TMV Inoculation of Tobacco Protoplasts in the Presence of Protoplast Fusion Agents

Alan C. Cassells

Department of Botany, University College, Cork, Ireland

and

Frances M. Cocker

Department of Horticulture, Wye College, University of London, Ashford, Kent, U.K.

Two methods used to induce protoplast fusion, involving polyethylene glycol (PEG) and high calcium/high pH, respectively, have been investigated in procedures for the inoculation of isolated tobacco protoplasts with TMV. It has been shown that inoculation in the presence of fusion agents involves two stages. Firstly, virus precipitation on to the protoplast surface and secondly, entrapment of virus particles between protoplasts in zones (putatively) of transient protoplast fusion.

Introduction

Virus inoculation of plant protoplasts, mediated by PEG, was first reported by Cassells and Barlass [1, 2] and subsequently for viral RNAs by Dawson *et al.* [3]. The use of this procedure was extended further by Maule *et al.* [4]. PEG causes both virus precipitation and protoplast fusion [5] at the concentrations used for virus inoculation of protoplasts.

Sarker *et al.* [6] also reported inoculation with viral RNA of plant protoplasts using high pH/high salt conditions. The latter procedure has also been modified for protoplast fusion [7].

This study was undertaken to clarify the relationship between protoplast fusion agents and virus inoculation of protoplasts with a view to elucidation and improvement of the PEG inoculation procedure.

Materials and Methods

Nicotiana tabacum cv. xanthi-nc plants were grown throughout the year in the glasshouse in 12.7 cm pots containing Irish Peat Moss supplied with a complete balanced fertilizer (Bio P Base; Pan Britannica Industries Ltd., Herts, U.K.). The plants were shaded in summer and provided with supplementary light in winter (HPLR 400 W; Philips Ltd., London, U.K.) to maintain approximately autumn light intensities (ca. $5-10$ MJ m^{-2} day^{-1}) throughout the year with a 15 h daylength. Plants were grown to the $10-20$ leaf stage and uniform leaves *c.* 22 cm laminar length used for protoplast isolation. Protoplasts were isolated by floating peeled leaves on 1% w/v Onozuka R10 cellulase, 0.05% w/v Macerozyme R10 in 0.66 M mannitol pH 5.8 for $1-2$ h at 25 °C in the dark. Full details of the isolation, washing and viability-determination are given in Cassells and Barlass [1].

Procedure 1 (standard procedure). To 0.4 ml of a suspension of 1×10^6 protoplasts per ml in 0.66 M mannitol in a 100 ml conical flask, was added 50 µl of TMV (12 mg/ml in 50 mM sodium phosphate buffer, pH 7.0) and the mixture gently swirled. Immediately 0.4 ml of 83.3 mM PEG (av. mol. wt.) 6000 in 0.66 M mannitol was added followed by 1.2 ml of 0.66 M mannitol in 50 mM sodium phosphate buffer pH 7.0. The solutions were gently swirled after each addition to ensure even mixing. The flasks were incubated at 25 °C for 1 h in the dark. After incubation, the protoplasts were pelleted under gravity, washed twice with 4 ml of minimal salt medium [9] pH 6.5 and then resuspended in 5 ml of minimal salt medium, transferred to 100 ml conical flasks and incubated in the growthroom (25 °C; 8.6 Wm^{-2}, 16 h photoperiod). Flasks were placed in the growthroom 8 h before the end of the light period.

Procedure 2. To the protoplast pellet (as above) was added 0.4 ml of 83.3 mM PEG and 1.2 ml PBM (0.66 M mannitol in 50 mM sodium phosphate buffer pH 7.0). The mixture was gently swirled and then incubated at 25 °C in the dark for 20 min. To this

was added 50 µl TMV solution (as above) and the mixture gently swirled. Care was taken to avoid disruption of the protoplast clumps. The flask was incubated for a further 1 h under the same conditions.

Washing and incubation were as for Procedure 1.

Procedure 3. To the protoplast pellet in a 100 ml conical flask (as. 1.) was added 50 µl of TMV solution and the flask gently swirled. Immediately, 1.5 ml of high pH/calcium fusion solution was added [7] (0.07 M calcium chloride, 0.66 M mannitol in 0.05 M glycine buffer, pH 10.4). The mixture was gently swirled and incubated for 45 min in the dark at 25 ° C.

The protoplasts were washed and incubated as in 1.

Procedure 4. As 3. above except that the protoplasts were aggregated in high pH/calcium solution for 20 min before addition of 50 µl of TMV solution. Following virus addition the mixture was gently swirled and incubated at 25 °C for 45 min in the dark.

