

## Assay for Viruses and Mycoplasmas Using Serologically Specific Electron Microscopy

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We thank the donors of viruses and antisera used in this report and the Department of Botany, Louisiana State University, for the use of electron microscope facilities.

Accepted for publication 23 December 1975.

### ABSTRACT

DERRICK, K. S., and R. H. BRLANSKY. 1976. Assay for viruses and mycoplasmas using serologically specific electron microscopy. *Phytopathology* 66: 815-820

Serologically specific electron microscopy (SSEM) was further developed as a diagnostic technique for plant viruses. The technique was shown to apply to a variety of plant viruses and to the corn stunt mycoplasma (CSM). Assays of barley yellow dwarf virus in crude extracts were easily done using a mouse antiserum produced with a partially purified virus preparation. The addition of sucrose (to 0.4 M) to virus extracts and washing buffers greatly reduced the amount of

debris on SSEM grids. The dilution of antiserum used to prepare SSEM grids did not have a significant effect on the number of virus particles attached, except at extremely high antiserum dilutions. In assays of young cultures of CSM and CSM-infected corn, filamentous forms were specifically attached to SSEM grids; in assays of older cultures only spherical forms, 0.1-0.2  $\mu$ m in diameter, were observed.

*Additional key words:* maize mosaic virus, tomato spotted wilt virus, maize chlorotic dwarf virus, maize dwarf mosaic virus.

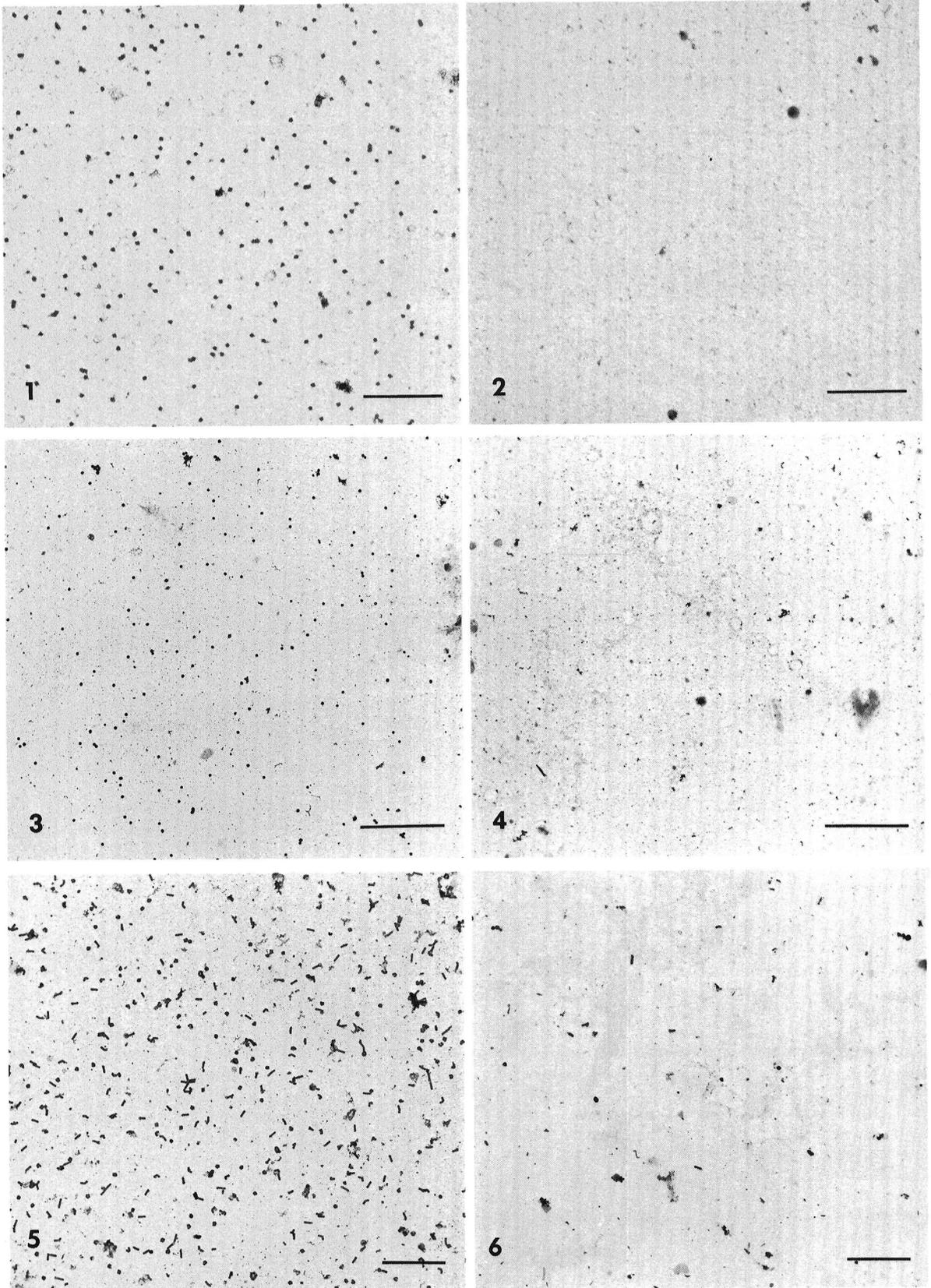
We have given reports (2, 5, 6) on a method, which has been named serologically specific electron microscopy (SSEM), that provides for specific attachment of virus particles to electron microscope grids. Grids with Parlodion films are coated with an adsorbed film of viral antiserum. Virus particles are attached and concentrated on the surface of the grids floated on extracts of virus-infected tissue. Salts and other components in the extract are removed by washing, and the virus particles are stained for examination with the electron microscope. In addition to being a very sensitive method for detection and identification of viruses, SSEM provides for quantitative assay of viruses in crude extract (5) and has been used to monitor aggregation and degradation of virus particles that may occur during purification procedures (6). The technique was used by Shalla et al. (9) to study viruslike particles in chloroplasts of plants infected with tobacco mosaic virus. Additional details on the use of SSEM as a diagnostic method are given in this report.

