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Electroporation - mediated infection of tobacco protoplasts with RNA of cucumber mosaic virus NT9 isolate

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Abstract. Tobacco (*Nicotiana tabacum* L. cv. Van-Hicks or W38) protoplasts prepared from mesophyll or suspension cells were inoculated with RNAs of NT9 isolate of cucumber mosaic virus (CMV) by electroporation. Immuno-assays including immunodot, enzyme-linked immunosorbent assay, immunofluorescent microscopy, as well as infectivity assay were used to measure the infection of protoplasts. Electroporation under optimal conditions resulted in the production of CMV in 70-90% of the surviving protoplasts. Optimal infection was achieved with 2 pulses of 500-1000 usec at a field strength of 1.3 KV/cm or 100-500 usec at 2.0 KV/cm. Optimal viral RNA concentration was about 40 μg/ml in a solution of 0.5 M mannitol.

Key words: Cucumber mosaic virus; Electroporation; Protoplast; Immuno-assays.

Introduction

Plant viruses usually do not infect plant cells synchronously which makes the detection of early events of virus replication difficult. Protoplasts provide a system for synchronous virus infection. Methods for the introduction of viral genes into plant protoplasts include the use of polycations, such as poly-L-ornithine (Aoki and Takebe, 1969), polyethylene glycol (Maule *et al.*, 1980), liposomes (Fukunaga *et al.*, 1981) and microinjection (Steinbiss and Stabel, 1983). Since electroporation has been used to introduce foreign genes into plant protoplasts (Fromm *et al.*, 1985), conditions for the introduction of plant viruses or viral RNA, such as: brome mosaic virus (BMV) (Watts *et al.*, 1987), BMV RNA (Watts *et al.*, 1987), cowpea chlorotic mottle virus (CCMV) (Watts *et al.*, 1987), CCMV RNA (Watts *et al.*, 1987), cucumber mosaic virus (CMV) (Okada *et al.*, 1986), CMV RNA (Nishiguchi *et al.*, 1986; Okada *et al.*, 1986), tobacco mosaic virus (TMV) (Okada *et al.*, 1986; Nishiguchi *et al.*, 1987), TMV RNA (Okada *et al.*, 1986; Nishiguchi *et al.*, 1986; Watanabe *et al.*, 1987), tobacco vein mottle virus (TVMV) RNA (Luciano *et al.*, 1987), tobacco etch virus (TEV) RNA (Luciano *et al.*, 1987) and potato virus Y RNA (Luciano *et al.*, 1987), etc. have been established. Nishiguchi *et al.* (1986) reported 46% of tobacco leaf protoplasts could be infected with CMV RNA by electroporation using a square wave pulse generator. Okada *et al.* (1986) described infection by CMV RNA in tobacco protoplasts by using the capacitor discharge method. The conditions they used were widely varied.

Recently, two local isolates of CMV, NT9 and M48, have been isolated and characterized (Hsu *et al.*, 1988; 1989). The former caused severe symptoms on all tested plants, whereas the latter with satellite RNA caused symptomless infection. By the pseudorecombination test, the symptom determinant resided on RNA 3 (Hsu *et al.*, 1988). These two isolates provide a good system to study the mechanism of symptom formation. In this report, we established the conditions for

the introduction of CMV-NT9 RNA into tobacco mesophyll or suspension protoplasts by a waveform type of electroporation. Approximately 60-80% of the protoplasts survived electroporation. Up to 80% of these were infected with CMV-NT9 RNA.

Materials and Methods

Isolation of Protoplasts

Sterilized seeds of *Nicotiana tabacum* var. Van-Hicks or Wisconsin 38 were grown in 1% agarose containing MS salt and 10g/l sucrose (Smith, 1986). Tobacco mesophyll protoplasts were isolated from 2 to 3-month-old seedlings according to the methods of Huang and Chen (1988). Suspension protoplasts were isolated from suspension cultures of tobacco according to Uchimiya and Murashige's method (1974).

Viral RNA

CMV-NT9 isolate was propagated on *N. tabacum* var. Van-Hicks (Hsu *et al.*, 1989). Virus purification and RNA extraction were described previously (Hsu *et al.*, 1989). The final RNA pellets were resuspended in sterile water and stored at -20°C.

