Purification and Serology of a Bacilliform Virus Associated with Banana Streak Disease

B. E. L. Lockhart

Department of Plant Pathology, University of Minnesota, St. Paul 55108/Département de Phytopathologie, Institut Agronomique et Vétérinaire Hassan II, Complexe Horticole, B.P. 438, Agadir, Morocco.

I wish to thank the Virology Section, Scottish Crop Research Institute, Invergowrie, Dundee, for use of electron microscope facilities, and Ian Roberts and Angela Lennon of SCRI for doing virus particle measurements and for helpful discussions. I also thank Mohamed Hallatou of the Tissue Culture Laboratory, Complexe Horticole, Agadir, for supplying virus-free banana explants.

This research was supported by funds from USAID/University of Minnesota Contract No. 608-0160-001.

Accepted for publication 1 May 1986 (submitted for electronic processing).

ABSTRACT

Lockhart, B. E. L. 1986. Purification and serology of a bacilliform virus associated with banana streak disease. Phytopathology 76:995-999.

Bacilliform particles measuring 119 × 27 nm were consistently associated with a chlorotic and necrotic leaf streak disease of banana (*Musa* sp.) in Morocco. The virus was detected by electron microscopy, immunosorbent electron microscopy (ISEM), and enzyme immune assay (EIA) in streak-diseased banana plants but not in symptomless plants or in plants infected solely with either cucumber mosaic virus (CMV) or banana bunchy top virus (BBTV). The disease was propagated vegetatively but was not transmitted through soil. Neither the disease nor the particles were transmitted mechanically to healthy banana or other test plants. The banana bacilliform virus was purified and an antiserum prepared. The disease, which occurs frequently in bananas in southern Morocco, was named banana streak disease, and the associated bacilliform virus is referred to as banana streak virus (BSV). In ISEM tests, BSV was trapped by its homologous antiserum but not by antisera to either cacao swollen shoot or rice tungro bacilliform viruses.

Bananas (*Musa* sp. [AAA group] 'Dwarf Cavendish' and 'Giant Cavendish') are produced commercially in the coastal area of southern Morocco. Since 1974, when virus disease surveys were first conducted in this banana-producing area, symptoms of a viruslike disease characterized by conspicuous leaf streaks that were initially chlorotic and became progressively necrotic (Fig. 1) were found to occur frequently in the field. In some plantings, more than 50% of the plants showed such symptoms. Attempts to identify or mechanically transmit a causal agent were unsuccessful. In the past 5 yr, banana cultivation in Morocco has expanded greatly, particularly with the development of banana production in large plastic greenhouses. A renewed attempt was therefore made to elucidate the etiology of the banana streak disease.

MATERIALS AND METHODS

Virus source. All studies were done with naturally infected field plants of Dwarf Cavendish and Giant Cavendish banana. Selected source plants showed conspicuous typical banana streak symptoms (Fig. 1) and were verified to be free of cucumber mosaic virus (CMV) infection by symptomatology (Figs. 2 and 3) and indexing by sap inoculation of test plants of Fordhook Zucchini squash (*Curcurbita pepo* L.), which was readily infected when inoculated with extracts of CMV-infected banana.

Mechanical transmission. Inoculum was prepared by grinding young banana leaf tissue showing prominent streak symptoms in cold 1% K_2HPO_4 containing 0.2% 2-mercaptoethanol. Carborundum was added to the extract, and the slurry was used to inoculate young healthy test plants of banana; *Zea mays* L. 'Golden Cross Bantam,' 'Earliking,' and 'Jubilee'; *Hordeum vulgare* L. 'Capri'; *Avena sativa* L. 'Clintland 64'; *Nicotiana benthamiana* Domin.; *N. edwardsonii* (Christie & Hall); *N. glutinosa* L.; and *N. tabacum* L. 'Xanthi.' Similar inoculations were done using partially purified extracts of banana leaf tissue infected with banana streak virus (BSV). They were used to inoculate test plants without being centrifuged on CsCl-sucrose gradients.

