Infectivity Neutralization Used in Serological Tests With Partially Purified Beet Curly Top Virus

James E. Duffus and A. H. Gold

Plant Pathologist, ARS, USDA, U.S. Agricultural Research Station, Salinas, California 93901; and Professor, Department of Plant Pathology, University of California, Berkeley 94720, respectively.

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ABSTRACT

A serological comparison of several isolates of beet curly top virus by infectivity neutralization with antiviral antibodies has established their relationship. Partially purified virus preparations were mixed with equal volumes of antisera and after incubation were fed to beet leafhoppers (*Circulifer tenellus*) through artificial membranes. One antiserum tested almost completely neutralized the infectivity of three curly top isolates, another only partially neutralized infectivity. Normal rabbit serum or antiserum against healthy shepherd's purse juice did not affect infectivity.

Partially purified curly top virus preparations from density-gradient electrophoresis fractions showed one type of particle in shadowed preparations, small, spherical, spongy appearing, and ca. 19-20 nm in diameter.

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The curly top viruses are known to occur in arid areas of the United States, South America, and Mediterranean Eurasia, but the geographical and ecological extent of their individual distribution is not known. These viruses resemble each other closely in symptoms induced on sugar beet, but their association with certain hosts and with the insects that transmit them are so specific and so different in the areas of their occurrence as to raise questions as to their taxonomic relationships.

Because plant and insect quarantines prevent direct comparison of the virus entities by transmission tests, serology was attempted as a first step to determine relationships of the curly top viruses.

MATERIALS AND METHODS.—Isolates of curly top virus used in these studies were maintained in beet leafhoppers, *Circulifer tenellus* (Baker), reared on diseased plants. Young adult leafhoppers of about the same age reared on healthy plants were used in membrane feeding tests. The nonviruliferous leafhoppers were obtained by placing about 200 nonviruliferous adults on individual beet plants for 2 days. The leafhoppers were removed, and the eggs were allowed to hatch. The resulting nymphs were all about the same age.

Membrane feeding tests were conducted, using feeding cages modified from earlier models (1, 7). The bottoms of plastic vials, 5 cm in diam, were cut, leaving a cylinder 5 cm long. To the cut end of the cylinder a fine-mesh screen was attached. The cap end of the vial was covered with a thin membrane of Parafilm (Marathon Products, Neenah, Wisconsin). Groups of 26 nonviruliferous leafhoppers were placed in these cages. Approximately 0.5 ml of the liquid extract to be tested was placed on the membrane and covered with another thin membrane of Parafilm. Extracts were adjusted to 15% sucrose by the addition of sucrose or dilution with buffer. Cages with leafhoppers and liquid extract were placed on white paper, membrane-side down, in a controlled temperature chamber maintained at 37 C. The leafhoppers were allowed to feed 4 hr on the extracts and then caged singly on seedling beets.

Extracts for antigen preparation and infectivity neutralization tests were derived from phloem exudate, collected from curly top-infected shepherd's purse, *Capsella bursa-pastoris* (L.) Medic (2). Exudate was clarified by low-speed centrifugation (10 min at 4,220 *g*) and frozen until used.

Density-gradient centrifugation was done in a SW-25.1 rotor for 10 hr at 53,819 *g*. Gradient columns were prepared by layering 4, 7, 7, and 7 ml of 10, 20, 30, and 40% sucrose, respectively, dissolved in 0.05 M phosphate buffer, pH 7.0, containing 0.01 M glycine. Columns were fractionated with an ISCO density-gradient fractionator.

The density-gradient electrophoresis apparatus used was similar to that described by van Regenmortel (13). The length of the column from electrode to virus source was 61 cm. The sucrose gradient occupied ca. 33 cm in the column. Phloem exudate was dialyzed against 35% sucrose in 0.005 M phosphate buffer, pH 8.0. The dialyzed phloem exudate was layered between 50% sucrose in 0.005 M phosphate buffer and the bottom of the gradient. The gradient column was formed with 0.005 M phosphate buffer and 35% sucrose in the same buffer. Electrophoresis was conducted at 7.5 mA for 22 hr. The columns were fractionated in 1-ml aliquots, and each fraction was assayed for virus infectivity by feeding leafhoppers on the extracts.

Healthy shepherd's purse antigen was prepared by clarifying crude extracts by low-speed centrifugation (10 min at 4,200 *g*) followed by ultracentrifugation (2 hr at 80,800 *g*). The pellets were resuspended in 1/150 of the original volume in 0.05 M phosphate buffer, pH 7.0, containing 0.01 M glycine.

