African Cassava Mosaic Virus (ACMV): Stability of Purified Virus and Improved Conditions for its Detection in Cassava Leaves by ELISA

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With 6 figures

Received April 25, 1988; accepted September 2, 1988

Abstract

African Cassava Mosaic Virus (ACMV) was purified by a method which allowed the separation of monomer from dimer virus particles.

Optimal conditions for storing purified virus to be used for immunization were determined by ELISA and inoculation on Nicotiana benthamiana. Purified virus could be stored without loss of infectivity and serological activity for more than 145 days at 4 °C or frozen at −20 °C, but not longer than 40 days in the presence of 50% redistilled glycerol.

Rabbit and chicken immunoglobulins were used to detect ACMV in cassava leaves by direct and indirect ELISA. To obtain the same absorbance values, it was necessary to use longer incubation times with the indirect method, but the virus detection end-point in sap from infected plants was the same for the two methods (U512).

Conditions for improving virus detection in cassava samples were determined. The virus was better detected when leaves from diseased plants were ground in 100 mM Tris-HCl containing 1% polyvinylpyrrolidone at pH 8.5 than in phosphate buffer. Plant inhibitors were the restricting factor in the detection of virus by ELISA, but this difficulty was avoided when leaves to be tested were harvested from the top of the cassava plants.

Zusammenfassung

African Cassava Mosaic Virus (ACMV): Stabilität des gereinigten Virus und verbesserte Bedingungen für dessen Entdeckung in Cassavablättern durch ELISA

Bei der Reinigung von african cassava mosaic virus (ACMV) wurde eine Methode angewandt, wodurch die Trennung der Monomer- und Dimerviruspartikeln möglich war.

Die optimalen Aufbewahrungsbedingungen des gereinigten Virus, das für Immunisationstudien benutzt werden soll, wurden durch ELISA und Inokulation an Nicotiana benthamiana ermittelt.

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Das gereinigte Virus ließ sich ohne Verlust an Infektivität oder serologischen Aktivität bei 4 °C oder eingefroren bei −20 °C länger als 145 Tage aufbewahren, jedoch nicht länger als 40 Tage in 50 %igen redestilliertem Glycerol.

Mit Hilfe von Kaninchen- und Hühnerimmunglobulinen wurde ACMV in Cassavablättern durch direkten oder indirekten ELISA ermittelt. Um die gleichen Absorptionswerte zu erreichen, waren längere Inkubationszeiten mit der indirekten Methode notwendig, jedoch wurde der gleiche Virusentdeckungspunkt (1/512) im Saft infizierter Pflanzen durch beide Methoden ermittelt.

Festgestellt wurden die Bedingungen, um die Virusentdeckung in Cassavaproben zu verbessern. Bessere Ergebnisse wurden erreicht, wenn Blätter von befallenen Pflanzen in 100 mM Tris-HCl, die 1 %ige Polyvinylpyrrolidon bei pH 8,5 enthielt, statt in Phosphatpuffer gerieben worden waren. Pflanzliche Inhibitoren waren die einschränkenden Faktoren bei der ELISA-Virusermittlung, diese Schwierigkeit konnte jedoch umgangen werden, indem die zu untersuchenden Blätter von der Spitze der Cassavapflanzen gesammelt wurden.

Résumé

La purification du virus de la mosaïque africaine du manioc est rendue difficile à cause de sa faible concentration et de sa localisation dans des tissus profonds de la plante. Une méthode utilisant du Triton X-100 à 1 % et un gradient de 20 à 50 % de saccharose nous a permis la purification de particules virales répondant aux caractéristiques des geminivirus et de séparer des formes monomères et dimères de ce virus. Des préparations de virus purifié peuvent être conservées plus de 145 jours à 4 °C ou à −20 °C sans perte d’infectivité ou d’activité sérologique. Des anticorps contre ce virus ont été préparés par immunisation de lapins et de poules.

Les inhibiteurs de la plante et la faible concentration du virus dans la plante rendent difficile la détection de ce virus par les méthodes immunoenzymatiques. Ces difficultés sont surmontées quand les feuilles à tester sont récoltées au sommet de la plante et broyées dans un tampon Tris-HCl 0,1 M à pH 8,5 contenant 1 % de polyvinylpyrrolidone. Les méthodes ELISA directe et indirecte permettent alors une bonne détection du virus. Pour obtenir les mêmes réponses en ELISA, il faut incuber le substrat plus longtemps pour la méthode indirecte. Le virus peut être détecté dans les feuilles de manioc jusqu’à une dilution de 1/512 pour les deux méthodes.

