Properties of Tobacco Yellow Dwarf and Bean Summer Death Viruses

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ABSTRACT

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Tobacco yellow dwarf and bean summer death diseases apparently are confined to Australia, and the causal pathogens are transmitted by the leafhopper *Orosius argentatus*. The diseases were shown to be caused by viruses. Purified preparations obtained from *Datura stramonium* plants, infected with either tobacco yellow dwarf virus (TYDV) or bean summer death virus (BSDV), contained viruslike particles occurring in pairs about 20 nm × 35 nm in size. On sedimentation in sucrose density gradients, the geminate particles of TYDV and BSDV each formed discrete zones which were associated with infectivity. TYDV preparations have an ultraviolet absorbance typical of a nucleoprotein, contain a single centrifugal component with a sedimentation coefficient of 76S, and yield a single structural protein species of molecular weight 27,500. The yield of TYDV was about 100–250 μg/kg of tissue, and of BSDV about 20–50 μg/kg of tissue. In addition to producing similar host reactions and having similar vector relations, TYDV and BSDV are serologically related, and are therefore considered likely to be strains of one virus. TYDV and BSDV have many properties in common with beet curly top virus (CTV), and a distant serological relationship was established between TYDV and CTV.

Additional key words: geminivirus.

Tobacco yellow dwarf (TYD) and bean summer death (BSD) diseases have been recognized in Australia for many years (1,18), and were once important factors in the production of tobacco (*Nicotiana tabacum* L.) and French beans (*Phaseolus vulgaris* L.). However, many French bean cultivars with resistance to BSD are now available, and although no commercial tobacco cultivars are resistant to TYD, the tobacco growing industry has been relocated away from areas of high TYD incidence. The diseases apparently are confined to Australia and have been reported from all mainland states (3,21,22). The pathogens can be transmitted by grafting and by the leafhopper *Orosius argentatus* Evans, and hence have long been considered to be viruses (1,10,18,19), though conclusive evidence for this assumption has been lacking.

Infectious extracts have been obtained from TYD and BSD-infected French bean or tobacco plants by using a variety of purification methods (30), and viruslike particles were sometimes detected when these preparations were examined under the electron microscope. However, only when *Datura stramonium* L. was used as the virus source was it possible to obtain appreciable yields of purified virus free from normal plant components. This paper describes the purification and some properties of tobacco yellow dwarf virus (TYDV) and bean summer death virus (BSDV). A preliminary account of part of this work has been published (31).

MATERIALS AND METHODS

One isolate of TYDV from tobacco (from Myrtleford, Victoria), and two isolates of BSDV from French bean (BSDV-1 from Brisbane, Queensland, and BSDV-2 from Dubbo, N.S.W.) were used in this work, and the isolates were maintained in tomato plants (*Lycopersicon esculentum* Mill. 'Grosse Lisse'). The BSDV-1 isolate was used by Bowyer and Atherton (10).

Initial experiments indicated that the virus concentrations were higher in *D. stramonium* than in Spartan Arrow or Redlands Pioneer French bean, Turkish tobacco or Grosse Lisse tomato. The viruses were therefore propagated in *D. stramonium* for purification. Plants were inoculated by side-cleft grafting with scions from infected tomato plants and usually developed symptoms 1–2 wk later. Expanding leaves, showing typical disease symptoms, were harvested and frozen at −18 C until use.

Low and high speed centrifugations were performed in a MSE High Speed 18 centrifuge and a Damon-IEC B-60 ultracentrifuge, respectively, and centrifugal forces were calculated at the outside tip of the centrifuge tubes. Both centrifuges were operated at 5 C. Because TYDV and BSDV have not been transmitted mechanically, infectivity of preparations was determined by using a leafhopper membrane-feeding technique. An infectivity index based on dilution of the virus preparation and the time taken for assay plants to show disease symptoms was employed (26). Spartan Arrow French bean was used as the assay plant because it rapidly produced marked disease symptoms, usually 7–8 days after exposure to infective leafhoppers.