Soil transmission. Healthy young Giant Cavendish explants, produced in tissue culture, were transplanted into soil collected from the root zones of streak-infected banana plants showing prominent symptoms. Soil samples were taken in a heavily streak-infected plantation in which there was evidence of spread of the disease to previously healthy plants.

Virus purification. Virus was purified from banana leaf tissue collected in the field. An initial antiserum was prepared using partially purified preparations and was used in enzyme immune assays (EIAs) to monitor tissue sources and extraction and clarification procedures in subsequent virus purification experiments. Purification procedures were also monitored by electron microscopic examination of extracts at each stage of the process. The procedure finally adpoted was to mince fresh, infected banana leaf tissue and then extract the tissue 1:2 (w/v) in a blender in cold 0.05 M Tris-citrate, pH 7.4, containing 0.5% (w/v) Na_2SO_3, 1% (w/v) polyvinylpyrrolidone (PVP, mol wt 40,000), and 1% (v/v) Triton X-100. The homogenate was squeezed through muslin and clarified by blending for 20 sec with 25% (v/v) chloroform followed by low-speed centrifugation (10,000 *g* for 10 min). The aqueous supernatant phase was collected and the virus concentrated by ultracentrifugation at 136,000 *g* for 1 hr. The high-speed pellets were resuspended in 0.01 M phosphate buffer, pH 7.2, and the virus was further purified by centrifugation for 4.5 hr at 116,000 *g* in a preformed 0–30% CsCl gradient in 10% (w/w) sucrose (4). The virus-containing, light-scattering band was removed with a syringe and dialyzed against 0.01 M phosphate, pH 7.2, to remove the cesium salt. The identity of the particles and the purity of the final preparation were verified by serology and electron microscopy.

Serology. Antisera against BSV were prepared in rabbits by intramuscular and subcutaneous injections of partially purified or purified virus suspensions emulsified with Freund's adjuvant. In both cases, initial multiple-site intramuscular and subcutaneous injections of virus emulsified with complete adjuvant were administered, followed 4 wk later by similar multiple-site injections of virus emulsified with incomplete adjuvant. Blood was collected starting 2 wk after the second series of injections. Immunodiffusion tests were done in 0.85% agarose, 0.1% NaN_3, in distilled water. Antiserum was diluted in 5% bovine serum in 0.05 M Tris-Cl, pH 7.2, 0.85% NaCl (13), for titering. EIAs were done

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

by the double-antibody sandwich method using alkaline phosphatase conjugate (3). Polystyrene plates were coated with 1 μg/ml purified BSV IgG. Leaf samples were extracted and diluted in phosphate-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 20 and 2% PVP (PBST-PVP). Enzyme conjugate was used at a 1/500 dilution. Incubations were done either at 37 C for 4 hr or at 4 C for 16 hr. Absorbances at 405 nm were determined spectrophotometrically with a Dynatech Minireader.

