An Improved Immunodiffusion Test for the Detection of Intact Cucumber Mosaic Virus in Crude Tobacco Sap

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ABSTRACT

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An improved immunodiffusion test for the detection and serological differentiation of intact capsids of cucumber mosaic virus (CMV) in crude tobacco sap was developed. The immunodiffusion medium was prepared by melting 0.8 g of agarose in 100 ml of 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 0.5% Triton X-100 and 0.01 M sodium thioglycolate. Sap from leaves of CMV-infected *Nicotiana tabacum* 'NC 95' or *N. rustica* was extracted with a leaf press 6–10 days after inoculation. Test antigens consisted of 200 μl of sap mixed with 50 μl of 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 15 mg of bentonite per milliliter and 1% formalin. Fifteen isolates of CMV produced intact capsid precipitin bands when tested by this method.

Devergne and Cardin (2) described an immunodiffusion test which resulted in the production of intact capsid precipitin bands (v-band) of cucumber mosaic virus (CMV) in crude sap. They used agarose prepared in 0.5 M citrate buffer, pH 6.5, and tobacco sap extracted by grinding leaves in the same buffer containing 2% formalin. Some of our CMV isolates (1) gave only the subunit precipitin band (s-band) reaction when tested by this method, which indicated instability of the isolates in crude sap even in the presence of formalin. Therefore, possible serological differences among CMV isolates could not be detected with crude sap because the s-band reactions of different CMV strains show only reactions of identity (18). We describe an improved method for obtaining CMV v-band reactions with crude sap in the Ouchterlony double-diffusion test.

MATERIALS AND METHODS

Virus isolates and antisera. The CMV isolates designated CMV-C (10) and Fulton's CMV-D (10) and an antiserum to CMV-C obtained from a rabbit that had received seven weekly intramuscular injections of a total of 45 mg of virus purified by Scott's method (15) were used throughout the study. In some tests, Arkansas isolates locally designated as CMV-233, CMV-81, CMV-23, CMV-78, CMV-EM, CMV-61, CMV-J, CMV-CL, CMV-14, and CMV-9 (1) and isolates CMV-Q (9) (ATCC PV 289), CMV-S (19) and Marchoux's CMV-D (12) (the last two were obtained from J. M. Kaper, Beltsville, MD) were also used. All isolates were maintained in *Nicotiana tabacum* 'NC 95.' Antiserum to CMV-S (ATCC PVAS 242a) was used only in our final procedure.

Preparation of agarose media. Agarose gel diffusion media were prepared by melting 0.8 g of agarose (MCB Manufacturing Chemists, Inc., Cincinnati, OH) in 100 ml of borate-EDTA buffer, pH 9.0, which was composed of combinations of 0.1, 0.05, or 0.005 M borate and 0.1, 0.05, or 0.005 M EDTA with 0.5 or 1% Triton X-100 (TX-100). The media were prepared by mixing stock solutions of 0.2 M sodium borate, 0.2 M EDTA and 20% TX-100 (v/v) in various proportions. Sodium thioglycolate or sodium diethyldithiocarbamate (DIECA) was incorporated into the buffer directly or from 0.1 M and 0.05 M stock solutions, respectively.

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Extraction and treatment of sap. *Nicotiana tabacum* 'NC 95' or *N. rustica* L. 6–10 days after inoculation was used as the source of CMV for all experiments. Crude sap was expressed from systemically infected leaves with a leaf press and was placed into the wells of immunodiffusion plates directly or after pretreatment with the same buffer used to prepare the immunodiffusion plates. In some experiments, buffer was added to leaf tissue during extraction. Bentonite and formalin were only incorporated into the buffer used for tissue or sap treatments but not into buffer used to

TABLE 1. Production of intact capsid precipitin bands (v-bands) and subunit precipitin bands (s-bands) by cucumber mosaic virus (CMV-C) in immunodiffusion tests using crude sap[a] and 0.8% agarose prepared in various concentrations of borate-EDTA buffer, pH 9.0

Concentration of borate	Concentration of EDTA	Addition of Triton X-100	Intensity of precipitin bands[b]	
			s-bands	v-bands
0.1 M	0.1 M	−	++	−
	0.05 M	−	++++	++
	0.005 M	−	++++	−
0.05 M	0.1 M	−	+	−
	0.05 M	−	+	−
	0.005 M	−	+++	−
0.005 M	0.1 M	−	−	−
	0.05 M	−	++	−
	0.005 M	−	++++	−
0.1 M	0.05 M	+[c]	+++	+++
	0.005 M	+	++++	++
0.05 M	0.005 M	+	++++	−
0.005 M	0.005 M	+	++++	−
0.1 M	0.02 M	+	+++	++
	0.01 M	+	+++	++
0.075 M	0.05 M	+	+++	++
	0.02 M	+	+++	++
	0.01 M	+	+++	+

[a] Sap extracted from CMV-infected tobacco leaf with a leaf press and placed directly into the wells of immunodiffusion plates.
[b] Intensity of v-bands and s-bands were recorded as no reaction (−), weak (+), moderately strong (++), strong (+++), or very strong (++++).
[c] Triton X-100 was added to the agarose medium at 1% concentration.

