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The Use of Rocket Immunoelectrophoresis to Detect Tobacco Mosaic Virus in Infected Pepper Tissues

By

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Rocket immunoelectrophoresis was used and compared with local lesion assay to detect tobacco mosaic virus in infected peppers. Hyperimmune mouse ascitic fluid was subjected to rocket immunoelectrophoresis for quantification of the content of tobacco mosaic virus in pepper plants. The rocket immunoelectrophoresis proved to be as sensitive as the local lesion assay in the case of the pepper plants.

Resistance to plant viruses is a major objective of pepper breeding programs. One of the most difficult problem is to determine virus concentration in different pepper lines after infection. This is due to the high inhibitor content of the host species. With due care local lesion assay can distinguish a difference of 10—20% between two similar preparations. However different pepper cultivars contain different amounts of inhibitor therefore this method is not generally satisfactory for testing breeding lines. It is known that many unwarranted conclusions may be drawn from local lesion assays. The major aspects are the wide variation in number of local lesions induced on different leaves by the same inoculum, the statistical requirements for making valid comparisons and the general nature of the curve relating dilution of inoculum and lesion number. To solve this problem we devised a method by which one can eliminate the effect of inhibitors on the virus content of peppers. This method combines the quantitative immunoelectrophoresis procedure (rocket assay) and a method which applies antibodies to tobacco mosaic virus (TMV) produced in ascitic mouse fluid (KIRIYAMA and OHSUMI, 1973). This combined method proved to be as sensitive but more reliable than the local lesion assays for detection of TMV in pepper tissue.

Materials and Methods

Purification of TMV. The Ul strain of TMV was propagated in *Nicotana tabacum* L. cv. Samsun. Plants were grown under greenhouse conditions and were used at the six to eight leaf stage. The virus was purified according to FRAENKEL-CONRAT (1966) modified to include a precipitation with polyethylene-glycol 6000 (GOODING

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and HEBERT, 1967). For immunization the virus concentration was adjusted to 10 mg/ml.

Antibody production. Ten six-week-old female CFLP mice were injected intraperitoneally with an emulsion of the antigen and Freunds incomplete adjuvant (0.1 ml each). Four identical booster injections were given at two weeks intervals. Two days after the last injection the animals received 5×10^6 Ehrlich Lettre ascites cells in 0.5 ml physiological saline solution by the intraperitoneal route in order to achieve immune ascitic fluid accumulation. Then days later when they showed distended abdomens, the mice were killed and the ascitic fluid was tapped. Fluids were pooled and immediately centrifuged at 4000 g for 30 min at 4 °C, to pack the cells. Immunoglobulins from the pooled ascitic fluid were purified by ammonium sulphate precipitation and on a DEAE 50 Sephadex A column (1 × 10 cm) according to HARBOE and INGILD (1973). The immunoglobulin preparation was concentrated to the original ascites fluid volume by dialysis in 0.1 *M* NaCl/15 *mM* NaN_3 solution containing 20% polyethylene-glycol 20 000 and stored at 4 °C. Protein content of the purified antibody preparation was determined by the microbiuret test (GOA, 1953) and adjusted to 5 mg/ml.

Rocket assays. The procedure was carried out according to (WEEKE, 1973) using a 1.5 mm layer of 1% agarose and barbital buffer, pH 8.6 at 0.02 ionic strength. The circular application wells had a diameter of 4.0 mm. The antibody content in the gels was 0.1 $\mu l/cm^2$ for the quantification of TMV samples containing 0.1 – 1.0 μg viral nucleoprotein or 0.5 $\mu l/cm^2$ for 1.0 – 4.0 μg TMV. An electric current of 1 V/cm was applied for 18 h at 15 °C. Immunoprecipitates were stained with Coomassie Brillant Blue G-250 (ethanol : water : acetic acid 45 : 45 : 10). Antigen (TMV) concentrations were determined using calibration curves based on known quantities of purified TMV. The average heights of the precipitates were calculated from five identical electrophoretic runs. Two different calibration curves (0.1 – 1.0 μg and 1.0 – 4.0 μg) were plotted. Local lesion assays were performed on *Datura stramonium* L. and *Nicotiana tabacum* L. cv. Xanthi-*nc*. with the same TMV preparations.

Hydroxylapatite column chromatography. For preparing intact long rod TMV particles, the purified TMV samples were loaded onto a 1 × 10 cm column of hydroxylapatite (BIO-GEL HTP) in 0.001 *M* potassium phosphate buffer, pH 7.8. The sample was eluted with a linear potassium phosphate gradient from 0.001 *M* to 0.20 *M* taking 1.5 ml fractions. The column was eluted with 80 ml of buffer. The nucleoprotein content of the fractions was determined spectrophotometrically at 260 nm. The fractions contaning intact particles were subjected to rocket immunoelectrophoresis and used for electron microscope (on carbon-backed Formvar-coated grids and negative stained with 2% of dodeca tungstophosphoric acid, pH 7.2).