Cassava was introduced into Africa from Brazil during the 16th century. A mosaic disease of cassava was first reported by Warburg (1894). The disease has been observed wherever cassava is grown in Africa and the adjacent islands (Dufrenoy and Hedon 1929). Two presumed virus diseases of cassava have been described in Africa many years ago: African cassava mosaic (Storey and Nichols 1938) and cassava brown streak (Storey 1936).

The disease caused by African cassava mosaic virus (ACMV) seriously decreases the yields of cassava crops. Reported losses range from 30 to 70 % (Dengel 1980). The causal agent, a geminivirus transmitted by the whitefly Bemisia tabaci was isolated from cassava leaves showing mosaic symptoms (Bock et al. 1977 and 1981, Walter 1980, Adejare and Coutts 1982, Robinson et al. 1984).

ACMV has been detected in crude cassava leaves by molecular hybridisation (Robinson et al. 1984, Roberts et al. 1984) and immunoenzymatic assay (Sequeira and Harrison 1982, Thouvenel et al. 1984, Thomas et al. 1986, Fargette et al. 1987).

However, the detection of virus in cassava leaves was limited by two difficulties: the low concentration of virus particles in the leaves and the presence of plant components which inhibit the ELISA reaction (Sequeira and Harrison 1982, Thouvenel et al. 1984, Fargette et al. 1987).
In the present paper we report experiments designed to improve the detection of ACMV by ELISA in cassava leaves.

**Material and Methods**

**Plants and Virus**

Cassava plants (*Manihot esculenta* Crantz and *Manihot glaziovii* Muell.) with mosaic symptoms (natural infection) and infected *Nicotiana benthamiana* were grown in an insect-proof greenhouse under controlled conditions (18–27 °C, 16 h light/day).

Cultivars TMS 30211 and TMS 30572 of *M. esculenta* were obtained from I.I.T.A. (Ibadan) and Kokobassié, Magnacle, Maloenda and Ouanga from O.R.S.T.O.M. (Abidjan).

The virus was purified from systemically infected *N. benthamiana* leaves harvested 20 days after inoculation, using a method similar to that described by LarSEN and Duffus (1984) except that after low-speed centrifugation of the polyethylenglycol (PEG)-treated solution, pellets were dissolved in the grinding buffer without Triton X-100. The virus particles were separated on a sucrose gradient (20–50 % in 0.1 M phosphate buffer - Na₂HPO₄-KH₂PO₄ - pH 7.2) after centrifugation for 6 h 1/2 in a MSE 6 x 38 ml rotor at 24,000 rpm (105,000 g Max.). Concentrations of purified particles were estimated by spectrophotometry, assuming a value of 7 for A₂₆₀ nm (1 mg/ml, 1 cm light path) (Robinson et al. 1984). Purified virus in sucrose solution in 0.1 M phosphate buffer pH 7.2 was stored at −20 °C or at +4 °C.

In some experiments, virus was pelleted from the gradient fractions and redissolved in the 0.1 M phosphate buffer.

**Electron microscopy**

Electronmicroscope observations were performed on carbon-coated grids floated on a drop of virus solution. After rinsing with TAM (10 mM Tris HCl pH 8, 10 mM NaN₃, 1 mM MgSO₄) and coloration with 2 % uranyl acetate, the preparations were observed using a Philips 300 microscope at the Biozentrum (Basel).

**Storage of purified virus**

The stability of purified virus in different storage conditions was examined. Purified virus in the sucrose solution (from gradient fractions) was distributed in different tubes containing each 2.5 ml. The contents of two tubes diluted, one with 2.5 ml of redistilled glycerol and the other with 2.5 ml of a 0.1 M phosphate buffer (pH 7.2) were kept at −20 °C. A third tube was held at 4 °C after dilution with 2.5 ml of 0.1 M phosphate buffer containing 0.03 % sodium azide. In a fourth tube, the virus solution was diluted with 2.5 ml of 0.1 M phosphate buffer and frozen in aliquots of 200 µl. The virus infectivity and the serological activity were examined weekly, respectively by inoculating two virus dilutions (41 and 0.6 µg/ml) to four *N. benthamiana* plants and by testing in ELISA serial dilutions of each virus preparation (from 600 to 2 ng/ml).

