Purification of banana bunchy top virus (BBTV).

M.L. ISKRA, M. GARNIER and J.M. BOVE

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ABSTRACT - Banana bunchy top disease (BBTD) is one of the most severe banana and plantain diseases. A virus, probably a member of the luteovirus group, is associated with it. The virus multiplies in the phloem tissue and is transmitted by the aphid *Pentalonia nigronervosa*. In this paper we report the development of a purification method which results in the isolation of isometric virus particles of about 28 nm in diameter from bunchy top affected banana plants.

PURIFICATION DU VIRUS DU BUNCHY TOP DES BANANIERS (BBTV).
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RESUME - La maladie du bunchy top des bananiers (BBTD) est l'une des plus sévères maladies atteignant les bananiers et plantains. Un virus, probablement un membre du groupe des lutéovirus, est associé à cette maladie. Le virus se multiplie dans les tissus du phloème, il est transmis par le puceron *Pentalonia nigronervosa*. Dans cet article, nous décrivons une méthode de purification qui a permis, pour la première fois, d'isoler des particules virales isométriques d'environ 28 nm à partir de plants de bananiers affectés par la maladie du bunchy top.

INTRODUCTION

Banana bunchy top disease (BBTD) was first reported in Fidji Island as early as 1889 (Magee, 1953). Since then the disease has been described in Australia, Asia, India, Pacific Islands and a few countries in Africa : Egypt, Gabon (E. Fouré, 1982), Congo ; for a review see Dale, 1987. BBTD is a severe disease of banana and plantain. A virus is thought to be associated with it. The banana aphid *Pentalonia nigronervosa* Coquerel transmits the disease in the field, in a persistent manner (Magee, 1927). The disease is also spread by the suckers from infected banana stools. The virus cannot be transmitted mechanically. It only multiplies in phloem tissues where it induces a disorganization (Magee, 1939). Diseased banana plants have yellow leaves bunching together at the apex of the plant with dark green streaks on the leaf veins, on the midribs and also on the petioles. These streaks are often referred as «morse code» pattern. Because of these characteristics BBTV is thought to be a member of the luteovirus group (Shepherd *et al.*, 1981 ; Matthews, 1982), although the virus has never been purified. In this paper we describe a purification procedure which allows repetitive extraction of virus particles from infected banana plants.

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MATERIALS AND METHODS

Plant material.

The affected banana plants used as initial sources of infected tissue for virus purification were collected in Gabon (Africa). They were multiplied vegetatively by *in vitro* culture (Escalant, 1987). The healthy banana plants were produced in the same way and both healthy and infected plants were maintained at 32°C in the greenhouse.

Virus purification.

Fresh leaves of infected banana plants (100 g) were homogenized in a waring blendor in the presence of 200 ml of pH 6.0 extraction buffer (0.1 M sodium citrate, 0.01 M. EDTA, 1% (w/v) thioglycerol, 0.1% (w/v) polyvinyl pyrrolidone K25 (PVP). Then 100 ml of extraction buffer containing driselase (Sigma) and sodium azide, filter sterilized, were added to the homogenate so that the final concentrations of driselase and sodium azide were respectively 1% and 0.02%. The homogenate was shaken during 16 hrs at 28°C (80 strokes/min). It was then passed through two layers of cheese cloth and centrifuged at 8,000 g for 20 min. The pellet was discarded and the supernatant was centrifuged at 140,000 g for 3 h through a 10% (w/v) sucrose layer which occupied about one quarter of the tube. The pellet from the high speed centrifugation was resuspended in 0.006 M phosphate buffer pH 7.0. The low and high speed centrifugation were repeated 3 times at a temperature of 7°C to 10°C. The final pellet was resuspended in