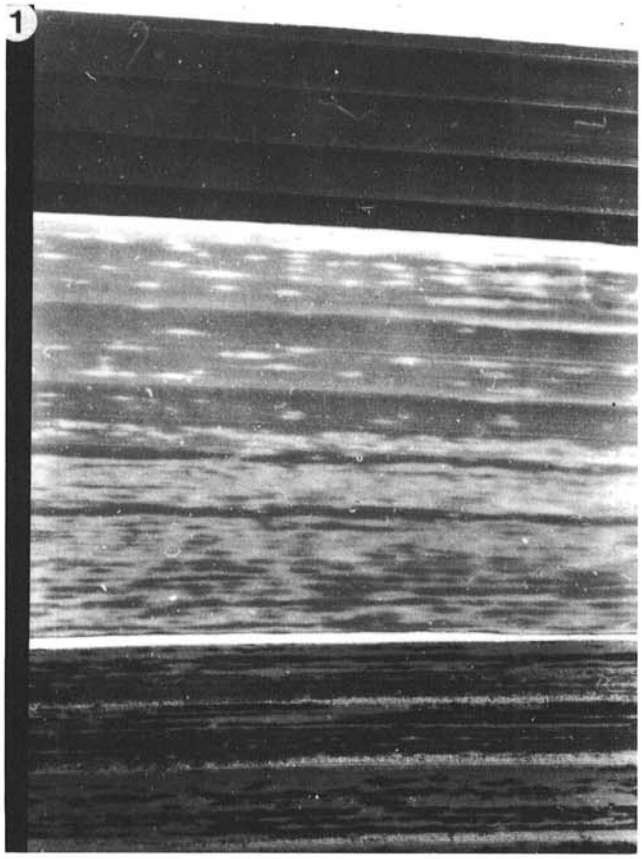
Electron microscopy. Leaf-dip, partially purified, and purified preparations were either negatively stained with 2% sodium phosphotungstate, pH 7.0 (PTA), or positively stained with saturated uranyl formate (UF). Virus particle measurements were made from electron micrographs of purified virus stained with UF. The lattice spacing of crystalline catalase (18) was used as an internal calibration standard. Particle dimensions were measured from projected electron micrograph negatives with an ID-TT-20 Digitizer (Summagraphics Corp., Fairfield, CT), and the measurements were processed by a 4051 Graphics System (Tektronix Inc., Beaverton, OR). For immunosorbent electron microscopy (ISEM) tests, carbon-coated grids were floated on drops of antiserum diluted 1:1,000 in 0.05 M phosphate buffer, pH 7.0, and incubated at 37 C for 15 min. After rinsing in the same phosphate buffer, the antibody-coated grids were floated on drops of partially purified BSV suspensions or BSV-infected crude leaf extracts and incubated at 4 C, then examined after incubation times of 1, 4, and 20 hr, respectively, after rinsing with phosphate buffer and staining with UF. Antisera to cacao swollen shoot virus (CCSV) (1) and rice tungro bacilliform virus (RTBV) (12) were supplied by I. M. Roberts.

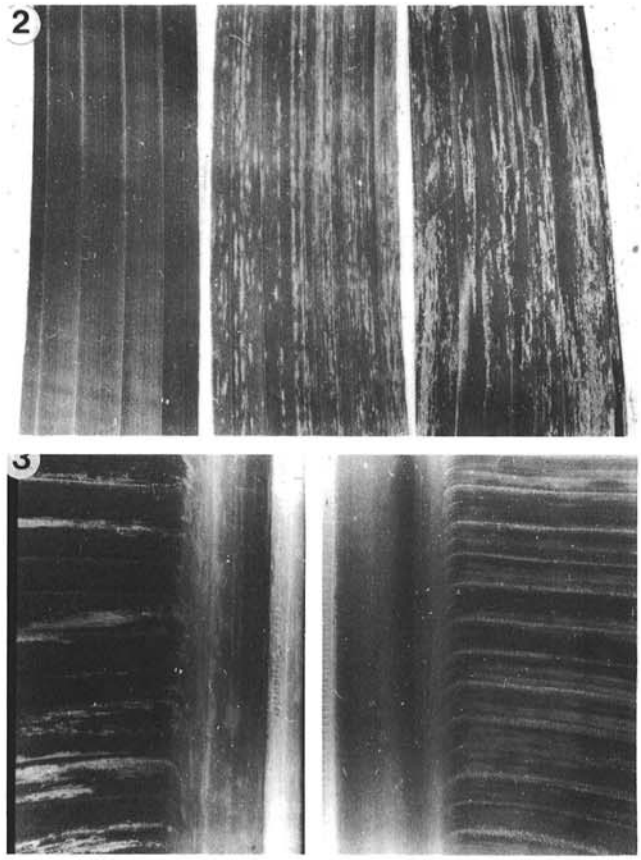
RESULTS

Disease occurrence and symptomatology. Banana streak disease was found in almost every established planting of field-grown Dwarf Cavendish bananas in southern Morocco. In some cases, infection exceeded 50%. Incidence of the disease was much lower in recently established open-field plantings derived from imported Giant Cavendish planting stock and was rarely seen in plastic greenhouse bananas derived from imported Giant Cavendish stock.