### MATERIALS AND METHODS

**Viruses and antisera.**—Potato virus Y (PVY) and tomato spotted wilt virus (TSWV) and antisera to PVY and TSWV (prepared by G. V. Gooding, North Carolina State University) were provided by L. L. Black, Louisiana State University. Maize chlorotic dwarf virus (MCDV) and antiserum were supplied by D. T. Gordon, Ohio Agricultural Research and Development Center. Maize mosaic virus (MMV) was collected in Louisiana by M. J. Giamalva, Louisiana State University. Antiserum to MMV (prepared by F. Herold) was provided by R. Lastra, Instituto Venezolano Investigaciones Cientificas, Caracas, Venezuela. Antiserum to maize dwarf mosaic virus (MDMV-A) was ATCC AS 58. Corn stunt mycoplasma (3, 10) in culture, in corn plants, and antiserum to it were gifts from R. F. Whitcomb,

Agricultural Research Service, Beltsville, Maryland. Barley yellow dwarf virus (BYDV) in 'Barsoy' barley was provided by M. A. Newman, West Tennessee Experiment Station. Mouse antiserum to BYDV was produced using partially purified virus prepared by the method of Brakke and Rochow (1) through the first cycle of differential centrifugation. Three white mice were given a total of four intraperitoneal injections of the virus preparation, suspended in Freund's incomplete adjuvant, at weekly intervals. Terminal bleedings were made 14 days after the last injection. Each mouse was injected with a total of 1 ml of the virus preparation, which represented the yield from 15 g of infected barley. A control serum was prepared using a similar preparation from healthy barley. Of the three antisera, the one used in this report was superior, one gave considerably less virus attachment, and one failed to give detectable virus attachment. Since it was not necessary to know accurately the dilution of a serum (provided it was less than 1:10,000) to use it in SSEM, trial bleedings from mice were made by tail clipping. The tails were clipped and immediately placed in a drop of Tris-NaCl; blood cells were allowed to coagulate and settle, and SSEM grids were prepared from the resulting drop of dilute serum. Terminal bleedings, by opening chest cavities, gave approximately one-half ml of serum, which is an adequate supply for SSEM assays.