**Immunization protocols**

Antisera were produced by injecting rabbits (8 injections) at 7 days intervals. For the first injection, a solution of 0.2 ml of purified virus at 290 µg/ml mixed with 0.2 ml of Freund’s complete adjuvant was subcutaneously injected into rabbits. For the following injections 0.5 ml of the same virus solution mixed with 0.5 ml of Freund’s incomplete adjuvant was used. After three injections (22 days after the first injection) rabbits were at regular intervals bled and sera collected.

Chickens were immunized following the same protocol as with the rabbits. The first injection was given with a solution of 150 µl purified virus at 290 µg/ml mixed with 150 µl of Freund’s complete adjuvant. For the subsequent injections 250 µl purified virus plus 250 µl of incomplete adjuvant were injected. Eggs were collected, labelled and stored at 4 °C until processed for extraction of immunoglobulins.
Extraction and purification of immunoglobulins

Rabbit immunoglobulins were prepared by rivanol precipitation according to the method of HARDIE and VAN REGENMORTEL (1977). The final solution containing immunoglobulins was dialysed against phosphate saline buffer (PBS) containing 0.02 % NaN₃ and adjusted to a concentration of 1 mg/ml in PBS (A₂₈₀ = 1.4). Purified rabbit IgG was stored at -20 °C with one volume glycerol. Chicken immunoglobulins (IgY) were purified from eggs following the method described by POLSON et al. (1980). Purified IgY at a concentration of 6 to 12 mg/ml was stored at -20 °C.

Preparation of IgG enzyme conjugate

Alkaline phosphatase (567.752, Boehringer Mannheim) was conjugated to rabbit IgG following the method described by AVRAMEAS (1969). Because of volume changes and possible losses of immunoglobulins during the conjugation procedure, amounts of conjugate used are expressed in terms of dilutions rather than absolute IgG concentrations.

ELISA

Direct ELISA method

Direct double antibody sandwich ELISA was done in polystyrene microtitre plates (Nunc Immunological II, 96 wells) as described by CLARK and ADAMS (1977).

Indirect ELISA method

The indirect ELISA method was that described by VAN REGENMORTEL and BURCKARD (1980) with few modifications. Microtitre plates were coated with 10 pg/ml of purified chicken IgY. Rabbit immunoglobulins were used at 2 pg/ml and the goat anti-rabbit globulin conjugate (Sigma A-8023) was diluted at 1/2,000.

Absorbance values were accepted as positive when they were greater than twice the mean absorbance of the virus free control sample.

All the ELISA values (diseased and healthy samples) are given without subtracting the absorbance of control buffer. The ELISA values of the healthy samples were usually similar to those of the control buffer.

Results

Virus purification

Virus particles were purified from infected N. benthamiana leaves harvested 20 days after inoculation. In our centrifugation conditions ultraviolet absorbance profiles always showed two peaks for the cassava cultivars TMS 30211 or Kokobassié (Fig. 1), whereas only the lower peak was obtained with cultivars Ouanga or TMS 30572.

Electron microscopic observation of material from the upper peak of the sucrose gradient showed monomer geminivirus particles of about 18 nm in diameter (Fig. 1/A) while the lower peak contained both single and paired (17—20 × 30—33 nm) particles. Sometimes trimer particles were observed (Fig. 1/B). When N. benthamiana plants were inoculated with each fraction, infectivity was associated with fractions 8 to 19 and was correlated with the ELISA response of each fraction (Fig. 1). The material in the two peaks had UV absorbance spectra typical of nucleoproteins. The A₂₆₀/A₂₈₀ ratio was 1.35—1.47 for the upper
Fig. 1. ACMV purification: Relative absorption at 254 nm (---) of material present in the 20—50 % sucrose gradient after centrifugation at 24,000 rpm for 6 h 1/2 in a 50 MSE rotor at 10 °C. Sedimentation was from left to right. The dotted line (---) is the ELISA response of different fractions of the sucrose gradient. Vertical bars represent the distribution of the infectivity of the sucrose gradient fractions inoculated on *N. benthamiana*. The infectivity was scored 18 days after inoculation.

Electron microscopy photographs of purified monomer (peak A) and dimer + monomer (peak B); (magnification: 240,000)
peak (monomer particles) and 1.45—1.65 for the lower peak corresponding to respectively 10—17 % and 16—25 % nucleic acid content (GIBBS and HARRISON 1976). The yield of purified virus was about 5 to 8 mg/kg *N. benthamiana* leaves.