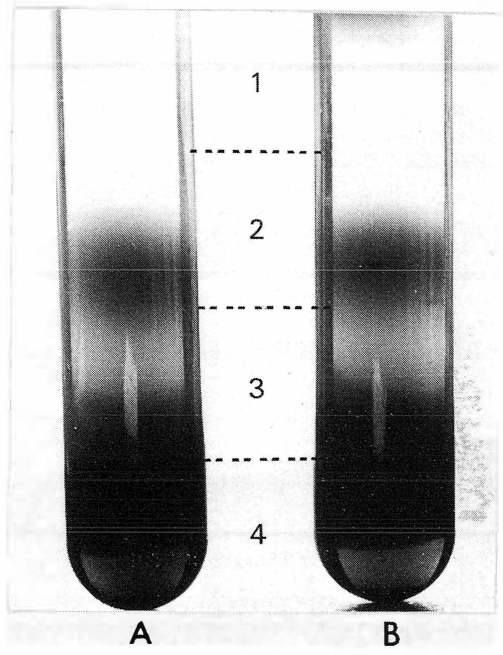

Figure 1 - First gradients from healthy (A) and infected (B) banana leaves. The numbers refer to the fraction to be layered on the second gradient.

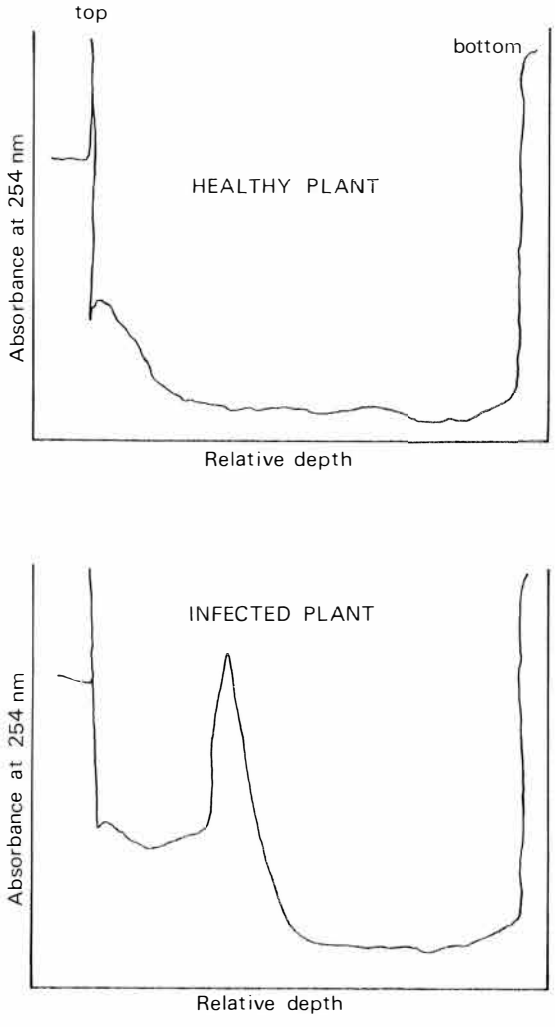

Figure 2 - OD recording of the 2nd gradients loaded with fractions 1 from the first gradients.

1 ml of 0.006 M phosphate buffer pH 7.0 and layered on top of a 10 to 40% sucrose gradient. Gradients were made by layering 2, 3, 3 and 2 ml of respectively 40, 30, 20 and 10% (w/v) sucrose. They were kept overnight at 4°C for diffusion to occur. The gradient was centrifuged at 150,000 g for 3 h. After centrifugation, four equal fractions were collected from the gradient. Each one was centrifuged at 130,000 g for 3 h, the resulting pellets were each resuspended in 1 ml of phosphate buffer and loaded on a second gradient identical to the first one. After centrifugation at 150,000 g for 3 h, the four gradients were scanned with an ISCO fractionator at 254 nm. The same procedure was applied to healthy leaves and the optical density (OD) profiles were compared. Negative staining of the fractions was used to confirm the presence of virus particles by electron microscopy.

