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Serodiagnosis of Tobacco Mosaic Virus By Cross Adsorption Method

Key words : Cross adsorption, DAS-ELISA, Serodiagnosis, *Tobacco mosaic virus*.

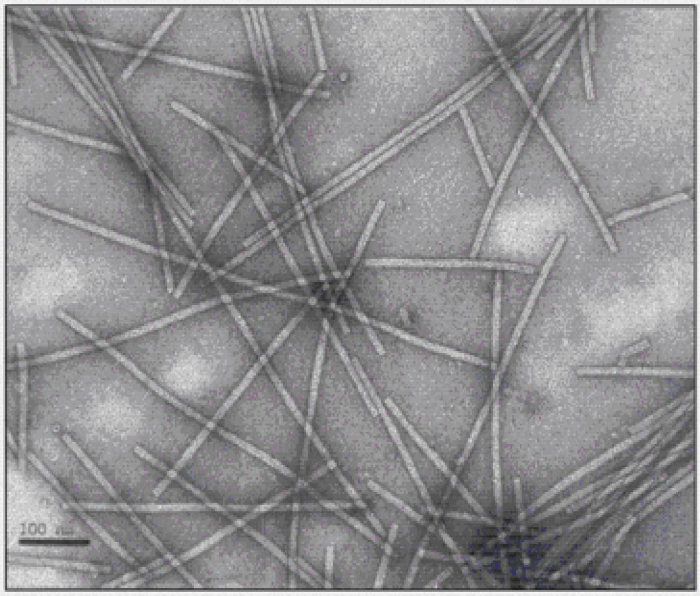
TMV was purified from systemically infected *Nicotiana tabacum* leaves by cross adsorption technique using antiserum raised against host proteins which is a boom for laboratories not having facilities for final purification. This preparation was used for immunization of rabbit. Electron microscopy of purified preparations of TMV showed numerous rigid rod shaped particles. Polyclonal antiserum was produced in rabbits against TMV. In slide agglutination test, the diseased sap reacted positive with antiserum produced against virus, which was evident from agglutination and no agglutination with healthy sap. γ globulins were purified from the produced antiserum by ammonium sulphate precipitin method and conjugated with alkaline phosphate enzyme by single step glutaraldehyde method. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was standardized and successfully used to detect *Tobacco mosaic virus* (TMV) in infected leaves using antiserum raised against TMV.

The tobacco environment has often provided ideal conditions for spread and multiplication of organisms which are later adapted as tobacco parasites. Bidi tobacco suffers from many abnormalities caused by a wide range of pathogens *viz*., fungi, nematodes, bacteria, viruses, flowering plant parasites and phytoplasma (Lucas, 1975). The losses due to these diseases are estimated to be in the range of 5 to 15 per cent depending on their intensity. Among all virus diseases, tobacco mosaic caused by *Tobacco mosaic virus* (TMV) causes leaf mosaic and severe crop losses. The infected tobacco plants showed mosaic symptoms as dark green islands surrounded by light green areas and reduced leaf size. It is most common on tobacco crop in India, appearing in every tobacco tract of the country. Since leaf is the final produce and no other chemical is effective, alternative approaches would be of better preposition with regard to its management.

