エンドウ葉肉細胞プロトプラストのクローバ黄斑モザイクウイルス感染系の改良

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An Improved System for Clover Yellow Mosaic Virus Infection of Pea Leaf Mesophyll Protoplasts

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Abstract

Isolation of pea (*Pisum sativum* L. cv. Alaska) mesophyll protoplasts by both the 'one-step' and the 'modified two-step' methods resulted in similar yields ($1–1.5 \times 10^7$ protoplasts per g fresh weight of leaves). The time of protoplast isolation was considerably reduced, from 2 hr to 35 min. by including Pectolyase Y23 in the digestion medium. Maximum infection, 57%, was obtained at concentrations of 1.0 to 1.4 μg/ml for poly-L-ornithine and 3 μg/ml for clover yellow mosaic virus (CYMV), using 0.24 M phosphate (K_2HPO_4-KH_2PO_4) buffer pH 6.3 as inoculation buffer. The replication of CYMV-RNA in protoplasts was followed by Northern blotting using specific cDNA probe. Synthesis of genomic RNA was detected from 12 hr after inoculation. Polyethylene glycol-mediated inoculation with CYMV-RNA resulted in about 35% infection of viable protoplasts.

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Key words: protoplast, pea, virus replication, cDNA.

INTRODUCTION

Clover yellow mosaic virus (CYMV), a member of the potexvirus group, is widespread in western North America[1,31], and it reduces winter hardiness and yield in clovers[32]. A natural CYMV infection of vetch in the Rocky Mountain House area has been reported as high as 90%[35]. The virus is extremely stable *in vitro*, easily sap-transmissible, and reaches a high concentration in the infected plant. Pea (*Pisum sativum* L. cv. Alaska) was chosen, as a host species in this study because of its high virus susceptibility. All introduction lines released by the United States Department of Agriculture were susceptible, and developed severe symptoms after CYMV infection[7].

The main objectives of this study are to improve protoplast methodology by critically evaluating selected procedures for the large scale isolation of pea leaf mesophyll protoplasts in an intact and viable state, and to examine certain factors affecting infection of the protoplasts with CYMV and CYMV-RNA.

MATERIALS AND METHODS

Plants. Seeds of Alaska pea were sown in a soil mix (3 parts loam : 2 parts peat : 1 part sand) in 10 cm clay pots in a greenhouse at 23 to 25 C. After germination, the plants were transferred to an incubator (Model E30, Percival Co., Boone, Iowa, U.S.A.) and kept at 23 C during a daily light period of 14 hr at approximately 8,000 lux supplied by 6 fluorescent tubes (F 24712 kw/40, Sylvania Ltd., Montreal, Canada) and two 40 watt incandescent bulbs (Westinghouse Canada Ltd.), and at 21 C during the dark period.

Isolation of protoplasts. After surface-sterilization of fully expanded leaves from 3-week-old plants with 70% ethanol and two washings with double-distilled water, the lower epidermis

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of the leaves was removed with a pair of forceps. The tissue was placed with its epidermis-stripped side down in a petri dish containing 0.05% Macerozyme R-10 (Kinki Yakult Manufact. Co.) and 1% Cellulose Onozuka R-10 (Kinki Yakult Manufact. Co.) in 0.7 M mannitol, pH adjusted to 5.5 with 2 N KOH. After 2 hr of incubation in a water bath at 30 C, the content of the petri dish was gently swirled and the incubation was continued for another 30 min. The mixture was filtered through a double layer of cheesecloth and the protoplasts were collected by centrifuging the filtrate for 2 min at $500 \times g$. The protoplasts were washed twice with 0.7 M mannitol and finally resuspended in 0.7 M mannitol. The percentage of intact protoplasts was determined by counting about 300 protoplasts, and their yield was calculated by using a haemocytometer.