Symptoms of banana streak consist of broken or continuous chlorotic streaks and spindle-shaped lesions that may be sparse or concentrated in distribution (Fig. 1, center). These chlorotic lesions become progressively necrotic and blackened and produce a characteristic black-streaked appearance in older leaves (Fig. 1, bottom). A conspicuous feature of the disease is the erratic distribution of symptoms, over individual leaves as well as on different leaves of the same plant. In general, leaves initiated or produced during periods of high temperature developed more pronounced symptoms. The symptoms of banana streak superficially resemble those caused by CMV infection (Fig. 2), but with practice, the two diseases can be readily distinguished. One characteristic feature of CMV infection was a midrib mosaic (Fig. 3) that was absent in leaves from plants infected with banana streak only. The isolate of CMV occurring in banana in Morocco was readily sap-transmitted from banana to herbaceous test plants and occurred in banana plantations in weed hosts such as *Bryonia dioica* Jacq. and *Malva* spp. CMV infection occurred in far lower frequency than did banana streak and did not cause the heart rot syndrome described for CMV infection elsewhere (16).

Mechanical and soil transmission. Neither streak disease nor BSV was transmitted mechanically from infected banana to healthy banana or to any of the other test plants, using either crude sap or partially purified extracts. Banana test plants were observed for up to 11 mo after inoculation. Failure to transmit BSV to any of the test plants was verified by negative results in ISEM and EIA using BSV antiserum. Soil transmission tests were also negative. Thirteen months after transplanting into soil taken from a streak-





Figs. 1–3. Leaf symptoms of banana streak disease and cucumber mosaic virus (CMV) infection in Dwarf Cavendish banana. **1,** Top: healthy banana leaf; middle: chlorotic streak symptoms of banana streak disease; and bottom: necrotic streak symptoms of banana streak disease. **2,** Comparison of foliar symptoms of (center) banana streak infection and (right) CMV infection; (left) healthy leaf. **3,** Comparison of symptoms caused by (left) CMV infection and (right) banana streak infection. Note midrib mosaic in CMV-infected leaf.

infected plantation, banana test plants showed no symptoms.

Virus purification. EIA established that BSV concentration in infected leaf tissue was related to intensity of symptoms and not to leaf age and that older leaves, with blackened, necrotic streaking, contained only slightly less virus than young leaves showing a similar degree of streaking. The choice of leaf tissue for virus purification was therefore based on symptom intensity, although younger leaf tissue was easier to manipulate. Virus yield was 10% higher from fresh than from frozen leaf tissue. EIA also showed that more virus was extracted in the pH range of 6.8–7.5 than at lower pH and that Tris-Cl or Tris-citrate extracts contained more virus than did those made in phosphate or citrate buffers. Markedly increased quantities of virus were extracted in the presence of PVP, as reported for citrus tristeza virus (10), and of Triton X-100.

Clarification using 8% (v/v) *n*-butanol, or acidification (11), resulted in loss of most of the virus, whereas concentrations of chloroform between 8 and 100% (v/v) caused relatively little loss of virus and eliminated tubular cellular material (Fig. 4) that was a major contaminant of virus preparations. The use of cellulolytic enzymes such as Driselase (15) and Celluclast 1.5 L (17) did not increase virus yield, although they eliminated pectic materials and other contaminating cellular components. Although the relative low cost of Celluclast (17) presents advantages in virus purification, the low pH optimum of the enzyme was considered a disadvantage to its use for purification of BSV.

Centrifugation of partially purified BSV preparations on CsCl-sucrose density gradients eliminated contaminating cellular material. The virus fraction collected after a single cycle of CsCl-sucrose density gradient centrifugation was often slightly contaminated with host plant material (Fig. 5). These contaminants were eliminated by a second cycle of CsCl-sucrose density gradient centrifugation (Fig. 6). Purified BSV preparations had a characteristic nucleoprotein UV absorption spectrum, although this was somewhat flattened, with no pronounced peak at 254 nm. The $A_{260/280nm}$ of the purified preparation was 1.26 (uncorrected for light scattering).

