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Isolation and characterization of a strain of African cassava mosaic virus from a local cassava in the Republic of Benin

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ABSTRACT : Gemini particles were purified from infected local cassava by a method which was developed and the virus was characterized. Virus particles measured ca. 36 × 23 nm. Purified virions or their nucleic acid transmitted to *Nicotiana benthamiana, N. debneyi, N. clevelandii,* and *Datura stramonium* plants showed difference in symptom expression compared to another strain of the virus received from the German Collection of Microorganisms in Braunschweig. Viral capsid protein was resolved by SDS-PAGE. Its estimated molecular-weight was 30 kD. A single nucleic acid of estimated molecular-weight of 0.7×10^6 D was isolated from virus particles and resolved by agarose gel electrophoresis. Thin-section electron microscopy of infected and healthy leaf of cassava and of *N. benthamiana* plants revealed no cytopathic effects common to whitefly-transmitted geminiviruses. Ultrastructural features like hypertrophy and segregation of nuclear components were observed in ACMV-infected leaves of cassava and of *N. benthamiana* plants, but they could also be detected rarely in healthy materials. With antisera prepared to ACMV, the virus content in different parts of casava plants were determined by ELISA. The virus isolated from the local cassava and the strain received from Braunschweig were identified with the monoclonal antibody SCR 23 as strains of the geminivirus group A (strains of West-Africa and West-Kenya).

Keywords : African cassava mosaic, geminivirus, capsid protein, cytopathic effects, ELISA

In the tropics and particularly in Africa which is the major area of cassava production many diseases limit cassava yield. Among them the virus-induced mosaic disease (ACMV) alone is often responsible for an yield loss of 30-95% (Anon, 1979; Seif, 1982; Dengel, 1981). The causal agent, a geminivirus, is found in India and Madagascar, but not in South-America, where the casava mosaic disease is caused by a potexvirus (Costa and Kitajima, 1972). The Aftican cassava mosaic virus is transmitted to cassava crops by its whitefly vector *Bemisia tabaci* Gennadius and also through cassava cuttings. The ACMV genome is a circular single-stranded DNA (Bock, *et al.,* 1978; Walter, 1980; Adejare and Coutts, 1981b). Since the first observation and description of the disease in Kenya (Warburg, 1894), many studies have been made : these include the virus host range, the transmission and isolation of the virus (Chant, 1958; Bock *et al.,* 1978; Bock and Guthrie, 1978; Walter, 1980), and epidemiology (Bock, 1983; Fargette *et al.,* 1985, 1986; Fargette and Fauquet, 1988; Fauquet *et al.,* 1988; Fauquet and Fargette, 1990), and ultrastructure of infected cells (Adejare and Coutts, 1981a). Here we report on a new method of isolation of a strain of the African cassava mosaic

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virus from West-Africa and on a partial characterization of this virus strain. We have recently reproted on the possibility of partially controlling the disease by mixed cropping (Ahohuendo and Sarkar, 1995).

MATERIALS AND METHODS

Maintenance of virus cultures

Virus cultures were maintained in *N. benthamiana* (Domin), *N. clevelandii* Gray and *Datura stramonium* L. plants. After inoculation, plants were kept at 25-30°C in an insect-proof glasshouse at the University of Hohenheim (Germany). The leaves were harvested 2 to 3 weeks after inoculation and frozen at -85°C (Gallenkamp Super Cold 85).

Ultrastructural studies

The leaf samples of infected and healthy *N. benthamiana* and casava (*Manihot esculenta* Crantz) plants were fixed in 5% glutaraldehyde in phosphate buffer of pH 7.2 for 90 min and then kept for 15 min under vacuum. After being rinsed three times, each for 10 min, in 0.1 *M* cacodylate buffer, leaf pieces were fixed for 90 min in 1% osmiumtetroxyde and then washed three times, each for 10 min, in bi-distilled water. The pieces of leaf were thereafter dehydrated in an ethanol series, embedded in Spurr's medium (Spurr, 1969) for 8 hrs at 70°C and thin-sectioned with an ultramicrotome (Omu, 2, Reichert, Vienna). Sections were double-stained in aqueous uranyl acetate and lead citrate for 10 min and examined under an electron microscope (Em 10; Zeiss, Oberkochen).

Purification of ACMV particles

For virus extraction deep frozen systemically infected symptom-bearing leaves of *N. benthamiana* were used. All steps in virus purification were carried out at 5°C after homogenisation of tissues in an electric blender. 30 g of *N. benthamiana* leaves were disrupted in 150 ml 0.1 *M* tris-phosphate buffer, pH 8.4 contianing 5 ml ß-mercaptoethanol. The extracts were then clarified by a 20-min centrifugation at 9000 rev/min (Vetter ZK-4, rotor A8 or beckman J2-21, rotor JA-20). The sediments were left in ice and the aqueous phase emulsified with chloroform 1:2 (v/v), stirred for 15 min. The mixture was submitted to a similar low-speed centrifugation and the supernant carefully removed and placed in ice. The sediments were resuspended in 100 ml tris-phosphate buffer, pH 8.4, clarified for 20 min at 9000 rev/min and the supernant centrifuged as above. The virus particles were further purified by ultracentrifugation (120 min at 35000 rev/min in a Beckman L-2 centrifuge, rotor Ti 50), the pellets were collected in resuspension buffer (0.01 *M* tris-phosphate pH 8.4 containing 10^{-3} *M* EDTA-Na_2). The virus suspension was clarified by centrifugation (20 min at 6000 rev/min) at 5°C. Finally, the virus suspension was kept overnight at -85°C, purified by two cycles of ultracentrifugation (120 min at 35000 rev/min), then eluted through a sephadex G 75 column. Virus-containing fractions from the eluted column were taken up in resuspension buffer, the virus particles were concentrated by ultracentrifugation as above and resuspended in a small volume of resuspension buffer.

Electron microscopy

Virus particles were contrasted with 2% phosphotungstic acid (PTA) on carbon-stabilized Formvar-coated grids.