Electroporation of Protoplasts

The protoplast pellets were suspended in 0.5 M mannitol to have a final concentration of 1.0×10^6 cells per ml. In each treatment, 0.2-0.4 ml of protoplasts were mixed with appropriate viral RNA and placed in a 24-well plate. Electroporation was performed by a Hoefer Pro-Genetor PG 101, which has three concentric electrodes spaced 1.5 mm apart. After electroporation, the protoplasts were kept on ice for 10 min and 0.6 to 0.8 ml of culture medium was added. Tobacco mesophyll protoplasts were cultured in a liquid medium of Huang and Chen (1988) and suspension protoplasts in a medium of Uchimiya and Murashige (1974). The protoplasts were cultured at 25°C in a growth chamber.

Immunoassays for Virus Infection

Various assays of viability and infection of protoplasts after electroporation were outlined in Fig. 1A. The detection of virus infection was routinely performed by immunodot assay. Protoplasts were usually harvested 72 hr after electroporation. After transferring the protoplasts to microfuge tubes, they were spun down at 2,000 rpm in a Kubota RA-150AM rotor for 5 min. The supernatant (designated as **Sup** hereafter) was saved, and the pellet was resuspended in 50 μl of distilled water. After repeatedly freezing, thawing and sonicating, the broken protoplasts were subjected to another low speed centrifugation. A full length of concentration and a 1:5 dilution of supernatant were saved and designated as $\mathbf{P_{S1X}}$ and $\mathbf{P_{S5X}}$, respectively. The pellet (designated as **PPt**) was again resuspended in 50 μl of distilled water. One microliter of each of the fractions were dotted on nitrocellulose membrane paper. The immunodot assay was conducted as described previously (Lin *et al.*, 1989), except the primary antibody 1:500 dilution of rabbit anti-CMV NT9 serum (Hsu *et al.*, 1989) and secondary antibody goat anti-rabbit IgG, alkaline phosphatase (AP) conjugates (Sigma), and their substrate BCIP and NBT (Bethesda Research Laboratories) used in stead. Procedures for obtaining the different fractions of protoplasts prepared for immunodot assays is outlined in Fig. 1B. For enzyme-linked immunosorbent assay (ELISA), the total of 50 μl $\mathbf{P_{S1X}}$ was coated on microtiter plates and indirect ELISA was performed as previously described (Lin *et al.*, 1989).

The percentage of infected protoplasts was determined by indirect fluorescent antibody staining. One drop of protoplast suspension was placed on a glass slide coated with 1% gelatin, fixed with 4% formaldehyde, and dried with a hair dryer. The slide was then immersed in acetone for 30 min at -20°C and washed with phosphate buffered saline (PBS), followed by a treatment of 1:500 dilution of anti-CMV-NT9 serum (Hsu *et al.*, 1989) for 1 hr at 37°C. After three washes with PBS, each for 10 min, a 1:75 dilution of rhodamine-conjugated goat anti-rabbit IgG (Sigma) in PBS was added for 1 hr at 37°C, followed by three more washes in PBS. The percentage of infection was examined under a Olympus BH-2 fluorescence microscope.

Viability and Infectivity Assay

Protoplast viability was determined by the Trypan Blue stain. The infectivity was assayed by the inoculation of protoplast extracts on local lesion host *Chenopodium quinoa* Willd.

Results and Discussions

In the preliminary test, protoplast viability was greatly affected by the combination of electric field