TMV was mechanically inoculated and maintained on tobacco plants raised in glasshouse. Leaves showing typical mosaic symptoms were collected and used for the purification of virus. TMV infecting tobacco was partially purified by the method reported by Hebert (1963) which is a modification of the polyethylene glycol (PEG) technique. Using this procedure partially or fully, other workers have also purified the virus (Gooding and Hebert, 1967; Steere, 1964; Asselin and Zaitlin, 1978; Reddy *et al.*, 1969; Narianiet *al*., 1977 and Shoba, 1996). One hundred gram inoculated tobacco leaves (*Nicotiana tabacum*) were collected and were frozen dried. The frozen leaves were homogenized in 0.5 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.2) containing 1 per cent 2-mercapto ethanol using 1 g of tissue per ml of buffer. The homogenate was then strained through muslin cloth. While stirring, 8 ml of n-butanol per 100 ml of extract was added. The extract was incubated for 15 min for chloroplast coagulation and centrifuged for 30 min at 10,000 g. After centrifugation, the supernatant was decanted. While stirring, 4.0 g of PEG (mol weight 6000) per 100 ml was added to the supernatant and after dissolution of PEG supernatant was centrifuged at 10,000 g for 15 min. Supernatant was discarded and the pellet was resuspended in 20 ml of 0.01 M PO_4 buffer (pH 7.2) and clarified at 10000 g for 15 min. Further, purification was obtained by a second precipitation with PEG (0.4 g PEG + 0.4 g of NaCl for every 10 ml). After the NaCl and PEG dissolution the mixture was centrifuged at 10000 g for 15 min. The supernatant was collected and which was light greenish in colour. The partially purified preparation is known to contain host proteins in addition to virus which were removed in final purification procedure. For final purification of *Tobacco mosaic virus*, to remove host proteins if any, the partially purified preparation was subjected to cross adsorption with antibodies raised against host proteins. The host proteins required for antibody production were purified by following the partial purification procedure used for virus using healthy leaf sample. The purified host proteins thus obtained were used for immunization of rabbit. Rabbit was immunized for the production of antiserum by injecting partially purified host proteins into the body. The antiserum

obtained was used for cross adsorption. Cross adsorption was done by mixing one ml of partially purified virus preparation which is known to contain host proteins and one ml of antiserum against host proteins. The mixture was incubated for 45 min at 37^0C for precipitation of host proteins. The coagulated host proteins were separated by low speed centrifugation at 5000 rpm for 5 min. The pellet was rejected and supernatant known to contain only virus was retained. The presence of virus in the final purified preparation was confirmed by electron microscopy. The antiserum against *Tobacco mosaic virus* was produced by immunizing rabbit with finally purified virus preparation, 0.5 ml of final purified virus preparation was emulsified with Freund's incomplete adjuvant (1:1 v/v) and was injected into the thigh muscle of rabbit. Three such injections were given at weekly interval and blood was collected from femoral vein of rabbit 10 days after fourth injection dose. It was allowed to clot at room temperature for 1 hour in slanting position and later centrifuged at 10000 rpm for 5 min to separate serum from other blood constituents. The antiserum thus obtained was used for further studies. A small drop of antiserum was kept on a microscope slide, to which a small drop of partially purified virus was added and incubated at 37^0C for 45 min. The method was also followed for controls containing buffer and healthy sap. After incubation, the mixture was observed under microscope for the presence or absence of agglutination in all the three slides. The method described by Clark and Adams (1977) was employed for the purification of IgG from polyclonal antiserum. The immunoglobulins were further purified by passing through cellulose column. The one step glutaraldelyde method (Clark and Adams, 1977) was followed for preparation of alkaline phosphatase homologous conjugate (TMV-IgG*^{AP}). After dialysis, Bovine serum albumin was added to obtain a concentration of 5 mg per ml. The DAS-ELISA procedure of Clark and Adams (1977) was employed for detection of TMV. DAS-ELISA procedure was standardized to know the optimum concentration of coating antibodies, conjugate and antigens for detection of TMV-T. Coating antibodies diluted at 1:100, 1:500 and 1:1000 were used. Partially purified virus at 1:10 and 1:100 and crude diseased leaf extract at 1:10 and 1:20 dilutions were used apart from buffer and healthy leaf extract as negative controls. Absorbance values were recorded at 405 nm after 45 min using a microplate reader.