Following incubation with virus, the protoplasts were washed and incubated in the growthroom, as 1. above.

Procedure 5. To the protoplast pellet (10^6 protoplasts in 0.4 ml of 0.66 M mannitol, in a 100 ml conical flask) was added 50 µl of TMV solution (as 1.). To this was added 0.1 ml of 41.4 mM PEG. After 1 min 1.5 ml of high pH/calcium fusion solution (see 3.) containing 6.6 mM PEG was added and the mixture gently swirled. The protoplast mixture was incubated for 45 min in the dark at 25 °C.

The protoplasts were washed and incubated after infection as in 1.

Procedure 6. To the protoplast pellet as above (1.) was added 1.5 ml of high pH/calcium fusion solution (as 3.) and the mixture gently swirled. After 20 min following aggregation, 50 µl TMV solution was added and 0.2 ml of 83.3 mM PEG. The protoplasts were incubated for 45 min at 25 °C in the dark and then washed and incubated in the growthroom as 1.

Appropriate controls were set up for each procedure.

After 24 or 48 h (as appropriate) the protoplasts were collected by gravity sedimentation, 10^6 protoplasts per 0.4 ml of medium. 0.15 ml of this protoplast suspension was removed and an aliquot stained with fluorescein diacetate to determine protoplast viability [1], a further aliquot was stained with fluorescent antibody to determine the percentage of infected protoplasts [8].

The remaining 0.25 ml of the protoplast suspension was diluted to 1 ml with 0.1 M sodium phosphate buffer pH 7.0. These protoplasts, and 1 ml volumes of the supernatant from the infected protoplast suspension, were dialysed overnight at 4 °C in phosphate buffered saline (0.1 M sodium phosphate buffer pH 7.0 containing 0.15 M sodium chloride).

After dialysis and before inoculation on to *N. tabacum* cv. xanthi-nc test plants, the samples were examined under the EM following negative staining with uranyl acetate [10] to estimate virus concentration. On the basis of the EM examination, the protoplast extracts were diluted to give approx. equivalent particle numbers to a 1:1000 dilution of the standard virus solution (12 mg/ml TMV in 50 mM sodium phosphate buffer, pH 7.00).

The samples in 0.3 ml containing 1 mg celite, were inoculated on to half leaves of xanthi-nc plants. The opposite half leaf was inoculated with standard virus preparation. Each sample was assayed 3 times. Local lesion development was in the growthroom (conditions as above).

The protoplast pellet (approx. 3×10^5 protoplasts in 0.15 ml) was fixed on the addition of 3 ml of Carnoy's Reagent (ethanol:chloroform:glacial acetic acid; 6:3:1; v/v/v). After 10 min the excess fixative was removed and 10 ml of acetone added. Decolourization was complete after 2 days at room temperature. The protoplasts and mannitol, which formed a flocculence, were collected by centrifugation at $500\times g$ for 10 min. The protoplasts were washed free of mannitol using distilled water. The protoplasts were then placed on slides precoated with Mayer's albumin and quickly dried in a stream of warm air. The staining and viewing conditions were as described for fluorescein conjugated antibody described previously [8].

Results

To test the hypothesis that infection results from the entry of particles in interprotoplast fusion zones, the standard PEG procedure was modified as below, and non-PEG fusion procedures, in the presence of low PEG (*i.e.* at which TMV precipitates but interprotoplast fusion does not occur), were investigated in relation to the initiation of infection.

Table I. The relation between virus precipitation, protoplast clumping and virus infection of tobacco mesophyll protoplasts. In procedures 1, 3 and 5, virus precipitation or entrapment between clumping protoplasts is possible. In procedures 2, 4, and 6, protoplast aggregation occurs before virus addition and/or precipitation. Full details of the procedures are given in "Methods"; the data are based on the mean of duplicate experiments. Protoplast viability was determined by staining with fluorescein diacetate, percentage protoplast infection by staining with TMV specific fluorescent antibody. Infectious virus production was determined by local lesion assay (full details in "Method").