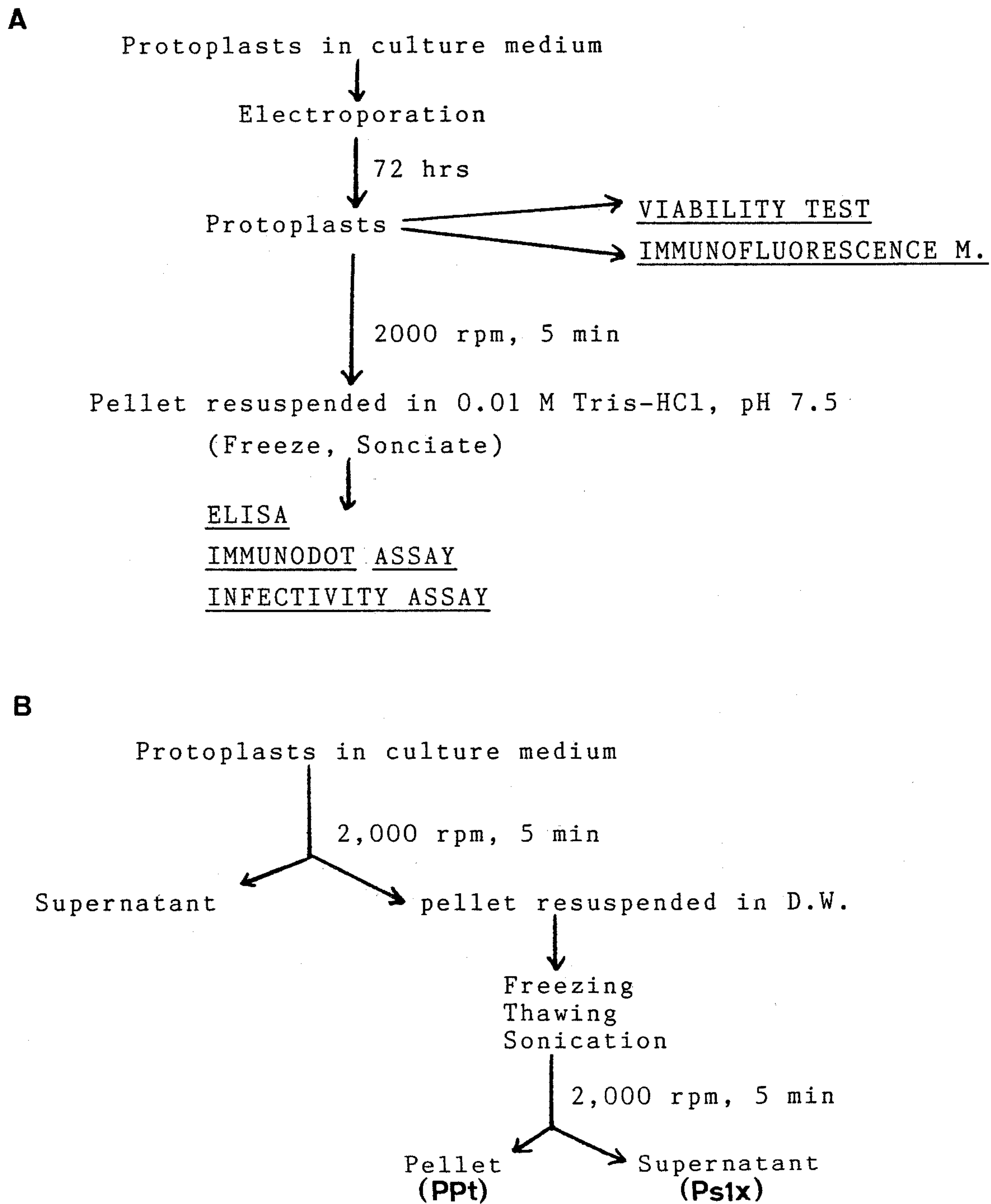

Fig. 1. (A) Outlines for assays of viability and infection of protoplasts 72 hr after electroporation. (B) Procedures for obtaining various fractions of protoplasts for immunodot assays.

strength and pulse duration. Thus, a mixture of protoplasts and CMV RNA were subjected to electroporation at different pulse voltages and pulse duration. Infection was first detected with 2 pulses each of 1 msec at a field strength of 1.3 KV/cm. Under this condition, protoplast viability was only slightly lower than the untreated ones. Even though it resulted in considerable detection of CMV infection, a field strength at 2.7 KV/cm for 1 msec or at 1.3 KV/cm for 10 msec generated irreversible damage to protoplasts. Under much milder conditions as shown in Fig. 2, a field strength at 2.0 KV/cm for a 100-500 usec pulse or at 1.3 KV/cm for 500-1000 usec generally resulted in 60-80% of the protoplasts surviving. Of the surviving protoplasts, 80% were infected by CMV-NT9 RNA. In considering the "dead space" in the pulsed wells as mentioned by Nishiguchi *et al.* (1986), the electroporation was routinely performed with two pulses. In some experiments, the percentage of infection was not significantly reduced when the number of pulses was conducted only once. However, the viability of protoplasts was not greatly affected whether 1 or 2 pulses were used. Therefore, we performed electroporation twice by changing the position of wells between the sets of pulses.

By immunofluorescent microscopy, the infected protoplasts were easily detected by showing a fluorescent red color when they were treated with anti-CMV-NT9 serum followed by a treatment of goat anti-rabbit IgG, rhodamine conjugates (Fig. 3). However, the uninfected protoplasts only showed weak autofluorescence. No reaction was observed when mock-inoculated protoplasts were treated with anti-CMV antibodies or when electroporated protoplasts were treated with normal rabbit serum (data not shown). Protoplasts prepared from suspension cells have advantages in immunofluorescent microscopic staining because of their weak autofluorescence.

