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Infection of cowpea mesophyll protoplasts by tobacco mosaic virus and tobacco necrosis virus

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Isolated protoplasts are being used in plant virology to answer questions about virus replication and the influence of virus infection on plants.

Many plants show a hypersensitive reaction to virus infection in producing local necrotic lesions. Necrosis, however, is not expressed in isolated protoplasts. An answer to the question why protoplasts fail to show any sign of necrosis could shed light on the mechanism of hypersensitivity, which is far from being understood yet.

As a model cowpea (*Vigna unguiculata* L.) Walp. cv. Blackeye Early Ramshorn was chosen in combination with a common strain of tobacco mosaic virus (TMV) and with a strain of tobacco necrosis virus (TNV) serotype A. Both viruses induce necrotic local lesions in cowpea.

We initiated a study of the TMV and TNV replication in cowpea mesophyll protoplasts. The first results are reported here.

For isolation of the protoplasts the method of Alblas and Bol (1977) was used. TMV was purified following the polyethylene glycol method of Gooding and Hebert (1967). The purification procedure for TNV has been described before (Wieringa-Brants, 1977).

For inoculation of the protoplasts a standard procedure was used. 10 ml inoculation medium, containing 9.3 ml mannitol 0.7 M; 0.1 ml poly-L-ornithine m.w. 122000 (200 µg/ml); 0.1 ml virus suspension (TMV: 2 mg/ml of TNV: 1.6 mg/ml) and 0.5 ml 0.2 M phosphate buffer pH 5.6, was pre-incubated for 10 min at 20°C. 6×10^5 protoplasts were sedimented from the 0.5 M mannitol solution and resuspended in the inoculation medium. After 15 min at 20°C the protoplasts were washed by three cycles of centrifugation at 200 *g* for 3 min to remove non-adsorbed virus and resuspended in sterile culture medium containing 0.5 M mannitol and 10 mM $CaCl_2$. To inhibit the growth of bacteria and fungi 20 µg/ml gentamycin sulphate (Sigma Chem. Comp.) and 50 µg/ml mycostatin (Squibb & Sons, N.Y.) were added. Portions of 10 ml were incubated in 100 ml Erlenmeyer flasks at 20°C under continuous illumination (about 3000 lux). Each portion was supplied with 10 µg mycostatin every 24 h. At 0, 1 and 2 days after inoculation protoplasts were collected by centrifugation at 300 *g* for 3 min. Pellet and supernatant were stored at –20°C. The pellets were resuspended in 1 ml water and homogenized using ultrasonification. The homogenate and supernatant were tested for virus activity in a local lesions assay on four half leaves of *Nicotiana tabacum* L. cv. Xanthi nc.

A slight infectivity was detectable in the pellet directly after the inoculated protoplasts had been washed (Table 1). One day after inoculation the infectivity had in-

Table 1. Virus production in cowpea-protoplasts after infection with TMV and TNV. Number of lesions on 4 half leaves of *Nicotiana tabacum* L. cv. Xanthi nc.

Days after inoc.	Sample	TMV: number of lesions		TNV: number of lesions	
		exp. 1	exp. 2	exp. 1	exp. 2
	inoculation-medium	14	88	135	145
0	supernatant	0	0	2	2
0	pellet	7	10	5	20
1	supernatant	0	2	50	13
1	pellet	199	347	86	126
2	supernatant	4	40	40	20
2	pellet	149	491	621	380

Tabel 1. Virusproduktie in cowpea-protoplasten na infectie met TMV en TNV. Aantal lesies op 4 halve blaadjes van Nicotiana tabacum L. cv. Xanthi nc.

creased, especially when TMV was used. Two days after inoculation the infectivity in protoplasts inoculated with TNV was high. In earlier experiments with bean leaves infected with different strains of TNV, however, we found that the first two days after inoculation the virus content of the leaves was low (Wieringa-Brants et al., 1978).

We did not prepare antisera with fluorescent antibodies, so it was not yet possible to determine the percentage of the protoplasts that became infected under the conditions described above. We may conclude, however, that it is possible to infect cowpea protoplasts with both TMV and TNV.

Samenvatting

Infectie van mesofylprotoplasten van cowpea door tabaksmozaïekvirus en tabaksnecrosevirus

Protoplasten werden geïsoleerd uit cowpeabladeren en konden worden geïnfecteerd met tabaksmozaïekvirus en tabaksnecrosevirus serotype A. De virusvermeerdering in de protoplasten werd tot twee dagen na de inoculatie onderzocht.

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