### Storage of purified virus

Purified ACMV particles could be stored for a long period without loss of infectivity or ELISA reactivity when kept frozen or at 4 °C, or when repeatedly frozen and thawed. *N. benthamiana* could still be infected when inoculated with the virus solution stored in these three conditions for 145 days. However, the virus stored in 50 % redistilled glycerol completely lost its infectivity after 20 days and no longer reacted in ELISA after about 35 days (Fig. 2 and Table 1).

### Influence of crude plant extract on virus reactivity in ELISA

Virus detection was lowered when purified virus was diluted in the sap of healthy cassava leaves compared to the absorbance readings obtained when the virus was diluted in PBS buffer (data not shown). The sensitivity of virus detection was lower when the virus was diluted in extracts from older leaves compared to extracts of younger healthy leaves.

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**Fig. 2.** ELISA response of purified virus after different periods of storage: The stability of the virus was tested by direct ELISA. Coating was done with 1 μg/ml IgG and the enzyme-conjugate was diluted 1/2,000. Before each experiment, virus was diluted to give a concentration range from 600 to 2 ng/ml. ELISA values are those obtained at 60 min with 66 ng/ml of virus.

- Buff: phosphate buffer
- F-T: storage by freezing and thawing the same fraction at X days intervals
- AL: storage by freezing 200 μl aliquots in each tube
- F: storage at 4 °C with 0.03 % sodium azide
- GLYC: freezing with 50 % redistilled glycerol
**Table 1**

Effect of storage conditions on virus infectivity

<table>
<thead>
<tr>
<th>Time</th>
<th>Storage conditions</th>
<th>Virus concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>AL</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>GLYC</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>F-T</td>
<td>4/4</td>
</tr>
<tr>
<td>7 days</td>
<td>AL</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>GLYC</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>F-T</td>
<td>1/4</td>
</tr>
<tr>
<td>21 days</td>
<td>AL</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>GLYC</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>F-T</td>
<td>4/4</td>
</tr>
<tr>
<td>56 days</td>
<td>AL</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>GLYC</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>F-T</td>
<td>1/4</td>
</tr>
<tr>
<td>77 days</td>
<td>AL</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>GLYC</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>F-T</td>
<td>0/4</td>
</tr>
<tr>
<td>145 days</td>
<td>AL</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>GLYC</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* number of infected plants/number of inoculated plants; nt = not tested

Infectivity on *N. benthamiana* of purified ACMV frozen (F-T; AL; GLYC) or kept at 4°C with 0.03% sodium azide (F)

F-T: storage by freezing and thawing the same fraction at X days intervals

AL: storage by freezing 200 µl aliquots in each tube

F: storage at 4°C with 0.03% sodium azide

GLYC: freezing with 50% redistilled glycerol

**Grinding buffer**

When two buffers — without additives — were compared for grinding cassava leaves (Tris-HCl and PBS-T), the Tris-HCl buffer was found to give the highest ELISA readings. Tris-HCl buffer at 0.1 M was used for routine ELISA.

The use of additives such as PVP, PEG, or sodium diethyldithiocarbamate (DIECA) in the grinding buffer did not give a significantly higher virus-specific absorbance. When PVP was added at high concentration (5%), virus detection by ELISA was inhibited, while at the concentration of 1% the ELISA response was slightly increased.
When the pH of the grinding buffer was 8.0 to 8.5, ELISA absorbance values were higher than at pH 7.5 (Fig. 3).

In conclusion, the optimal grinding buffer was Tris-HCl 0.1 M, 1 % PVP, pH 8.5 buffer.

Because of the low concentration of viral antigen in cassava leaves of any cultivar tested here, it was important to determine the optimal conditions for grinding the leaves. The limit of detectability was obtained when one gram of cassava leaves was ground in 32 ml of grinding buffer (Fig. 4). While the detection was possible when one gram of leaves was ground in one milliliter of grinding buffer, the highest ELISA absorbance values were obtained by grinding one gram of cassava leaves in 2 to 4 ml of buffer.

**Conditions for improving ACMV detection in cassava crude sap by ELISA**

Antigen in cassava leaves could be detected when a dilution of 1/81,000 of rabbit antiserum was used. When plates were coated directly with the antigen, detection was possible with rabbit antisera diluted at 1/27,000.

In direct ELISA, IgG used for coating the plates was diluted at 1 μg/ml and the IgG enzyme conjugate was at 1/4,000.

