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Virus content of leaves of cassava infected by African cassava mosaic virus

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SUMMARY

African cassava mosaic virus (ACMV) was detected in cassava leaves by ELISA. Some normal constituents of cassava leaves interfered with virus detection but leaf extracts of *Nicotiana benthamiana* did not. The symptom pattern was determined early in the growth of a leaf and subsequently changed little. ACMV was found only in the yellow or yellow green areas of the mosaic pattern. Virus content of the leaves increased with increasing symptom intensity, but decreased with leaf age and ACMV was not detected in mature leaves. Most whiteflies were found on young growing cassava leaves and the number decreased progressively with leaf age. This distribution will aid both the acquisition and inoculation of the virus.

INTRODUCTION

African cassava mosaic disease seriously decreases the yields of cassava (*Manihot esculenta* Crantz) crops in Africa. The causal agent, African cassava mosaic virus (ACMV), is transmitted by grafting and by the whitefly *Bemisia tabaci* (Storey & Nichols, 1938; Chant, 1958). The virus was first isolated in East Africa and named cassava latent virus (Bock, Guthrie & Meredith, 1978) and only later was it shown to be the cause of cassava mosaic and renamed (Bock & Woods, 1983). The same pathogen has been found in the Ivory Coast (Walter, 1980), Angola (Sequeira & Harrison, 1982) and Nigeria (Adejare & Coutts, 1982). It is a member of the geminivirus group (Harrison *et al.*, 1977; Bock *et al.*, 1978).

Although the localisation within plant tissues of several viruses causing diseases of the mosaic type has been extensively studied (Matthews, 1973; 1980), there is little information about the location and concentration of ACMV in the infected leaf in relation to symptom development and stage of growth of the leaf. However, ACMV concentration in cassava has been assessed by ELISA (Sequeira & Harrison, 1982; Thouvenel, Fargette, Fauquet & Monsarrat, 1984).

MATERIALS AND METHODS

Leaf tissue. Experiments with cassava were done with leaves from the CB cultivar. Plants derived from healthy cuttings were subsequently infected by whiteflies. Leaf position was counted from the youngest unfolded leaf (12 cm mean leaflet length) downward to the older leaves. Symptom intensity was divided into five classes by the following adaptation of Cours' scale (Cours, 1951): 0, no symptoms; 1, slight mosaic covering less than 20% of the leaf area without leaf distortion or size reduction; 2, mosaic covering less than 50% of the leaf area without obvious size reduction but with some distortion; 3, mosaic covering most of the leaf surface with leaf distortion and some leaf reduction; 4, well developed mosaic all over the leaf with blistering, severe distortion and stunting either all over the leaf or mainly in the basal area.

Preparation of antiserum. The ACMV isolate was obtained from diseased cassava plants of the ORSTOM Experimental Station of Adiopodoumé (Ivory Coast), propagated in *Nicotiana benthamiana* and purified by the procedure of Walter (1980). A rabbit was given six subcutaneous injections of 1–2 mg of virus fixed in 1% glutaraldehyde and mixed with an equal volume of Freund's incomplete adjuvant. The antiserum had a titre of 1/256 when tested by agar double diffusion.

Enzyme-linked immunosorbent assay (ELISA). Partially purified immunoglobulins (Ig) were prepared by precipitation with ammonium sulphate and dialysis against half-strength phosphate buffered saline (Phosphate-buffered saline (PBS) = 0.15 M Nacl in 0.02 M phosphate buffer, pH 7.4). The Ig at 1 mg/ml total protein (A_{280} = 1.4) was conjugated with alkaline phosphatase (Type VII, Sigma Chemical Co., St Louis, MO) at an enzyme/Ig ratio of 2:1 (w/w) using 0.06% glutaraldehyde. After dialysis and addition of 5 mg/ml of bovine serum albumin, the conjugate was stored at 4°C.

Methods for ELISA were essentially as described by Clark & Adams (1977) although we found that using longer incubation times and more concentrated reagents for some stages of the procedure gave a greater distinction between infected and healthy samples. Wells in Microelisa plates were coated by incubating 200 µl of unlabelled Ig diluted 1: 100 in 0.05 M sodium carbonate, pH 9.6 at 37°C for 3 h. After incubation, the plates were rinsed three times with distilled water. Samples were prepared by grinding leaves with a mortar and pestle in PBS, containing 0.05% Tween 20, 2% polyvinylpyrrolidone (PVP, mol. wt 40 000) (PBS-Tween-PVP) at a ratio of 1 g of leaf material in 5 ml of buffer. Sometimes, leaf extracts were clarified by adding 1 ml of chloroform to 3 ml of leaf extract, shaking for 5 min and collecting the supernatant fluid after centrifuging for 5 min at 4000 *g*. Antigen preparations were incubated overnight at 6°C in the coated, rinsed wells. After further rinsing, antibody conjugate, diluted 1/500 in a buffer mixture containing PBS, 0.05% Tween 20, 2% PVP and 0.02% ovalbumin was added and incubated for 6 h at 37°C. Unreacted conjugate was removed by rinsing, and specific antibody-antigen reactions were assessed by adding 250 µl p-nitrophenyl phosphate at 1 mg/ml in 1 M diethanolamine buffer pH 9.8. Reactions were stopped after 4 h by adding 50 µl of 3 M sodium hydroxide per well. Absorbance at 405 nm was measured using a Titertek Multiskan spectrophotometer. We considered an A_{405} value twice that of the background to be positive.