The purification method of Bock et al (8) for maize streak virus (MSV) was modified by including a polyethylene glycol MW6000 (PEG) precipitation step. This modified method was used for TYDV and will be termed method 1. After extraction and clarification, the virus was precipitated by adding 10% PEG and 0.2 M NaCl and stirring for 1 hr at 5 C. The precipitate was collected by centrifugation and the pellets were resuspended in 0.01 M phosphate buffer (pH 7.7) containing 1% Triton X-100 and stirred for 30 min at room temperature. Following a low speed centrifugation, the schedule was continued as reported by Bock et al (8).

An alternative purification schedule for TYDV, and the only successful method for BSDV-1, was based on that reported by Mumford (26) for beet curly top virus (CTV), and will be termed method 2. Frozen *D. stramonium* tissue was homogenized in 0.01 M phosphate buffer (pH 7.0), containing 0.001 M ethylenediaminetetra-acetic acid (EDTA) and 0.01 M Na_2SO_3, at the rate of 2 ml/g of tissue. The homogenate was strained through cheesecloth, then one part cold chloroform/butanol (1:1,v/v) to two parts sap was slowly added and stirred for 5 min at room temperature. The mixture was then centrifuged at 6,000 *g* for 15 min, the resulting aqueous phase was removed, and 10% PEG and 0.2 M NaCl were added to it. The mixture was stirred for 1 hr at 4 C. The schedule was continued as for method 1, except that 0.001 M phosphate buffer (pH 7.0), containing 0.001 M EDTA was used.

Partially purified virus preparations were layered onto 10–35% linear sucrose density gradients, and after centrifugation at 88,000 *g* for 4 hr, the gradients were fractionated with an ISCO Model 183 fractionator connected to an ultraviolet (UV) absorbance monitor.

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Virus-containing fractions were pooled, diluted twofold with 0.01 M phosphate buffer (pH 7.7), and concentrated by centrifugation at 250,000 *g* for 1 hr.

A preparation of TYDV, purified according to method 1, was subjected to analytical ultracentrifugation in a Beckman Model L2 65 ultracentrifuge, fitted with an An-D rotor and schlieren optics. The virus preparation was suspended in 0.75 ml 0.01 M phosphate buffer (pH 7.7) and had an UV absorbance of 0.52 units at 260 nm. Centrifugation was performed at 20 C and at a rotor speed of 14,350 rpm.

Gel electrophoresis of TYDV coat protein was performed in 7.5% continuous polyacrylamide-sodium dodecyl sulphate (SDS) gels (32) or discontinuous polyacrylamide-SDS gels composed of a 3% stacking gel and a 10% separating gel (24). TYDV protein was extracted by mixing equal volumes of purified virus suspension and dissociating mixture, and boiling for 1 min. The dissociating mixture contained 10 ml running buffer, 2 ml of 10% SDS and 50 μl mercaptoethanol, to which 4.65 g sucrose and 0.2 ml of 0.05% bromophenol blue were added. The following proteins were used as standards and were prepared in the same manner as the virus protein: bovine serum albumin (MW 68,000), catalase (MW 60,000), aldolase (MW 40,000), pepsin (MW 35,000), trypsin (MW 23,300), tobacco mosaic virus coat protein (MW 17,500), and myoglobin (MW 17,200). Twenty-five or 50 μl samples were layered onto the gels and electrophoresed at 5 mA/gel for ~4 hr. Protein bands were detected by staining with 0.1% Coomassie Brilliant Blue in a mixture of methanol, water, and acetic acid (5:5:1,v/v) for 1 hr at room temperature. The gels were then destained in a mixture of methanol, water, and acetic acid (5:100:7, v/v).

Virus preparations were negatively stained with 0.5% aqueous uranyl acetate on carbon-stabilized formvar or parlodion-coated grids and examined under a Philips EM201 or JEOL 100 C electron microscope.

An antiserum against TYDV was prepared in a rabbit, by giving the animal three intramuscular injections, each of 1 ml of purified virus (from a sucrose density gradient) emulsified with an equal volume of Freund's complete adjuvant. The first injection, containing 0.24 A_{260nm} units of TYDV, was followed by two more injections, 8 days (0.18 A_{260nm} units) and a further 12 days (0.17 A_{260nm} units) later. The rabbit was bled 14 days after the final injection, and thereafter at approximately 14-day intervals.

