Improved Methods for Infection of Plant Protoplasts with Viral Ribonucleic Acid

J. R. O. Dawson, P. E. Dickerson, Janet M. King, F. Sakai *, A. R. H. Trim, and J. W. Watts

John Innes Institute, Colney Lane, Norwich

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Infection of tobacco protoplasts with RNA of tobacco mosaic virus and the bromoviruses has been studied. The quality of the RNA was very important. Ribonuclease activity was controlled by incorporating 5 mM $ZnCl_2$ in the inoculum. Up to 60% infection has been observed with bromovirus RNA but infection with TMV RNA was much less efficient. A procedure for inoculating in 40% polyethylene glycol was developed. High levels of infection were obtained with all the viruses and viral RNA examined.

Introduction

Plant protoplasts are increasingly used in the study of virus infection; they can be efficiently and synchronously infected with a variety of viruses, and levels of infection approach 100% [1]. The results obtained with free viral RNA have, however, been much less encouraging. Aoki and Takebe [2] reported 3–7% infection of tobacco protoplasts inoculated with tobacco mosaic virus (TMV) RNA at 1 mg/ml. Similar levels of infection were observed when tobacco protoplasts were infected with RNA of cowpea chlorotic mottle virus (CCMV) [3] or brome mosaic virus (BMV) [4]; this work indicated that when poly-L-ornithine was present at 1 μg/ml during inoculation, the concentration of RNA should not exceed about 2 μg/ml. Modification of the procedure increased the infection with CCMV RNA to 25% [5]. A radically new procedure using inoculation at high pH under conditions similar to those that promote fusion of protoplasts [6] gave 70–100% infection of tobacco protoplasts with TMV RNA [7] but difficulties have been experienced in repeating this work and the method has not found general application. Beier and Bruening [8] have reported up to 65% infection of cowpea protoplasts with cowpea mosaic virus RNA, using protamine sulphate as the polycation in the inoculum, but rather high concentrations of RNA (50 μg/ml) were needed. We have attempted to define and control some of the variables that influence infection with RNA in order to be able to infect tobacco protoplasts to useful levels (greater than 30%) routinely.

* Present address: Institute for Plant Virus Research, Ministry of Agriculture & Forestry, Tsukuba Science City, Yatabe, Ibaraki 300-21, Japan.

Requests for reprints should be sent to J. W. Watts, John Innes Institute, Colney Lane, Norwich NR4 7UH.

Materials and Methods

Protoplasts of *Nicotiana tabacum* (cv. White Burley) were prepared and inoculated with virus and cultivated by the methods of Motoyoshi *et al.* [3]. Percentage infection was determined by staining the protoplasts with fluorescent antibodies [3]. The method of inoculation with viral RNA was initially that of Watts *et al.* [5]; equal weights of RNA and poly-L-ornithine were mixed at 0 °C to give a final concentration of 500 μg of each/ml. This was at once diluted with ice-cold 0.01 M potassium citrate (pH 5.2) in 0.7 M mannitol to a concentration of 1 μg RNA/ml and added to a freshly sedimented pellet of protoplasts (10^5 protoplasts/ml of inoculum) at 0 °C. After 2–3 min the mixture was transferred to a water-bath at 25 °C for 5 min and the protoplasts were subsequently washed with 0.7 M mannitol and cultured [3].

When $ZnCl_2$ was used to control ribonuclease activity 20 μg poly-L-ornithine and 20 μg RNA were mixed in 1 ml 0.02 M potassium succinate buffer (pH 5.4) containing 0.05 M $ZnCl_2$ and kept at 25 °C for 5 min. After dilution with 8 ml 0.02 M succinate buffer containing 0.7 M mannitol, the mixture was added to 10^6 protoplasts freshly suspended in 10 ml 5 mM $ZnCl_2$ in 0.7 M mannitol and incubated for 10 min at 25 °C. The protoplasts were then washed and cultured as before.

Inoculation in polyethylene glycol (PEG) was carried out by resuspending freshly pelleted proto-

plasts (10^6) in 2 ml 40% (w/v) PEG 1500 in 3 mM $CaCl_2$ containing 25 – 50 μg viral RNA or virus at 25 °C. After about 10 sec, 20 ml 0.7 M mannitol was gradually added and mixed well. The protoplasts were left 15 – 30 min and then washed and cultivated as before.

RNA was prepared by treating virus with sodium dodecyl sulphate and phenol following published methods [9, 10].

