# The Use of Lissamine Rhodamine B Conjugated Antibody for the Detection of Tobacco Mosaic Virus Antigen in Tomato Mesophyll Protoplasts

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A method is described for the preparation and use of Lissamine Rhodamine B conjugated antibody for the detection of Tobacco Mosaic Virus antigen in mesophyll protoplasts of tomato.

The development of methods for the isolation of cell protoplast from the leaves of higher plants [1, 2] and the demonstration that protoplasts can support virus replication [3] have provided plant virologists with synchronous systems in which cellular events in plant virus replication can be studied. It is anticipated that the techniques which have been developed by animal virologists will be applied by their plant colleagues. Such an application was made by Takebe and Otsuki [3] who used fluorescein isothiocyanate (FITC) conjugated antibody to detect Tobacco Mosaic Virus (TMV) antigen in infected tobacco (*Nicotiana tabacum* cv. Xanthi) mesophyll protoplasts as a parameter of the number of protoplasts infected. This note describes the use of Lissamine Rhodamine B (RB200) conjugated antibody for the detection of TMV antigen in infected tomato (*Lycopersicon esculentum* cv. Potentate) mesophyll protoplasts and the advantages of the latter fluorochrome over FITC in the tomato protoplast system.

The Rothamsted culture of the type strain of TMV was purified from tobacco (*Nicotiana tabacum* cv. White Burley [4]) except that mercaptoethanol was not used. The purity of protein from the virus preparation was examined on sodium dodecyl sulphate-acrylamide gels [5] on which, even which deliberately overloaded, only a single band was seen, representing a polypeptide of molecular weight $18\,000 \pm 1000$. The molecular weight markers used were cytochrome c, $\beta$-lactoglobulin, lactate dehydrogenase and ovalbumin.

Antibody was prepared in rabbit as follow: the purified virus preparation was dialysed against buffered saline (PBS – 0.9% NaCl in 10 mM phosphate buffer pH 7.0), the protein concentration was determined by the method of Lowry *et al.* [6]. Purified TMV (1 ml of 1 mg/ml virus protein) was emulsified with an equal volume of Freund's incomplete adjuvant [7] and injected subcutaneously. Two further injections were given at four-day intervals, followed by an intravenous injection 14 days after the last of the subcutaneous injections. The serum was collected after a further 14 days and concentrated by the method of Tenenhouse and Deutsch [8]. The titre of the antibody was 1/8192 [9]. No cross reaction was detected with sap from healthy tomato plants using microimmunodiffusion tests [7].

RB200 conjugated antibody was prepared from the thionyl chloride derivative as previously described [10]. The reaction was terminated and excess reagent removed on a column of BioGel P6; the elution buffer was PBS. The labelled antibody-containing solution was frozen at $-17\,^{\circ}$C for 15 hours then thawed out and centrifuged at $20\,000 \times g$ for 30 min. Following freezing and thawing to remove unstable protein, the degree of conjugation was determined to be 3 mol RB200/mol of $\gamma$-globulin based on an extinction coefficient of $73 \times 10^3$ for RB200 [11] and a molecular weight of 160 000 for $\gamma$-globulin [12]. The conjugated antibody was diluted to a titre of 1/819 with PBS and stored in 1 ml aliquots at $-17\,^{\circ}$C.

RB200 conjugated antibody is stable when thus stored at $-17\,^{\circ}$C for several months. However, the thawed antibody must be centrifuged before use or considerable non-specific fluorescence results. No further reduction in non-specific binding results from treating the centrifuged preparation with powdered tissue. The removal of denatured protein by centrifugation does not result in any reduction in titre.

Protoplasts were isolated from the 5th and 6th leaves of 6 week-old tomato plants which had been inoculated with a 1:1000 dilution of purified TMV at the cotyledon stage. Protoplasts were also prepared from non-inoculated plants. Both inoculated and non-inoculated plants were checked for the presence of TMV antigen using microimmunodiffusion tests [7]. Protoplasts were produced by a one-step method [1] using a solution of 1% Macerozyme, 4% Onozuka Cellulose SS (Kinki-Yakult, 8.21 Shingikancho, Nishinomiya, Japan) in 0.6 M mannitol. 0.1 mM $CaCl_2$ pH 5.8. The leaves were floated on enzyme solution for 15 hours at 25 °C in the dark.

