Beet Pseudo-Yellows Virus: Purification and Serology

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ABSTRACT

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Beet pseudo-yellows virus (BPYV) has been purified from BPYV-infected *Nicotiana clevelandii*. Purified preparations had an $A_{260/280nm}$ ratio of 1.315 and contained long, flexuous rod-shaped particles approximately 12 nm wide and 1,500–1,800 nm long. Virus yield ranged from 100 to 400 μg/kg of leaf tissue. An extinction coefficient of 3 $(mg/ml)^{-1} \times cm^{-1}$ at A_{260nm} was used. An antiserum to BPYV was prepared that has enabled us to diagnose BPYV-infected plants by the indirect enzyme-linked immunosorbent assay (ELISA) but not the direct ELISA test. The disease, transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum*), has been diagnosed previously only by transmission and host range tests. This is the first report of the description of the particle morphology of BPYV and the production of an antiserum that can be used for serodiagnosis.

The greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), is one of the most important pests of greenhouse crops throughout the world. However, the most important damage by this insect, damage that has been virtually undiagnosed, is induced by a virus transmitted by the whiteflies. The virus, termed beet pseudo-yellows virus (BPYV), was described by Duffus in 1965 from the United States (3). BPYV as originally described has a wide host range of crops, weeds, and ornamentals. The virus was subsequently found in France (13,14), the Netherlands (19), Australasia (5), Italy (16), Bulgaria (8,9), and Spain (18). A similar virus was found in greenhouse cucumber and melon in Japan (20). The virus induces stunting, unthriftiness, and interveinal yellowing or reddening. The symptoms are very similar to what agriculturalists expect from low fertility, lack of moisture or deficient conditions, insect feeding, and natural aging or ripening. The disease on many vegetables and ornamentals looks similar to nutrient deficiency symptoms caused by low iron, magnesium, or manganese.

Beet pseudo-yellows virus has been severe for the past several years in greenhouse cucumbers from several areas of California and Ohio. Johnson (10) reported an outbreak in the Encinitas area of San Diego County. Three growers were affected and all crops were essentially a complete loss. Since 1982 in Spain, the incidence of BPYV in melon (*Cucumis melo* L.) crops, cultivated under plastic, has continually increased to the extent that the commercial viability of the crop is threatened (J. E. Duffus, *unpublished*). BPYV has become increasingly important and appears to be spreading rapidly. The increasing importance of whitefly-transmitted yellowing viruses in protected environments and in agricultural regions of the world has shown the critical need for more effective assay procedures for this group of viruses. Before this report only one virus, lettuce infectious yellows virus (LIYV), had been characterized as to particle morphology and an effective serological assay developed for its identification (6). Unknown whitefly-transmitted agents, including BPYV, could only be tentatively identified by whitefly transmission to indicator plant species. These methods can and have led to a confusing array of conclusions. This paper describes the purification, particle morphology, serology, and a serological method for BPYV identification. A preliminary report of this research has been published (12).

MATERIALS AND METHODS

Virus source and whitefly maintenance. The virus isolates used for this study were obtained from field-infected dandelion (*Taraxacum officinale* Wigg.) plants collected in the Salinas Valley of California. The isolates were maintained in cheeseweed (*Malva parviflora* L.) and transferred from plant to plant via inoculation with the whitefly vector. The greenhouse whitefly, *T. vaporariorum*, used in these tests was collected originally in the greenhouse and reared on *Physalis alkekengi* L., in muslin-covered cages maintained in growth rooms at 24–26 C. Viruliferous whiteflies were reared on virus-infected plants of *M. parviflora*. Seedlings of *Nicotiana clevelandii* Gray (3 wk old) were inoculated using about 50 viruliferous whiteflies per plant, and a 24-hr inoculation access period. After inoculations with viruliferous whiteflies, the plants were sprayed with resmethrin. They then were maintained in the greenhouse at 26–32 C until symptoms appeared. All plants were grown in screened greenhouses fumigated at weekly intervals with dichlorvos and resmethrin.