Serology. BSV antiserum did not react with crude sap from infected plants in immunodiffusion tests. However, virus concentrated by a single cycle of ultracentrifugation produced a single precipitin line (Fig. 7). Using such partially purified virus extracts as antigen and similarly prepared healthy leaf extracts as controls, BSV antiserum prepared using purified virus had a homologous titer of 1/512 in immunodiffusion tests.

In EIA, BSV was consistently detected in plants showing streak symptoms only as well as in plants doubly infected with streak and CMV or banana bunchy top virus (BBTV). In a typical experiment, spectrophotometric readings, with healthy controls taken as 0.00, were 0.74 for BSV-infected tissue, 0.01 for CMV-infected tissue, 0.08 for BBTV-infected tissue, and 0.82 for a mixed BSV-BBTV infection. Coating polystyrene microtiter plates with BSV IgG for 4 hr at 37 C and for 16 hr at 4 C gave no differences in final spectrophotometric readings, but incubation of test antigen for 16 hr at 4 C produced significantly higher final absorbances than did the shorter incubation time at higher temperature. Leaf tissue samples were extracted 1:2 (w/v) in PBST-PVP for routine EIAs. Although BSV could be reliably detected in crude leaf extracts at a 1:10 dilution, higher dilutions gave less clear results. Brief centrifugation of tissue extracts (10,000 *g* for 10 min) before incubation in coated plates significantly reduced the healthy nonspecific background absorbance.

Electron microscopy. The bacilliform particles of BSV contrasted poorly and were not readily observed in leaf-dip preparations. The particles appeared to be stable in PTA and UF as well as in 2% ammonium molybdate (AM), pH 6.8. Particles were rare in leaf-dip preparations but could be more readily seen after trapping on BSV antiserum-coated grids (Fig. 4). An initial BSV antiserum, prepared using partially purified virus, trapped 10 times as many BSV particles from a partially purified preparation as did uncoated grids. Expressed on a standard area basis (1,000 μm^2) (14), the number of particles trapped by BSV antiserum-coated grids was 1,282 compared with 131 for uncoated grids over the same incubation period (20 hr at 4 C). Particles of BSV were

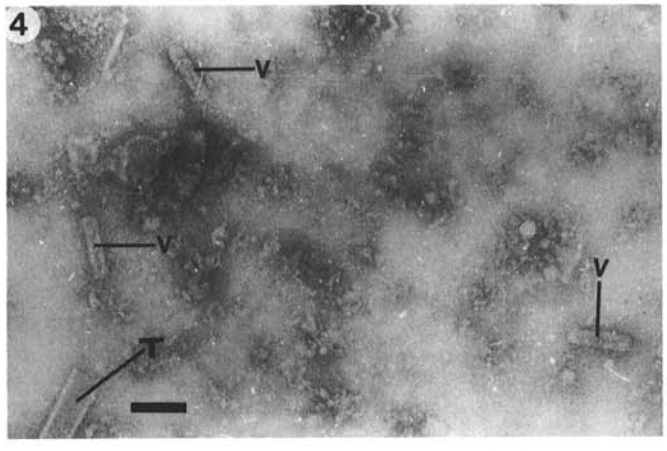
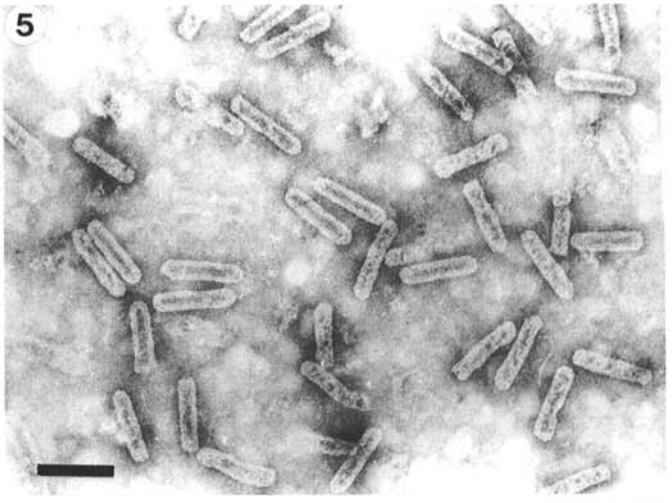
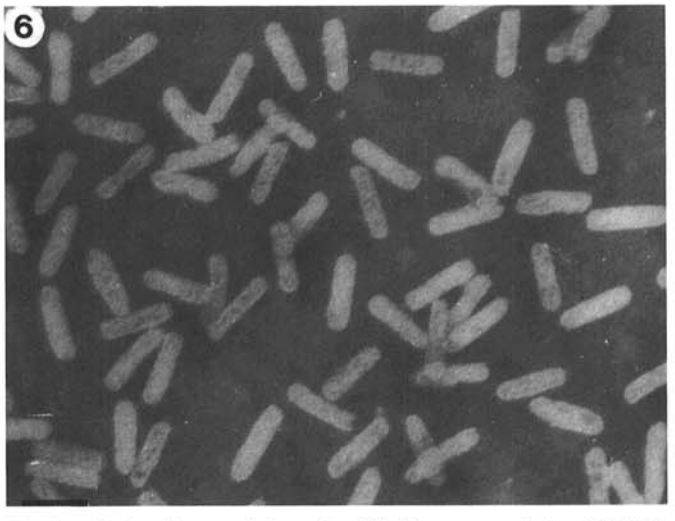