Results

Physiological condition of the protoplasts

Fig. 1 summarizes experiments with several different batches of protoplasts, comparing the percentage infections by virus and viral RNA. There is some correlation between the susceptibility to infection by virus and RNA: the highest infections by RNA occurred with those protoplasts most readily infected with virus. The highest infection with RNA did not however exceed 50% although infection with the virus approached 100%. It is reasonable to conclude therefore that good infections with RNA will occur only when the protoplasts have maximum receptivity, but that this is not the only factor involved. It is not known precisely what factors are responsible for high susceptibility to infection; seasonable variation is observed in plants grown in the glasshouse, and some workers have therefore used plants grown in controlled environment cabinets to reduce the problem [11]. One possibility that must not be ignored when using RNA is the level of ribonuclease that may be present in the medium during inoculation [12]; this would be much greater when the protoplasts were fragile and easily broken, so reducing the chances that RNA would successfully infect before being inactivated although the susceptibility of the protoplasts might be very high.

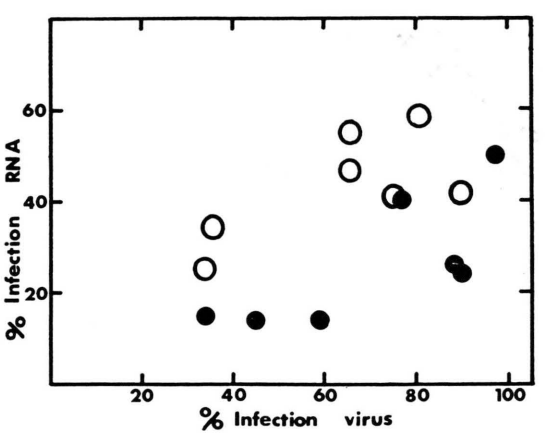

Fig 1. Comparison of the infection of protoplasts with virus and viral RNA. The same batch of protoplasts was inoculated with either CCMV or its RNA at pH 5.2 – 5.4 in presence of poly-L-ornithine (see Methods). ●, inoculation by original method [5]; ○, inoculation with RNA in presence of 5 mM $ZnCl_2$.

The quality of RNA

CCMV RNA used in earlier experiments was prepared by the method of Bancroft *et al.* [13]; this procedure gives very low recoveries of RNA (around 30% usually) and appears to entail preferential loss of high molecular weight RNA (RNA 1 and 2) [10]. It could not be preincubated with poly-L-ornithine at room temperature without inactivation, presumably because of the presence of traces of ribonuclease. RNA prepared by a modification of this procedure [10] became available during the present experiments and proved sufficiently free from ribonuclease to allow preincubation at 25 °C. This at once gave a useful increase in the percentage infection, *e. g.* infection was increased in one experiment from 25% to 35% by preincubation of RNA and poly-L-ornithine for 10 min at 25 °C.

Use of inhibitors of ribonuclease

The effects of several inhibitors of ribonuclease activity were examined. Diethylpyrocarbonate [14] was too toxic to be used at concentrations that might give useful inhibition of ribonuclease. The best results showed an increase in infection from 15% in the controls to 22% in the presence of 0.005% diethylpyrocarbonate, but the effect was erratic and possibly fortuitous. The effect of zinc salts was much more encouraging. Zn^{2+} is known to be an effective inhibitor of plant leaf ribonucleases [15]. It cannot be used with the citrate and phosphate buffers normally employed in inoculation of protoplasts because of precipitation. Initial experiments were therefore done without a buffer using 0.7 M mannitol adjusted to pH 5.2 with 0.1 N-NaOH, and showed useful and consistent increases in infection, *e. g.* from 15% infection in the controls to 22% in presence of 5 mM $ZnCl_2$ without toxic effects on the protoplasts.

Several buffers were investigated: citrate, phosphate and maleate were discarded because of pH changes when $ZnCl_2$ was added. Cacodylate was

Table I. The effect of 5 mM $ZnCl_2$ on infection with CCMV RNA.

	$ZnCl_2$ (±)	% Infection (24 h)
Experiment 1		
Control (virus, 1 μg/ml)		90
RNA (1 μg/ml)	−	13
RNA (1 μg/ml)	+	42
Experiment 2		
RNA (1 μg/ml)	−	15
RNA (1 μg/ml)	+	25 *
RNA (1 μg/ml)	+	36 +

* RNA and poly-L-ornithine were preincubated 5 min at 4 °C before inoculation.

\+ RNA and poly-L-ornithing were preincubated 5 min at 25 °C before inoculation.
Poly-L-ornithine was present at 1 μg/ml during inoculation; 0.01 M potassium succinate buffer (pH 5.4) was used in all inoculations.

satisfactory as a buffer but appeared to be toxic. Potassium succinate buffer was finally chosen as the most satisfactory. Table I and Fig. 1 show some of the results using $ZnCl_2$ in succinate-buffered mannitol. These data do not represent the highest levels of infection obtained under these conditions; experiments using fractionated RNA have given infections of 60% under favourable circumstances.