RESULTS

Sucrose density gradient centrifugation of extracts from TYDV-infected *D. stramonium* obtained by either purification method, produced a UV-absorbing zone that was not obtained from healthy control tissue (Fig. 1). When material in this zone was fractionated and concentrated by centrifugation, a quite pure preparation of paired (geminate) viruslike particles, measuring about 20 nm × 35 nm, was obtained (Fig. 2). When paired particles thus isolated were recentrifuged on a similar sucrose density gradient, a single UV-absorbing zone, containing geminate viruslike particles, was detected. This zone, to which paired particles were confined, also represented the zone of maximum infectivity (Fig. 1). The paired particles are believed to be virus particles, and to be the causal agents of TYD disease. In the concentration stages of the purification schedules, TYDV was precipitated by 10% PEG and 0.2 M NaCl, but not by 4% PEG and 1% NaCl, as judged by the presence or absence of virous zones in the sucrose density gradients.

Sucrose density gradient centrifugation of extracts from BSDV-1-infected *D. stramonium*, obtained by using method 2, also produced a discrete UV-absorbing zone, containing geminate virus particles indistinguishable from those of TYDV.

No suitable schedule for the purification of BSDV-2 was found. However, very small numbers of paired viruslike particles were obtained when frozen leaves of BSDV-2-infected *D. stramonium* were treated as follows. The tissue was extracted twice in 0.1 M Na_2HPO_4 containing 0.9% thioglycollic acid (pH 4.5) and the homogenate was clarified with one-quarter volume of chloroform and low speed centrifugation. The extract was then concentrated by PEG precipitation and subjected to ultracentrifugation and sucrose density gradient centrifugation. No discrete UV-absorbing zone of virus particles was present in the gradient. Geminate virus particles were detected by electron microscopy, however, when fractions of the gradient were sampled, assuming that BSDV-2 had a similar sedimentation rate to BSDV-1.

Purified TYDV, after two cycles of sucrose density gradient centrifugation, had a UV absorbance typical of a nucleoprotein. The UV scan exhibited an absorbance maximum at 261 nm and a minimum at 245 nm. The $A_{260/280}$ ratio was 1.25.

A gradual decrease in virus yield from expanding TYD-infected *D. stramonium* leaves, with increasing age of the plant, occurred. Plants infected for 6 mo yielded about 20% less virus, on the basis of

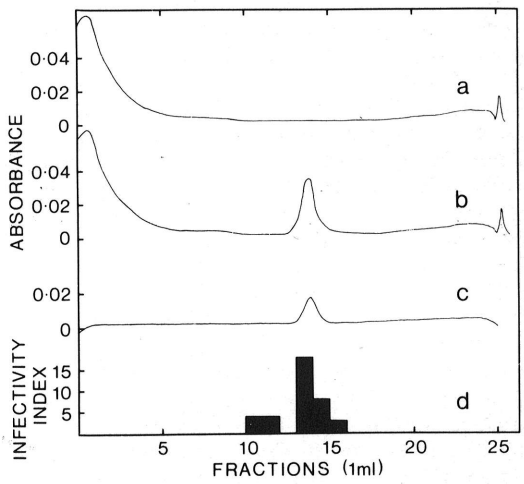

Fig. 1. A diagram illustrating the correlation of infectivity with the presence of paired viruslike particles in sucrose density gradients. Ultraviolet absorption profiles of 10–35% linear sucrose density gradients after centrifugation of: curve a, of an extract from healthy *Datura stramonium*; curve b, of an extract from tobacco yellow dwarf virus-infected *D. stramonium*; curve c, of material from virus-specific zone in the gradient represented by curve b, and bar graph d, of infectivity detected in fractions of the gradient represented in curve c.