For immunodot assays, the fractions of protoplast extract were prepared as described in Fig. 1B. If an

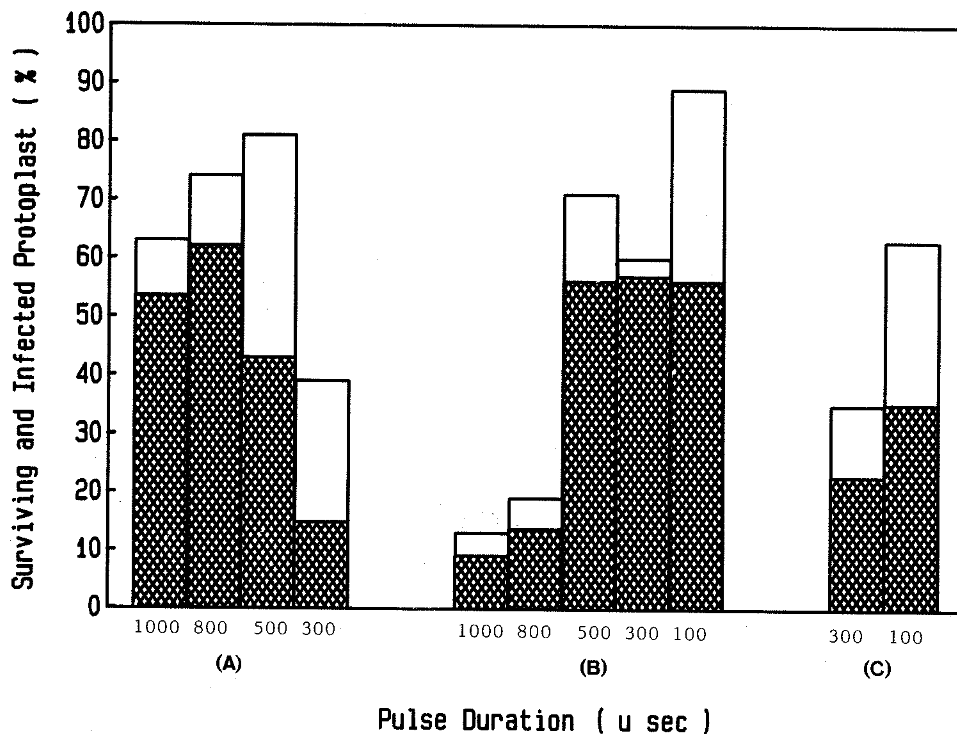

Fig. 2. Effect of field strength and pulse duration on the infection of tobacco protoplasts with CMV-NT9 RNA. Electroporation was performed in 0.5M mannitol in the presence of 40 μg/ml of CMV−NT9 RNA with 2 pulses of various pulse duration at a field strength of (A) 1.3 KV/cm (B) 2.0 KV/cm (C) 2.7 KV/cm.

: surviving protoplasts; : infected protoplasts.