Fig. 4. Reaction of banana streak virus (BSV) with its homologous antiserum in immunosorbent electron microscopy (ISEM). BSV particles were trapped by antiserum-coated carbon grids from a crude leaf extract. V = virus particles. T = tubular material occurring in both healthy and infected banana. Scale bar = 100 nm.





Figs. 5 and 6. Particle morphology of purified banana streak virus (BSV). **5,** Purified BSV after a single cycle of CsCl-sucrose density gradient centrifugation (stained with uranyl formate) **6,** Purified BSV after a second cycle of CsCl-sucrose density gradient centrifugation. Stained with 2% sodium phosphotungstate, pH 7.0.

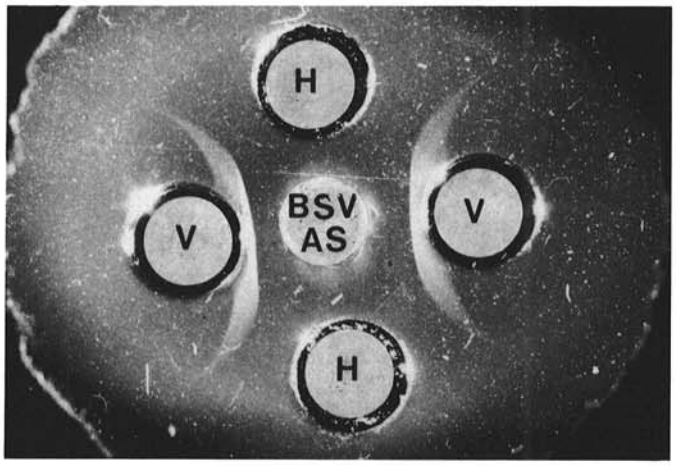


Fig. 7. Reaction of banana streak virus (BSV) with its homologous antiserum in immunodiffusion. Reaction is between undiluted BSV antiserum in center well and a partially purified BSV preparation (V). H = healthy banana leaf extract after identical partial purification.