TABLE 2. The effect of sodium thioglycolate or sodium DIECA on the occurrence and intensity of v-bands of two isolates of cucumber mosaic virus (CMV) in immunodiffusion tests[a] with crude tobacco sap

Treatment[b]	Sodium thioglycolate		Sodium DIECA		Occurrence and intensity of v-bands produced by CMV isolates	
	Presence in buffer[c]	Concentration in agarose	Presence in buffer	Concentration in agarose	CMV-C	CMV-D[d]
1	No	0.0	No	0.0	++[e]	−
2	No	0.0	No	0.0	+++	−
3	No	0.0	No	0.0	+++	−
4	Yes	0.0	No	0.0	+++	−
5	No	0.0	Yes	0.0	+++	+
6	Yes	0.01 M	No	0.0	++++	++
7	Yes	0.05 M	No	0.0	++++	++
8	Yes	0.1 M	No	0.0	++++	++
9	No	0.0	Yes	0.005 M	++++	−
10	No	0.0	Yes	0.01 M	++++	++
11	No	0.0	Yes	0.02 M	++++	+

[a] Tests were in 0.8% agarose prepared in 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 0.5% Triton X-100.
[b] Treatments were as follows: treatment 1—Crude sap extracted from CMV-infected tobacco leaf with a leaf press and placed directly into wells of immunodiffusion plates; treatment 2—same as in treatment 1 but 0.1 M borate-0.05 M EDTA buffer, pH 9.0, was added to leaf tissue at the rate of 200 µl of buffer to 0.6 g of tissue during crude sap extraction; treatment 3—buffer was added to crude sap at the rate of one volume of buffer to four volumes of sap immediately after sap extraction; treatments 4–11—same as in treatment 3, but the buffer contained 0.01 M sodium thioglycolate or 0.01 M sodium DIECA.
[c] Buffer was used to treat leaf tissue during sap extraction or to treat sap immediately after extraction.
[d] Fulton's CMV-D isolate (10).
[e] Intact capsid precipitin band reactions (v-bands) were recorded as no reaction (−), weak reaction (+), moderately strong reaction (++), strong reaction (+++) and very strong reaction (++++).

TABLE 3. The effect of bentonite and/or formalin treatment of virus-containing sap on the occurrence and intensity of v-bands produced by cucumber mosaic virus (CMV) isolates in immunodiffusion tests[a]

Virus isolates	Intensity of v-bands of CMV isolates			
	Control[b]	Formalin[c]	Bentonite[d]	Bentonite plus formalin[e]
CMV-C	++++[f]	++++	++++	++++
CMV-D[g]	++	++	++++	++++
CMV-233[h]	+++	+++	++++	++++
CMV-81	++++	++++	++++	++++
CMV-23	++++	++++	++++	++++
CMV-78	++	+++	+++	++++
CMV-EM	++	++++	+++	++++
CMV-61	++	+++	+++	++++
CMV-J	+++	ND[i]	ND	++++
CMV-CL	+++	ND	ND	++++
CMV-9	+++	ND	ND	++++
CMV-14	+++	ND	ND	++++
CMV-Q	++	+++	++++	++++
CMV-D[j]	ND	ND	ND	++++
CMV-S	ND	ND	ND	++++

[a] Tests were in 0.8% agarose prepared in 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 0.01 M sodium thioglycolate and 0.5% Triton X-100.
[b] Control consisted of treating crude sap with 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 0.01 M sodium thioglycolate.
[c] Same as in a) except that the buffer contained formalin at 1% final concentration.
[d] Same as in a) except that the buffer contained 15 mg/ml bentonite suspension.
[e] Same as in a) except that the buffer contained both bentonite and formalin at final concentrations of 15 mg/ml and 1%, respectively.
[f] For explanation refer to Table 2.
[g] Fulton's CMV-D isolate (10).
[h] Ahmad (1).
[i] Not determined.
[j] Marchoux's CMV-D isolate (12).

prepare the immunodiffusion medium. Details of these treatments are explained in the Results section.