Beet curly top virus antigen was prepared in two ways. In one method, crude phloem exudate was clarified and injected into rabbits. With a second method, the most infectious zones of several density-gradient electrophoresis runs were used as the antigen.

Sera were taken after six intramuscular injections of rabbits at weekly intervals, using Freund's complete adjuvant (Difco-Bacto).

RESULTS.—*Correlation of particles and infectivity.*—The curly top virus is extremely stable, but attempts to study the nature of the infectious particle have been hampered by its apparent highly adsorptive qualities (3). Phloem exudate, because of its high virus content and its usefulness as a leafhopper-feeding extract (1) was used as a starting material to establish a correlation between particles and infectivity.

Density-gradient centrifugation of clarified phloem exudate resulted in infectivity in all layers in the density-gradient columns. Moreover, no unique UV-absorbing components associated with infectivity were found in the columns by scanning with the ISCO density-gradient fractionator and scanner.

Because of the difficulty in obtaining one infective band by density-gradient centrifugation, dialyzed phloem exudate was subjected to density-gradient electrophoresis. The virus, as evidenced by infectivity tests, migrated rapidly compared to noninfective opalescent zones in the electrophoresis columns. Although the electrophoresis fractions containing virus varied somewhat in their position in the columns in different runs, the zone with the highest infectivity appeared to be completely separated from other particulate material as judged by electron-microscope observation. Infectivity was generally found 6-24 cm from the origin, with the region of highest infectivity 14-17 cm from the origin.

The region of highest infectivity showed one type of particle in shadowed preparations—small, spherical, spongy-appearing particles ca. 19-20 nm in diam and occurring mostly in clumps (Fig. 1). Particle size determinations were based on rough estimates of the size of the smallest units observed in the aggregates. The particles are similar to particles found by K. M. Smith & C. W. Bennett in phloem exudate passed

through gladocol membranes with estimated pore sizes of ca. 25 nm (3). Characteristic particles were absent from zones with no infectivity.

Infectivity neutralization.—Serological neutralization of infectivity (11) was conducted in two ways. In the first, insects were fed directly on the virus-antiserum reactants. This technique demonstrates virus neutralization. In the second method, the virus-antiserum reactants were subjected to density-gradient centrifugation before the feeding of insects. Since earlier work with density-gradient centrifugation had indicated infectious zones scattered throughout the gradient columns, all zones were fed to insects. This technique also demonstrates virus neutralization. The procedure for the first method consisted of mixing clarified phloem exudate, or partially purified electrophoresis fractions with either an equal volume of buffer, normal rabbit serum, antiserum to the juice of healthy shepherd's purse, or antiserum to the virus. After incubation for 2 hr at 37 C, the mixture was fed to nonviruliferous beet leafhoppers through membranes. The beet leafhoppers were caged individually on sugar beet seedlings. Infectivity of three curly top virus isolates was almost completely neutralized with antiserum CT-1 (produced against curly top phloem exudate), but only partially with the lower titer antiserum CT-E (produced against curly top electrophoresis zones) (Tables 1, 2). Normal rabbit serum or antiserum against healthy shepherd's purse juice had little effect on infectivity.

Another experiment was designed to measure the titer of antiserum (CT-1) against strain 11 of the curly top virus (Table 3). The reaction was carried out using phloem exudate diluted to 15% sucrose with buffer. Antiserum was diluted with 0.85% NaCl in series to 4^{-5} the original concentration. The dilutions of antiserum were incubated with the diluted phloem exudate for 2 hr at 37 C and fed to leafhoppers. Dilutions of antiserum to 2^{-1} effectively neutralized this concentration of the curly top virus. The dose of antiserum that neutralized half the infectivity was determined to be a dilution between 4^{-2} and 4^{-3}.

