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Is a helper factor necessary for infection of cowpea protoplasts with blackeye cowpea mosaic virus?

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The potyvirus group is the largest, and economically very important, group of plant viruses (Edwardson, 1974). Potyviruses possess flexuous virions, about 720 nm long, consisting of a single-stranded RNA molecule of approximately 3.2×10^6 Da and a coat protein of about 33 000 Da, which induce characteristic pinwheel inclusions in cells of infected plants (Hollings and Brunt, 1981). Goffinet and Verhoyen (1979) were the first to report successful inoculation of protoplasts with a potyvirus (potato virus Y) (PVY). They had shown by bio-assay, serology (microprecipitation test) and electron microscopy that PVY had multiplied in mesophyll protoplasts of tobacco *N. tabacum* 'White Burley'). In our preliminary studies we had repeated their experiments with PVY and tobacco protoplasts, but we could not achieve any infection. There was also no evidence of infection of cowpea (*Vigna unguiculata*) protoplasts with blackeye cowpea mosaic virus (BlCMV) purified according to their method for PVY. Zhengkaixu et al. (1984), following the procedure of Goffinet and Verhoyen (1979), were also unable to get infection of tobacco (*N. tabacum* 'Xanthi') protoplasts with another potyvirus (tobacco vein mottling virus). This paper describes the conditions required for infection of cowpea protoplasts with BlCMV.

Two isolates of BlCMV were used: one originally isolated from yard-long bean (*V. unguiculata* subsp. *sesquipedalis*) at Wageningen (BlCMV-W) and the other from diseased soybean plants (*Glycine max*) in Indonesia (BlCMV-Ind). The viruses were mostly propagated in plants of *N. benthamiana* and purified from diseased leaf material by the procedure of Lima et al. (1979) with some minor modifications. Purified virus suspensions were stored in liquid nitrogen. For comparison we also used a purified suspension of cowpea mosaic virus (CPMV). For preparation of virus RNA a BlCMV suspension (4 ml), containing 10 mg ml^{-1}, was added to 16 ml of a solution containing 8 ml 0.2 M Tris-HCl buffer pH 9.0 with 0.01 M EDTA and 0.1 M NaCl, 2 ml 10% (w/v) 4-amino-salicylic acid and 2 ml double-distilled water. This mixture was incubated at room temperature for 15 min, extracted with phenol twice and the RNA was precipitated with ethanol and stored under 70% (v/v) ethanol at -20 °C.

Mesophyll protoplasts were isolated from the primary leaves of 10 to 11-day-old

cowpea (*V. unguiculata* subsp. *unguiculata* 'California Blackeye') as described by Van Beek et al. (1985), except that four half leaves devoid of lower epidermis were floated on 50 ml 0.8% (w/v) unpurified Cellulase Onozuka R-10 and 0.1% (w/v) Macerozyme R-10 in 0.5 M mannitol. To increase the number of intact, viable mesophyll protoplasts the gentle isolation method described by Fannin and Shaw (1982) was also used. With both methods protoplast yield was approximately 10×10^6 protoplasts g^{-1} of leaf (fresh weight) and the percentage of viable protoplasts, as assessed by staining with fluorescein diacetate (Larkin, 1976), was approximately 80%.

The protoplasts were inoculated with either purified BlCMV-W or -Ind (V) (150 μl at 0.3 to 2.0 mg ml^{-1}) mixed with 100 μl deionized water containing 25 mg mannitol, or a virus-containing supernatant fluid (VS) (250 μl), or a mixture of V (150 μl) and VS (100 μl), or a mixture of V (150 μl) and a virus-free supernatant fluid (S) (100 μl), or a mixture of V (150 μl) and VS exposed to UV-irradiation for 4 h (UVS). Supernatant VS was obtained by centrifugating sap squeezed from leaves of diseased *N. benthamiana* plants for 10 min at approximately 6500 *g* and S by centrifugating sap squeezed from leaves of diseased *N. benthamiana* plants for 90 min at 100 000 *g*. For inoculation with BlCMV-RNA, 150 μl containing 0.2 to 1.0 mg ml^{-1} RNA in 0.4 M mannitol were used. Mock-inoculated protoplasts (with 150 μl 0.5 M mannitol only) and protoplasts inoculated with a mixture of V (150 μl) and supernatant fluid (HS) (100 μl) obtained after low-speed centrifugation of sap from uninfected *N. benthamiana* plants served as controls. In each experiment inoculations were also done with a purified suspension of CPMV (4 μl virus at 18 mg ml^{-1} and 100 μl 0.5 M mannitol) to test the ability of the protoplasts to support virus multiplication.