Bentonite suspended in magnesium phosphate buffer, pH 7.4, was prepared according to Dunn and Hitchborn (4). Since Mg^{++} causes aggregation of CMV (17), the bentonite was repelleted and washed once with 0.005 M borate-0.005 M EDTA buffer and once with 0.1 M borate-0.05 M EDTA buffer, pH 9.0. The pellet was resuspended in the latter buffer to produce a stock bentonite suspension containing 40 mg/ml.

RESULTS

Effects of different concentrations of borate-EDTA buffer. Concentrations of borate and EDTA in the agarose medium profoundly affected the appearance of the precipitin bands (Table 1). High concentrations of borate and EDTA were more suitable for CMV-C than low concentrations. In 0.1 M borate-0.05 M EDTA both v-bands and s-bands were formed, whereas in 0.005 M borate-0.005 M EDTA only s-bands were obtained.

Effect of addition of Triton X-100. TX-100 at 1% improved the intensity of CMV-C v-bands when it was incorporated into agarose prepared in 0.1 M borate-0.05 M EDTA buffer, pH 9.0 (Table 1). Moreover, v-bands were formed in a shorter time in the presence of TX-100 than in its absence. Maximum intensity of v-bands was attained after 24–48 hr of incubation. Further experiments showed that 0.5% TX-100 was as effective as 1% in increasing the intensity of CMV-C v-bands. Reduction of the concentrations of borate to 0.075 M and EDTA to 0.02 or 0.01 M resulted in lower intensity of v-bands and higher intensity of s-bands even in the presence of 1% TX-100. At this stage, a medium consisting of 0.8% agarose, 0.1 M borate-0.05 M EDTA buffer, pH 9.0, and 0.5% TX-100 was selected.

Effect of sodium thioglycolate and sodium DIECA. Fulton's CMV-D produced only s-bands when tested in the medium described above indicating degradation of virus particles. Hence, reducing agents were incorporated into 0.1 M borate-0.05 M EDTA buffer, pH 9.0, in an attempt to induce the production of v-bands with CMV-D and to increase the intensity of v-bands with CMV-C. Sodium thioglycolate and sodium DIECA were incorporated into the borate-EDTA buffer to a concentration of 0.01 M. Initially, the buffer containing sodium thioglycolate or sodium DIECA was used to treat sap but was not used to prepare immunodiffusion plates. Two hundred microliters of this buffer were added to 0.6 g of leaf tissue from which sap was extracted with a leaf press, or one volume of the buffer was added to four volumes of sap immediately after it was extracted. Both treatments improved the intensity of the CMV-C v-bands but were ineffective in inducing the formation of CMV-D v-bands (Table 2).

The incorporation of 0.01 M, 0.05 M or 0.1 M sodium thioglycolate or 0.01 M, 0.02 M, or 0.05 M sodium DIECA into the agarose medium improved the intensity of CMV-C and induced the formation of Fulton's CMV-D v-bands (Table 2). One drawback of sodium DIECA was that it caused a slight cloudiness in the agarose and formed small crystals around the wells of the immunodiffusion plates after 2–3 days of incubation. For this reason 0.01 M sodium thioglycolate was chosen for incorporation into the agarose