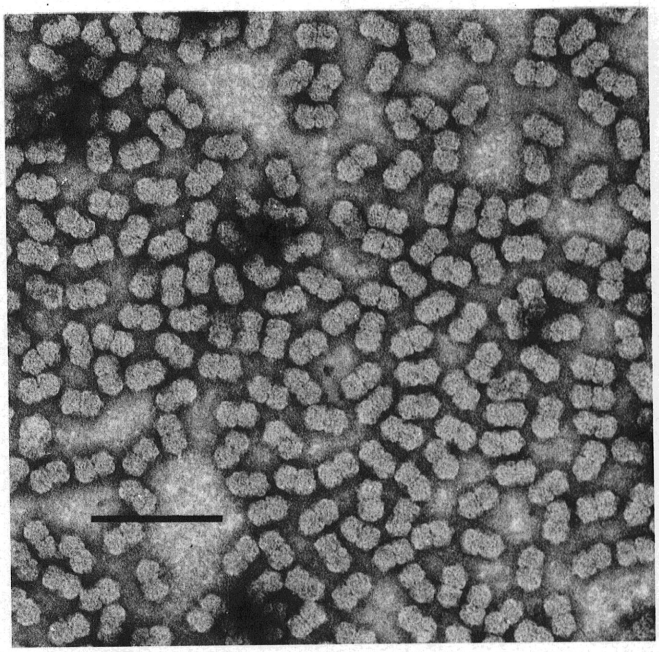

Fig. 2. An electron micrograph of a purified preparation of tobacco yellow dwarf virus, negatively stained with 0.5% uranyl acetate. Bar represents 100 nm.

A_{260nm} data, than did plants infected for 1–3 mo. Tissue frozen for 3 mo prior to extraction yielded about 15% less TYDV than did tissue frozen for only a few days. No purified TYDV could be extracted from *D. stramonium* plants infected for more than 10 mo. Similar yields of TYDV were obtained by using either of the purification methods described.

TYDV appears to be morphologically similar to the geminivirus bean golden mosaic virus (BGMV) (14), and both viruses contain a single structural protein species (BGMV, MW 31,000; TYDV, MW 27,500, reported later in this work). If TYDV is assumed to have an extinction coefficient similar to that of BGMV ($E^{0.1\%}_{1\,cm}$ 260 nm, = 7.7) (14), then yields of TYDV in the present work would be of the order of 100–250 μg/kg of tissue. The yields of BSDV-1 were only about 20% that of TYDV (ie, about 20–50 μg/kg of tissue).

TYDV that had been purified by using method 1, sedimented as a single boundary and had an estimated sedimentation coefficient of 76S at 20 C in 0.01 M phosphate buffer (pH 7.7). Although BSDV-1 and BSDV-2 were not sedimented in an analytical ultracentrifuge, their sedimentation rates were very similar to that of TYDV during rate zonal sucrose density gradient centrifugation.

TYDV protein for electrophoresis was extracted from virus preparations purified by using either of the methods described. A major component with an estimated molecular weight of 27,500 ± 1,000 (mean of ten determinations using three virus preparations) was detected. A very minor component with an estimated molecular weight greater than 100,000 was detected in every TYDV protein preparation. The significance of this component is not known.

In gel diffusion tests, purified TYDV reacted with its homologous antiserum to produce a single, sharp precipitation line (Fig. 3), and in such experiments a titer of 1/128 was obtained. Purified BSDV-1 also reacted strongly with TYDV antiserum (As), the heterologous titer being at least 1/8. However, insufficient purified BSDV-1 was available for antiserum production or to determine the heterologous titer of TYDV As against BSDV-1. Highly purified, concentrated preparations were needed to obtain strong serological reactions, and sap from diseased plants, or partially purified virus preparations failed to give positive reactions. TYDV As did not react with the sap from healthy *D. stramonium* plants.

TYDV reacted with an antiserum against CTV to produce a single, somewhat diffuse, precipitation line in gel diffusion tests (Fig. 3). This antiserum had a heterologous titer of 1/16 against TYDV. When the same virus preparations were reacted simultaneously with TYDV As, an homologous titer of 1/128 was obtained. No reaction was obtained when the same batch of TYDV As was reacted with purified CTV (D. Mumford, *personal communication*). In this reciprocal test the homologous titer of CTV As was 1/256.

No reaction was obtained between TYDV and antisera to the geminiviruses MSV and Chloris striate mosaic virus.