Polyacrylamide gel electrophoresis (PAGE) of viral protein

Electrophoresis of heat-denaturated viral protein was performed according to Laemmli (1970) on a discontinuous vertical 8 cm wide gel composed of 1.5 cm long, 6.25% stacking and 5.5 cm long, 12.5% separating gel, stained with Coomassie blue. The molecular-weights of viral proteins were determined as described by Weber *et al.* (1972) using as standards (molecular-weights in brackets), phosphorylase b (94.0 kD), bovine serum albumin (67.0 kD), ovalbumin (43.0 kD), carbonic acid anhydrase (30.0 kD), trypsine inhibitor (20.4

kD), and a-lactalbumin (14.4 kD).

DNA extraction and agarose gel electrophoresis

Viral DNA was prepared from concentrated viral preparations and also from infected plant materials, using sodium dodecyl sulfate (SDS) (Adejare and Coutts, 1981b). Partially released nucleic acid was then extracted with phenol, precipitated with 2 volumes of absolute ethanol, analysed by electrophoresis in 1% continuous agarose gel and stained with toluidine blue. As a control Brome mosaic virus nucleic acid was prepared and co-electrophoresed. Presence of DNA in the nucleic acid preparations was confirmed by diphenylamine treatment (Dische, 1930).

Serology

To prepare antiserum, 2.4 mg purified ACMV was emulsified with Freund's incomplete adjuvant (Freund, 1947) and used in three subsequent intramuscular immunizations of a rabbit. Samples of blood were collected and processed at weekly intervals starting 12 days after the final injection. For the double-antibody sandwich form of enyzme-linked immunosorbent assay (DAS-ELISA), we used the method of Clark & Adams (1977). The distribution of ACMV in different parts of the infected plants was analysed by ELISA.

RESULTS

Symptom expression

The ACMV strain isolated from the local variety BEN 86002 (ACMV-Be) differed strongly from another one recieved from the German Collection of Microorganisms in Braunschweig ACMV-Br) in inducing less stronger dwarfing and deformation of leaf in *N. benthamiana* plants (Figures 1A and 1B). With ACMV-Br, symptoms developed within 7 days after inoculation as compared to 14 days after inoculation with ACMV-Be. In *D. stramonium* plants, the ACMV-Br caused necrotic local lesions 14 days after inoculation. In contrast, *D. stramonium* plants inoculated with the ACMV-Be showed no visible symptoms. The plants were probably not infected. Also, plants of *N. clevelandii* and *N. debneyi* inoculated with both strains showed no visible symptoms even about three weeks after inoculation.

Ultrastructural studies

Under the electron microscope, hypertrophied and segregated nucleolus, as shown in Figures 2A-2H, were observed in preparations of infected plant cells, but also sometimes in healthy cells. In many instances cell ultrastructure, such as mitochondria, cytoplasm, nuclei walls, cell vacuoles, in virus-infected and virus-free leaf tissues were well preserved (Figures 2A-2H). No cytopathic effects characteristic of ACMV and other whitefly-transmitted geminiviruses (ring-shaped electron-opaque structures, viruslike particles in nuclei) were found.

Virus purification

ACMV was purified successfully from inoculated *N. benthamiana* plants but not from our cassava plants, according to the procedure described above. The deep freezing (-85°C) of *N. benthamiana* leaf after harvest and the first differential centrifugation obviously denatured most of the host plant protein. Different gradient centrifugations, glycerol-, sucrose- and caesium chlorid-gradient, were compared, but didn't give satisfactory results. The ACMV geminate particles isolated from infected *N. benthamiana* plants (Figures 3A and 3B) after elution of viral suspensions through sephadex G 75 and stained with 2% PTA measured about 36 × 23 nm.

Detection of a virus-associated protein

The viral protein was visualized by gel electrophoresis after disruption of purified viral preparations by SDS and heat. Two protein bands appeared in fractions C, F (ACMV-Br), D, G (ACMV-Be), (Figure 4A), in fractions B, E (ACMV-Br), C, F (ACMV-Be), (Figure 4B). Bands with a molecular-weight of 30 kD were associated with the presence of viral particles. The smears of high-molecular-weight (between 67 kD and 94 kD) seen in fractions obtained from partially purified virus preparations, indicated the presence of host