Protoplasts were also isolated by the method of Nagata and Ishii[23]. Pea leaf tissues, with or without stripped lower epidermis, were placed in a petri dish containing a solution of 0.1% Pectolyase Y23 (Kikkoman Shoyu Co. Ltd., Noda, Chiba-ken, Japan) and 2% Cellulose R-10 in 0.7 M mannitol, pH 5.5. After 35 to 45 min of incubation at 30 C, with occasional gentle swirlings, most of the cells released protoplasts which were then collected and washed as described earlier.

In some experiments protoplasts were also isolated by the method of Watts and King[44].

Virus. Purification of the vetch strain of CYMV was performed as described previously[35].

Inoculation of protoplasts. Fresh preparations of CYMV stored at 4 C for no longer than 2 weeks were used for inoculation of the protoplasts. Unless otherwise stated, the virus was diluted to a concentration of 10 μg/ml in 0.05 M phosphate buffer, pH 6.3, containing 0.7 M mannitol, and then poly-L-ornithine (PLO) (mol wt 150,000, Pilot Chemical Co., Boston, Mass., U.S.A.) was added to a concentration of 2 μg/ml. This mixture was incubated for 20 min at 25 C and then added to an equal volume of a protoplast suspension. Thus, the final concentrations of virus, PLO and protoplasts were 5 μg/ml, 1 μg/ml, and approximately 2.5×10^5/ml, respectively.

For the direct method of inoculation, the virus was diluted to a concentration of 5 μg/ml in 0.025 M phosphate buffer, pH 6.3, containing 0.7 M mannitol, and then PLO was added to a concentration of 1 μg/ml. The inoculation mixture, with the final concentrations of the virus, PLO and protoplasts as in the indirect method, was incubated at 25 C for 20 min prior to suspending freshly pelleted protoplasts. Except for an increased mannitol concentration of 0.85 M, all other conditions were the same as in the indirect method for inoculation by the osmotic shock method[25].

In all the methods used for inoculation, the protoplasts were incubated with the inoculum for 20 min at 25 C with occasional swirlings and then they were separated from the unadsorbed virus particles by three washes in 0.7 M mannitol containing 10 mM $CaCl_2$. Washed protoplasts were resuspended in the incubation medium of Aoki and Takebe[2], at a concentration of approximately 2.5×10^5/ml. They were incubated in 10 ml portions in 125 ml Erlenmeyer flasks at 25 C under continuous illumination (approximately 1,000 lux) from fluorescent tubes. Inoculation of protoplasts with purified viral RNA was carried out essentially as described by Maule *et al.*[18] A pellet of protoplasts (1×10^5) was directly suspended in 60 μl of 30% polyethylene glycol (PEG) (mol wt 6,000) containing 25 μg of CYMV-RNA, and after 10 seconds it was diluted with 0.7 M mannitol to a final volume of 1 ml. The protoplasts were kept for 20 min at room temperature and collected by centrifugation followed by two washes with 0.7 M mannitol before suspending in the incubation medium.

Infectivity assay. After the required incubation times, inoculated protoplasts were collected by centrifugation at $100 \times g$ for 2 min, washed once with 0.7 M mannitol, repelleted and frozen at -20 C for infectivity assay as described previously[33,34].

Fluorescent antibody staining. The proportion of infected protoplasts was assessed after different periods of incubation by both direct[27] and indirect[24] fluorescent antibody staining.