**Procedure for serologically specific electron microscopy.**—Cohen-Pelco handle grids, 74  $\mu$ m (200-mesh), were dipped in a 1% solution of polybutene in xylene and allowed to dry. Films were put on the grids using a 0.5% solution of Parlodion in amyl acetate. The grids were carbon-coated in a vacuum evaporator. The grids were floated on drops of antiserum diluted 1:1,000 to 1:10,000 with Tris buffer (0.05 M, pH 7.2) for 30 minutes. Unadsorbed serum proteins were removed by floating the grids (five times) on drops of Tris buffer or by placing the grids in a stream of buffer from a wash bottle. Prepared SSEM grids have been washed with distilled



**Fig. 1-6.** Electron micrographs of serologically specific electron microscopic assays. (1-2) Extract of corn infected with maize chlorotic dwarf virus (MCDV) on: 1) a MCDV-antiserum grid, 2) a control grid prepared using maize dwarf mosaic virus antiserum. (3-4) Extract of *Datura stramonium* infected with tomato spotted wilt virus (TSWV) on: 3) a TSWV-antiserum grid, 4) a control grid prepared using tobacco mosaic virus antiserum. (5-6) Extract of corn infected with maize mosaic virus (MMV) on: 5) a MMV-antiserum grid, 6) a control grid prepared using MCDV antiserum. The scale bars equals: (1-2) 0.5  $\mu\text{m}$ , (3-6) 2.0  $\mu\text{m}$ .

water, allowed to dry, and successfully stored for up to two weeks at 4 C. The grids used in these studies were not allowed to dry, but immediately after washing were placed on virus extracts. Control grids were prepared using normal serum or antiserum to a virus that was not serologically related to the virus being assayed.

Samples of virus-infected leaf tissue were ground with a mortar and pestle with extraction buffer. All viruses in this study, except TSWV, were extracted and diluted with Tris buffer (0.05 M, pH 7.2) containing 0.15 M NaCl (Tris-NaCl). Extractions and dilutions of TSWV were made with 0.02 M phosphate, pH 7.2, containing 0.01 M sodium sulfite. SSEM grids were placed on drops of diluted virus extract. Following reaction times of 1-24 hours, the grids were washed with extraction buffer and distilled water. The grids were given a positive stain by floating them on 1% uranyl acetate in 50% ethanol for 10 minutes, followed by washing with 50% ethanol. Excess liquid was blotted from the grids, and they were allowed to dry. All operations were done at room temperature.

### RESULTS AND DISCUSSION

The SSEM technique has been used in our laboratory as a diagnostic method for a variety of plant viruses. The results given here are typical; the method should work for most, if not all, viruses. It has proven to be very useful for identification of MCDV, a leafhopper-transmitted virus that is not transmitted manually. Virus particles, which appear as dark spheres at low magnification when given a positive stain with uranyl acetate, were readily detected in extracts of MCDV-infected corn or Johnsongrass. In an assay of an extract from corn diluted 1:100 and using a 4-hour reaction time, virus particles were readily seen on a MCDV-antiserum grid (Fig. 1) but not on a control grid (Fig. 2).

In initial SSEM assays of TSWV, virus particles were fixed by floating grids on 1% glutaraldehyde prior to staining (2). The results shown here were obtained by assaying fresh tissue immediately upon extraction and without fixing with glutaraldehyde. An extract of TSWV-infected *Datura stramonium* was diluted 1:100 with 0.02 M phosphate, pH 7.2, containing 0.01 M sodium sulfite and assayed, using a 4-hour reaction time. Numerous spherical virus particles were observed on a TSWV-antiserum grid (Fig. 3); no particles were found on a control grid (Fig. 4).

An antiserum to MMV, reported to be of low titer and produced using a partially purified preparation (7), gave specific attachment of rhabdovirus particles from a virus isolate collected in Louisiana that was suspected to be MMV (Fig. 5). The virus extract was diluted 1:10, and a 1-hour reaction time was used. There was detectable nonspecific attachment of virus particles to the control grid (Fig. 6), but at low magnification it was difficult to distinguish the few virus particles that were present from debris.

Positive staining of virus particles with uranyl acetate proved to be easier and more consistent than negative staining with phosphotungstate or ammonium molybdate. This was especially true if a large number of grids were being processed. Isometric virus particles with a positive stain were easily distinguished from debris with very modest-resolution electron microscopy.