Virus purification. BPYV-infected *N. clevelandii* plants were harvested 2–3 wk after inoculation. Plants were ground in a meat grinder with cold 0.5 M sodium citrate buffer (pH 6.5) plus 5 mM EDTA and 0.5% 2-mercaptoethanol. The plant material was then homogenized with a ball mill for 2 hr at 4 C. The homogenate was expressed through cotton muslin and chloroform was added to a concentration of 10%. The slurry was stirred 10 min at 4 C, and then subjected to low-speed centrifugation for 5 min at 8,000 *g*. The virus was precipitated from the supernatant with polyethylene glycol (MW 8,000) at the rate of 10 g/100 ml of extract in 0.2 M NaCl by stirring for 1 hr at 4 C. The precipitate was pelleted for 30 min at 10,000 *g*. The virus containing pellets were resuspended in 1/10 of the original volume with 0.1 M phosphate buffer, pH 7.0, and Triton X-100 added to a final concentration of 2%, and then stirred for 30 min at 4 C. The virus preparations were centrifuged for 15 min at 2,000 *g* and the supernatant centrifuged for 1 hr and 15 min at 149,000 *g*. Pellets were resuspended in 0.01 M phosphate buffer, pH 7.0, and the suspension centrifuged for 10 min at 3,000 *g*. After an additional cycle of high- and low-speed centrifugations, the pellets were resuspended in 0.01 M phosphate buffer, pH 7.0. The suspension was centrifuged for 5 min at 3,000 *g* and the supernatant brought to a final concentration of 40% cesium sulfate (w/v). The virus-cesium sulfate preparation was centrifuged at 20 C for 20–22 hr at 84,200 *g*. Light-scattering bands were extracted with a bent needle and syringe, and dialyzed three times in 0.01 M phosphate buffer, pH 7.0, at 4 C.

In several experiments in which the same extraction method was used, viral preparations were layered onto sucrose density gradients (10–35% in 0.1 M phosphate buffer, pH 7.0) instead of cesium sulfate gradients and centrifuged at 4 C for 2 hr at 120,000 *g*. Gradients were analyzed with an ISCO (Lincoln, NE) Model 640 fractionator equipped with a Model UA-5 analyzer.

Electron microscopy. A small drop of 2% potassium phosphotungstate (PTA), pH 6.0, was placed on a carbon-coated Formvar grid and a piece of infected leaf tissue of *N. clevelandii* about 2 mm in width was dipped in the stain several times. Excess stain was removed by touching the edge of the grid with a filter paper. The grid was then examined in a Zeiss EM 109 electron microscope (Thornwood, NY) at 80 kV. Light scattering bands from the cesium sulfate preparations were examined for virus particles; a drop of a 3% glutaraldehyde-fixed suspension (15) was placed on carbon-coated Formvar grids, stained with 2% PTA. The grids were examined in the electron microscope.

Infectivity assays. The purified virus preparations for infectivity assays were dialyzed against 0.01 M phosphate buffer (pH 7.0). The extracts were adjusted to 25% sucrose and dyed yellow with liquid food coloring before being fed to whiteflies through Parafilm membranes (2). Fifty whiteflies per cage were used in attempts to detect virus infectivity in *N. clevelandii*.

Antiserum production and serology. Purified virus in aliquots of 0.5 ml (0.5 A_{260nm} units) was prepared for six intramuscular injections into a rabbit. Each aliquot was emulsified with 0.5 ml of Freund's complete adjuvant (Difco) just prior to injection. Injections were administered weekly and bleedings were begun at week seven. Immunoglobulins were precipitated with ammonium sulfate, purified by DE 22 cellulose chromatography, and conjugated to alkaline phosphatase (Type VII-NA, Sigma Chemical Co., St. Louis, MO). Enzyme-linked immunosorbent assays (ELISA) methods employed were the direct double-antibody sandwich method described by Clark and Adams (1), and the indirect method described by Koenig (11). In the indirect ELISA tests, the crude antigen dilution in coating buffer was 1:20 and the antiserum dilution was 1:1,000. The enzyme-labeled goat anti-rabbit antibodies (Sigma Chemical Co., St. Louis, MO), were diluted 1:1,000. Results of the experiments were analyzed by the Mann-Whitney U test (17) at $P \leq 0.05$.

RESULTS

Virus purification. Light-scattering bands were not detected in sucrose density gradients from either infected or healthy plants. A single band at 35–38 mm from the meniscus was observed in cesium sulfate gradients of partially purified virus. This band was absent in gradients from healthy plant material subjected to the same purification techniques. The density of the virus band after cesium sulfate equilibrium centrifugation was 1.335 g/ml. The band, after dialysis, had an absorption spectrum typical of viral nucleoprotein. The $A_{260/280nm}$ value for the purified virus was 1.315. Virus yield ranged from 100 to 400 μg/kg of leaf tissue using an extinction coefficient of 3 $(mg/ml)^{-1} \times cm^{-1}$ at 260 nm.

Infectivity assays. Nonviruliferous whiteflies that were allowed to feed on the purified preparations from cesium sulfate gradients failed to transmit BPYV to more than 100 test plants.

Electron microscopy. Sap from BPYV-infected leaves of *N. clevelandii* with interveinal yellowing symptoms contained long, flexuous rod-shaped particles (Fig. 1). Most of the particles were 1,500–1,800 nm long and approximately 12 nm wide. Healthy leaves of *N. clevelandii* did not contain the particles. Purified virus preparations after cesium sulfate equilibrium centrifugation contained aggregates of similar particles (Fig. 2).

