Reprinted from PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE,
1963, v114, 750-754

The Bentonite Flocculation Test for Detection of Plant Viruses and Titration of Antibody. (28801)

JOHN BOZICEVICH, HOWARD A. SCOTT AND MONROE M. VINCENT
(Introduced by Harry Eagle)
Microbiological Associates, Inc., Bethesda, Md., and Plant Virology Laboratory, Crops Research Division, Beltsville, Md.

This report describes the application of the bentonite flocculation test to detection of plant viruses in both crude plant saps and purified preparations. In previous studies, a bentonite suspension sensitized with antigen has been used for detection of antibodies(1, 2,3,4). The data presented here demonstrate that the system can be employed in reverse for detection of antigen without decreasing the sensitivity or reliability of the test. A method for titrating antisera with bentonite particles coated with virus antigen is also described.

Serological techniques for detection and identification of plant viruses have only recently been widely applied. Most of these techniques are limited either by lack of sensitivity or because of nonspecific reactions due to contaminating plant components. The complement fixation test, although extremely

sensitive, is rarely used, both because of its complexity and the problem of interfering substances present in many plants(5). The methods most frequently used are various types of the precipitin test. The ring precipitin test requires a minimum of materials, but falsely positive tests are often encountered (6,7), while the tube test requires large quantities of reactants. With certain viruses, particularly those characterized by flexuous rods, the chloroplast agglutination test has been applied, in which a drop of crude sap from a virus-infected plant is mixed with a drop of antiserum. This test is the least sensitive of all(5), and is further complicated by spontaneous agglutination of the chloroplasts. Except for this last procedure, all serological tests for plant viruses so far described require some manipulation of the plant extract, such as clarification by centrifugation, alternate freezing and thawing, or heating, before the test can be performed.

Materials and methods. Sources and preparation of test antigens. The purified virus preparations and the source plants were: Tobacco mosaic virus (TMV), *Nicotiana tabacum L.* (Samsun)(8); southern bean mosaic virus (SBMV), *Phaseolus vulgaris L.* (Black Valentine)(9); and tobacco ringspot virus (TRSV), *Cucurbita pepo L.* (Caserta)(10).

In addition, studies were carried out with crude sap extracts prepared by grinding infected leaves in a mortar with pestle and squeezing the sap through gauze (44 × 36 mesh). For such tests, the following viruses were studied in addition to those listed above: *N. tabacum L.* (Samsun) infected with alfalfa mosaic virus (AMV) or with potato virus X (PVX), and *Hordeum vulgare L.* (Atlas) infected with barley stripe mosaic virus (BSMV). Extracts from uninoculated plants were used as controls in all tests involving crude sap.

Sources of antisera. The antisera were prepared by Microbiological Associates (Bethesda, Md.), in cooperation with the Committee on Virus Type Culture Collection, American Phytopathological Society(11).

Preparation of stock bentonite suspension. The stock bentonite suspension was prepared as described by Bozicevich *et al*(3), and contained 0.64 mg/ml dry bentonite.

Preparation of antibody-sensitized bentonite suspension. Five ml of saturated ammonium sulfate were added to 1 ml of antiserum which had been diluted to 10 ml with distilled water. After low-speed centrifugation (750-800 *g* for 15 minutes), the supernatant was discarded. The globulin precipitate was resuspended in 5 ml of buffered saline, pH 7.3 (8 g NaCl, 0.5 g NaH_2PO_4, 3 g Na_2HPO_4 in 1 liter of distilled water) and dialyzed 4 hours against 2 changes of 50 ml buffered saline. The dialyzed antiserum globulin was diluted to 10 ml with buffered saline and centrifuged at low speed as above for 15 minutes. The supernatant was mixed with 20 ml of the stock bentonite suspension and allowed to stand overnight at 4°C. Methylene blue (2.0 ml of a 0.1% solution) was added to facilitate visualization. This suspension was then centrifuged at 750-800 *g* for 5 minutes, the pellet of bentonite was washed twice with buffered saline, and suspended in a final volume of 10 ml with distilled water. As a stabilizing agent, 0.2 ml of 1% polyvinylpyrrolidone* was added and 0.2 ml of 3% aqueous merthiolate as a preservative. The final product can be refrigerated or stored at room temperature, but must not be frozen.