Identification of a virus was based more on the random distribution of virus particles at low magnification rather than upon distinguishing particle structure of a few particles at higher magnification. Electron micrographs shown in this report are not selected areas; they are representative of entire grids. Staining with solutions of uranyl acetate in water or ethanol (50 or 95%) gave similar results; with the ethanol solutions the stain was possibly a little more intense. Shadow casting of rod viruses gave results equal to positive staining; the latter is preferred in routine work because it is easier to apply.

Two antisera to PVY were used to determine the effect of titer and dilution of antiserum upon the attachment of virus particles to SSEM grids (Table 1). Serum one gave a strong reaction to PVY in sodium dodecyl sulfate (SDS) immunodiffusion tests and a dilution of 256 in microprecipitin tests. Serum two (which was prepared in this laboratory) failed to give a positive reaction in either test. Grids were prepared using the two PVY antisera and normal serum, diluted as indicated in Table 1, and were placed on a crude extract of PVY-infected tobacco diluted 1:100 for one hour. The particle counts shown represent the total number of virus particles on 7 × 8 cm negatives taken at ×2,500. Obviously, the high dilutions that can be used to prepare SSEM grids is not a reflection of the titer of the antiserum. The number of attachment sites on a grid will be constant for a given antiserum without regard to the dilution of antiserum used to prepare the grid, as long as it is sufficient to completely coat the grid with a film of serum proteins, within the adsorption time being used. Antiserum dilutions of 1:1,000 to 1:10,000 are recommended with an adsorption time of 30 minutes. There seems little point in using dilutions greater than 1:10,000, but a particularly valuable serum could be used at even higher dilutions.

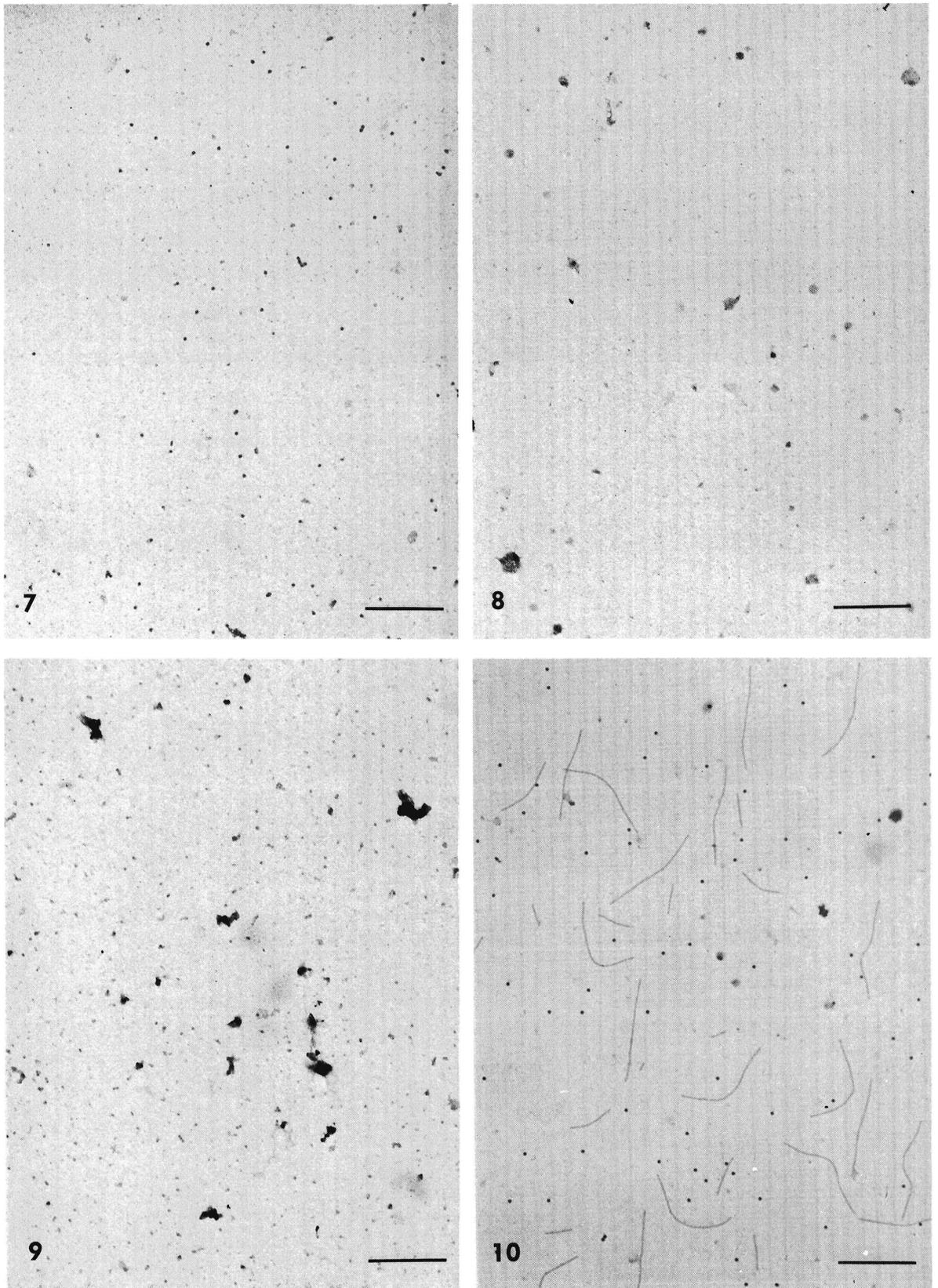
Observations that SSEM required small amounts of antisera and worked well using antisera with a low titer and containing antibodies to host antigens led to efforts to produce antisera in white mice using partially purified virus preparations. Mouse antisera, suitable for use in SSEM, have been made to several plant viruses using virus preparations that had been subjected to one cycle of differential centrifugation.