Northern hybridization. After thawing the protoplast pellets, total nucleic acids were extracted with water-saturated phenol, precipitated with 75% ethanol and resuspended in sterile distilled water. The nucleic acid preparations were subjected to denaturation by heating at 65 C for 2 min in the presence of 50% (v/v) formamide, 6% (w/v) formaldehyde and electrophoresis buffer (20 mM N-[2-hydroxyethyl]-piperazine-N′-3-propane-sulfonic acid, 10 mM sodium acetate and 0.1 mM ethylenediaminetetraacetic acid, pH 8.0). The samples were then electrophoresed in 1.5% agarose-formaldehyde gel[17] prepared in electrophoresis buffer and were transferred to GeneScreen Plus transfer membrane (NEN Research Products, Lachine, Canada) by capillary blotting[43]. The membrane was baked at 80 C for 60 min and prehybridized at 42 C using a shaker-waterbath for 4–6 hr in plastic bags containing 50% formamide (v/v), 5×SSPE (1×SSPE=0.12 M NaCl, 0.015 M sodium citrate, 0.013 M Na_2HPO_4 pH 6.5, 2 mM EDTA), 4× Denhardt's solution (1×Denhardt's=0.02% each of bovine serum albumin, Ficol 400 and Polyvinylpyrrolidone 40), 100 μg/ml of denatured calf thymus DNA and 0.1% SDS. Hybridization was carried out at 55 C for 24 hr in the same but fresh solution containing 10% dextran sulfate and a ^{32}P-labeled cDNA probe specific to CYMV-RNA made by random priming using reverse transcriptase[42]. The probe was used at a concentration of 10^6 cpm/ml of hybridization solution. Following hybridization, the membrane was washed, according to Klessig and Berry[12], three times for 5 min each at room temperature in 2×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and 0.1% SDS, and twice for 30 min each at 55 C in 1×SSC and 0.5% SDS, and two times for 30 min each at 55 C in 0.1×SSC and 0.1% SDS. The membrane was air-dried and exposed to Kodak X-OMAT X-Ray film at −20 C overnight and developed according to the manufacturer's recommendations.

RESULTS

Factors affecting CYMV infection

Yields of pea mesophyll protoplasts, isolated by both the 'one-step' and the 'modified two-step' methods, amounted to approximately 1 to 1.5×10^7 protoplasts per g fresh weight of leaves (Table 1) and 78 to 85% of the protoplasts isolated appeared intact. Although liberation time for the protoplasts from mesophyll cells differed in the two isolation methods (Table 1), their infection rates with CYMV remained approximately the same.

An increasing number of protoplasts were infected as the pH of 0.025 M phosphate buffer was raised from 5.7 to 6.3, which was an optimal range, and the number of infected protoplasts decreased sharply at higher pH values (Fig. 1).

Percentages of CYMV infection from three experiments for 0.01 M citrate buffer pH 6.0/0.025 M phosphate buffer pH 6.3 were 37/52, 40/57 and 38/53, indicating that the presence of phosphate in the inoculum results in higher infection than that of citrate.

High rate of infection occurred at the optimum concentration of PLO 1.0 to 1.4 μg/ml (Fig.

Table 1. Comparison of different methods used for isolation of pea leaf mesophyll protoplasts

Methods	Lower epidermis peeled	Macerozyme R10 (%)	Pectolyase Y23 (%)	Cellulose R10 (%)	Yield protoplasts/g[a)]	Time required for liberation of protoplasts
A[b)]	Yes	—	0.1	2	$1\text{-}1.5\times10^{7}$[d)]	35 min
A	No	—	0.1	2	$1\text{-}1.5\times10^{7}$	45 min
A	Yes	0.05	—	1	$1\text{-}1.5\times10^{7}$	2 hr
B[c)]	Yes	1	—	2	$1\text{-}1.5\times10^{7}$	3 hr

a) Fresh weight of leaves used.
b) One-step method.
c) Modified two-step method.
d) Protoplasts were counted, after washing twice with 0.5 M mannitol, using a haemocytometer.

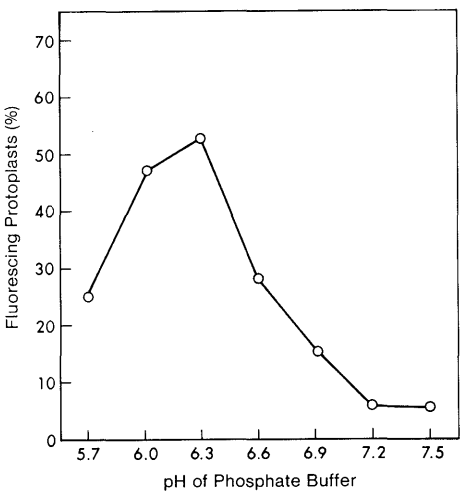

Fig. 1. Effect of pH of inoculation buffer on CYMV infection of pea mesophyll protoplasts. The protoplasts were inoculated with 5 μg/ml CYMV in the presence of 1 μg/ml poly-L-ornithine. The protoplasts were stained with fluorescent antibody specific to CYMV after 44 hr of incubation.