Inoculation at high pH

The method of Sarkar *et al.* [7] used an inoculum of RNA in a solution containing 0.34 M KCl, 0.05 M $MgCl_2$ and 1% glycine (pH 9.0). Repeated attempts over several years failed to give levels of infection approaching those observed by Sarkar *et al.* [7], with TMV, CCMV or their RNA although conventional methods of inoculation with poly-L-ornithine at pH 5.2 gave infections of 4 – 8% with TMV RNA and very much greater infection with CCMV RNA. Some infection by TMV or its RNA was normally observed at pH 9.0 or higher, but this seems in part to be due to charge effects caused by high pH. The presence of 2% dimethylsulphoxide, the highest safe concentration, failed to increase infection. The best result was 12% infection with TMV RNA at pH 9.5 but the response in the range pH 9.0 – 10.5 either with TMV or its RNA seldom exceeded the level obtained by conventional inoculation with TMV RNA (about 5%). Many efforts to inoculate with CCMV or its RNA at high pH were completely unsuccessful.

Inoculation with polyethylene glycol

Polyethylene glycol (PEG) has been widely used to promote fusion of plant protoplasts [16] and, more recently, animal cells [17]. It has also been used to induce protoplasts to take up particles like chloroplasts and bacteria [18, 19]. It is therefore a suitable candidate for promoting infection by viruses and their RNA. Table II shows an experiment using three concentrations of PEG; at the highest concentration (40%), PEG caused rapid aggregation of the protoplasts and inoculation was terminated by dilution with 0.7 M mannitol after 30 sec. The lower concentrations (20 and 30%) did not cause rapid aggregation. It can be seen that the procedure was very successful at the highest concentration of PEG but much less successful at the lower concentrations. The procedure was even more successful with CCMV RNA and equally successful with intact viruses. The presence of $CaCl_2$ was essential for infection.

Table II also shows the relationship between the concentration of RNA in the PEG inoculum and the

Table II. The effect of PEG on infection with virus and RNA.

Experiment 1 *		% Infection (24 h)
TMV + PLO (pH 5.2)		43
TMV RNA + PLO (pH 5.2)		4
TMV RNA + PLO + 2% DMSO (pH 5.2)		8
TMV + 2% DMSO (pH 9.0)		1
TMV RNA + 2% DMSO (pH 9.0)		1
TMV RNA (50 μg) in 1 ml 40% PEG, 3 mM $CaCl_2$ for 1 min		52
Experiment 2		
TMV RNA		
(40 μg) in 2 ml 20% PEG, 3 mM $CaCl_2$ for	30 sec	11
20%	2 min	13
30%	30 sec	5
40%	30 sec	57
CCMV RNA (25 μg)		
40%	30 sec	69
Experiment 3		
BMV RNA		
(1 μg) in 1 ml 40% PEG, 3 mM for 20 sec		5
(2 μg)	„	5
(5 μg)	„	9
(10 μg)	„	14
(25 μg)	„	33
Experiment 4		
BMV RNA		
(20 μg)	„	27
(40 μg)	„	35
(60 μg)	„	23
(80 μg)	„	45
(100 μg)	„	59

* Except where stated virus or RNA and PLO were at 1 μg/ml.

percentage infection. The BMV RNA used in experiments 2 and 4 was a batch of inferior quality which gave low levels of infection when used to inoculate by conventional methods using poly-L-ornithine. There was a clear cut concentration dependence.

If protoplasts were washed and stored in culture medium for several hours after preparation they became progressively more refractory to inoculation in PEG; this problem was largely avoided if the protoplasts were washed immediately before inoculation. Prolonged culture (18 h) reduced infectibility to relatively low levels which may in part be due to wall formation. Treatment with cellulase at this stage rapidly digested the new cell wall, but did not enhance the level of infectivity. It would appear that changes occurred in the membrane that made the protoplasts more resistant to infection.

Discussion

The lower specific infectivity of TMV RNA compared with that of CCMV RNA is presumably due partly to the greater length of its RNA and consequently greater disorder in the free state which will obscure initiation sites. The greater length will also result in more mechanical breakage during preparation and manipulation. When protoplasts are inoculated in presence of poly-L-ornithine at pH 5 – 6 there is an optimal concentration of RNA, approximately the same as the polycation on a simple weight basis. This concentration is adequate to allow high infections with CCMV RNA but not with TMV RNA.

The results obtained with CCMV RNA show that the quality of the RNA, and control of ribonuclease activity during inoculation are very important; the physiological condition of the protoplasts is therefore also extremely important. Once these variables can be controlled, it is possible to obtain above 50% infections with low concentrations of CCMV RNA. This is never the case with TMV RNA. High infections with TMV RNA appear to require a procedure that will cause much larger amounts of RNA to enter the protoplasts. The high pH method of Sarkar *et al.* [7] is one such procedure, whilst PEG has produced similar results in the present work. The effect of PEG is almost certainly to increase uptake of RNA by at least an order of magnitude. The process is dependent on aggregation of the protoplasts which suggests that inoculum may be trapped between the protoplasts during aggregation and taken up into the cytoplasm during subsequent washing.

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