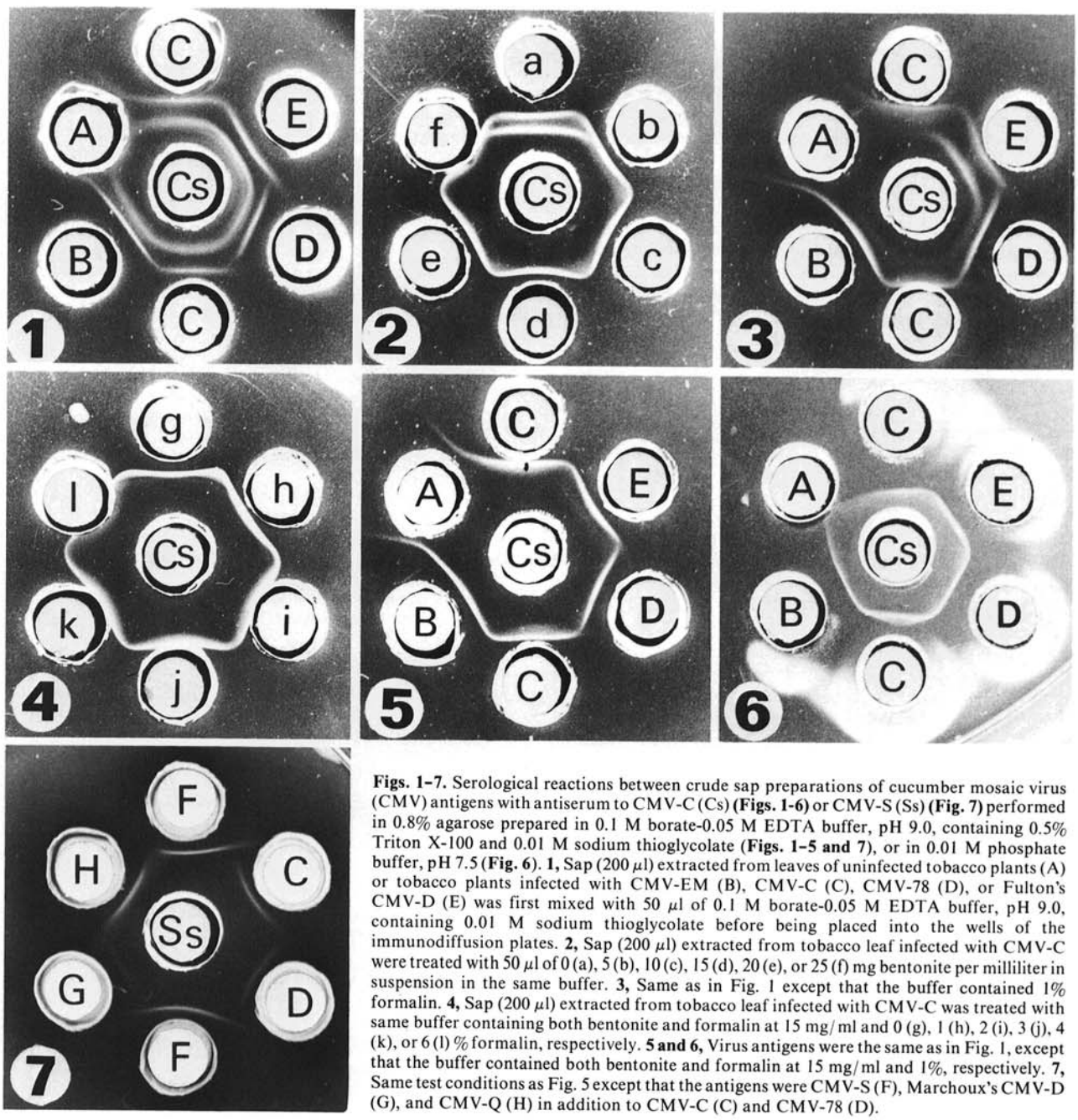
medium, although strong s-bands were still observed (Fig. 1). Moreover, with the exception of CMV-C, CMV-81, and CMV-23, all other isolates gave only weak to moderately strong v-band reactions in the immunodiffusion tests using this medium (Table 3).

Effects of treatment of sap with bentonite or formalin. Sap mixed with 20 mg/ml bentonite suspension at the proportion of four volumes of sap to one volume of bentonite produced stronger v-bands of CMV-C and CMV-D than the controls consisting of sap treated with buffer only. Further experiments showed that 10–20 mg/ml bentonite was sufficient to induce the various CMV isolates to produce strong to very strong v-band reactions. The optimum concentration was 15 mg/ml bentonite. Treatment of sap with bentonite at a concentration of 5 mg/ml resulted in the appearance of s-bands and weaker v-bands indicating degradation of the virus particles, but treatment with 25 mg/ml resulted in weaker v-bands, suggesting aggregation and binding of the virus particles by bentonite (Fig. 2). Six of the nine CMV isolates tested showed very strong v-bands when treated with buffer containing 15 mg/ml bentonite (Table 3).

Bentonite treatment helped stabilize intact virus particles. This was shown by the results of an experiment in which sap from CMV-infected tobacco leaves was stored at room temperature for 10, 20, 30, or 60 min before being treated with bentonite suspension. Sap without bentonite treatment and sap to which bentonite suspension was added immediately following extraction were used as controls. The delay in the addition of bentonite did not appreciably decrease the intensity of v-bands. However, the intensity of the s-bands increased with delay in adding bentonite suspension to the sap.

Treatment of sap with buffer containing 1% formalin resulted in slight improvement in the intensity of v-bands of some isolates of CMV but did not prevent partial degradation of the virus particles (Fig. 3, Table 3).