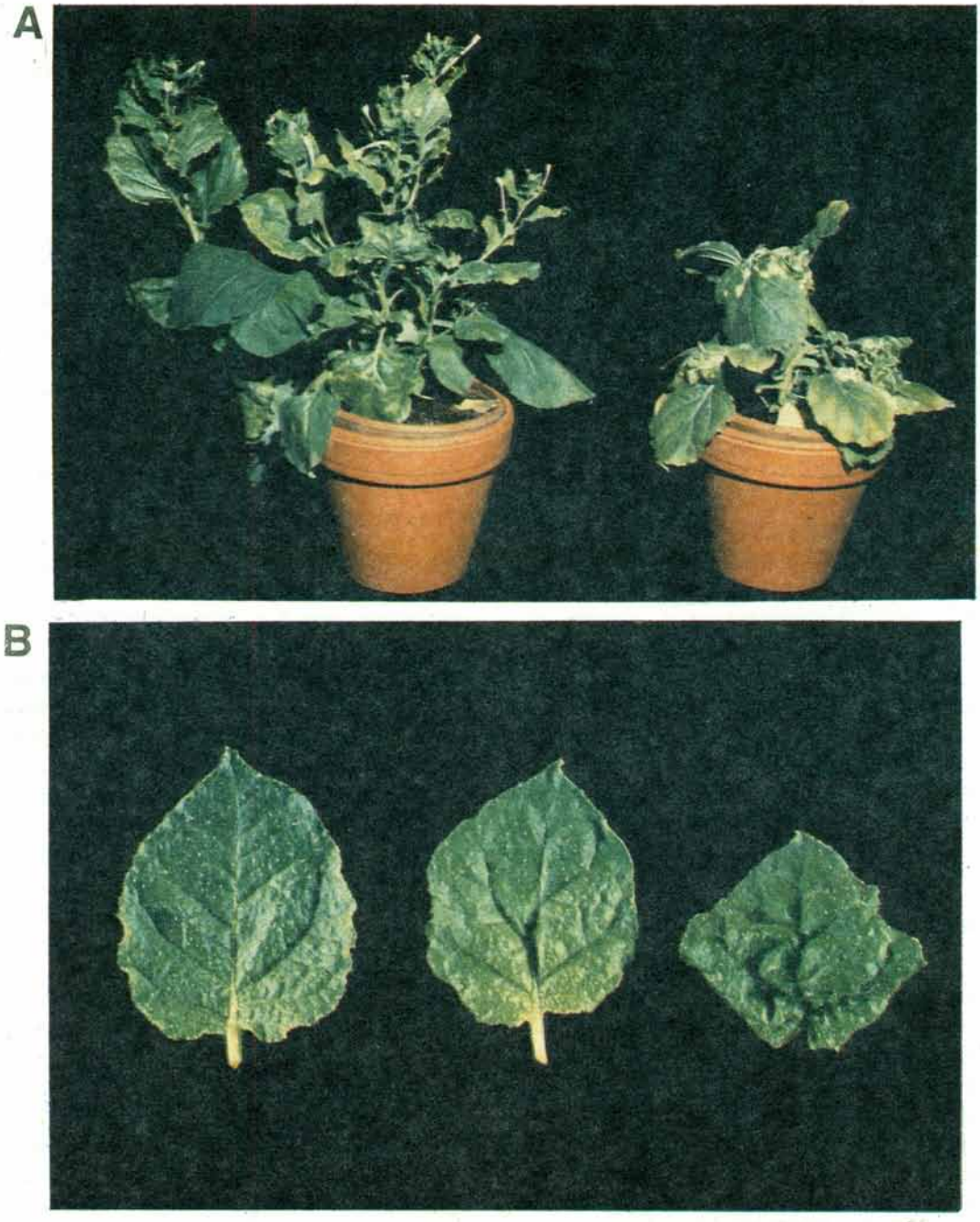

Fig. 1. Symptoms of ACMV in *N. benthamiana* plants (34 days after the inoculation). A= with ACMV-Be infected plants (left), with ACMV-Br infected plants (right) and symptoms of ACMV in leaves of *N. benthamiana* (18 days after the inoculation).B = healthy leaf (left), with ACMV-Be infected leaf (middle), with ACMV-Br infected leaf (right).

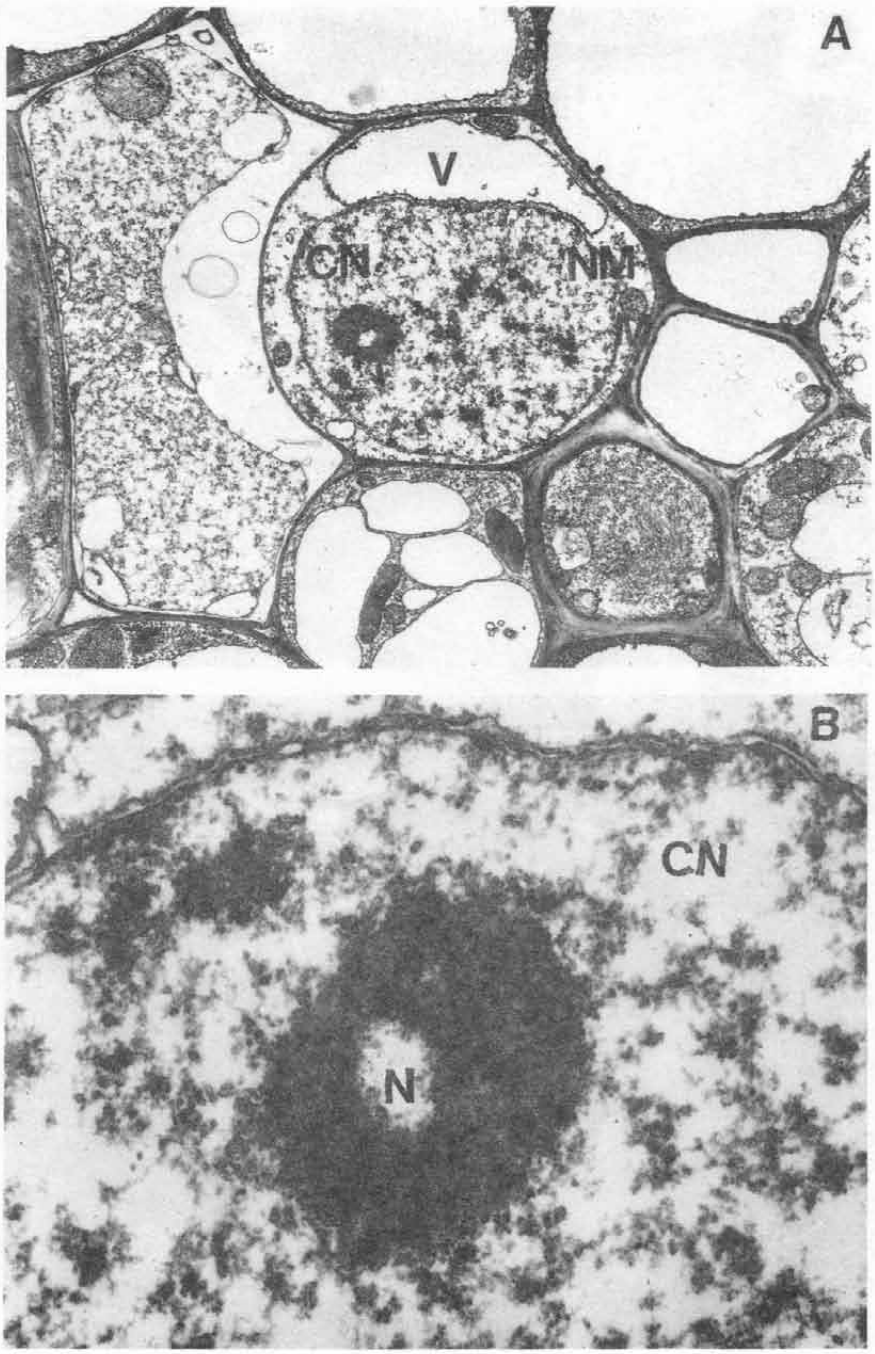

Fig. 2. Healthy cells of *Nicotiana benthamiana* leaf observed under electron microscope. A = X 7500 and B X 40000. Legend : CN = cell nucleus; NM = nucleus membran; M = mitochondria; V = vacuole; N = nucleolus.