RESULTS

Figure 1 shows the first sucrose density gradients from healthy (A) and bunchy top affected banana leaves (B.). No differences were observed between the two gradients. Dark green scattering zones preventing OD recording were present in both preparations. Four equal fractions (1 to 4 on figure 1) from each gradient were collected and worked out separately as described in material and methods. After the second gradient no difference in the elution profiles from healthy and infected material was observed with fractions 2-3-4 (results not illustrated). Negative staining of various fractions along these gradients failed to reveal any virus particles. However, as shown on figure 2, the elution profiles of the second gradients layered with fractions 1 (figure 1) showed the presence of a peak in the OD recording from infected plants only. This peak corresponds to a fine opalescent zone located 1.5 cm from the top of the gradient. However in some experiments, this band is not visible in the gradient tube but can be picked up by OD recording. The fraction corresponding to the peak was collected and the presence of virus particles was investigated by electron microscopy. As shown on figure 3, virus particles were indeed present ; they were isometric and had a diameter of about 28 nm after negative staining. Other fractions from this gradient were also examined by electron microscopy. No virus particles could be detected in any of them. Virus concentration was estimated by comparison of the peak areas obtained in the same conditions with 20 μg of TYMV (turnip yellow mosaic virus). TYMV which has comparable characteristics with those of the luteovirus group (isometric structure of 30 nm, RNA MV about 2×10^6 d, coat protein MV about 20 Kd, $S_{20}W$ = 117) has been used as a standard. From the peak of figure 2, about 30 μg of virus were obtained from 200 g of infected leaves. This purification method has now been used repeatedly and found consistently to yield from 66 to 170 μg purified virus for 1 kg of infected leaves.