For SSEM assays of all viruses, with the exception of BYDV, it was possible to use crude extracts of virus-

TABLE 1. Effect of dilution and titer of potato virus Y antiserum (PVY-AS) used to prepare serologically specific electron microscopy grids on the number of virus particles attached.

Serum dilution	Number of virus particles		
	PVY-AS 1	PVY-AS 2	Normal Serum
1:100	1,187	32	7
1:1,000	1,020	47	... <sup>a</sup>
1:2,000	935	...	...
1:4,000	1,050	28	...
1:8,000	1,130	21	3
1:16,000	989	17	...
1:32,000	819	25	...
1:320,000	206	7	...
1:3,200,000	43	...	...
Infinite	4	...	...

<sup>a</sup>Dots indicate assay was not made.



**Fig. 7-10.** Electron micrographs of serologically specific electron microscopic assays. (7-8) Extract of barley infected with barley yellow dwarf virus (BYDV) assayed with 0.4 M sucrose added to the extract and washing buffer on: 7) a BYDV-antiserum grid, 8) a control grid prepared using antiserum to an extract of healthy barley. 9) Extract of barley infected with BYDV assayed without added sucrose on a BYDV-antiserum grid. 10) Extract of Johnsongrass infected with maize chlorotic dwarf virus (MCDV) and maize dwarf mosaic virus (MDMV) on a grid prepared using a mixture of MCDV and MDMV-A antisera. The scale bar equals 0.5  $\mu\text{m}$ .

infected plants diluted 1:10 or 1:100. This dilution greatly reduced the background debris on the grids. Additional reduction of debris, although not absolutely necessary for successful assays, was considered desirable. Vigorous washing of grids after the reaction step, which is recommended and which does not remove virus particles, will remove a considerable amount, but not all, of the debris. Washing grids with a 1% solution of sodium dodecyl sulfate (SDS) gave very clean grids, but virus particles were also removed in direct proportion to the contact time with SDS. The addition of sucrose (to 0.4 M) to virus extracts and washing buffers greatly reduced the amount of debris on grids. This was first observed in assaying for corn stunt mycoplasma where 0.4 M sucrose was necessary to preserve the integrity of the organism, as has been recommended by Cole et al. (4) for *Spiroplasma citri*. The reduction of debris on grids by the addition of

sucrose made a dramatic difference in SSEM assays. This was particularly true when minimal dilution of extracts was considered necessary for assaying tissue with a low titer of virus. Electron micrographs shown in Fig. 1-6 and 9 are of assays in which sucrose was not added to extracts and washing buffers; Fig. 7, 8, and 10-14 show results with added sucrose.