Effect of treatment of sap with bentonite plus formalin. When four volumes of crude sap were mixed with one volume of buffer containing 15 mg/ml bentonite and 1% formalin, the intensity of the v-bands was improved (Table 3) and the intensity of s-bands produced by some isolates was reduced. The optimum



Figs. 1–7. Serological reactions between crude sap preparations of cucumber mosaic virus (CMV) antigens with antiserum to CMV-C (Cs) **(Figs. 1-6)** or CMV-S (Ss) **(Fig. 7)** performed in 0.8% agarose prepared in 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 0.5% Triton X-100 and 0.01 M sodium thioglycolate **(Figs. 1–5 and 7)**, or in 0.01 M phosphate buffer, pH 7.5 **(Fig. 6)**. **1,** Sap (200 μl) extracted from leaves of uninfected tobacco plants (A) or tobacco plants infected with CMV-EM (B), CMV-C (C), CMV-78 (D), or Fulton's CMV-D (E) was first mixed with 50 μl of 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 0.01 M sodium thioglycolate before being placed into the wells of the immunodiffusion plates. **2,** Sap (200 μl) extracted from tobacco leaf infected with CMV-C were treated with 50 μl of 0 (a), 5 (b), 10 (c), 15 (d), 20 (e), or 25 (f) mg bentonite per milliliter in suspension in the same buffer. **3,** Same as in Fig. 1 except that the buffer contained 1% formalin. **4,** Sap (200 μl) extracted from tobacco leaf infected with CMV-C was treated with same buffer containing both bentonite and formalin at 15 mg/ml and 0 (g), 1 (h), 2 (i), 3 (j), 4 (k), or 6 (l) % formalin, respectively. **5 and 6,** Virus antigens were the same as in Fig. 1, except that the buffer contained both bentonite and formalin at 15 mg/ml and 1%, respectively. **7,** Same test conditions as Fig. 5 except that the antigens were CMV-S (F), Marchoux's CMV-D (G), and CMV-Q (H) in addition to CMV-C (C) and CMV-78 (D).

concentration of formalin was 1%, since increasing it to 2% or higher resulted in progressive reduction of the intensity of v-bands (Fig. 4). Spurs were formed among the v-bands of certain isolates with antisera to CMV-C (Fig. 5) or CMV-S (Fig. 7). These bands were observed in immunodiffusion plates prepared in 0.1 M borate-0.05 M EDTA buffer, pH 9.0, containing 0.5% TX-100 and 0.01 M sodium thioglycolate (Figs. 5 and 7), but were not observed in immunodiffusion plates prepared in 0.005 M borate-0.005 M EDTA, pH 9.0, or in 0.01 M phosphate buffer, pH 7.5 (Fig. 6). In these two media only s-bands were formed.

DISCUSSION

An immunodiffusion test is needed for detecting the v-band reactions of CMV isolates using crude sap. The method described provides a system for detecting CMV antigens in crude sap and for demonstrating serological differences among CMV isolates without having to use concentrated (3) or purified virus (1). The salient feature of this method is the utilization of certain additives. For example, sodium thioglycolate was incorporated into the immunodiffusion medium because it has been used by many workers as a reducing agent during homogenization of leaf tissue for CMV purification (11,13,15–17). TX-100 has been used in the purification of CMV isolates that could not be purified by methods employing organic solvents for the clarification step (8,13,16) since it disrupts plant membranes and causes better release of virus (11,14). Sodium ethylene diaminetetraacetate is used in the purification of CMV for chelation of divalent cations which cause aggregation of virus particles (17).

Bentonite has been used in the purification of peanut stunt virus (20) but not CMV. We found, however, that it is an essential factor for intensifying the appearance of v-bands. Formalin treatment of crude sap also enhances the production of v-bands even though the production of s-bands was not eliminated. A combined bentonite-formalin treatment was better than each individual treatment probably because the mechanisms by which they stabilize virus particles are different. Formalin stabilizes the protein shells of the virus particles without protecting the RNA (7) which remains susceptible to RNase even though the protein shells show resistance to degradation. Bentonite also prevents degradation of virus particles by binding and inactivating the enzyme responsible for the degradation of RNA of the virus (4,5). Bentonite and formalin, therefore, complement each other and prevent the loss of both the protein-protein and RNA-protein interactions which are responsible for maintaining the integrity of virus particles (6,9).

The addition of only one volume of the bentonite-formalin suspension to four volumes of crude sap minimized the chance of diluting the CMV antigen in crude sap to a concentration below the limit of detectability in immunodiffusion tests for CMV (3). The CMV s-band reactions which are produced even when crude sap is mixed with an equal volume of 0.5 M citrate buffer containing 2% formalin (2) were also eliminated. However, bentonite-formalin treatment of crude sap produced only s-bands when placed in immunodiffusion media containing borate and EDTA of low molarities. Thus, high molarity borate-EDTA buffer, TX-100, and sodium thioglycolate are all important components in the immunodiffusion medium for detection of CMV intact capsids in crude tobacco sap.

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