Serology. Preliminary serological studies demonstrated that antiserum prepared against purified preparations of BPYV formed white flocculent precipitates. The titer of the antiserum was

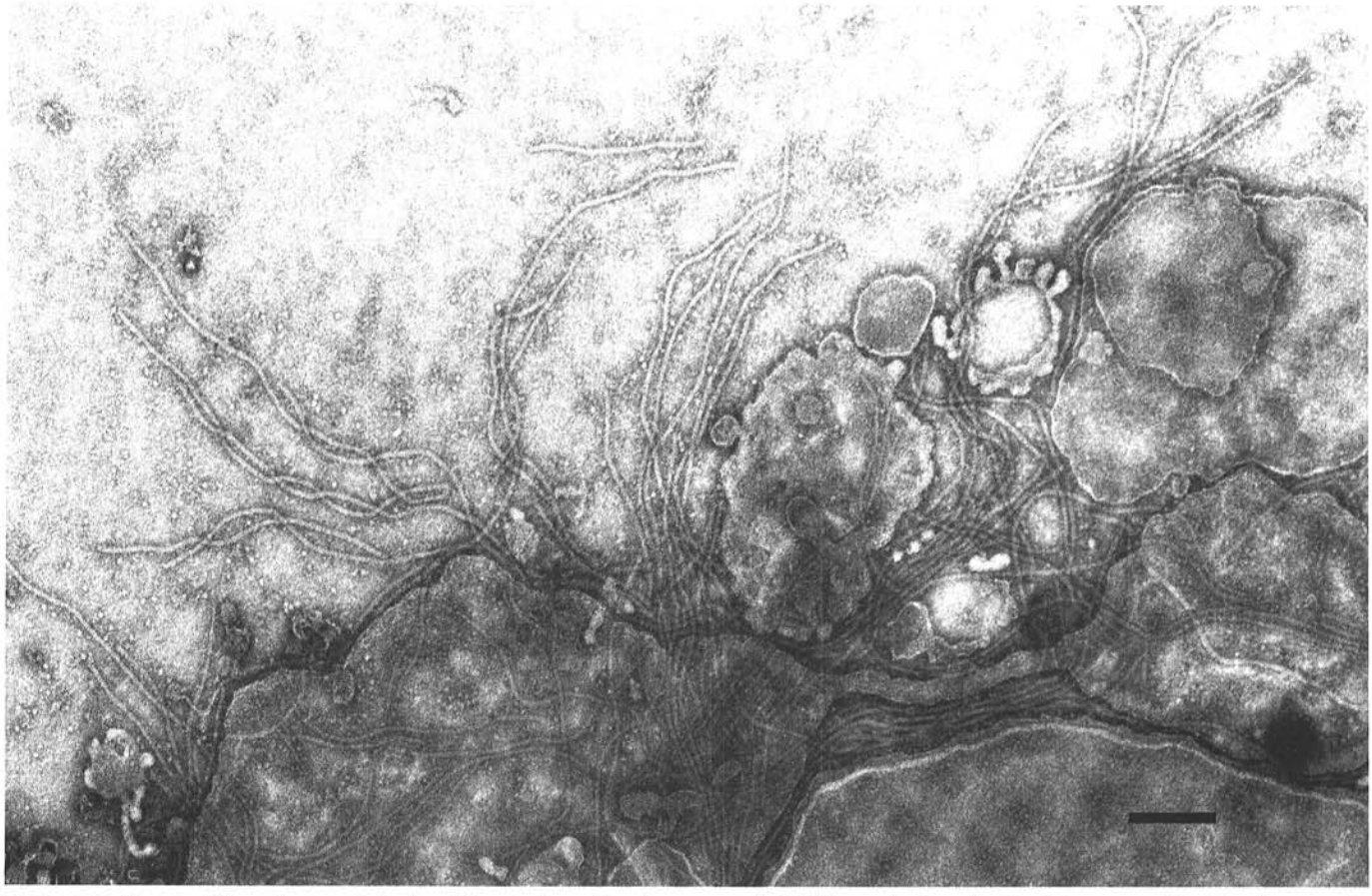

Fig. 1. Long, flexuous filamentous particles in a 2% potassium phosphotungstate (pH 6.0) negatively stained leaf dip preparation from beet pseudo-yellows virus-infected *Nicotiana clevelandii* Gray. Bar represents 200 nm.