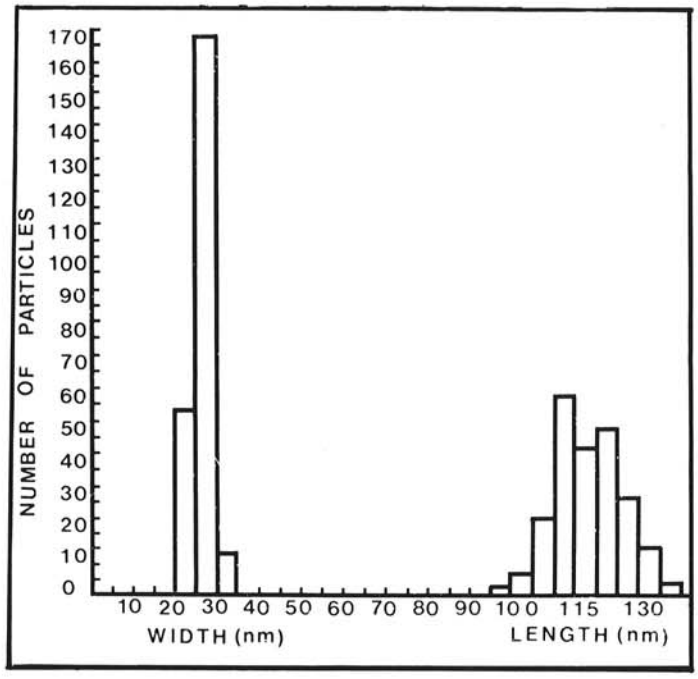


Fig. 8. Particle dimensions of purified banana streak virus (BSV). Composite graphical data for particle width and length measurements of purified BSV. Stained with uranyl formate.

not trapped by grids coated with antisera to either CCSV or RTBV. Virus particles in purified preparations contrasted well in UF (Fig. 5) and PTA (Fig. 6). Measurement of 501 particles from electron micrographs of a purified BSV preparation stained with UF gave mean particle dimensions of $119 \pm 8 \times 27 \pm 3$ nm (Fig. 8).

DISCUSSION

The bacilliform virus described and named banana streak virus (BSV) was shown to be consistently associated with the chlorotic and necrotic leaf streak disease of banana. In EIAs conducted over a 10-mo period, all of more than 50 streak-infected banana plants reacted positively with BSV IgG. This included plants that also showed symptoms of mixed BSV-CMV or BSV-BBTV infection. No positive results were ever obtained in EIAs using test samples lacking streak symptoms. The disease could clearly be distinguished from CMV and BBTV infection symptomatologically and serologically by antiserum to BSV. Despite the constant association of BSV with banana streak disease, however, the etiological role of the bacilliform particles will be unequivocally established only when a method is found to transmit the virus to healthy bananas and reproduce the symptoms. No other viruslike particles were observed in either crude or purified preparations from streak-infected bananas, and current evidence at least suggests that BSV is the agent responsible for the disease. Failure to transmit BSV by mechanical inoculation using crude leaf extracts may be due to either low in situ virus concentration or to virus localization with infected tissues. CCSV, which is mealybug-transmitted, is also mechanically transmitted (2), whereas RTBV, which is leafhopper-transmitted (12), is phloem-limited and not transmitted mechanically. Failure of cellulolytic enzyme treatment to improve virus yield in purification of BSV suggests that BSV may not be phloem-limited, and tissue extract concentrations and A_{405nm} readings in EIA indicate that BSV occurs in low concentration in infected banana leaf tissue.

In the transmission tests reported, banana streak disease was not soilborne. All suckers taken from infected mother plants developed the disease, and it appears that vegetative propagation is probably the principal means of spread. A similar conclusion was reached for the apparently identical disease in the Ivory Coast (8,9). Recent observations have indicated that in two separate plantings, the disease has spread to previously healthy plants. The newly infected plants developed typical symptoms, and BSV was readily detected in those plants by EIA. This lends further support for the suggestion that BSV is the causal agent of the disease and suggests that the virus may have an aerial vector.

On the basis of symptoms, Moroccan banana streak disease appears to be similar or identical to the dotted-line mosaic (*mosaique en tirets*) of bananas reported from the Ivory Coast (8,9). The latter disease was reported to be widespread, to cause yield losses of 4.5–30 t/ha, and to also adversely affect plant growth and fruit quality. No viruslike particles were associated with that disease, which was also not transmitted mechanically (9,19). Symptoms similar to those occurring on bananas in Morocco were observed on bananas in the Canary Islands and in Jordan. The presence of BSV in these countries was not confirmed. If the symptoms observed in Morocco, the Ivory Coast, and elsewhere are due to the same disease and the same causal agent, banana streak may prove to be a virus disease of significant economic importance to banana cultivation in some areas.