The purification of the virus was done by using tobacco leaves showing systemic mosaic symptoms raised in glasshouse. The partial purified preparation was light greenish in colour indicating presence of some host proteins along with virus. The final purification of the virus was done by cross adsorption method as discussed in materials and methods. The partial purified preparation was mixed with equal quantity of antiserum and incubated at 37^0C for 45 min. The partially purified virus preparation which was light green in colour changed to clear suspension with coagulated proteins settling in the bottom of the tube. The precipitated proteins were then pelleted at 5000 rpm centrifuged for 5 min. The clear supernatant known to contain only virus was decanted. The presence of virus particles in the final purified preparation was confirmed by electron microscopy and the virus particles are typical rod shaped and the preparation was free from host proteins (Plate 1). Chester (1936), Dunin (1937) and Hebert (1961) have purified the virus by cross adsorption technique. The antiserum against *Tobacco mosaic virus* was produced by immunizing rabbit with finally purified virus preparation. Reddy *et al.* (1969) have produced antiserum in rabbits against TMV and the antiserum had a titre of 1/3200 as determined by tube precipitation test. Nariani *et al.,* (1977) and Shankar *et al.,* (1971) have also produced antiserum against TMV and had the titre of 1/2048 and 1/4096 in precipitin tests. The IgG of TMV were partially purified by saturated ammonium sulphate method and final purification was done by passing through cellulose column. The fractions collected from cellulose column were monitored at 280 nm in spectrophotometer and absorbances were recorded (Fig1). The fractions with OD/280 values of more than 1.4 viz., number 7, 8 and 9 were pooled and the strength of immunoglobulins were adjusted to 1.4 OD using half strength phosphate buffer saline to give 1 mg per ml concentration. One ml (= 1 mg) purified IgG was successfully conjugated with enzyme alkaline phosphatase (ALP, Bangalore Genei) by following one step glutaraldehyde method. The conjugate thus prepared was used for DAS-ELISA for standardization and detection of TMV. To know the optimum conditions for DAS-ELISA for detection of TMV, DAS-ELISA was performed with coating antibodies at 1, 0.5 and 0.1 μg dilutions, sample at 1:10 and 1:20 dilutions and conjugate at 1:100, 1:500 and 1:1000 dilutions were used. The results presented in Table 1 indicated that DAS-ELISA with optimum conditions such as coating antibody at 1:1000, infected sap at 1:20 and conjugate at 1:1000 dilutions gave positive reaction when compared with the buffer and healthy controls. Thus the antiserum produced against TMV can be used for testing of virus in the test samples.



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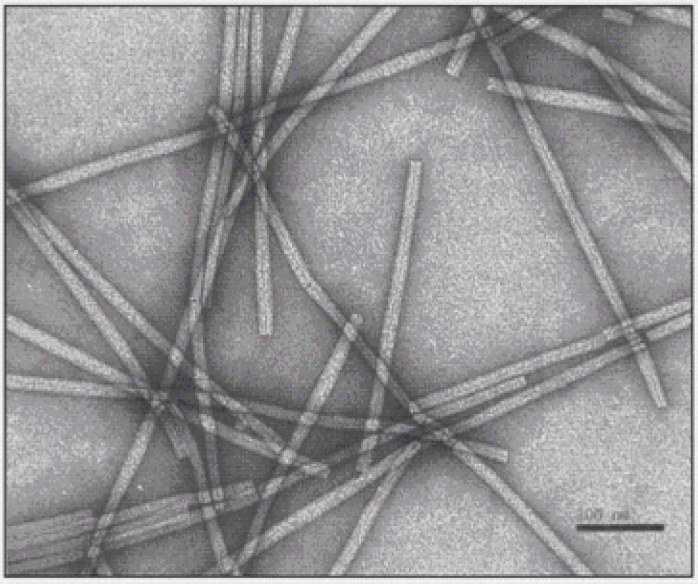

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Plate1 : Electron micrograph of typical rod shaped particles of TMV in final purified preparation

