the increased infectivity 8 h after infection. The RNA from infected protoplasts was extracted 6 h after inoculation by the phenol method, either directly from the protoplasts or these were first extracted in buffer, left overnight at 20 °C to destroy any free virus RNA, and then extracted with phenol. No evidence for the presence of free virus RNA was obtained. Using carborundum, the infectivity of both extracts was small, each averaging 4 lesions/half leaf as against 1 lesion produced by the phenol extract of the 0 h sample. In this experiment the final virus concentration was 100 μg/10^6 protoplasts.

Delaying infection

Normally protoplasts were infected immediately after washing away the enzymes. Delaying infection by 4 h halved the amount of virus, as compared with protoplasts that were infected immediately and there was no virus in protoplasts where infection was delayed 24 h. The protoplasts that were infected immediately after washing produced 112 μg virus/10^6 protoplasts. By contrast, we found that released protoplasts could be left in the enzyme solution for 4 h without decreasing the eventual virus concentration.

Chemical composition of the media

In the 'two-step' method 0·3% potassium dextran sulphate is added to the macerating medium together with 0·5% Macerozyme (Takebe, Otsuki & Aoki, 1968). Recently, Motoyoshi *et al.* (1973) increased the dextran concentration to 0·5%. This polyanion was added because it markedly improved the yield of intact protoplasts. In the mixed enzyme method we found it made no difference to the yield of intact protoplasts or the virus yield. The amount of virus produced by 10^6 protoplasts 48 h after infection was 316 μg with potassium dextran sulphate and 356 μg without. We therefore omitted this rather expensive chemical.

The infection medium is buffered with potassium citrate and because the protoplasts are exposed to this chelating substance we thought they might require the presence of $CaCl_2$ and $MgSO_4$ in the incubation medium. However, when we substituted sodium acetate for potassium citrate much less virus was produced. At pH 5·2, 48 h after infection 10^6 protoplasts produced 112 μg of virus with potassium citrate, but only 20 μg with sodium acetate.

Calcium chloride is used in the washing and incubating solutions after the protoplasts are infected. In the incubation medium Takebe *et al.* (1968) used 0·1 mM-$CaCl_2$ but Aoki & Takebe (1969) increased this to 10 mM without explaining why. We detected no virus multiplication when $CaCl_2$ was omitted from the washing and incubating solutions, even when the protoplasts were infected using sodium acetate buffer, but they appeared in good

condition even 48 h after infection. We obtained 500 μg of virus from 10^6 protoplasts when using 0·1 mM- and 10 mM-$CaCl_2$, respectively, during washing and incubating, 440 μg of virus when the concentrations were respectively 0·1 mM and 50 mM, and 280 μg of virus when they were 10 mM and 10 mM. Thus the concentrations of 0·1 mM and 10 mM for washing and incubation, respectively, as recommended by Aoki & Takebe (1969), gave most virus. Although $CaCl_2$ is needed for virus multiplication it can be replaced to some extent by $MgSO_4$. In two experiments where 10 mM-$MgSO_4$ was substituted for 10 mM-$CaCl_2$ the amount of virus was reduced to a fifth.

To test whether $CaCl_2$ influences the yield of virus, we delayed adding it to the incubation medium for 4 or 24 h. After incubating inoculated protoplasts for 4 or 24 h without $CaCl_2$, the incubation medium was changed to one with $CaCl_2$. Extracts, from protoplasts kept for 24 h after inoculation without $CaCl_2$, produced no lesions even when tested undiluted. Yields were 2 μg and 112 μg of virus/10^6 protoplasts, respectively, when the $CaCl_2$ was delayed for 4 h or was present all the time. The effect therefore is on an early stage of infection.

The incubation medium of Aoki & Takebe (1969) contained 1 mM-$MgSO_4$, but we found more virus was produced when only 0·1 mM-$MgSO_4$ was used; yields were 250 μg of virus/10^6 protoplasts with 0·1 mM-$MgSO_4$ and 180 μg virus with 1 mM-$MgSO_4$. In another experiment the virus yield was unaffected by omitting $MgSO_4$ from the incubation medium, but nevertheless we continued to use 0·1 mM-$MgSO_4$.