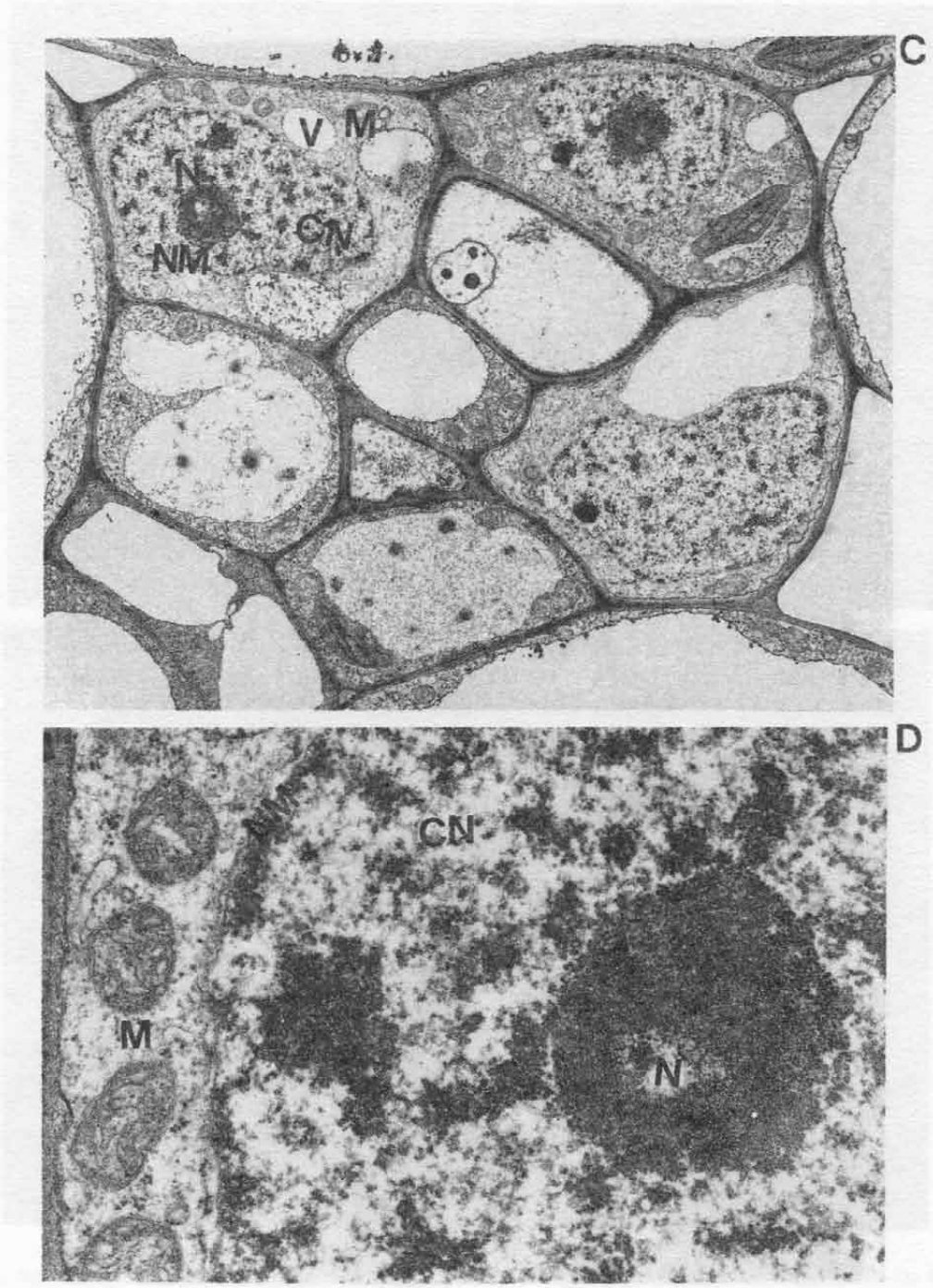

Fig. 2. Infected cells of *N. benthamiana* leaf observed under electron microscope. C = X 7500 and D = X 40000. Legend see und 2A and 2B.