BSV appears to belong to a group of viruses that include cacao swollen shoot (1), *Colocasia bacilliform* (6), rice tungro bacilliform (12), rubus yellow net (7), and yam internal brown spot bacilliform (5) viruses, among others. Apart from similarity in particle morphology and dimensions, however, no other criteria are currently available for establishing relationships among these viruses. Further characterization of BSV and other viruses of this type is very likely to provide a basis for their classification within a single group.

LITERATURE CITED

1. Adomako, D., Lesemann, D.-E., and Paul, H. L. 1983. Improved methods for the purification and detection of cacao swollen shoot virus. Ann. Appl. Biol. 103:109-116.
2. Brunt, A. A. 1970. Cacao swollen virus. No. 10. Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England. 4 pp.
3. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
4. Gumpf, D. J., Bar-Joseph, M., and Dodds, J. A. 1981. Purification of citrus tristeza virus (CTV) on sucrose-cesium sulfate cushion gradients and estimation of its RNA size. (Abstr.) Phytopathology 71:878.
5. Harrison, B. D., and Roberts, I. M. 1973. Association of virus-like particles with internal brown spot of yam. Trop. Agric. 50:335-340.
6. James, M., Kenten, R. H., and Woods, R. D. 1973. Virus-like particles associated with two diseases of *Colocasia esculenta* (L.) Schott in the

Solomon Islands. J. Gen. Virol. 21:145-153.
7. Jones, A. T., and Roberts, I. M. 1976. Ultrastructural changes and small bacilliform particles associated with infection by rubus yellow net virus. Ann. Appl. Biol. 84:305-310.
8. Lassoudière, A. 1974. La mosaique dite "à tirets" du bananier 'Poyo' en Côte d'Ivoire. Fruits 29:349-357.
9. Lassoudière, A. 1979. Mise en évidence des répercussions économiques de la mosaique en tirets du bananier en Côte d'Ivoire. Possibilitiés de lutte par eradication. Fruits 34:3-34.
10. Lee, R. F., Garnsey, S. M., and Brlansky, R. H. 1981. An improved purification procedure for citrus tristeza virus. (Abstr.) Phytopathology 71:235.
11. Lockhart, B. E. L., Khaless, N., El Maataoui, M., and Lastra, R. 1985. Cynodon chlorotic streak virus, a previously undescribed plant rhabdovirus occurring in the Mediterranean area. Phytopathology 75:1094-1098.
12. Omura, T., Saito, Y., Usugi, T., and Hibino, H. 1983. Purification and serology of rice tungro spherical and rice tungro bacilliform viruses. Ann. Phytopathol. Soc. Jpn. 49:73-76.
13. Purcifull, D. E., and Batchelor, B. L. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant viral inclusions. Fla. Agric. Exp. Stn. Bull. 788 (Tech). 39 pp.
14. Roberts, I. M., Tamada, T., and Harrison, B. D. 1980. Relationship of potato leafroll virus to luteoviruses: Evidence from electron microscope serological tests. J. Gen. Virol. 47:209-213.
15. Singh, S. K., Anjaneyulu, A., and Lapierre, H. 1984. Use of pectinocellulolytic enzymes for improving extraction of phloem-limited plant viruses as exemplified by the rice tungro virus complex. Agronomie 4:479-484.
16. Stover, R. H. 1973. Banana, Plantain and Abaca Diseases. Commonwealth Mycological Institute, Kew, Surrey, England. 497 pp.
17. Waterhouse, P. M., and Helms, K. 1984. Purification of particles of subterranean clover red leaf virus using an industrial-grade cellulase. J. Virol. Methods 8:321-329.
18. Wrigley, N. G. 1968. The lattice spacing of crystalline catalase as an internal standard of length in electron microscopy. J. Ultrastruct. Res. 24:454-464.
19. Yot-Dauthy, D., et Bové, J. M. 1966. Mosaique du bananier. Identification et purification de diverses souches du virus. Fruits 21:449-465.