The titer of BYDV in crude extracts is below the detection limits of conventional serological techniques (8). Prior to making observations on the effect of sucrose, SSEM assays of BYDV were difficult. Since it was necessary to assay undiluted sap or sap diluted 1:2, there was always a considerable amount of debris on the grids, but areas could usually be found in which virus particles could be distinguished. Repeated freezing and melting of plant tissue and of expressed sap, which has been shown to aid in purification of BYDV (1), also was required for

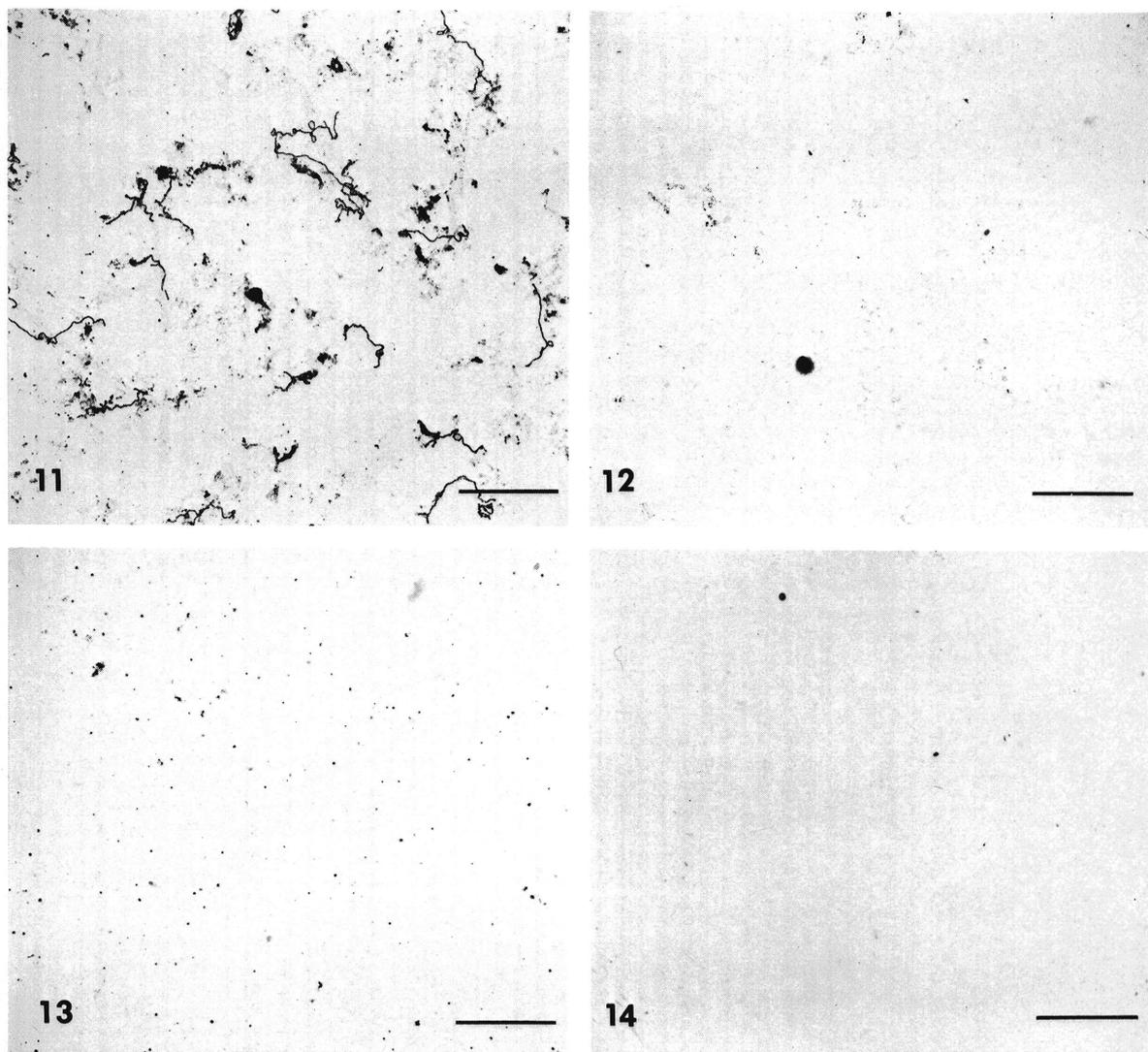


Fig. 11-14. Electron micrographs of serologically specific electron microscopic assays of corn stunt mycoplasma (CSM). (11-12) A 9-day-old culture of CSM on: 11) a CSM-antiserum grid, 12) a normal-serum grid. (13-14) A 24-day-old culture of CSM on: 13) a CSM-antiserum grid, 14) a normal-serum grid. The scale bar equals 5.0  $\mu\text{m}$ .