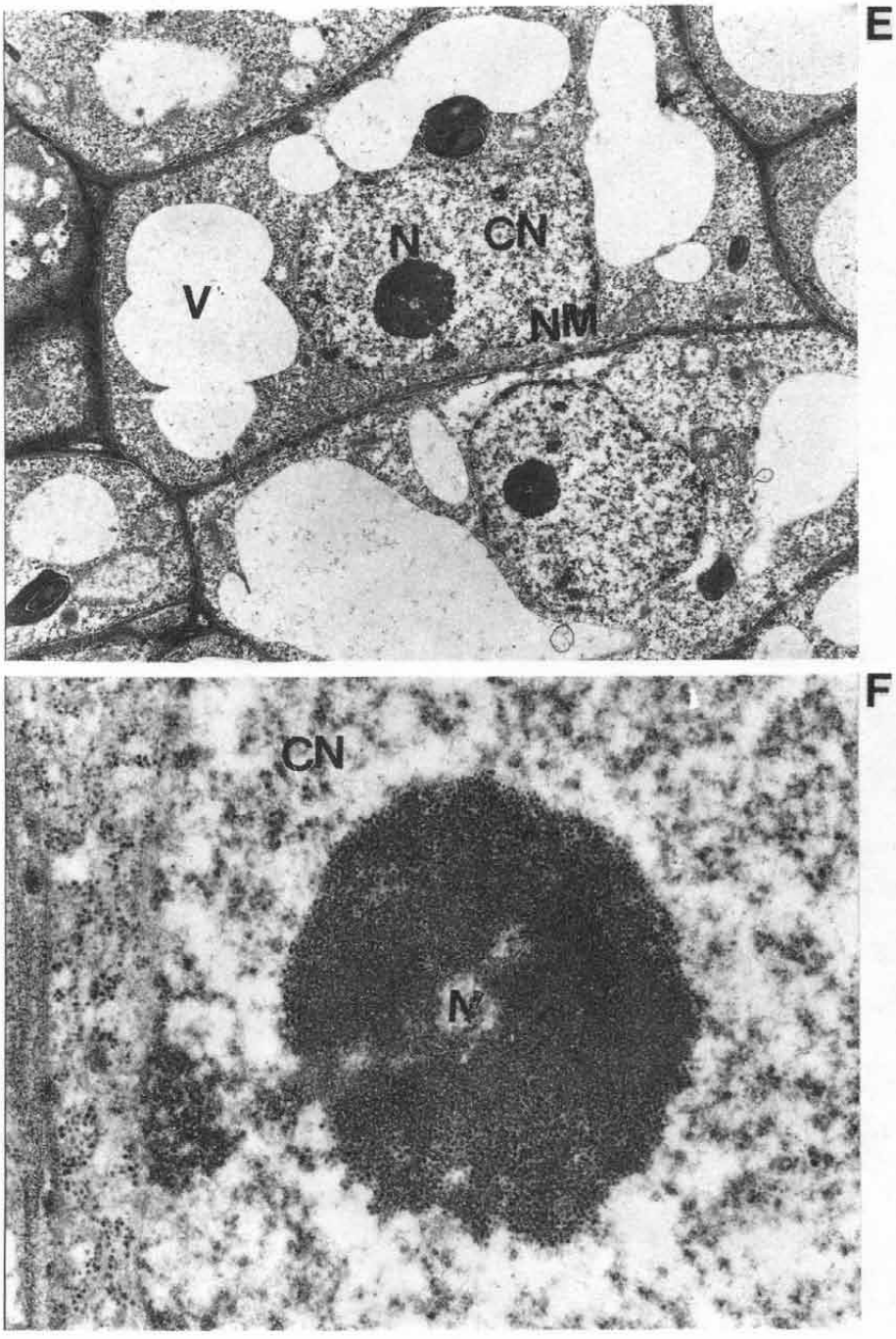

Fig. 2. Healthy cells of cassava leaf observed under electron microscope. E = X 7500 and F = X 40000. Legend see und 2A and 2B.

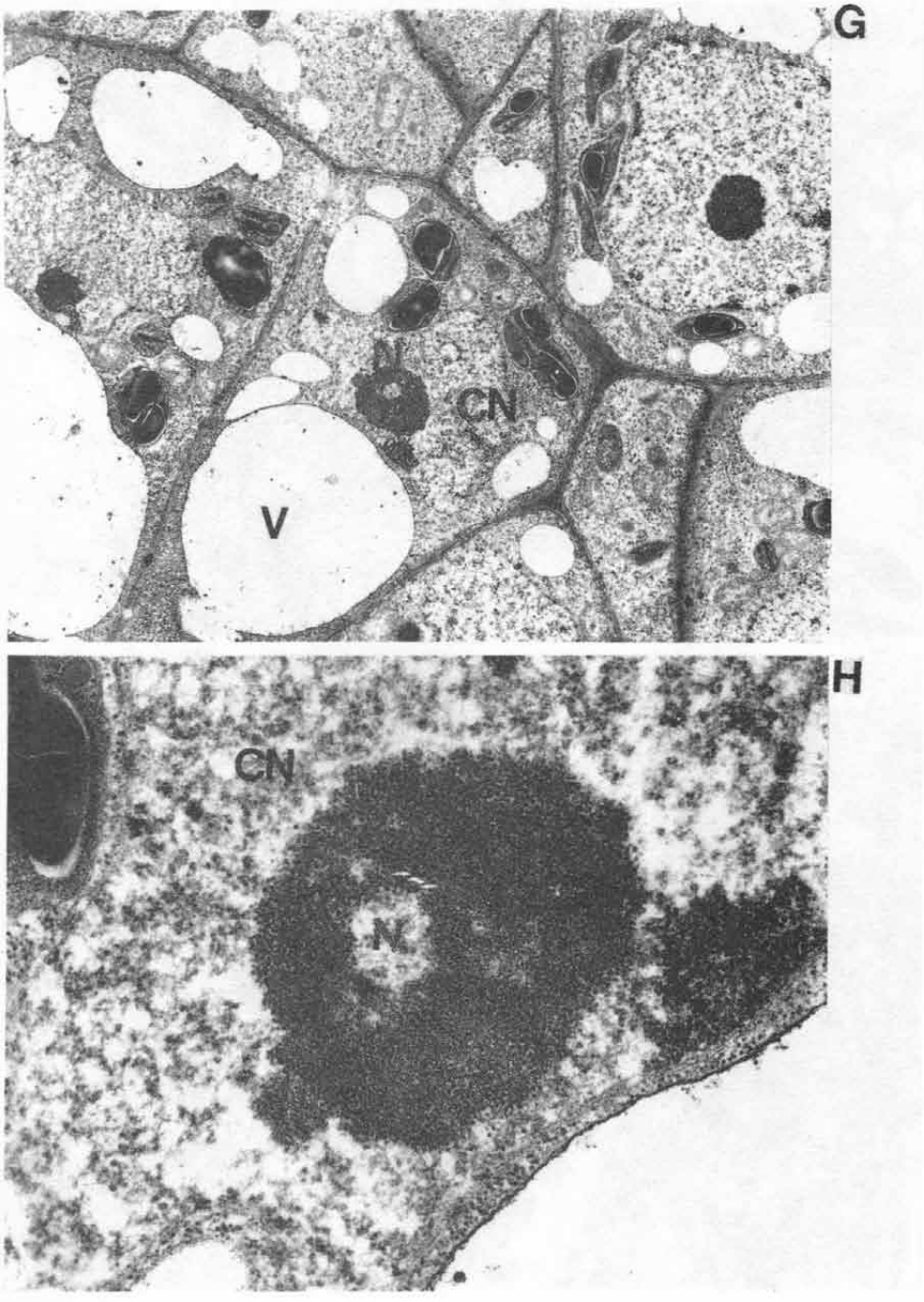

Fig. 2. Infected cells of *N. benthamiana* leaf observed under electron microscope. G = X 7500 and H = X 40000. Legend see und 2A and 2B.