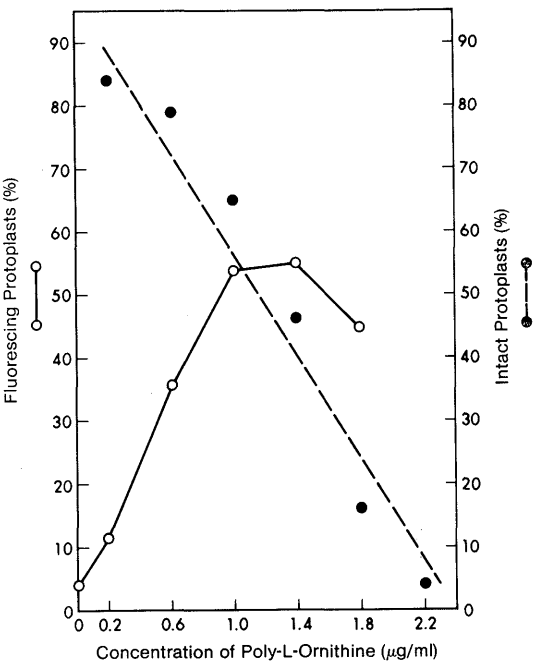

Fig. 2. Effect of poly-L-ornithine concentrations in the inoculum on survival of pea mesophyll protoplasts and their infection with CYMV. Inocula contained 0.025 M phosphate buffer, pH 6.3, 5 μg/ml CYMV and various concentrations of poly-L-ornithine. The protoplasts were stained 44 hr after inoculation.

2). As a higher percentage of protoplasts was damaged at 1.4 μg/ml than 1.0 μg/ml, the protoplasts were inoculated at a PLO concentration of 1.0 μg/ml in subsequent experiments.

The 'indirect method'[30], 'direct method'[20], and the 'osmotic shock method'[25] were compared for the efficiency of infection in the CYMV-pea protoplast system. In three experiments, the average percentages of infection by the 'indirect method', the 'direct method' and the 'osmotic shock method' were 44, 43, and 46, respectively, showing little difference amongst them. Therefore, the 'direct method' was used subsequently.

Frequency of infection of the protoplasts increased with the concentration of CYMV in the inoculum up to a concentration of 3 μg/ml, the highest infection rate being 57% (Fig. 3). Further increase in the virus concentration did not improve the rate of infection.

In a series of experiments in which the protoplasts were incubated for different periods of time (0 to 60 min) with the mixture of CYMV and PLO that had been pre-incubated for 20 min, 10 min of incubation gave a relatively good infection rate, and prolonging incubation further, up to 60 min, did not change the rate significantly (Fig. 4).

Multiplication of CYMV

The extent of multiplication of CYMV in the protoplasts was determined by two methods; namely, infectivity assay of protoplast extracts and fluorescent antibody staining of inoculated protoplasts. In a time course study of the virus multiplication assayed by the infectivity in the protoplasts at various times after inoculation, a small amount of CYMV infectivity was detected immediately after inoculation which could be attributed to the virus particles simply adsorbed to