In the indirect ELISA, microplates were coated with 10 μg/ml IgY. In order to minimize the background absorbance reading observed with healthy samples, purified rabbit-IgG was used at 2 μg/ml and the goat anti-rabbit enzyme conjugate (GAR) was diluted at 1/2,000.
African Cassava Mosaic Virus

Fig. 4. Effect of volume of grinding buffer on virus detection by ELISA: two cassava cultivars were tested (TMS 30211 and Ouanga). Direct ELISA was done on plates precoated with 1 μg/ml IgG. The IgG conjugate was diluted 1/4,000. ELISA values were read 60 min after addition of substrate.

The absorbance values of the wells receiving sample extract and substrate only were similar to those of wells containing extracts from virus-free tissue and extraction buffer controls (MOWAT and DAWSON 1987). The variation in absorbance readings between replicate wells was consistently low. For example, using 6 replicate wells, the standard deviation was 0.025 for a mean absorbance of 1.79 (virus containing sample) and 0.005 for a mean of 0.172 (virus-free sample) 60 min after addition of substrate.

The sensitivity of ACMV detection in cassava leaves was about the same by the direct and indirect ELISA methods. When serial dilutions of cassava crude sap

<table>
<thead>
<tr>
<th>Sap dilutions</th>
<th>Direct ELISA (45 min)</th>
<th>Indirect ELISA (60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>H</td>
</tr>
<tr>
<td>1/2</td>
<td>1.77</td>
<td>0.14</td>
</tr>
<tr>
<td>1/8</td>
<td>1.89</td>
<td>0.16</td>
</tr>
<tr>
<td>1/32</td>
<td>1.52</td>
<td>0.15</td>
</tr>
<tr>
<td>1/128</td>
<td>0.73</td>
<td>0.13</td>
</tr>
<tr>
<td>1/512</td>
<td>0.32</td>
<td>0.14</td>
</tr>
<tr>
<td>1/2048</td>
<td>0.19</td>
<td>0.15</td>
</tr>
</tbody>
</table>

D = sap of diseased cassava
H = sap of healthy cassava

For direct ELISA plates coated with 1 μg/ml IgG were incubated for 2 h at 37 °C and the IgG-conjugate was diluted at 1/4,000. Indirect ELISA was done as described in Material and Methods. O.D. at 405 nm were read at 45 min (direct ELISA) and 60 min (indirect ELISA) after adding substrate.
were tested by the two methods, the detection end-point was the same: 1/512 (Table 2), although colour development occurred much faster by the direct method.

![Graph showing the effect of incubation time with IgG used for coating the plates in direct ELISA. Purified virus was incubated overnight at 4 °C; enzyme conjugate at 1/4,000 for 3 hours at 37 °C; readings of O.D. at 405 nm were at 60 min after addition of substrate.](image)

**Fig. 5.** Effect of incubation time with IgG used for coating the plates in direct ELISA: purified virus was incubated overnight at 4 °C; enzyme conjugate at 1/4,000 for 3 hours at 37 °C; readings of O.D. at 405 nm were at 60 min after addition of substrate.

![Bar chart showing ELISA response of different parts of cassava plants of two cultivars tested by direct ELISA.](image)

**Fig. 6.** ELISA response of different parts of cassava plants of two cultivars tested by direct ELISA. Plant tissues were crushed in a mortar using Tris-HCl 0.1 M buffer at pH 8.5 containing 1 % PVP (1 g/2 ml). Plates were coated with 1 μg/ml IgG and the conjugate was diluted 1/4,000. ELISA values were read 60 min after adding substrate.
It was not possible to use higher concentrations of reagents in the indirect method because of high background reactions with virus-free samples.

**Incubation times of IgG coating and enzyme conjugate**

The incubation times for coating the plates with IgG and adding enzyme conjugate were determined by the direct ELISA method using purified ACMV as described by McLAUGHLIN et al. (1981). When the plates were coated with IgG at 1 to 5 μg/ml, purified virus could be detected after one hour incubation at 37 °C (Fig. 5). When the IgG concentration was lower (0.2 μg/ml), the optimal incubation time was 4 to 5 hours. The optimal incubation time of enzyme conjugate at 1/4,000, was 3 hours at 37 °C (data not shown).

**Virus distribution in the cassava plant**

When different tissues of the plant were compared, cassava leaves were found to produce higher ELISA readings than the petiols or the stems. Furthermore the youngest leaves harvested at the top of the stems gave the best results (Fig. 6).