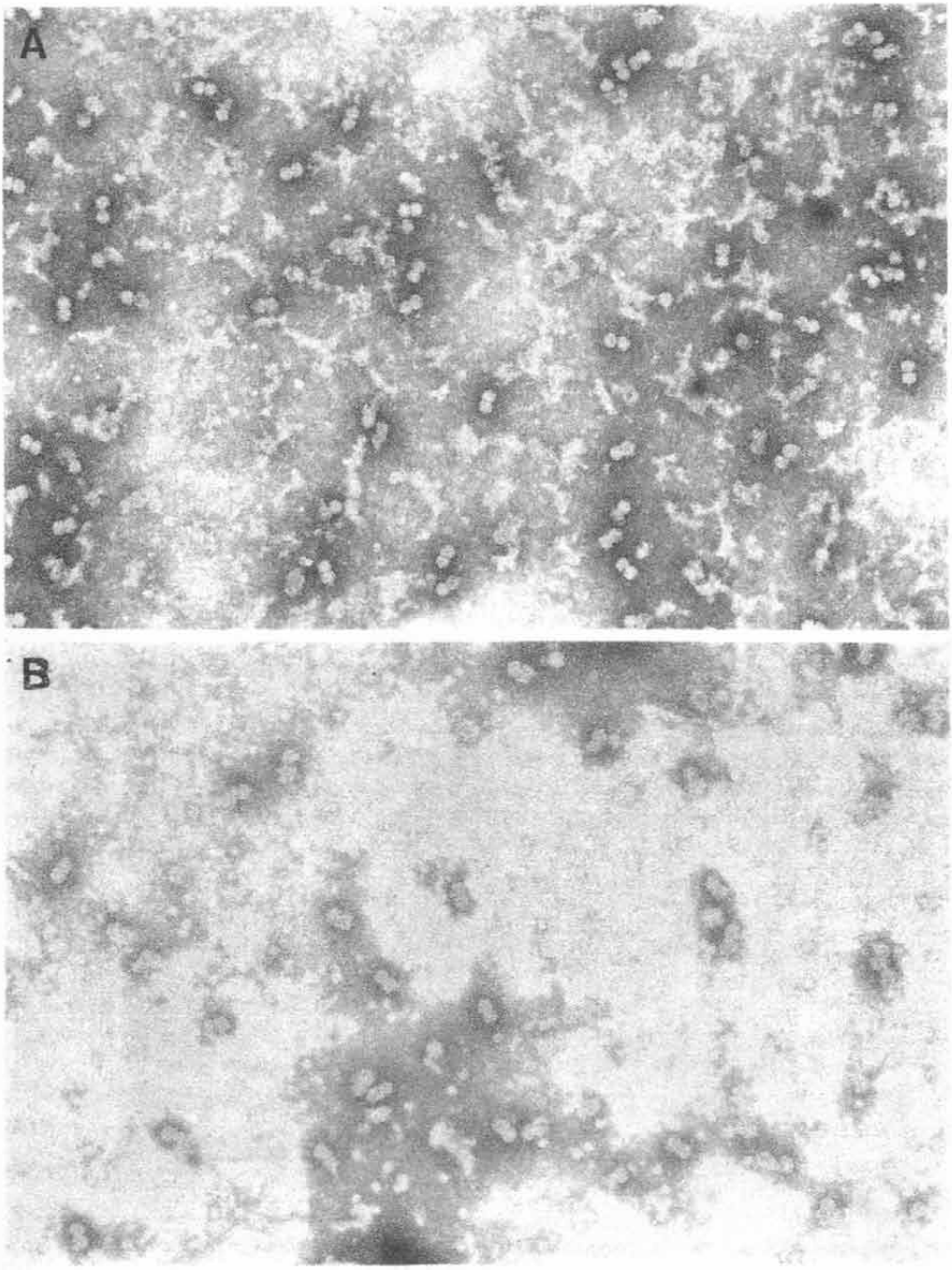

Fig. 3. African cassava mosaic virus particles observed under the electron microscope. A = ACMV-Be particles (X 12000); B=ACMV-Br particles (X 12000). Negative contrasted with 2% PTA.