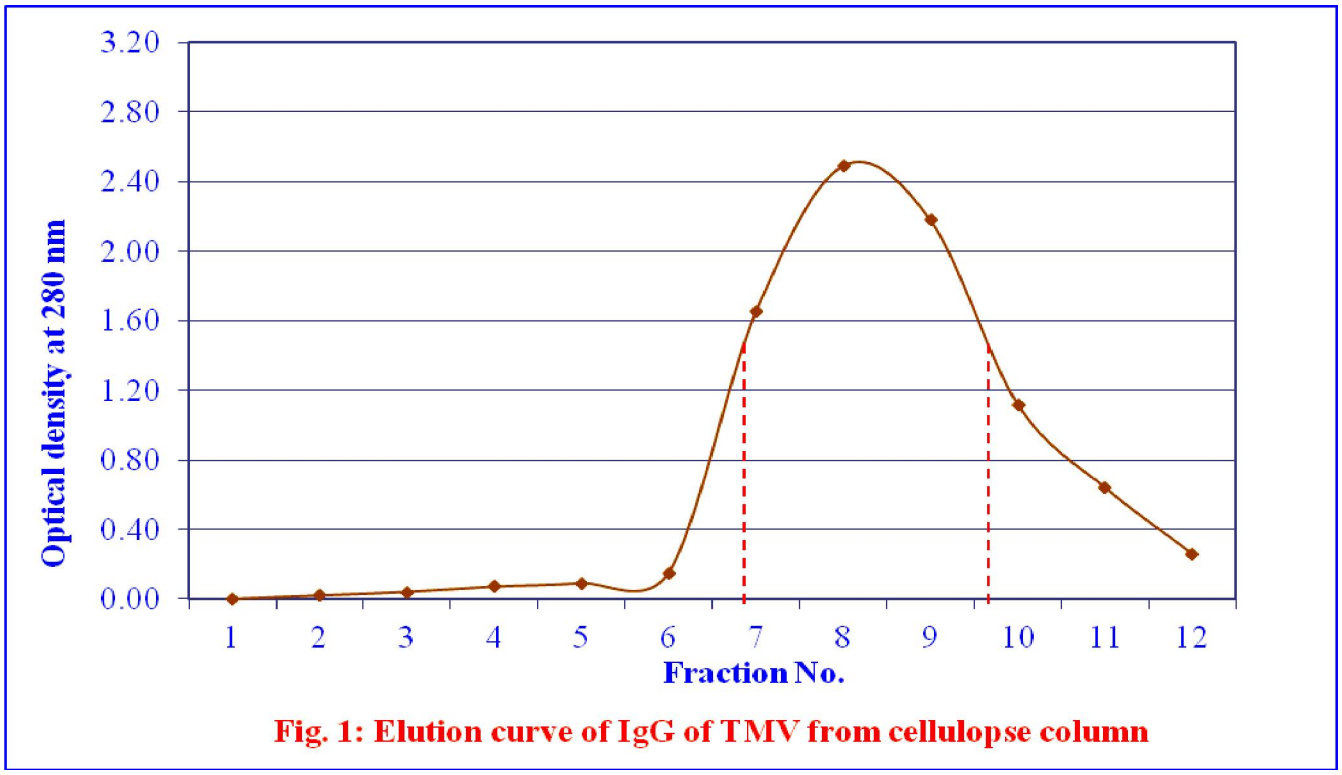

Fig. 1: Elution curve of IgG of TMV from cellulopse column

Table 1. Determination of optimum concentration of coating and conjugate antibodies for the detection of TMV by DAS-ELISA

Sl. No.	Treatments	Antigen dilution	Mean absorbance (at 405 nm)								
			Coating antibody dilution								
			1 µg			0.5 µg			0.1 µg		
			1 µg	0.5 µg	0.1 µg	1 µg	0.5 µg	0.1 µg	1 µg	0.5 µg	0.1 µg
1.	Buffer		0.005	0.003	0.002	0.004	0.003	0.002	0.003	0.002	0.002
2.	Healthy	1:10	0.027	0.024	0.019	0.021	0.017	0.015	0.017	0.014	0.021
3.	Diseased sample	1:10	0.186	0.167	0.154	0.143	0.133	0.108	0.126	0.117	0.102
4.	Diseased sample	1:20	0.123	0.114	0.101	0.109	0.102	0.095	0.092	0.087	0.075
5.	Partial purified virus	1:10	1.685	1.466	1.395	1.328	1.297	1.235	1.246	1.235	1.167
6.	Partial purified virus	1:100	1.055	0.965	0.936	0.852	0.814	0.763	0.742	0.669	0.632

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Department of Plant Pathology
College of Agriculture, Dharwad
University of Agricultural Sciences,
Dharwad- 580 005

B Sivaprasad
S Jahgirdar
A S Byadgi
S Kulakarni

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