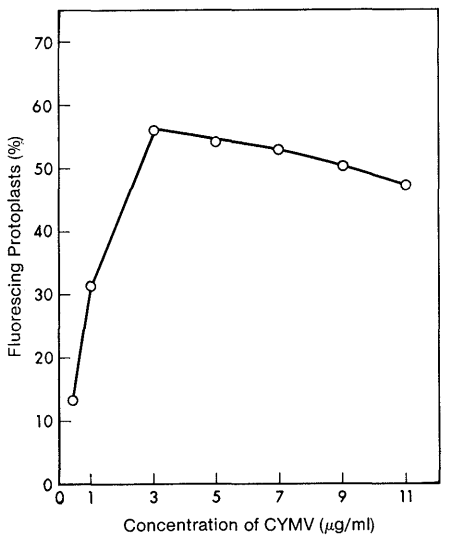

Fig. 3. Effect of CYMV concentration in the inoculum on infection of pea mesophyll protoplasts. The inoculum mixtures contained various concentrations of CYMV, 1 μg/ml poly-L-ornithine and 0.025 M phosphate buffer, pH 6.3. The percentage of infected protoplasts was determined after 43 hr of incubation.

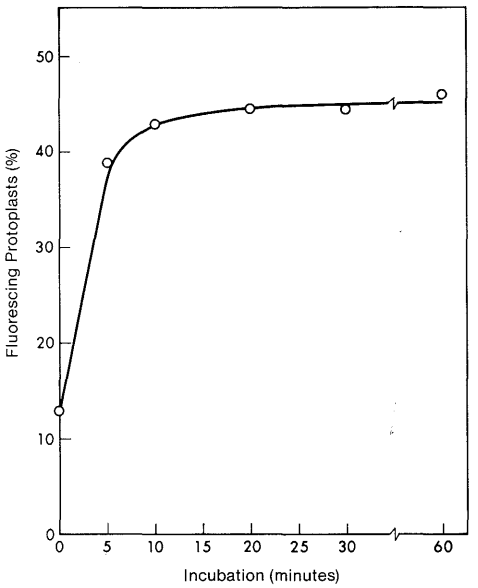

Fig. 4. Effect of time of contact between inoculum and protoplasts on infection of pea mesophyll protoplasts with CYMV.

the protoplasts. This initial infectivity was followed by a decrease in the infectivity 6 hr after inoculation. It increased very rapidly between 12 hr and 24 hr and then slowed down (Fig. 5). In the determination of the percentage of infected protoplasts by fluorescent antibody staining, the first fluorescence as small specks was observed 12 hr after inoculation at a 10% level. During the following 12 hr the percentage increased very rapidly to 38%, and then the increase slowed (Fig. 5). After 40 hr of incubation the fluorescence was distributed throughout the cytoplasm, and it appeared as a network due to unstained dark chloroplasts. No fluorescence was observed in uninoculated controls.

Replication of CYMV-RNA

The replication of CYMV-RNA in protoplasts was followed by Northern blotting using specific cDNA probe. Synthesis of CYMV-RNA was detected from 12 hr after inoculation and continued thereafter, until the end of probe assay which was terminated at 48 hr after incubation. Mock-inoculated samples did not show any CYMV-specific RNA (Fig. 6). The PEG-mediated infection of protoplasts with CYMV-RNA resulted in 35% infection as assessed by indirect fluorescent antibody staining 24 hr after inoculation.

DISCUSSION

All the methods evaluated for isolating protoplasts resulted in similar protoplast yields but differed in the liberation time of protoplasts (Table 1). Use of Pectolyase Y23 considerably reduced the time of protoplast isolation from 2 hr to 35 min.

In this investigation, up to 57% of pea mesophyll protoplasts were infected with CYMV.