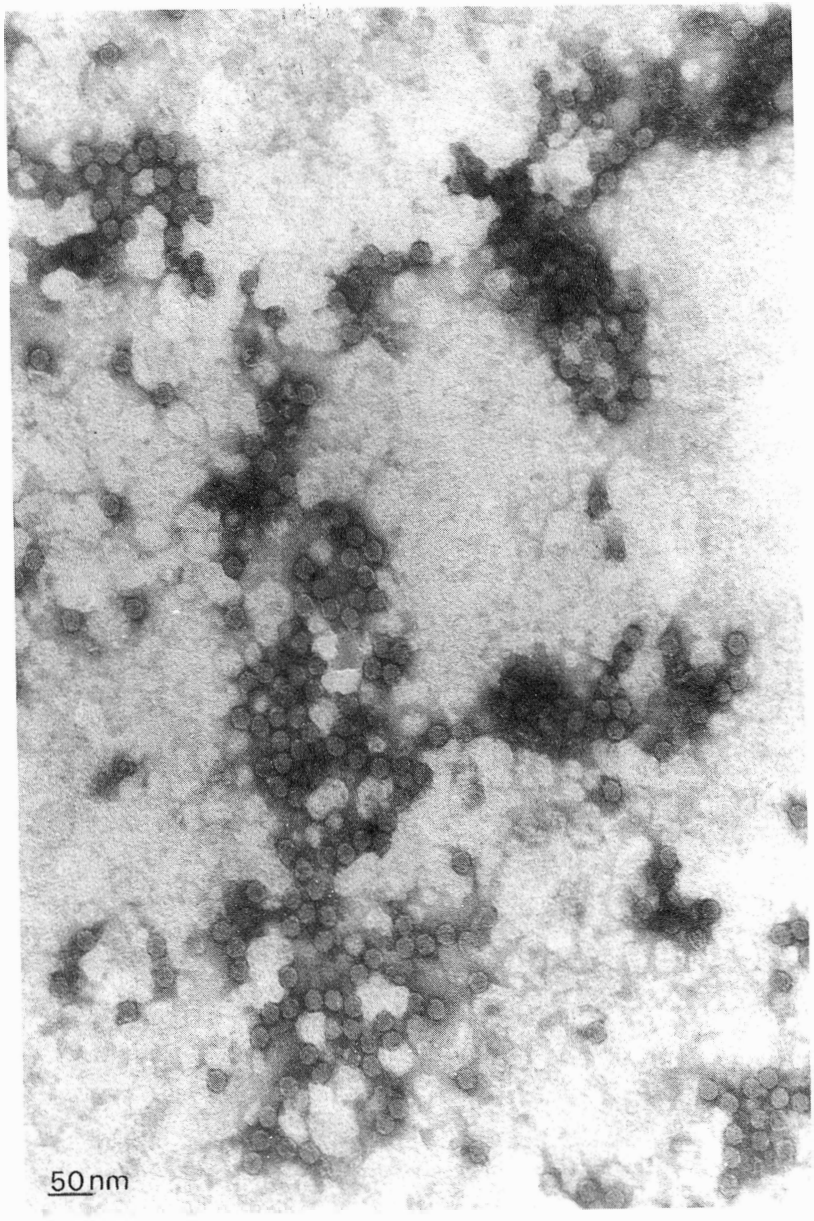

Figure 3 - Electron micrograph of negatively stained banana bunchy top virus particles.

DISCUSSION

The method of purification that we have developed, has allowed us to isolate an isometric virus from bunchy top affected banana leaves. With other purification procedures using various organic solvents such as chloroform or n-butanol and precipitation with PEG no virus was obtained. Our purification method is a gentle procedure in which the enzymatic step probably results in a good release of the virus. Such an enzymatic step has been used previously by Waterhouse and Singh (Waterhouse *et al.*, 1981 ; Singh *et al.*, 1984) and was found to increase the yield of other luteoviruses. Because luteoviruses are located only in the phloem tissue, their purification generally gives poor yields. However, amounts ranging from 200 µg to 1 mg are generally obtained from 1 kg of leaves with viruses, such as potato leafroll virus (1.3 mg/kg) (Takanami *et al.*, 1979) ; carrot red leaf virus (0.5 to 1 mg/kg) (Waterhouse *et al.*, 1981) ; tobacco necrotic dwarf virus (300 to 400 µg/kg) (Kubo *et al.*, 1979) ; soybean dwarf virus (166 µg/kg yellowing strain ; 369 µg/kg dwarfing strain) (Kojima *et al.*, 1976) and pea leafroll virus (668 µg/kg) (Ashby *et al.*, 1979). In contrast the yield obtained with the banana virus by our purification procedure seems rather low. This is probably because banana plants are much more difficult to grind and digest than the other plants used to propagate luteoviruses.

Our results give for the first time direct evidence that a virus is associated with banana bunchy top disease. Even though the purification procedure leads to relatively poor yields it should be sufficient to produce serological and/or genomic probes for further characterization of the virus and improved field diagnosis.

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LÄUTERUNG DES BUNCHY TOP VIRUS DER BANANENPFLANZE (BBTV).

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KURZFASSUNG - Die Bunchy Top Krankheit der Bananenpflanzen (BBTD) zählt zu den schwersten Krankheiten der Bananenpflanzen und Mehlbananen. Ein Virus, das wahrscheinlich zu der Luteovirengruppe gehört, wird mit der Krankheit in Zusammenhang gebracht. Das Virus vermehrt sich in den Geweben des Phloëms und wird von der *Pentalonia nigronervosa*-Laus übertragen. In vorliegendem Artikel beschreiben wir eine Methode der Virusläuterung, die es zum ersten Mal ermöglicht hat, isometrische Viruspartikel von etwa 28 nm aus Bananenpflanzen zu isolieren, die an Bunchy Top erkrankt waren.

PURIFICACION DEL VIRUS DEL BUNCHY TOP DE LOS BANANOS (BBTV).

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RESUMEN - La enfermedad del bunchy top de los bananos (BBTD) es una de las enfermedades más severas que afectan a los bananos y plátanos. Un virus, probablemente un miembro del grupo de los luteovirus, se asocia a esta enfermedad. El virus se multiplica en los tejidos del floema, es trasmitido por el pulgón *Pentalonia nigronervosa*. En este artículo describimos un método de purificación que ha permitido por primera vez aislar partículas virales isométricas de 28 nm aproximadamente, a partir de plantas de banano afectados por la enfermedad del bunchy top.