Preparation of antigen-sensitized bentonite. One ml of purified virus, either TMV (5 mg), TRSV (2 mg), or SBMV (5 mg), diluted with 5 ml buffered saline, was added to 10 ml aliquots of the stock bentonite suspension and allowed to adsorb overnight at 4°C. After adsorption, methylene blue and merthiolate were added as for the preparation of antibody-sensitized bentonite described above.

Testing procedure for detection of antigen. Dilutions in 0.85% saline of purified or crude sap antigens (0.1 ml volume) were placed on ringed microscope slides as described by Bozicevich *et al*(3) and 0.05 ml antibody-sensitized bentonite was added. The mixture was rotated at 120 oscillations per minute for 20 minutes at room temperature.† The cri-

* Grade K-30, General Aniline & Film Corp.

† Boerner-type rotator.

TABLE I. Results of Bentonite Flocculation Test Using Purified Virus Antigens.

Antigens, μg/.1 ml ↓	Bentonite suspension sensitized with: Anti-TMV serum tested against			Anti-TRSV serum tested against			Anti-SBMV serum tested against		
	TMV	TRSV	SBMV	TMV	TRSV	SBMV	TMV	TRSV	SBMV
20.0	—*	0†	—	—	0	0	—	0	—
5.0	0	—	0	0	—	0	0	—	—
2.0	—	0	—	—	+	—	—	—	—
.5	+++	—	0	0	—	0	0	—	—
.2	++++	0	0	0	+++	0	0	0	++++
.1	++++	0	0	0	++	0	0	0	++++
.05	+++	0	0	0	±	0	0	0	+++
.03	++	0	0	0	0	0	0	0	+
.015	±	—	0	0	—	0	0	0	0
.008	0	0	0	—	0	0	0	0	0

* — = Not done. † 0 = Negative.

TABLE II. Results of Bentonite Flocculation Test with Dilutions of Crude Sap from Virus-Infected Leaves.

Reciprocal of crude sap dilutions	Reactivity of crude extracts of infected plants with homologous antibody-sensitized bentonite *					
	TMV	TRSV	PVX	AMV	SBMV	BSMV
10	0†	+++	++++	0	—	0
100	0	+++	++++	+	—	+++
1,000	±	±	+++	+++	—	++++
2,000	+++	—‡	++	+++	++	++++
4,000	+++	—	±	++	+++	++++
8,000	++++	—	0	+	±	++++
16,000	+++	—	—	—	—	+++
32,000	++	—	—	—	—	++
64,000	+	—	—	—	—	—

* No cross reactions were observed when 1) antigens were tested against heterologous antibody-sensitized bentonite or when 2) extracts from corresponding healthy leaves were tested.
† 0 = Negative.
‡ — = Not done.

TABLE III. Inhibition of Flocculation of TMV-Sensitized Bentonite by Addition of TMV to Antiserum, and Its Failure to Inhibit Flocculation of TRSV- or SBMV-Sensitized Bentonite.

μg of TMV added to antiserum prior to addition of antigen-sensitized bentonite	Flocculation of TMV-sensitized bentonite by 1:100 TMV antiserum	Flocculation of TRSV-sensitized bentonite by 1:100 TRSV antiserum	Flocculation of SBMV-sensitized bentonite by 1:100 SBMV antiserum
2,500	—	++++	++++
1,250	—	++++	++++
625	—	++++	++++
312	—	++++	++++
156	—	++++	++++
79	—	++++	++++
39	+	++++	++++
20	++	++++	++++
10	++++	++++	++++
5	++++	++++	++++
2.5	++++	++++	++++
1.25	++++	++++	++++

Antisera were diluted 1:100 and dispensed in 0.1 ml amounts. Into each tube, 0.1 ml of serial dilutions of TMV antigens was added and allowed to react for 10 min. One-tenth ml from each tube was placed on a slide and rotated for 20 min prior to reading.

TABLE IV. Titration of TMV, TRSV, and SBMV Antisera with TMV-Sensitized Bentonite Particles.

	Reciprocal of serial 2-fold dilution of antisera											
	2	4	8	16	32	64	128	256	512	1024	2048	4096
TMV	+	+	+	+	+	+	+	+	+	+	+	−
TRSV	−	−	−	−	−	−	−	−	−	−	−	−
SBMV	−	−	−	−	−	−	−	−	−	−	−	−

teria for reading the test were as previously described(3).