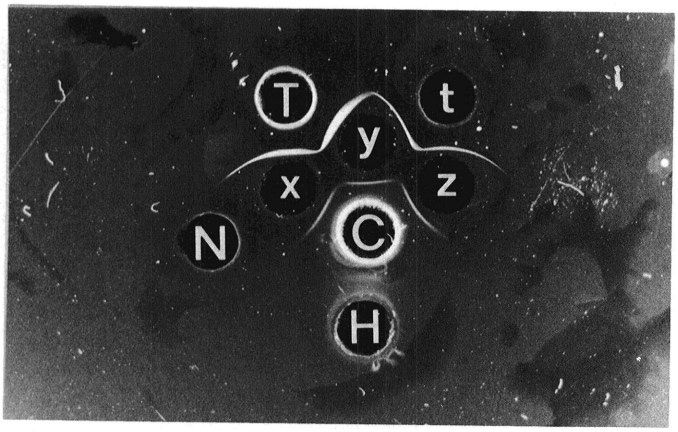

Fig. 3. Serological reactions of tobacco yellow dwarf virus (TYDV) in an agar gel double diffusion test. Undiluted antiserum against TYDV in well T and a fourfold dilution in well t; undiluted antiserum against beet curly top virus in well C, normal serum in well N, healthy *Datura stramonium* L. sap in well H, and undiluted, twofold and fourfold diluted TYDV in wells x, y, and z, respectively.

DISCUSSION

Infectivity has been correlated with the presence of geminate viruslike particles in purified preparations obtained from plants infected with either the bean summer death or tobacco yellow dwarf pathogens. The particles are thus considered to be viral and to be the causal agents of the diseases.

Because only minute amounts of purified BSDV could be obtained, most investigations were conducted with TYDV. TYDV is a very stable virus. A purified preparation was still infectious after storage at 4 C for 26 days, during which time the preparation dried, and infectivity was detected in French bean tissue which had been frozen for 11 mo. Certain steps in the purification schedules were performed at room temperature, with no apparent adverse effect on virus stability. The virus retained infectivity in solutions throughout a wide range of pH values and containing any of several reducing or chelating agents.

During purification procedures, the advantages of virus stability are offset by the apparently low virus concentration in diseased plants. The sap from diseased plants does not react with TYDV As and virus particles have not been observed in negatively stained leaf dip preparations viewed under the electron microscope. Yields of TYDV were about 100–250 μg/kg of tissue and of BSDV-1 20–50 μg/kg of tissue, and these compare with the low yields reported for members of the luteovirus group. Purified preparations of barley yellow dwarf virus usually contain less than 100 μg of virus/L of sap (28) and yields of soybean dwarf virus are usually 100–400 μg/kg of tissue (29). The yield of TYDV obtained using either purification method was similar, and if need be, either schedule could be completed in one day.

The possibility that TYDV and BSDV are related was first proposed by Bowyer (9). The viruses are transmitted by the same leafhopper vector and produce similar symptoms in several host plants. In addition, the present work shows that the viruses are serologically related and have morphologically indistinguishable geminate particles. However, the isolates display some differences in the severity of symptoms produced in certain hosts (30) and in the yields of purified virus obtained. These similar, but distinct, isolates are thus likely to be strains of one virus.

An extensive correlation between the resistances of French bean cultivars to BSDV and CTV, and the similarity of symptoms produced in French bean by these viruses, has been noted (1–4). It is now known that both diseases are caused by morphologically similar geminate viruses (26, and this work). Although the host range of BSDV has not been examined in detail, the related TYDV does have an extensive host range, 32 plant species in seven families having been recorded as hosts (17,20,30). CTV also has an extensive host range, including more than 300 species in 44 plant families (5). TYDV, BSDV, and CTV all have deltocephalid leafhopper vectors, in which they have a persistent, probably circulative, relationship (5,10,19, and J. E. Thomas *unpublished*). In addition, the present work shows that TYDV and CTV have a distant serological relationship. The failure of CTV to react in the reciprocal tests with TYDV As may reflect the low titer of the TYDV As that was used.

On the basis of the properties reported here, TYDV and BSDV appear to be members of a newly recognized group of plant viruses, the geminiviruses (16). These viruses have isometric particles, usually 17–20 nm in diameter, which occur predominantly in pairs (8,13,14,16,25–27), and have a persistent relationship with their leafhopper (5,7,10,15) or whitefly (6,11) vector. Several members have been shown to have a single-stranded DNA genome (14,16), and to accumulate in the nuclei of infected plant cells (8,12,13,23,27).

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