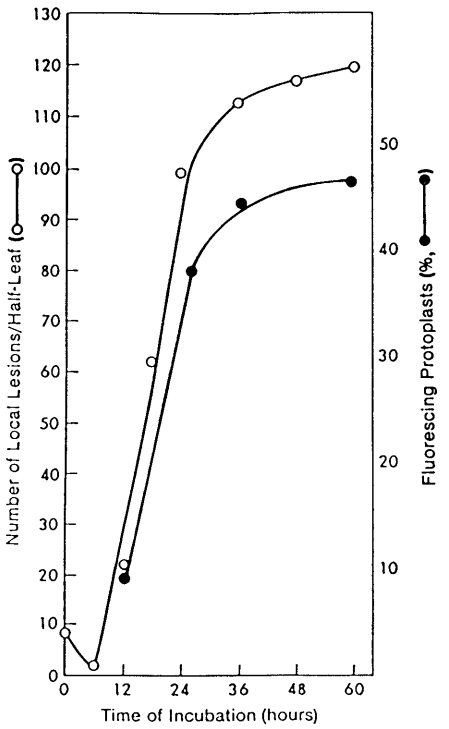

Fig. 5. The time-course of CYMV multiplication in pea mesophyll protoplasts as determined by infectivity assays on *Chenopodium amaranticolor* leaves and by fluorescent antibody staining. The survival rate of protoplasts after 60 hr of incubation was about 60 to 70%.

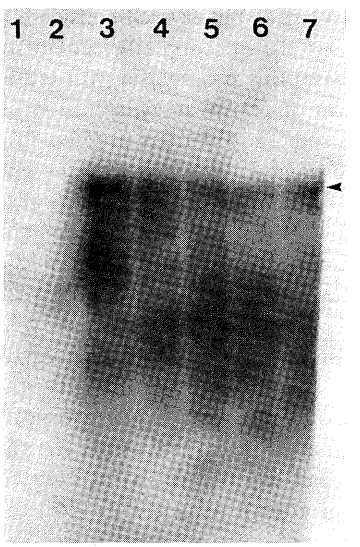

Fig. 6. Autoradiogram of a Northern blot of RNA extracted from protoplasts at different times after inoculation with CYMV-RNA. Lane 1: mock-inoculated protoplasts after 48 hr incubation. Lanes 2 to 6: protoplasts sampled after 0, 12, 24, 36, and 48 hr incubation. Lane 7: CYMV-RNA. The survival rate of protoplasts after 48 hr of incubation was about 60%.

The observations that virus antigen was detectable 12 hr after inoculation, and that extensive fluorescence was observed 40 hr after inoculation indicated that the protoplasts inoculated *in vitro* supported active virus multiplication. The synchronous nature of infection and virus multiplication was evident from a rapid increase in infectivity between 12 hr and 24 hr, and from the observation that a majority of the cells fluoresced within 24 hr of inoculation. The result of a time-course study of virus multiplication in the protoplasts was similar to that of some other virus-protoplast systems[26,40].

In the investigation of the effect of some factors on infection of pea protoplasts with CYMV, PLO was found necessary for infection (Fig. 2) as reported in many other virus-protoplast combinations[3,33,40]. While many viruses that require PLO for infection were negatively charged at the pH values tested for inoculation of the protoplasts, some other viruses that were positively charged at their inoculation pHs do not require PLO for infection[21,26]. In addition to the charge on virus particles that influences the requirement for PLO, the amount of negative charge of the protoplast membrane may also be involved. While negatively charged cowpea mosaic virus and tobacco mosaic virus (TMV) did not require PLO for infection of cowpea protoplasts[9], the same viruses required PLO for infection of tobacco protoplasts[11,41]. The mechanism of the PLO-enhanced infection is not very well understood, although it appears that PLO acts both on virus

particles and protoplasts. Upon mixing, it forms a complex with the negatively charged viruses[15,33,38], facilitating the virus adsorption to the plasmamembrane. It also seems to enhance the uptake of virus particles through enhanced pinocytotic activity of protoplasts[6,10,30], or through plasmamembrane lesions that serve as the sites for virus binding[4,5]. A recent study with bromoviruses suggests that infection of protoplasts depends primarily on physical interactions caused by electrical charges of virus particles[45].

The kind of buffer used considerably influences the infection[13,37,39]. Various buffers were used to test the infection efficiency of protoplasts[13,19,22]. Phosphate buffer increased infection of pea mesophyll protoplasts with CYMV to a higher percentage than did citrate buffer. The mechanism of phosphate-enhanced virus infection of protoplasts is not completely understood, although some postulations were made, including an increased number of sites of infection and favorable effects of phosphate on the plasmamembrane after virus attachment[13,15].