Testing procedure for detection of antibody. Two-fold serial dilutions of uninactivated antiserum in 0.85% saline in a volume of 0.1 ml were mixed with antigen-sensitized bentonite as described above.

Results. Table I summarizes results obtained in the detection of purified TMV, TRSV, and SBMV with antibody-sensitized bentonite. There were no cross-reactions; but prozone inhibition was observed at high concentrations of antigen. TMV could be detected in quantities as little as 0.015 μg in 0.1 ml, SBMV at 0.03 μg and TRSV at 0.05 μg. The slight differences in the minimum detectable amounts of antigen may be due to differences in antibody content of the several antisera.

Table II shows results of similar tests using crude sap from virus-infected leaves. Results are expressed as the reciprocals of the highest detected dilution of crude material. Dilutions of 1:10, 1:100, and 1:1000 were used for initial screening tests, followed by a second test with 2-fold serial dilutions in the appropriate range. As a control, sap from non-infected plants was similarly tested. All antigens were tested against each of the antibody-sensitized bentonite preparations. Flocculation occurred only in homologous systems. The negative results with higher concentrations of TMV and AMV were attributed to antigen excess, and not to the presence of sap components, since PVX and TRSV extracts obtained from the same type of source plant, gave positive readings with the appropriate specific antisera in the higher concentrations.

The specificity of the bentonite flocculation test was borne out by inhibition studies (Table III). Thirty-nine μg of TMV inhibited the flocculation of the homologous antibody-sensitized bentonite, but similar amounts of TMV added to TRSV and SBMV did not affect flocculation in their respective bentonite systems.

Table IV shows that the bentonite flocculation test may be employed for antibody titration. The test is highly sensitive and specific.

Discussion. The bentonite flocculation test should find wide application in plant virus studies, and particularly in detecting the presence of suspect virus in plants. Once the reagents are prepared, the test requires only 20 minutes, and no specialized equipment is necessary. The antibody-sensitized bentonite

is stable for at least 3 months at room temperature without loss of sensitivity or specificity.

The bentonite test can be applied to purified virus or sap from infected plants. The presence of sap components from bean, tobacco, and barley in high concentrations neither inhibited specific flocculation nor caused nonspecific flocculation. The sap extracts need not be clarified nor treated in any way except for dilution. Preliminary evidence indicates that undiluted sap may be used provided the virus antigen is not in sufficient excess to cause a prozone effect.

The presence of potato virus "X" in tubers from infected potato plants was also detected by the bentonite flocculation test. This has not hitherto been possible with other serological tests(12). There is reason to believe that the list of viruses similarly detectable in crude plant or seed extracts can be greatly extended.

Summary. A flocculation test using a highly stable antibody-sensitized bentonite was used to detect plant viruses in both crude and purified preparations. Conversely, antisera could be titrated with bentonite suspensions coated with purified virus. In both systems, the bentonite flocculation test proved rapid, sensitive and specific.

1. Bozicevich, J., Tobie, J. E., Thomas, E. H., Hoyem, H. M., Ward, S. B., *Public Health Rep.,* 1951, v66, 806.

2. Bozicevich, J., Bunim, J. J., Freund, J., Ward, S. B., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 180.

3. Bozicevich, J., Nasou, J. P., Kayhoe, D. E., *ibid.,* 1960, v103, 636.

4. Diena, B. B., Wallace, R., Greenberg, L., *Canad. J. Microbiol.,* 1963, v9, 221.

5. Matthews, R. E. F., *Plant Virus Serology,* 1957, 128 pp, Cambridge Univ. Press.

6. Zaitlin, M., Schechtman, A. M., Bald, J. G., Wildman, S. G., *Phytopath.,* 1954, v44, 314.

7. Bancroft, J. B., Moorhead, E. L., Tuite, J., Lin, H. P., *ibid.,* 1960, v50, 34.

8. Steere, R. L., *Adv. Virus Res.,* 1959, v6, 1.

9. Scott, H. A., Vincent, M., Zaumeyer, W. J., *Phytopath.,* 1961, v51, 755.

10. Steere, R. L., *ibid.,* 1956, v46, 60.

11. Committee on Virus Type Culture Collection, *ibid.,* 1960, v50, 428.

12. Beemster, A. R. B., *Proc. First Triennial Conf. European Assn. Potato Res.,* 1960, 62.

Received September 30, 1963. P.S.E.B.M., 1963, v114.