Procedure sequence	Sample time (h post infection)	Protoplast viability [%]	Infected protoplasts [%]	Infectious virus prodn. (particles/protoplast)
Procedure 1				
Protoplasts + TMV	24	75	52	0.31×10^6
+ 10% PEG	48	60	70	1.60×10^6
Procedure 2				
Protoplasts + 10% PEG;	24	80	0	0
TMV added after protoplast clumping	48	70	0	0
Procedure 3				
Protoplasts + TMV	24	60	0	0.009×10^6
+ high pH/calcium	48	55	20	0.09×10^6
Procedure 4				
Protoplasts + high pH/calcium;	24	60	0	0
TMV added after protoplast clumping	48	55	0	0
Procedure 5				
Protoplasts + TMV + 4% PEG	24	60	48	0.41×10^6
+ high pH/calcium	48	50	75	1.12×10^6
Procedure 6				
Protoplasts + high pH/calcium	24	55	0	0
TMV + 4% PEG added after protoplast clumping	48	40	0	0

In the standard infection procedure (Procedure 1), 70% of the protoplasts were shown to contain TMV antigen at 48 h postinfection; 1.6×10^6 virus particles were produced per infected protoplast. When virus was added after protoplast clumping (Procedure 2), no virus antigen was detected nor was any progeny virus formation detected by local lesion assay. In the non-PEG, high pH/calcium fusion procedure, when virus was added before the addition of the fusion solution (Procedure 3), 20% of the protoplasts were shown to contain virus antigen and mean virus production per infected protoplasts was estimated to be 0.09×10^6 particles. When the sequence of addition was reversed in the high pH/calcium procedure *i.e.* aggregation of protoplasts before TMV addition (Procedure 4), no infection was detected.

A combination of virus addition to the protoplast pellet followed by low PEG addition which caused virus precipitation but no visible clumping, resulted in infection on sequential addition of high pH/cal-

cium fusion solution (Procedure 5). 75% of the protoplasts were shown to contain virus antigen and 1.12×10^6 progeny particles were formed per infected protoplast. In contrast, when clumping preceded TMV and low PEG addition, no infection occured (Procedure 6).

The full data for antibody staining and local lesion assay of infectious virus production at 24 and 48 h postinfection for the mean of duplicate experiments are given in Table I. (See also Fig. 1.)

The above studies suggested that the initiation of virus infection in protoplasts in the presence of PEG could be separated into two stages, viz. virus precipitation on the protoplast surface followed by inter-protoplast fusion leading to infection. Consequently, the infection procedure was separated into these two stages in an attempt to improve the efficiency.

Using the modified procedure shown in Fig. 2, virus inoculum was reduced five fold without reducing either the percentage of protoplasts infected or progeny virus production.
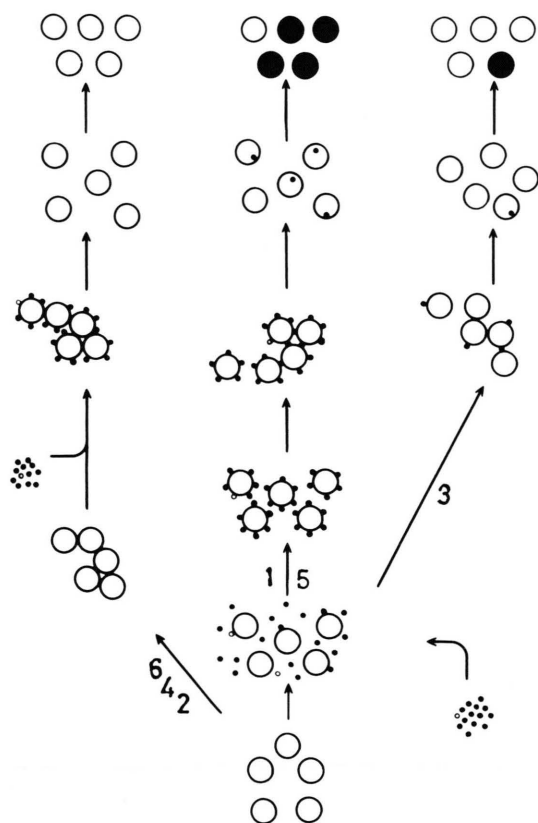
Fig. 1. The relation between virus precipitation, protoplast clumping and virus infection of tobacco mesophyll protoplasts. In procedures 1, 3 and 5, virus precipitation or entrapment between clumping protoplasts is possible. In procedures 2, 4, and 6, protoplast aggregation occurs before virus addition and/or precipitation. Full details of the procedures are given in "Methods"; the data are based on the mean of duplicate experiments (Table I).

Fig. 2. Modified inoculation procedure.