Comparison of the rocket assay and local lesion assays. At the eight leaf stage, the four lower leaves of pepper plants (*Capsicum annuum* L. cv. Javított cecei) were inoculated with 0.1 mg/ml TMV. At different times after inoculation pepper leaves were homogenized with a mortar and pestle (part fresh weight of tissue and 2 parts

of 0.01 *M* phosphate buffer, pH 7.0) and the cell-debris was removed by centrifugation at 5 000 *g* for 30 minutes. Samples containing 15 μl of the supernatant (or dilutions thereof) were subjected in duplicate to rocket assay and local lesion assays on Xanthi-nc tobacco or *Datura stramonium*. On the basis of precipitate peak heights, the TMV concentration was calculated and expressed in μg per mg fresh weight of the infected tissue. Samples were taken from different parts of plants at different intervals after infection (3, 5, 7, 10 and 14 days). For these experiments the local lesion assay was done using ten 10 mm leaf discs per sample. These were ground as before with a mortar and pestle and applied onto hypersensitive responding tobaccos.

Results

All mice survived the immunization schedule and a total of 120 ml of ascitic fluid was collected. The antibody titre of the purified immunoglobulin preparation was 12 820 μg/ml^{-1} as determined by the rocket assay according to HARBOE and INGILD (1973). The purified antibody preparation was tested for reactions with healthy tobacco, and pepper sap, as well as with purifed viruses which also infect the peppers. These included alfalfa mosaic, cucumber mosaic and tomato aspermy viruses. No serological reactions occurred in these tests indicating that the antibody preparation was specific for TMV. Using this ascitic fluid for the rocket assay we compared this procedure to the local lesion assay for both sensitivity and precision. The results shown in Fig. 1 indicate that both procedures respond in approximately linear fraction to increasing amounts of the virus. However, the precision of the

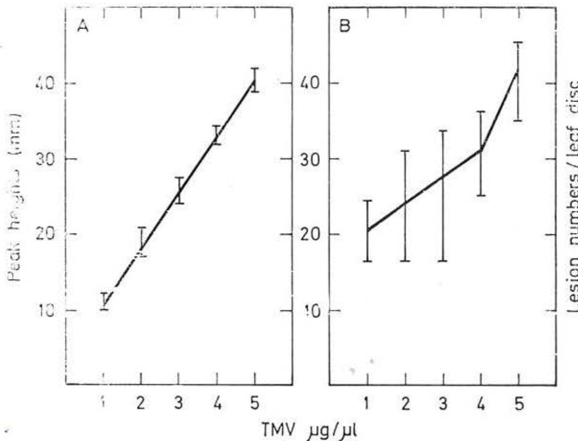

Fig. 1. Comparison of rocket assay with the local lesion assay. (A) Rocket immunoelectrophoresis of TMV preparation at 1, 2, 3, 4 and 5 μg. The antibody content in the gel was 0.5 μl/cm^2. Data represent of five replications. (B) Local lesion assay of TMV preparation at 1, 2, 3, 4 and 5 μg on Xanthi-*nc* leaf discs. Data were plotted from five replications

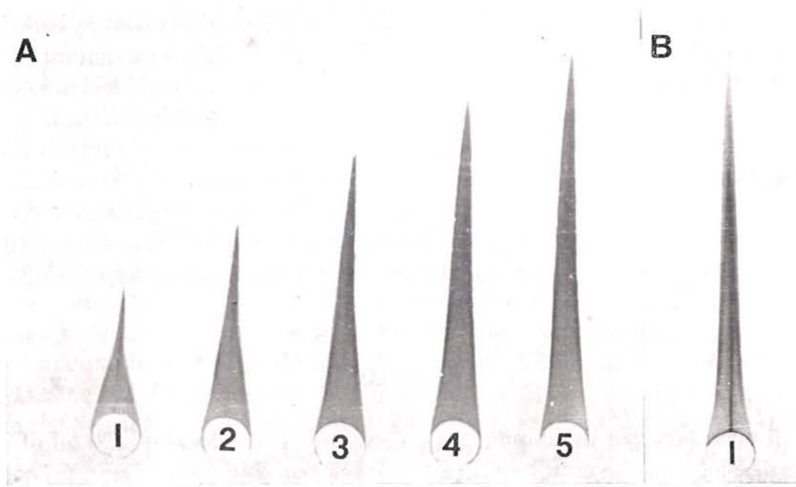

Fig. 2. Rocket immunoelectrophoresis of TMV preparation purified on hydroxylapatite column. (A) The antibody content in the gel was 0.5 μl/cm^2. Wells 1–5 from the left, 1, 2, 3, 4, and 5 μg TMV. Anode at the top. (B) Crude TMV preparation was subjected to the rocket assay in the same condition at 5 μg concentration. (B 1)