The response of pea protoplasts at different pH values of inoculation medium towards infection with CYMV was considerably different from most other virus-protoplast combinations. The optimal pH value of 6.3 for infection of pea protoplasts was higher (Fig. 4) than that for the infections of certain protoplasts with TMV[40] and several other viruses[16,20,28]. A higher pH value of phosphate buffer was also optimal for infection of tomato protoplasts with TMV[22], tobacco protoplasts with tobacco rattle virus[14], and cowpea protoplasts with cymbidium ringspot virus[37]. The effect of pH on virus infection of protoplasts is interpreted as being due to its influence on interactions between virus and PLO, and between virus or a virus-PLO complex and protoplasts. It seems that at pH 6.3, the virus particles assume optimal negative charge so as to form a positively charged virus-PLO complex for the maximum adsorption to the negatively charged protoplast surface. By virtue of the increased pH, phosphate buffer indirectly improves the stability of the protoplasts, which are unstable at lower pH values[22,28].

The extent of infection of protoplasts also depends on the concentration of virus in the inoculum. In this investigation, the percentage of infected protoplasts increased with the increase in CYMV concentration up to 3 μg/ml (Fig. 3). Further increases in the virus concentration did not improve the infection efficiency. This reduction may be explained by a shift in the net charge of the virus-PLO complex from positive to negative charge due to increased virus concentrations in the inoculum. Alternatively, it may be due to a need for some increase in PLO concentration in the inoculum. However, the effect of increased PLO concentrations was not examined, as the survival rate of the protoplasts declined sharply at higher PLO concentrations (Fig. 2). For the maximum infection of protoplasts, CYMV required an inoculum virus concentration higher than that of some other viruses[14,41]. It is interesting to note that potato virus X, the type members of the potex group, also required a high inoculum virus concentration[29].

The synthesis and the subsequent accumulation of CYMV-RNA, as detected by a Northern blot method[43], showed the replication of CYMV during the period of incubation. The percentage of infection with RNA in comparison with that of intact virus was low and it could be due to the lesser proportion of survived protoplasts following the treatment with PEG. However, for us, it is the method of choice for inoculating the protoplasts with viral RNA since PLO failed to give infection with RNA (data not shown).

In summary, this investigation has demonstrated that large proportions of populations of pea protoplasts are susceptible to infection by intact CYMV particles and, to a lesser extent, CYMV-RNA. Thus, the pea protoplast system seems to be suitable for studying molecular events in the replication of CYMV. Further improvement in infection efficiency with RNA may be achieved by other approaches such as electroporation[8] and electroinjection[36].

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和 文 摘 要

Piara S. BAINS・Hanumantha R. PAPPU・比留木忠治: エンドウ葉肉細胞プロトプラストのクローバ黄斑モザイクウイルス感染系の改良

エンドウ (*Pisum sativum* L. cv. Alaska) の葉肉細胞由来プロトプラストの分離について，一段法と改良二段法を比較した結果，同程度の収量（葉組織生重 1 g 当り $1\sim1.5\times10^7$ のプロトプラスト数）が得られた。分離に要する時間は Pectolyase Y23 を処理液に含めることによって通常法による 2 時間から 35 分まで短縮された。0.24 M りん酸緩衝液 (K_2HPO_4-KH_2PO_4) pH 6.3 を接種に用いて，クローバ黄斑モザイクウイルス (CYMV) 濃度を 3 μg/ml とし，poly-L-ornithine の存在下 (1.0～1.4 μg/ml) で最高感染率 57% が得られた。CYMV-RNA のプロトプラスト中における複製はノーザンブロット法によって確認された。ゲノム RNA の生成は接種 12 時間後に見いだされた。polyethylene glycol を用いた CYMV-RNA 接種法では生存したプロトプラストの約 35% が感染した。