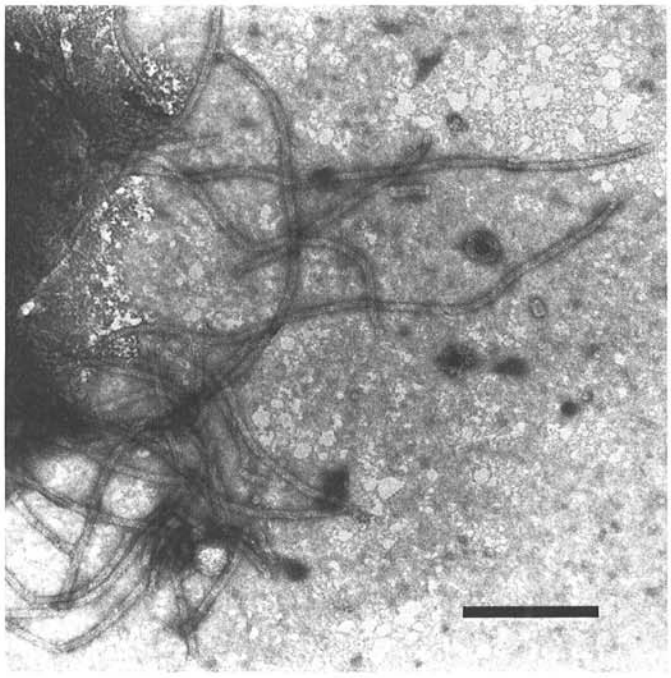

Fig. 2. Electron micrograph of purified beet pseudo-yellows virus fixed in 3% glutaraldehyde and stained in 2% potassium phosphotungstate (pH 6.0). Bar represents 200 nm.

1/32 in microprecipitin serology tests. Indirect ELISA tests demonstrated that BPYV antisera reacted with purified BPYV but not with purified LIYV and gave a positive reaction to BPYV-infected tissue from cucumber, watermelon, cantaloupe, and a number of weed species (Table 1). Our antisera did not react in direct ELISA tests.

DISCUSSION

Whitefly-transmitted yellowing viruses of crops and ornamentals have increased dramatically in recent years. BPYV, first described in the United States in the early 1960s, was subsequently found throughout the world (3,5,8,9,13,14,16,18,19). The diagnosis of BPYV was based on transmission by *Trialeurodes* and host range tests. In two instances *Trialeurodes*-transmitted entities, cucumber yellows virus (CYV)(20) and muskmelon yellows virus (MYV)(14), appeared to have host ranges somewhat distinct from BPYV and were thus considered as distinct entities. Recent evidence (21) indicated that the insect transmission and host range characteristics of CYV have been shown to be identical to BPYV. CYV reported from Japan, as distinct from BPYV based on host range, should now probably be considered to be BPYV. MYV was reported to be restricted to the Cucurbitaceae, but the authors indicated that improvement of transmission techniques could perhaps modify the results (14). Thus, the causal agent of the melon yellowing disease in France appears similar to BPYV. BPYV could only be positively identified previously by means of the whitefly-vectored host range tests. However the development of an antiserum provides a more accurate and efficient detection method.

Lettuce infectious yellows virus, transmitted by *Bemisia*, was the first of these closteroviruslike entities to be characterized as to particle morphology and serology (6). Serologically distinct from BPYV and other closterovirus, it has been found, so far, only in California, Arizona, and Mexico. A *Bemisia*-transmitted yellowing and stunting disorder (YSD) of cucurbits, found in countries of the Middle East since the early 1980s, has been recently shown to be distinct from LIYV and BPYV (7).

The particles found in BPYV-infected plants were few in number and restricted to certain tissues in their host plants. Extraction of the virus particles from the diseased plants was very difficult because of their fragility and tendency to aggregate. For these reasons, they have not been detected readily from diseased plants. Our purified preparations contained only low numbers of virus particles, so the titer of our antiserum to BPYV was low. However, the antiserum produced could be used for reliable indirect ELISA tests to detect BPYV infection (Table 1). For some unknown reasons, our antisera did not react in direct ELISA tests. Similar observations were also been reported (11). This is the first report on the production of an antiserum to BPYV and its use for serodiagnosis of BPYV.

TABLE 1. Indirect enzyme-linked immunosorbent assay (A_{405nm})[a] values obtained with leaf extracts from healthy and beet pseudo-yellows virus (BPYV)-infected plants

Test plants	Healthy	BPYV-infected
Capsella bursa-pastoris	0.011	0.031*[b]
Citrullus vulgaris	0.049	0.118*
Cucumis melo	0.065	0.233*
Cucumis sativus	0.042	0.135*
Malva parviflora	0.098	0.166*
Nicotiana clevelandii	0.043	0.114*
Purified BPYV	0.026[c]	0.190*[d]
Purified LIYV	0.030[c]	0.032[d]

[a]Average of six experiments.
[b]* = Significantly different from healthy control at $P \leq 0.05$ with the Mann-Whitney U test.
[c]Sample buffer.
[d]Purified virion.

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