The incubation medium normally contains 0·2 mM-KH_2PO_4 and 1 mM-KNO_3. Because callus tissue cultures grow better in high concentrations of N, P and K (Murashige & Skoog, 1962), we tested the effect of adding five times the usual concentration on virus yield of the protoplasts. Yields were 160 μg of virus/10^6 protoplasts with normal amounts of N, P and K and 130 μg with five times as much.

DISCUSSION

Power & Cocking (1970) used mixed enzymes and found that most protoplasts were released in 4 h incubation with 0·4 % pectinase and 4 % cellulase. Overnight incubation with these enzymes released nearly all protoplasts but these could not be infected with TMV. In the summer and early autumn we found 0·3 % Macerozyme and 0·6 % cellulase were sufficient to release the protoplasts of nearly all the cells overnight. There was important seasonal variation in the yield of protoplasts and in virus yield. Although the leaves looked more tender in late autumn, we had to increase the concentration of Macerozyme to 0·4 % and cellulase to 1·2 % to maintain the protoplast and virus yields we had in summer. These seasonal changes were gradual so that during the winter the concentration may need to be increased further. Power & Cocking (1970) reported a need to use more enzyme during winter.

Virus concentration was halved when inoculation was delayed for 4 h after washing. No virus was produced when inoculation was delayed for 24 h, possibly because a layer of cellulose had then been deposited around the protoplasts. The effect of delaying inoculation until some hours after washing away the enzymes seems not to have been tried before, but protoplasts prepared by the 'two-step' method may behave similarly.

We prefer to incubate overnight in mixed enzymes because the protoplasts are then ready for infection during the morning, and the rest of the day is available for experiments; also, shaking is unnecessary and a greater number of intact protoplasts are obtained. The physiological state of the plants is important for infection but is less critical than with the 'two-step' method. Our method and the 'two-step' method of Takebe & Otuski (1969)

gave similar final virus yields (2×10^6 virus particles/protoplast) and with both there was a rapid increase in virus 8 h after inoculation, but we could not show a drop in the residual infectivity between 0 and 4 h after inoculation. We could not find free virus RNA just before new whole virus was detectable, although tobacco necrosis RNA can be detected in leaves 6 h after inoculation and 2 h before whole virus appears (Kassanis & Welkie, 1963). The RNA from TMV is difficult to detect because it has less than 1 % of the infectivity of the same amount of RNA in nucleoprotein particles, whereas RNA from tobacco necrosis virus has 20 %. We had hoped that the synchronous infection of protoplasts would overcome this difficulty; in this respect however, protoplasts seem no better than leaves.

Potassium dextran sulphate was not required when using mixed enzymes. We still do not know how this polyanion protects protoplasts, although Takebe *et al.* (1968) suggested that the Macerozyme used contains some basic proteins that are toxic to tobacco cells and dextran sulphate prevents these from causing damage by binding them electrostatically. This explanation cannot be correct because, using the same Macerozyme, we obtained stable protoplasts without dextran. It is possible that the mixed enzyme method is more gentle and therefore the protective action of dextran is not needed.

Calcium chloride is needed for multiplication even when the chelator, potassium citrate, is omitted from the infection medium. Inoculated protoplasts produced hardly any virus when $CaCl_2$ was delayed, even for 4 h. This suggests that Ca is needed for the initiation of infection. Calcium is essential for the retention of ions and its absence quickly deranges membrane structure and function, causing leakage of ions (Epstein, 1971). However, these effects can seldom be remedied by replacing Ca with Mg, whereas we have shown that virus can multiply to some extent when $CaCl_2$ is replaced with $MgSO_4$. A number of authors have implicated Ca or other divalent metals in the infection of various phages (Fildes, Kay & Joklik, 1953). Possibly therefore TMV infection also requires the presence of a divalent cation, a requirement that could not be tested using plants instead of protoplasts.

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