The infectivity of the inocula used was assayed on *Chenopodium amaranticolor* and *C. quinoa*. Depending on the susceptibility of the *Chenopodium* species the number of local lesions ranged from 100 to 200 per leaf of *C. quinoa* for the inocula containing V or V+VS and from 50 to 100 per leaf for the inocula containing VS or virus RNA. No infection occurred when S or UVS from diseased *N. benthamiana* plants was used as inoculum. Inocula with CPMV induced 50 to 200 local lesions per leaf of *C. quinoa*. Leaves of *C. amaranticolor* usually gave fewer lesions, both with BlCMV and with CPMV.

Inoculation and incubation of protoplasts were done by both the methods of Van Beek et al. (1985) and Fannin and Shaw (1982).

For infectivity assays protoplasts were washed in 0.35 M KCl and centrifuged for 3 min at 100 *g*. The pellet was triturated in a roughened watch-glass with a few drops of 0.1 M phosphate buffer pH 7.0. The ground material was mixed with 300 μl of the same phosphate buffer and inoculated onto one plant each of *C. amaranticolor* and *C. quinoa*, two plants of *N. benthamiana* and three plants of *V. unguiculata*. In some experiments only the *Chenopodium* species were used. Local lesions on *Chenopodium* species were counted 10 days after inoculation and symptoms on the other plant species were assessed 10 to 14 days after inoculation.

For electron microscopy protoplasts were fixed, embedded, sectioned and stained as described by Rezelman et al. (1982). Ultrathin sections were made only of protoplasts belonging to batches which had reacted positively in bio-assay.

Pinwheel inclusions were purified from BlCMV-infected *N. benthamiana* leaves, as described by Dougherty and Hiebert (1980) for cylindrical inclusions induced by tobacco etch virus and pepper mottle virus. The amount of protein was determined

by the method of Lowry et al. (1951). Yields up to 10 mg protein per 100 g leaf tissue were obtained.

For preparation of antiserum to pinwheel inclusions a rabbit was immunized intramuscularly with a suspension containing 0.2 mg pinwheel inclusion protein in 1 ml 0.02 M Tris-HCl buffer pH 8.2 emulsified with 1 ml of complete Freund's adjuvant. A second injection with 0.8 mg was given two weeks later. Two weeks thereafter the rabbit was bled and the antiserum was absorbed with purified virus.

Fluorescent antibody staining was done as described by Van Beek et al. (1985) with a 1/100 dilution of the gamma globulin fraction (1 mg ml^{-1}) of antiserum to BlCMV-Ind (titre 128), to BlCMV-W (titre 512), to pinwheel inclusions or to CPMV (titre 2048).

In preliminary experiments results obtained with BlCMV-W were similar to those with BlCMV-Ind, therefore the virus is referred to solely as BlCMV, whereby the results of both BlCMV isolates are presented.

With V as inoculum, no infection of protoplasts could be established, in contrast to the controls inoculated with CPMV. Bio-assay of protoplasts only rarely yielded an infected plant, both in the series of protoplasts assayed immediately after inoculation and in those sampled at 48 h and 65 h after inoculation. These protoplasts stained with fluorescent antibodies to virus particles showed fluorescent string- and thread-like structures on, and in between, the protoplasts. This fluorescense clearly was due to virus particles still attached to the protoplasts (even after washing and incubation) as specimens stained with fluorescent antibodies to pinwheel inclusions did not show any fluorescence. Protoplasts assayed immediately after inoculation with CPMV did not show fluorescence when stained with fluorescent antibody to CPMV, indicating the absence of attached virus particles. In samples taken 48 h after inoculation, the protoplasts infected with CPMV showed a bright green fluorescing network and could easily be distinguished from the dull green uninfected ones. About 50 to 80% of the protoplasts were infected with CPMV.

No infection could be established with V-RNA as inoculum; bio-assay of inoculated protoplasts never yielded infected plants.

In contrast to the experiments with V and V-RNA, protoplasts in most of the experiments with V+VS as inoculum became infected. Bio-assay of protoplasts sampled both 48 h and 65 h after inoculation yielded systemically infected plants of *N. benthamiana* and *V. unguiculata* and local lesions on *C. amaranticolor* and *C. quinoa* (approximately 10 per leaf). With fluorescent antibody to pinwheel inclusions, there was no fluorescence in the series immediately after inoculation, but in the 48 h and 65 h series protoplasts showed a bright fluorescence in the shape of distinct angular spots and an infection percentage of approximately 18% was established. Ultrathin sections of protoplasts sampled 48 h after inoculation, revealed the presence of pinwheel inclusions (Fig. 1).

When VS was used as inoculum, infection of protoplasts also occurred, as shown by bio-assay, by immunofluorescence and by electron microscopy. There was no difference in infection when VS or V+VS was used as inoculum. No infection was observed either with V+S, V+UVS, or with V+HS. The above-mentioned results were obtained with protoplasts prepared and inoculated by both the methods of Van Beek et al. (1985) and Fannin and Shaw (1982).