An experiment on the effect of the age of the leaves on the detection of the virus was carried out using three cassava cultivars (TMS 30572, Maloenda and Kokobassié) from which leaves to be tested were harvested from the top to the bottom of the stems. One cassava plant (TMS 30572) had two stems, one without symptoms and one with typical mosaic symptoms. In the leaves without symptoms no virus was detected by direct ELISA. In leaves presenting typical symptoms, the amount of viral antigen decreased from the top to the bottom of the stem (Table 3).

**Table 3**

ACMV detection by direct ELISA in different parts of infected cassava leaves

<table>
<thead>
<tr>
<th>Cassava leaves</th>
<th>Top</th>
<th>Bottom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>T30572*</td>
<td>0.24</td>
<td>0.20</td>
</tr>
<tr>
<td>T30572*</td>
<td>1.16</td>
<td>1.02</td>
</tr>
<tr>
<td>Maloenda*</td>
<td>&gt; 2</td>
<td>1.91</td>
</tr>
<tr>
<td>Koko*</td>
<td>&gt; 2</td>
<td>1.99</td>
</tr>
</tbody>
</table>

* without symptoms
* with mosaic symptoms
** these leaves showing symptoms (1 to 4) belong to one stem of the same plant as that apparently healthy (first line in the table)

Cassava sap (1 g leaves/2 ml grinding buffer) was incubated overnight at 4 °C on plates coated with 1 μg/ml IgG. The conjugate was diluted 1/4,000. O.D. was read 60 min after addition of substrate

**Discussion**

Geminivirus particles occur in two forms, single monomer particles and paired particles (BOCK 1982, GOODMAN 1981, HARRISON 1985). LARSEN and DUFFUS (1984) showed that the monomeric and dimeric forms of Curly Top Virus
could be separated by gradient centrifugation. Using their virus purification method, it was possible to separate single monomer from the paired particles of African cassava mosaic virus, although complete separation was not obtained since paired and monomer particles were still mixed in the lower peak (Fig. 1). Electron microscopy of the material of the upper peak showed only the presence of monomer particles. Such a separation was not achieved in earlier studies of geminivirus isolated from cassava. Bock et al. (1978) obtained three light-scattering zones on sucrose gradients: the upper zone contained fragmented material which was not infectious, while both middle and lower zones contained numerous paired particles and were infectious. The lower zone contained “pairs of pairs” of aggregated virus particles. The same authors did not obtain paired-particle virus from the “coastal strain”.

In our experiments, monomer particles were obtained in the case of virus extracted from cassava cultivars TMS 30211 or Kokobassié. With other cultivars such as Ouanga or TMS 30572, or with M. glaziovii, we only obtained dimer particles. The monomer particles we observed may have resulted from the virus purification method used since geminivirus particles are not always stable, especially in high salt concentration (Abouzid and Jeske 1986).

Attempts made to test the infectivity of monomer particles on healthy cassava plants failed, as did all trials to transmit the virus back to cassava plants by mechanical inoculation or by microinjection (Dick and Longman 1985).

Our results show that when the virus is kept frozen at -20 °C or at 4 °C when additioned with NaN3, it is possible to stock purified ACMV for more than 145 days without loss of infectivity or antigenic activity.

Both direct and indirect ELISA methods can be successfully applied to the detection of ACMV in cassava leaves. Since geminivirus particles occur in cassava at a relatively low concentration (Walter 1980, Robinson et al. 1984) and because of the presence of plant inhibitors in cassava (Sequeira and Harrison 1982, Thouvenel et al. 1984, Fargette et al. 1987) virus detection in plant sap by ELISA may present problems. By using young leaves and an appropriate grinding buffer these difficulties can be overcome and virus detection can be readily achieved in cassava crude sap. The ELISA method should be used for detecting virus in the field in conjunction with a search for resistance to ACMV in cassava cultivars.

We thank Dr. M. Wurtz (Biozentrum, Basel) for the help with electron microscopy, Mr. G. Stocky (INRA, Colmar) for technical assistance and also Dr. M. H. V. Van Regenmortel for helpful discussions. We also thank the European Economic Community, the Government of Congo and INRA for financial support.

**Literature**


African Cassava Mosaic Virus

AVRAMEAS, S., 1969: Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 6, 43—52.


LARSEN, R. C., and J. E. DUFFUS, 1984: A simplified procedure for the purification of Curly Top Virus and the isolation of its monomer and dimer particles. Phytopathology 74, 114—118.


WALTER, B., 1980: Isolation and purification of a virus transmitted from mosaic diseased cassava in the Ivory Coast. Pl. Disease 64, 1040—1042.