successful assays. In typical SSEM assays of BYDV frozen tissue was ground, and sap was expressed from the resulting pulp. The sap was frozen and melted three times and diluted 1:2 with Tris-NaCl or with Tris-NaCl containing 0.8 M sucrose. Serologically specific electron microscopy grids that had been prepared using mouse antiserum to BYDV or to healthy barley extract, were floated on drops of the extracts for 2 hours. The grids were washed with Tris buffer or Tris buffer containing 0.4 M sucrose and distilled water prior to staining. Virus particles were readily seen on the BYDV-antiserum grid when sucrose was added to the extract (Fig. 7). No virus particles were seen on the control grid of the same extract (Fig. 8). Virus particles could be detected on the BYDV antiserum grid when sucrose was omitted from the extract (Fig. 9), but, as previously noted, considerable scanning was necessary to find areas in which virus particles could be distinguished from debris.

Viruses that differ in particle morphology can be assayed on the same SSEM grid by using a mixture of antisera. For example, MCDV and MDMV commonly occur in corn, sorghum, and Johnsongrass; often in mixed infections. A positive reaction to both viruses was observed in assaying an extract of Johnsongrass diluted 1:100 with Tris-NaCl containing 0.4 M sucrose (Fig. 10). The reaction time used was 1 hour, and the grid had been prepared using a mixture of equal volumes of MCDV and MDMV-A antisera, previously diluted 1:1,000.

Assay of a 9-day-old culture of corn stunt mycoplasma (CSM) resulted in numerous filaments being attached to a SSEM grid prepared using an antiserum to CSM (Fig. 11). No filaments were seen on the control grid (Fig. 12); considerable scanning of the grid failed to reveal any organisms. In an assay of a 24-day-old culture, only small spherical forms, 0.1-0.2  $\mu\text{m}$  in diameter, were attached to a CSM-antiserum grid (Fig. 13); few, if any, of these spherical forms were seen on the control grid (Fig. 14). The results shown were from assays of undiluted cultures, with the following modifications in procedure. Following 1-hour reaction times, the grids were washed repeatedly with neutral 0.1 M phosphate containing 0.4 M sucrose. The grids were not washed with distilled water at this point, but were washed with a 5% solution of uranyl acetate in water and allowed to float on a drop of the staining solution for 10 minutes. The grids were then washed repeatedly with distilled water, blotted, and allowed to dry. Attempts to assay CSM using solutions without 0.4 M sucrose for dilution or washing (prior to staining with uranyl acetate) were unsuccessful. Organisms were readily detected in young cultures diluted 1:100 with neutral 0.1 M phosphate containing 0.4 M sucrose, and in juice expressed from the midveins of CSM-infected corn leaves diluted with an equal volume of neutral 0.1 M phosphate containing 0.8 M sucrose.

Of a variety of techniques used to prepare SSEM grids, best results have been obtained using grids with carbon-coated films prepared by floating Parlodion films off microscope slides. Cohen-Pelco tabbed grids offer an

obvious advantage, due to the extensive handling of grids that is required. Coating grids with 1% polybutene solution in xylene makes the Parlodion film stick to the grids. The film may even be torn badly in some areas of the grid due to extensive handling, but invariably some good openings can be found. When polybutene is not used, occasionally the entire film will separate from a grid, usually during a washing step.

Repeated efforts to prepare SSEM grids using Formvar films, both with and without carbon-coating, were unsuccessful. Apparently serum proteins are not adsorbed to Formvar, at least not under the conditions tried, which included using serum diluted in Tris buffer, pH 7.2, or 0.05 M carbonate, pH 9.5. The difference between carbon-coated Formvar and carbon-coated Parlodion was not expected and is not readily explained.

The omission of 0.15 M NaCl, which is required for optimum reaction in most serological systems, from buffers used for extraction of tissue for SSEM does not decrease the number of virus particles attached to grids. This provides for serological studies of viruses which may be degraded, aggregated, or otherwise modified by 0.15 M NaCl.

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