1. *Virus precipitation on Protoplast Surface:* 0.4 ml 0.66 M Mannitol pH 5.8 containing 10^6 protoplasts

+

0.1 ml 83.3 mM PEG.6000 in 0.66 M Mannitol and 10 μl TMV (12 mg/ml) in 0.5 ml of 0.005 M Phosphate buffered 0.66 M Mannitol, pH 6.5

↓ incubate 4 °C, 20 min

2. *Protoplast Fusion:* 0.3 ml 83.3 mM PEG.6000 in 0.66 M Mannitol

+

0.7 ml 3.5 mM Phosphate buffer pH 6.5 containing 11 mM $CaCl_2$ in 0.66 M Mannitol

↓ incubate 1 h, 25 °C

3. *Fusion/Wash:* Allow protoplasts to precipitate under gravity

+

Resuspend p'plast pellet in 50 mM $CaCl_2$ in 0.66 M Mannitol pH 10.5

↓ precipitate under gravity (approx. 10 min)

resuspend pellet in Takebe's liquid medium, pH 6.5

↓ allow to resettle under gravity

4. *Post Infection Incubation:* Resuspend protoplasts (10^6) in 5 ml Takebe's liquid medium

↓ incubate in growthroom (22 °C, 16 h photoperiod, 8.6 Wm^{-2}).

Discussion

The proposed model (Fig. 1) for the initiation of TMV infection in tobacco protoplasts in the presence of PEG, involves three stages, — a) precipitation of TMV particles on the protoplast surface; b) a slower aggregation of protoplasts and c) incorporation of virus particles into localized areas of membrane fusion or destabilization between adjacent protoplasts and putatively, initiation of infection follows. Infection can also occur when protoplasts clump in the presence of virus e.g. in the presence of high pH/calcium, but under these conditions virus concentration at the plasmalemma is lower and the process is less efficient (see Table I).

Evidence for this model comes from the present study and earlier work [2]. Previously it was shown that variation in input TVM inoculum concentration over a 20 fold range did not alter mean virus production per infected protoplast (suggesting that multiplicity of infection was not changed), but mean percentage protoplast infection was reduced at the lowest inoculum concentration. A significant percentage of the protoplasts did not join in clump formation and mean percentage infection may be related to the percentage of protoplasts aggregating. Observations of protoplasts following PEG treatment reveal few points of contact per protoplast. This latter finding suggests that the point(s) of virus entry into PEG treated protoplasts are few (contrast with polycation infection procedure discussed below).

The PEG (and other fusion-type) inoculation procedures outlined above differ in at least one major aspect from the polycation procedure [11]. In the latter, relatively large numbers of virus particles are seen in vesicles at the plasmalemma and in the cytoplasm in the early stages of infection. No vesicle formation was detected in the early stages of the PEG infection procedure (Cassells and Cocker, unpublished). Considerable controversy surrounds the mechanism of infection in the polycation procedure. Some workers believe that particles taken up by pinocytosis initiate infection [11] while others [12, 13] consider that virus entering through polycation-induced membrane lesions may also initiate infection.

The polycation and PEG/fusion procedures, assuming that they are fundamentally different mechanisms, provide systems for the study of the processes in plant virus replication. Their further investigation may provide an insight into the initiation process itself, with the caveat, that the cell wall may play a significant role in the initiation of infection in the intact plant.

[1] A. C. Cassells and M. Barlass, Physiol. Plant. **42**, 236–242 (1978 a).

[2] A. C. Cassells and M. Barlass, Virology **87**, 459–462 (1978 b).

[3] J. R. Dawson, P. E. Dickerson, J. M. King, F. Sakai, A. R. H. Trim, and J. W. Watts, Z. Naturforsch. **33 c**, 548–551 (1978).

[4] A. J. Maule, M. I. Boulton, C. Edmunds, and K. R. Wood, J. Gen. Virol. **47**, 199–203 (1980).

[5] K. N. Kao and F. Michayluk, Planta **115**, 355–367 (1974).

[6] S. Sarkar, M. D. Upadhya, and G. Melchers, Molec. Gen. Genet. **135**, 1–9 (1974).

[7] W. A. Keller and G. Melchers, Z. Naturforsch. **28 c**, 737–741 (1973).

[8] A. C. Cassells and A. A. Gatenby, Z. Naturforsch. **30 c**, 696–697 (1975).

[9] T. Nagata and I. Takebe, Planta **99**, 12–20 (1970).

[10] D. Noordam, Identification of plant viruses pp. 140, Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands 1973.

[11] I. Takebe, Ann. Rev. Phytopathol. **13**, 105–125 (1975).

[12] C. Burgess, F. Motoyoshi, and E. N. Fleming, Planta **112**, 323–332 (1973).

[13] B. Kassanis, R. F. White, R. H. Turner, and R. D. Woods, Phytopath. **88**, 215–228 (1977).