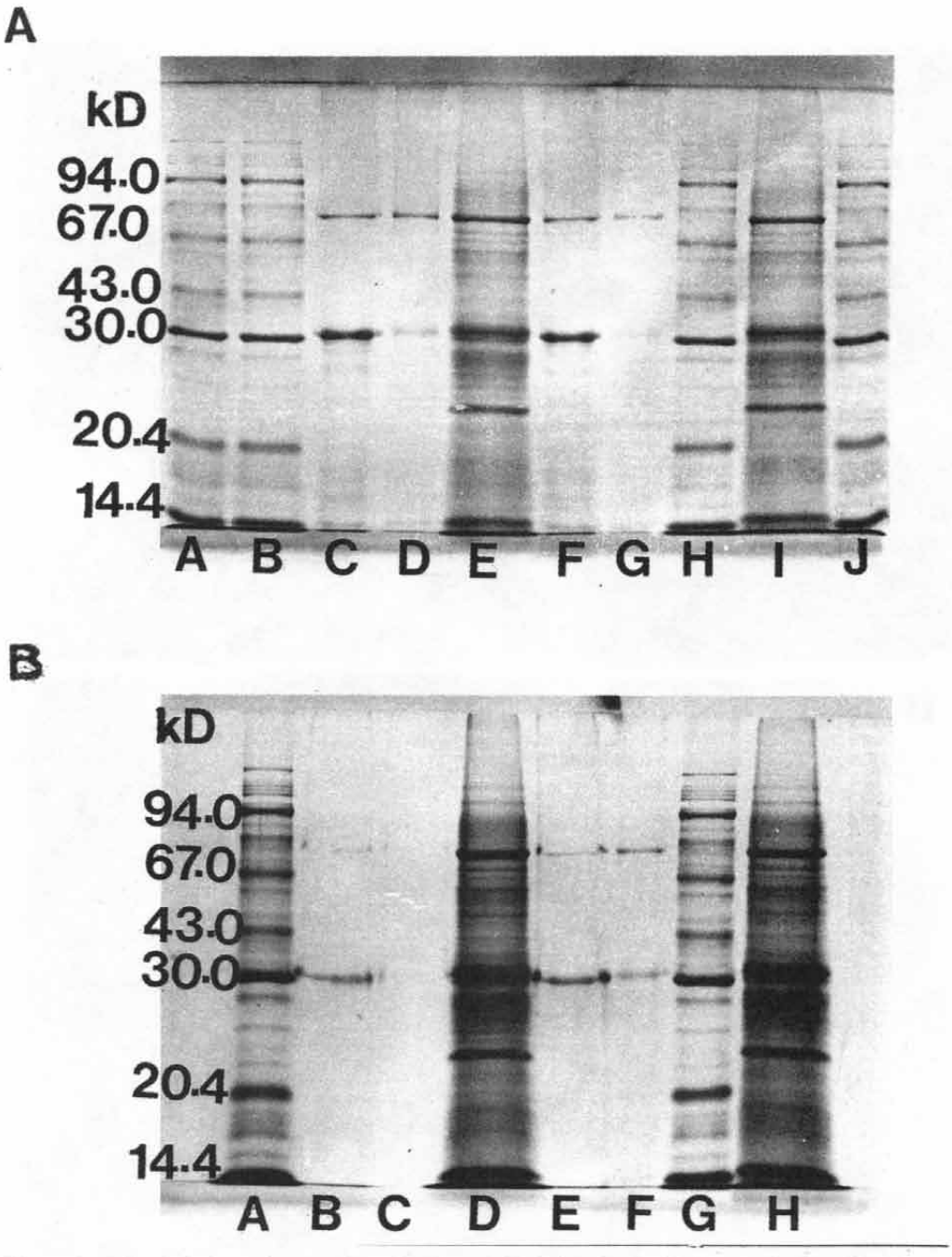

Fig. 4. Electrophoresis of viral protein on polyacrylamide gel (12.5%) stained with Coomassie blue. GEL A = standard molecular-weights (A, B, H and J), partially purified ACMV-Br (C and F), partially purified ACMV-Be (D and G), healthy *N. benthamiana* leaf (E and I). Gel B = standard molecular-weights (A and G), partial purified ACMV-Br (B and E), partially purified ACMV-Be (C and F), ACMV-Br from plants sap (D), ACMV-Be from plants sap (H). For details see tests.

protein (Figures 4A and 4B). In infected leaf samples, no additional bands were found. As Figures 4A and 4B showed, in virus-infected and virus-free leaf tissues, protein bands with molecular-weight of 30 kD appeared. However, bands in virus-infected leaf samples were broader than those of the control (healthy *N. benthamiana*). This is due to the presence in plant extracts of a protein of the same molecular-weight as the viral protein.

Detection of a virus-associated nucleic acid

The viral nucleic acid was visualized by agarose gel electrophoresis. A single nucleic acid band appeared in fractions B, E (ACMV-Br), in fractions C, F (ACMV-Be). Relative to the Brome mosaic virus RNA, the estimated molecular-weight of ACMV nucleic acid was 0.7×10^6 D (Figure 5).

Serological characterization

Using a sap dilution of 1:10 (w/v) from systemically infected *N. benthamiana* leaf and an antiserum dilution of 1/200, the virus could be detected in direct ELISA. It was not possible to detect the presence of ACMV in different parts of the cassava plants using the polyclonal antibody raised in a rabbit (Table 1). However, the virus could be assayed in infected *N. benthamiana* plants using the monoclonal antibody SCR 23 (Table 2). The Benin-strain of ACMV as well as ACMV-Br were found to belong to the geminivirus group A (strains of West-Africa and West-Kenya) as defined by Harrison *et al.* (1986).

DISCUSSION

Symptoms of ACMV in the local cassava variety BEN 86002 were very similar to those described by Chant (1958) and others. The symptom produced by ACMV-Be in *N. benthamiana* was different from that of ACMV-Br. Adejare and Coutts (1981b) found some difference between three strains of the ACMV-virus in studying the host range of the virus and concluded that this was probably due to experimental conditions. However, all our test plants were grown under the same experimental conditions. Recently, Morris *et al.* (1991) reported that by manipulation of one of the three open reading frames (AC_3) of the DNA-1 strand of ACMV, symptom appearance in *N. benthamiana* plants was delayed and decreased in intensity. Such a mutation could occur also in nature. The mild symptom of ACMV-Be, as compared to that of ACMV-Br, observed on *N. benthamiana,* points to such a possible difference in their genomes.

The cause of the African cassava mosaic disease was unknown for a long time, although the symptoms and the damage to the crop were recognized very early. Many different methods were tried during attempts to purify ACMV and other

Table 1. ELISA Absorbance values of different parts of healthy and infected cassava plants

Plant parts	Healthy plants	Diseased plants
Top leaf	0.066 ± 0.002	0.070 ± 0.005
Top leafstalk	0.100 ± 0.004	0.103 ± 0.005
Lower leaf	0.061 ± 0.009	0.072 ± 0.005
Lower leafstalk	0.068 ± 0.004	0.089 ± 0.003
Top stalk parts	0.089 ± 0.002	0.099 ± 0.002
Middle stalk parts	0.086 ± 0.005	0.098 ± 0.007
Roots	0.078 ± 0.005	0.085 ± 0.005

Buffer (0.044 ± 0.002)