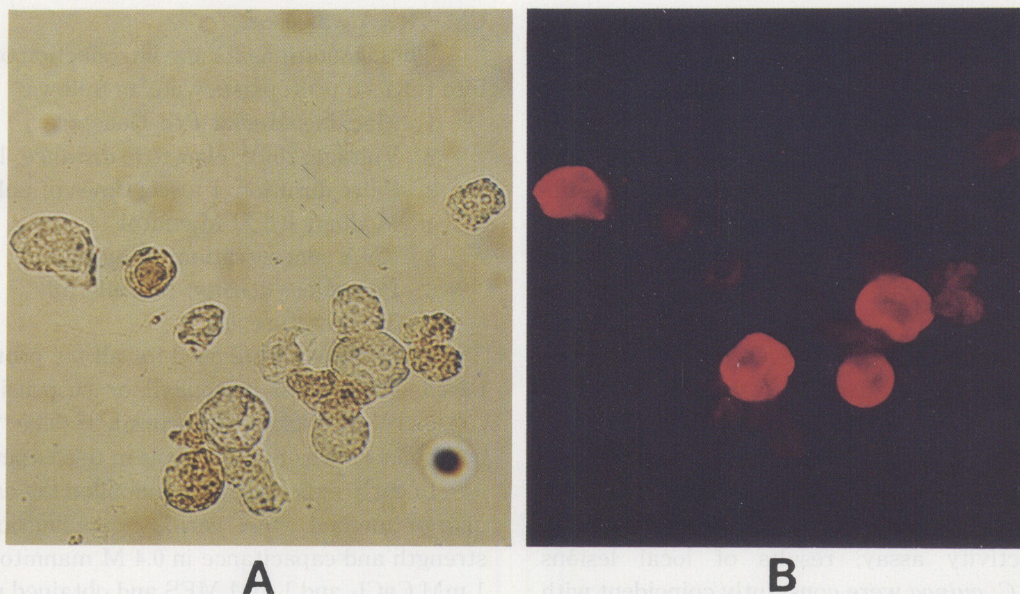

Fig. 3. Photographs of light microscopy (A) and immunofluorescent microscopy (B) of isolated protoplasts from suspension cells. Protoplasts were electroporated in the presence of 40 μg/ml of CMV-NT9 RNA with 2 pulses of 1 msec at a field strength of 1.3 KV/cm. The protoplasts were then immunostained with 1: 500 dilution of anti-CMV-NT9 serum, followed by goat anti-rabbit IgG, rhodamine conjugates (magnification $\times$ 240).

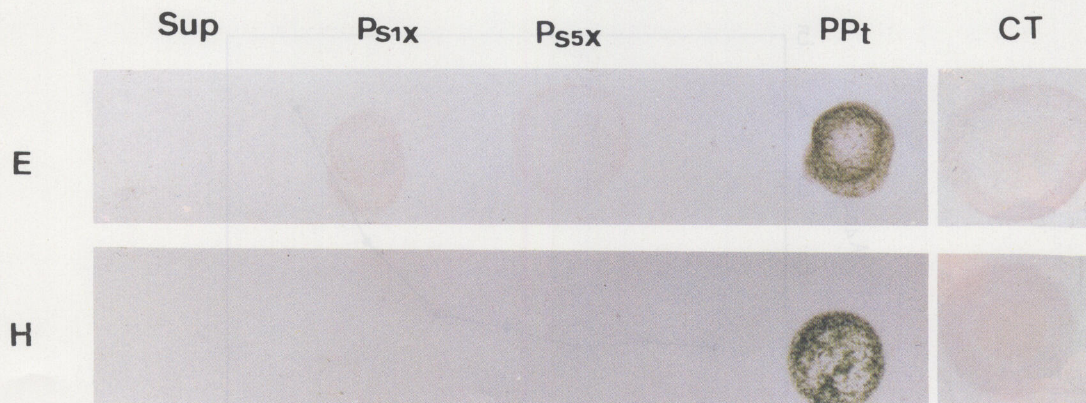

Fig. 4. Immunodot assays of various fractions of mesophyll protoplasts with (E) and without (H) electroporation using anti-CMV antiserum followed by goat anti-rabbit IgG- alkaline phosphatase. Fractions designed for **PPt**, $\mathbf{P_{S1X}}$, $\mathbf{P_{S5X}}$ and **Sup.** were described in the text. Immunostained dots of purified CMV viruses were used as a control (CT).

infection succeeded, the viral antigen was always detectable in the pelleted supernatant both in full length concentrations (e.g. **P** S1X) and in 1:5 dilutions (e. g. **P** S5X). They showed purplish spots on the blots (Fig. 4), whereas the pellet (**PPt**) of broken protoplast debris showed a dark green color (from mesophyll protoplast preparation) or a slightly gray color (from suspension protoplast preparation). These spots were easily distinguishable from the purplish spots of a true reaction. Viruses were never detected in the culture medium as seen in supernatant (**Sup**), indicating that no viruses were released into the medium after infection. In some experiments, immunodot could even detect the infection in the 1:25 dilution of protoplast extract. On the basis of sensitivity of the dot blot using the AP system between 20-50 pg (Promega Biotec, product description "ProtoBlot"), the amount of viral antigen per protoplast was between 0.195-0.487 pg.

By infectivity assay, results of local lesions produced on *C. quinoa* were constantly coincident with those of immunoassays. Generally, mixtures of **Psx** and **PPt** produced local lesions on *C. quinoa,* whereas the fractions of **Sup** did not. In a mixture of protoplasts and CMV RNA for 72 hr incubation without electroporation, none of the fractions produced any lesions.