The results obtained by bio-assay, immunofluorescence and electron microscopy showed that infection of protoplasts occurred only when the latter had been in-

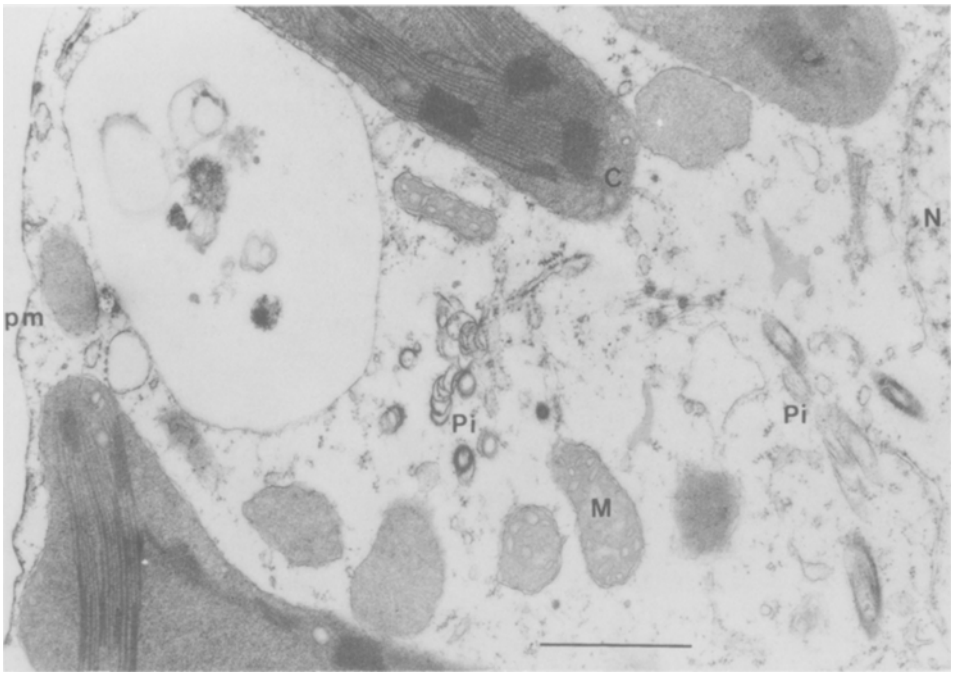

Fig. 1. Electron micrograph of a mesophyll protoplast of *Vigna unguiculata* infected with BlCMV, 48 h after inoculation with a mixture of purified virus and supernatant fluid obtained after low-speed centrifugation of sap from BlCMV-infected plants of *Nicotiana benthamiana*. C, Chloroplast; M, Mitochondrion; N, Nucleus; Pi, Pinwheel inclusions; pm, protoplast membrane. Bar marker represents 1 μm.

oculated with a mixture of V and VS, or with VS alone and not with purified virus alone. Although V was even more infective in bio-assays than VS, infection of protoplasts could only be established with VS. Therefore, it would appear that VS contains a factor essential for the infection of protoplasts. It is conceivable that this factor is changed in one of the steps in the purification procedure. Our results with RNA are in line with those reported by Zhengkaixu et al. (1984), but conflict with those of Barker and Harrison (1984). The former authors were unable to achieve infection of 'Xanthi' tobacco protoplasts with unencapsulated RNA of tobacco vein mottling virus, but approximately 15% of the protoplasts became infected when they used RNA encapsulated in liposomes as inoculum. Barker and Harrison (1984), however, reported that they had obtained infection of mesophyll protoplasts of 'Xanthi' tobacco and potato with unencapsulated RNA of PVY. We have no explanation to offer for these conflicting results. For the negative results with unencapsulated potyvirus RNA, we may hazard that there is a similarity with the inability of aphids to transmit viruses after probing through membranes into solutions of viral RNA (Pirone and Megahed, 1966), but this needs further investigations.

Samenvatting

Is een helper-factor nodig voor infectie van 'cowpea'-protoplasten met 'blackeye cowpea mosaic virus'?

Bij inoculatie van mesofyl protoplasten van 'cowpea' met een zeer infectieuze, gezuiverde suspensie van 'blackeye cowpea mosaic virus' (BlCMV), een potyvirus, kon een infectie niet worden geconstateerd. Wanneer echter de bovenstaande vloeistof, verkregen na centrifugering bij laag toerental van sap van BlCMV-geïnfecteerde planten, als inoculum werd gebruikt, dan kon vermeerdering van het virus in de protoplasten worden vastgesteld door middel van infectiositeitsproeven, immunofluorescentie en elektronenmicroscopie. Werd deze bovenstaande vloeistof ontdaan van zijn infectiositeit, hetzij door bestraling met UV-licht, hetzij door centrifugering bij hoog toerental, en vervolgens gemengd met gezuiverd virus, dan trad er geen infectie van de protoplasten op.

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