Protoplasts were prepared for staining by modification of the method of Otsuki and Takebe [3]. One drop of protoplast suspension, containing not less than $1 \times 10^6$ protoplasts, was placed on a glass slide which had been smeared with a layer of Mayer's albumin. The slide was dried rapidly in a stream of warm air and fixed in dry acetone at room tem-

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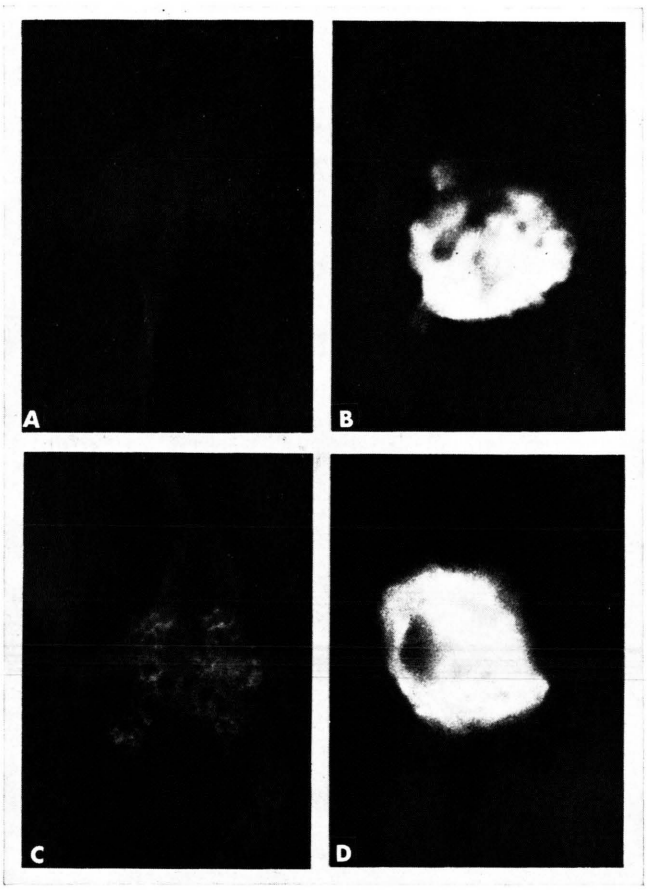


Fig. 1. Tomato mesophyll protoplasts from healthy (A), (C) and TMV infected leaves (B), (D), stained for virus antigen with RB200 conjugated antibody (A), (B) and FITC conjugated antibody (C), (D), (Magnification × 800).

perature for 15 hours. The slide was then washed in PBS for 2 hours with four changes of buffer. RB200 conjugated antibody solution was thawed and centrifuged at 20 000 × *g* for 30 min. One drop of the supernatant was placed on each slide and the slide incubated at 37 °C in a humid chamber for 2 hours. Unreacted antibody was removed by washing as above. Two control slides were incubated, one in PBS and the second in unconjugated antibody followed by conjugated antibody. Slides were examined with a Vickers M41 Photoplan, incident light, fluorescence microscope equipped with an HBO 200 mercury vapour lamp, a BG 38 suppressor filter, a TRITC 1 Balzer exciter filter and OG 590 and RG 610 barrier filters.

FITC conjugated antibody was prepared [3] and used, with the appropriate filters, as for RB200 conjugated antibody above.

As shown in Fig. 1 * RB200 conjugated antibody provides a sensitive method for the detection of TMV antigen in tomato protoplasts. This fluorochrome has an advantage over FITC in that while the extinction coefficients for RB200 and FITC are of the same order [10], the conditions for FITC fluorescence produce a higher protoplast autofluorescence in tomato than those for RB200. Consequently FITC fluorescence is subject to interference and is relatively insensitive for the detection of low levels of TMV antigen.

The FITC and RB200 conjugated antibody systems were further compared by staining the 5th and 6th leaves of 6-week-old plants inoculated with the M 11 – 16 symptomless strain of TMV [13] purified in *Lycopersicon esculentum* cv. Potentate as above. Using RB200 conjugated antibody, 60 – 70% of the protoplasts from type strain infected and 40 – 50% of those from symptomless strain infected showed the presence of antigen. The fluorescence of protoplasts from both type and symptomless strain inoculated plants was of the same intensity. The results for FITC conjugated staining of the same material differed in that the fluorescence of the symptomless strain infected protoplasts was less than that of the type strain infected ones, and consequently with the higher background fluorescence in this system, quantitative measurements of the same accuracy could not be made. Staining with FITC conjugated antibody confirmed that 60 – 70% of the protoplasts from type strain inoculated plants contained TMV antigen.

It is suggested that both FITC and RB200 conjugates may be used together for the detection of virus antigen in protoplasts simultaneously infected with different viruses or virus strains due to the differences in the spectral properties of these fluorochromes. Work is in progress to test this latter proposition.

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* Fig. 1 see Table on page 696 b.


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