To examine the effect of CMV RNA concentration on the level of infection, conditions for electroporation were set at a 1.3 KV/cm field strength for 1 msec pulse. ELISA was performed in this study. Fig. 5 shows that a few protoplasts were infected even at 0.6 μg/ml CMV RNA. Maximal infection was reached when 40 μg/ml CMV RNA was used.

Thus, conditions for the introduction of NT9 RNA into tobacco protoplasts were as follows:

1. Machine: Hoefer Pro-Genetor
2. Voltage: 200V; electrode distance: 1.5 mm
3. Pulse duration: 1 msec; times of pulse: 2
4. Medium: 0.5 M Mannitol
5. RNA concentration: 40 μg/ml
6. Protoplast density: 10^6 cells/ml
7. Temperature: 0°C

These conditions were used for all the protoplasts prepared either from mesophyll or suspension cells, of tobacco Van-Hicks or Wisconsin 38. The "host-RNA" combination was not stringent in this experiment.

In early experiments, we applied the capacitor discharge method with various combinations of field strength and capacitance in 0.4 M mannitol containing 1 mM $CaCl_2$ and 1 mM MES and obtained no infection. Infection was eventually obtained by using a square wave pulse generator under the conditions as described. The field strength we used (1.3-2.0 KV/cm) was much lower than that of Nishiguchi *et al.* described (5.0-10.0 KV/cm) (1986) and the level of infection was even higher. Of the surviving protoplasts, 70-90% were infected. The immunoassays employed here including immunodot, ELISA and immunofluoresent microscopy showed the synthesis and accumulation of

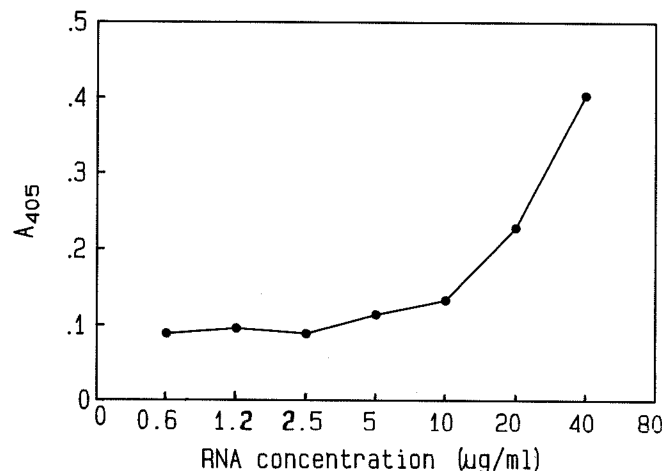

Fig. 5. Effect of concentration of CMV RNA on the infection of tobacco protoplasts by electroporation. Protoplasts were electroporated with 2 pulses of 1 msec at a field stretgth of 1.3 KV/cm. Homogenates from 2×10^5 protoplasts were assayed for CMV using ELISA (expressed as absorption at 405 nm).

capsid protein of CMV-NT9 in electroporated cells after their RNA delivery. Local lesion bioassay on *C. quinoa* also indicates that replication of viral RNA occurs. Electroporation indeed is a much more efficient and simple method when compared to conventional methods (Muhlbach, 1982; Takebe, 1983; Sairder and Mertes, 1984). This will facilitate the analysis of RNA and protein synthesis of CMV-NT9 and -M48 RNA at an early stage of infection at a single cell level. Furthermore, it will also help in investigating the *in vivo* interactions between RNA 5 and genomic RNAs, or in determining the cistron(s) of RNA 3 controlling the symptom formation.

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電導菸草原生質體感染胡瓜嵌紋病毒 NT9 分離株之 RNA

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從菸草(萬國士或 W38)葉肉細胞或組織培養細胞懸浮液分離而得之原生質體，以電導方式感染胡瓜嵌紋病毒 NT9 分離株之 RNA。本文以免疫偵測法中之如墨點法、酵素聯結吸附法和螢光顯微鏡法，以及感染力測定法來偵測原生質體之感染情形。在適當之條件下，經電導法可使存活之原生質體有 70-90%之感染率。此條件爲電場強度 1.3KV/cm，電擊 500-1000 微秒，或於 2.0KV/cm，電擊 100-500 微秒，共電擊二次。所用 RNA 之最適濃度爲 0.5M 甘露醇溶液中含 40μg/ml。