DISCUSSION.—A systematic study of the geographic distribution and interrelationships of the curly top viruses, which cause serious disease losses

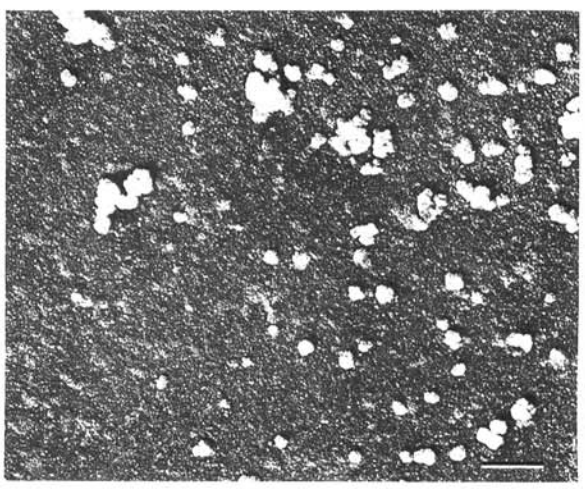

Fig. 1. Electron micrograph of shadowed sugar beet curly top virus particles. Scale bar represents 100 nm.

TABLE 1. Infectivity neutralization of beet curly top virus isolates

Sample tested	Infectivity of isolates after incubation with the indicated diluent		
	Strain 11	Isolate 8	Isolate 22
Buffer + virus[a]	76[b]	72	84
Normal serum + virus	79	69	79
ASHSP[c] + virus	69	75	74
ASCT-1 + virus	1	0	0
ASCT-E + virus	31	28	41

[a] Dialyzed phloem exudate was subjected to density-gradient electrophoresis. The most highly infectious zones in bands 14-17 cm from the origin were pooled and frozen until use. The virus sample was diluted with buffer to 15% sucrose, then mixed with an equal volume of the indicated diluent, and incubated for 2 hr at 37 C.

[b] Number of plants infected out of 200 inoculated by individual beet leafhoppers fed through a membrane on each sample.

[c] Antiserum to healthy shepherd's purse (ASHSP); antiserum to Strain 11 curly top virus prepared against phloem exudate (ASCT-1); antiserum to Strain 11 curly top virus prepared against density-gradient electrophoresis fractions (ASCT-E).

TABLE 2. Infectivity neutralization of beet curly top virus followed by density-gradient centrifugation

Density-gradient zones[a]	Infectivity of zone after incubation with the indicated diluent		
	Buffer	Normal serum	ASCT-1
2	2[b]	1	0
4	3	3	0
6	2	2	0
8	4	1	0
10	3	3	0
12	8	3	0
14	6	12	0
16	7	6	0
18	4	6	0
20	6	8	0
22	3	4	0
24	4	4	0
26	3	2	0
28	1	3	0
30	0	1	0

[a] Dialyzed phloem exudate was diluted to 15% sucrose with buffer, and the virus sample was mixed with an equal volume of the indicated diluent and incubated for 2 hr at 37 C. The virus-antiserum reactants were subjected to density-gradient centrifugation and fractionated.

[b] Number of plants infected out of 16 plants inoculated in two tests by individual beet leafhoppers fed through a membrane on each sample.

TABLE 3. Effect of serum dilution on infectivity neutralization of beet curly top virus

Sample tested	Infectivity of sample after incubation with the indicated serum dilution
ASHSP[a] + virus[b]	72[c]
ASCT-1 + virus	3
ASCT-1 2^{-1} + virus	0
ASCT-1 4^{-1} + virus	18
ASCT-1 4^{-2} + virus	28
ASCT-1 4^{-3} + virus	57
ASCT-1 4^{-4} + virus	70
ASCT-1 4^{-5} + virus	63

[a] Antiserum to healthy shepherd's purse (ASHSP); antiserum to Strain 11 curly top virus prepared against phloem exudate (ASCT-1). Serum dilutions were made with 0.85% NaCl.

[b] Virus sample was phloem exudate diluted with buffer to 15% sucrose.

[c] Number of plants infected out of 145 inoculated by individual beet leafhoppers fed through a membrane on each sample.

on several major world food crops (12), could be economically and scientifically important. The viruses appear to occur in several well defined areas (4, 5, 6). They show several strong similarities, yet there are differences that raise questions concerning their true relationships and their areas of origin.

A serological approach to a geographic study of the various curly top viruses would be especially valuable. The studies reported herein indicate that curly top virus is immunogenic and may be tested by one of the most specific of all virus-antibody reactions—neutralization. Neutralization of infectivity by immune sera has greatly aided in the clarification of some of the interrelationships of the yellowing viruses of the beet western yellows group (8, 9, 10, 11). It is a sensitive test and can be used for demonstrating serological relationships where the precipitation test cannot be applied. It also has the advantage that antibodies to normal plant constituents do not affect the usefulness of the serum, and that it detects active virus.

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