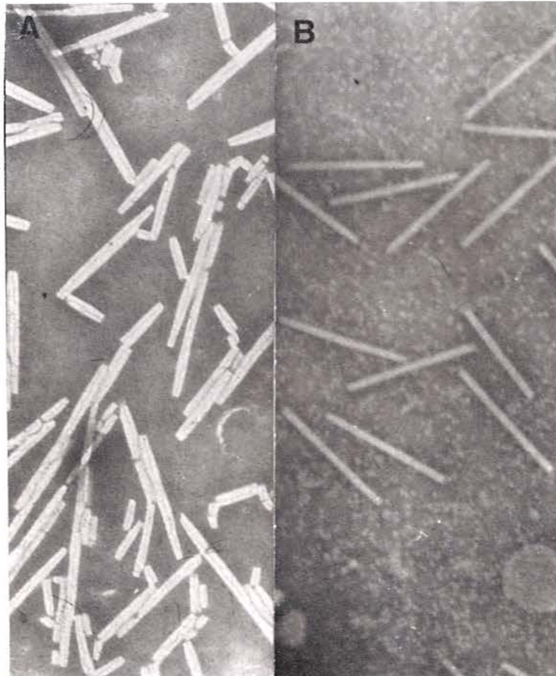

Fig. 3. Electron microscopic pictures of TMV samples before passing (A) and after (B) the column. (1 × 56,000) magnification

rocket assay is much greater. When a crude preparation of TMV was subjected to the rocket assay three peaks were observed suggesting the presence of three electrophoretically different fractions. Further purification of our virus preparation on the hydroxylapatite column resulted in electrophoretically homogenous peaks (Fig. 2 A + B). These peaks also demonstrated that the aggregation of the TMV disturb the precipitate peak heights. (Compare Fig. 2 A and B.) By means of the rocket assay TMV infected samples containing 0.1 – to 4.0 μg viral nucleoprotein can be quantitated at the accuracy of 99.2%. We controlled our TMV samples by using electron microscope before and after passing through of hydroxylapatite column. In our preparation we always observed breakdown products, aggregations, and incomplete particles. However, by using the hydroxylapatite column we were able to eliminate most of TMV antigens that were less then full length particles (Fig. 3).

To test assay method for the pepper breeding program we studied the virus movement and virus content in pepper plants following infection. Samples were taken from the inoculated leaves, and those above, from the top and from the roots. The virus content was measured using the rocket assay and simultaneously in local lesion test. Data are summarized in Fig. 4.

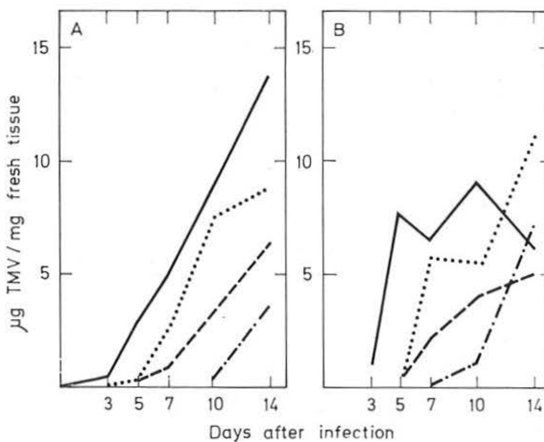

Fig. 4. TMV content of Javított cecei pepper plants after infection. Measured by rocket assay (A) and by local lesion assay (B) on Datura leaf discs. Data were summarized from three experiments. Samples were taken from inoculated leaf (solid line) and those above (broken line) from the top (dotted line) and from the roots, (alternate dots and dashes)

Discussion

The major advantage of using mouse ascitic fluid is the ease with which antibody pools can be obtained. The use of pools in any kind of serological studies is strongly advisable because of the high individual differences in the immune response of the animals (Herbert, 1973). Mouse ascitic fluid antibodies have been prepared

against TMV antigen (KIRIYAMA and OHSUMI, 1973) however the use of immune ascitic fluid in quantitative immunoelectrophoresis was only reported in the field of fungal serology (HORNOK and SZÉCSI, 1977). The use of the rocket assay for quantitative estimation of TMV is economical rapid. Thus, the method is suitable for routine purposes such as estimating virus content and testing varietal susceptibility. For the preparation of the 10 × 20 cm immunoplate containing 0.5 μl antibody solution/cm² 100 μl of ascitic fluid is needed. This means that the peritoneal fluid of an average mouse is sufficient for 100 to 120 plates. On such a plate as many as 40 samples can be detected in infected tissue at a lowest concentration of 0.067 μg/μl. The sensitivity of the method can be increased by applying larger sample volumes.

Considering the fact that serological methods have some disadvantages; i.e. they measure the virus protein antigen, instead of the amount of infective virus, in the case of rod shaped viruses as TMV end to end aggregation can markedly affect the results, and applyied only to limited number of plant viruses (MATTHEWS, 1970). In the case of the above described method we were able to eliminate the first two disturbing factors. Further experiments are in progress to determine if other plant viruses may be assayed by this method.

From our test experiment, using pepper plant which contains different amount of inhibitors, it turned out that our method is as sensitive as the local lesion assay and it is much more accurate as compared to it. This method proved to be a very useful, and rapid assay in the case of such species as pepper, that contains high amount of virus inhibitors.

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