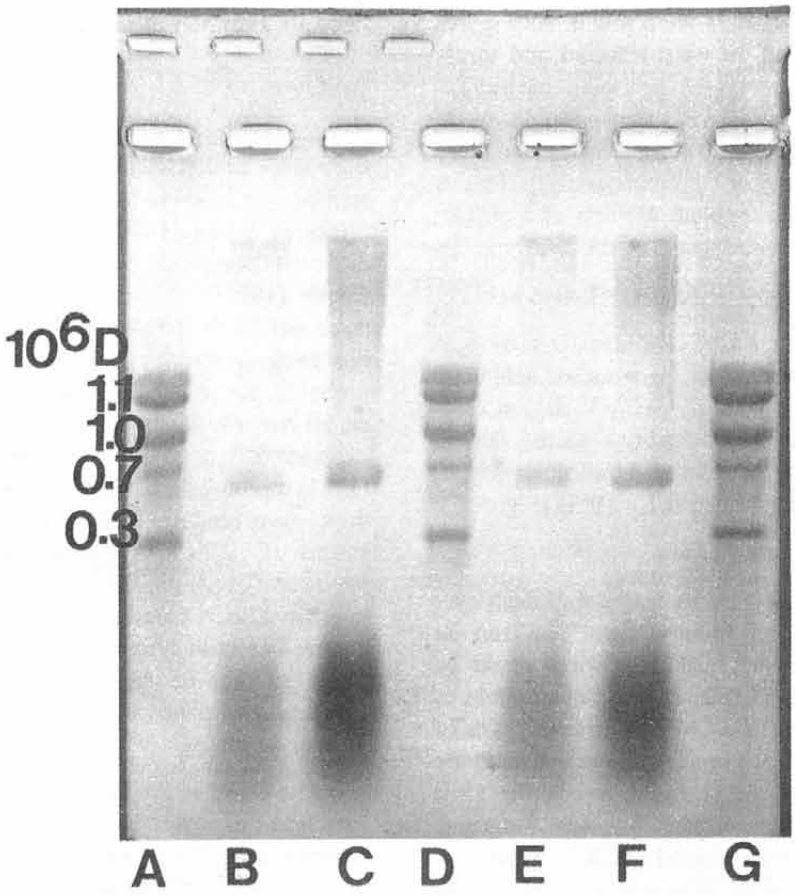

Fig. 5. Electrophoresis of viral nucleic acid on agarose gel (1%) stained with toluidine blue. Standard molecular-weights (A, D, and G), ACMV-Br (B and E), ACMV-Be (C and F).

Table 2. ELISA-Absorbance values of Sap from infected *Nicotiana benthamiana* plants using the monoclonal antibody SCR 23

Samples	Absorbance values
ACMV-Br	0.650 ± 0.120
ACMV-Be	0.550 ± 0.070
Healthy leaf	0.087 ± 0.005
Buffer	0.072 ± 0.005

geminiviruses (Goodman, 1977; Bock *et al.,* 1978; Francki *et al.,* 1979; Thomas and Bowyer, 1980; Sequeira and Harrison, 1982; Abouzid and Jeske, 1986). In all these attempts, the virus was submitted to differential centrifugation in different buffer solutions, with or without clarifying agents,

antioxydants and polyethylene glycol. In 1981 Bock *et al.* failed to purify 4 strains of ACMV (CLV-c, CLV-m, CLV-n, CLV-t) by the same method. For the strain CLV-c, the extraction buffer was modified and an antioxydant was added. During the present investigation, the addition of ß-mercaptoethanol, an antioxydant, was very useful. The method of ACMV isolation described by Sequeira and Harrison (1982) did not give satisfactory results in our laboratory. This is probably due to a low virus concentration in our plants. Freezing of viral suspensions at or near -85°C proved to be an important step and led to the isolation of ACMV particles free from most of the host components. The virus particles observed under electron microscope were similar in size and morphology to those previously described (Bock *et al.,* 1978; Walter, 1980; Adejare and Coutts, 1981b and others). Several workers have described cytopathic effects caused by ACMV. These inlcude ring-shaped electron-opaque structures, viruslike particles, hypertrophied nuclei, cytoplasmic inclusion body (Lastra and Gil, 1981; Thongmeearkon *et al.,* 1981; Kim and Martin, 1982; Cherif and Russo, 1983; Kim and Fulton, 1984; Kim *et al.,* 1986; Christie *et al.,* 1986; Roberts, 1989 and others). These features were not observed in preparations during our study, except hypertrophied and segregated nucleoli, which we could, however, find also sometimes in healthy material. According to our experience, therefore, we do not consider a segregation of nuclei as a reliable diagnositc feature of an ACMV infection.

Analysis of partially purified viral suspension by SDS-Page after denaturation by heating with 2% SDS and 10% ß-mercaptoethanol showed the presence of the viral capsid protein with an apparent molecular-weight of 30 kD. This molecular-weight was similar to those found by Bock *et al.* (1977), Mathew and Muniyappa (1992) for ACMV and other geminiviruses. On 1% agarose gel electrophoresis, the viral nucleic acid behaved as a single component of a molecular weight of 0.7×10^6 D. The nature of the nucleic acid of the isolated ACMV was found to be a DNA (DISCHE-test). This agreed with the previous observations on ACMV and other geminiviruses of Goodman (1977, 1981), Adejare and Coutts (1981b). However, it was not possible to demonstrate the two strands of the ACMV nucleic acid. This is due to the small difference in the molecular-weights of the two strands : 0.93×10^6 D and 0.91×10^6 D (Stanley and Gay, 1983). Adejare and Coutts (1981b) reported a molecular-weight of 0.77×10^6 D for a strain of the ACMV. The exact size of the nucleic acid of ACMV-Be, must be further analysed. Although ACMV-Be virions were easily inoculated to *N. benthamiana* plants from diseased cassava plants, attempts to detect the virions in obviously heavily infected cassava plants by ELISA were unsuccessful (Table 1). Sequeira and Harrison (1982) and Fargette *et al.* (1985) investigated on the virus content of cassava leaf. They could not always demonstrate the presence of virus in all the cassava leaves. Our results suggest that a failure in demonstrating the presence of the virus in cassava plants could be due to an interfering effect of a high latex content of the cassava plants. In is known that the indexing of a virus in plant sap by ELISA can be influenced by various components of the host tissue (Clark, 1981). In case of ACMV it may be necessary to develop some preliminary purification (or clarification) of the plant sap before applying a simple or a modified ELISA test. Since both ACMV-Be and ACMV-Br reacted well to the monoclonal antibody, they have some epitope(s) in common. The results justify the inclusion of these two strains in the group A, as mentioned already, whereas the virus strains belonging to the group B (strains from East-Africa) and group C (strains from India and Sri Lanka) have wide serological differences (Harrison *et al.,* 1986).

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