RESULTS

ELISA response curves. Fig. 1 plots A_{405} values against dilution for purified ACMV, serially diluted in PBS and Fig. 2 plots A_{405} values for infected *N. benthamiana* and cassava (*Manihot esculenta*) leaf extracts. With purified preparations and infected *N. benthamiana* tissue extracts, A_{405} values decreased with dilution and reached the background (value obtained with healthy *N. benthamiana*) at dilution 1/1024. By contrast, with infected cassava leaf extracts, the A_{405} values initially increased with dilution to reach a maximum at 1/4 (Fig. 2); the values then decreased to reach the background (value for healthy cassava leaf extract) at 1/256. Absorbances for healthy *N. benthamiana* and cassava were about 0.07 irrespective of dilution. These results suggested that ELISA was inhibited by cassava sap and experiments were designed to investigate this effect.

Effect of chloroform clarification of cassava extracts on ELISA inhibition

Fourfold serial dilutions of samples from young infected cassava leaves, clarified with chloroform or unclarified, were tested by ELISA.

In eight of nine tests, the A_{405} values for an unclarified extract increased when the sample was diluted two to four times. Fig. 3 illustrates a typical result. Chloroform treatment increased the absorbances for undiluted extracts from 0.38 to 0.84 in Fig. 3 and from a mean of

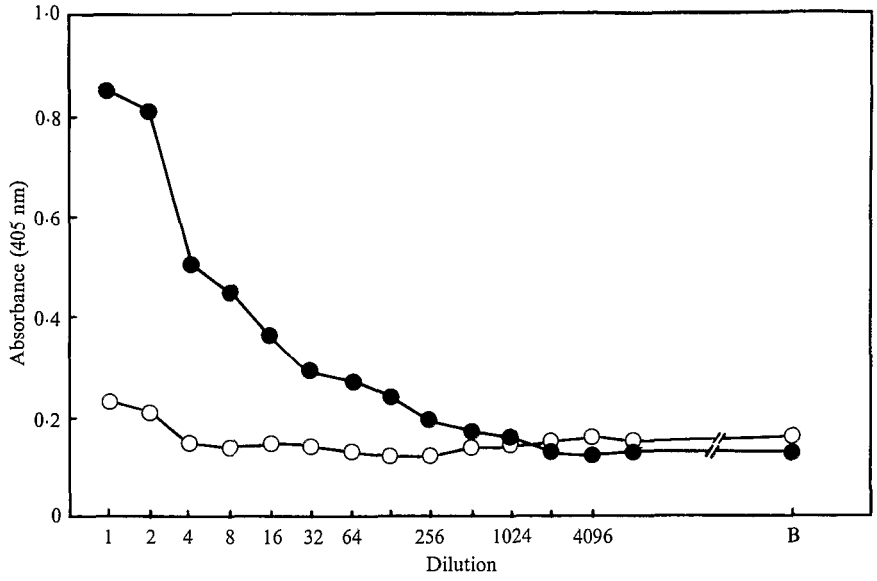

Fig. 1. Absorbances (405 nm) obtained in ELISA with a two fold dilution series in PBS of ACMV purified from *Nicotiana benthamiana* (●) and of concentrated healthy *N. benthamiana* leaf extracts (○). B indicates the absorbances obtained with PBS.

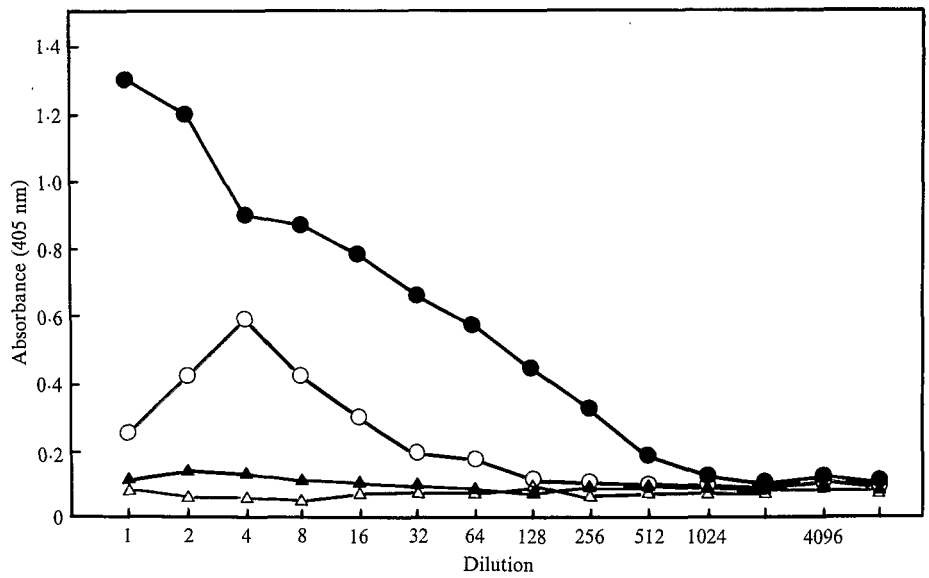

Fig. 2. Absorbances (405 nm) obtained in ELISA with a two fold dilution series in PBS of extracts of infected (●) and healthy (▲) *N. benthamiana* leaves and infected (○) and healthy (△) cassava (*M. esculenta*) leaves.

0.27 to 0.59 for the nine replicates. Chloroform treatment was more effective than extraction in PBS-Tween-PVP together with 1 mg/ml bentonite or with 1 mg/ml nicotinic acid, or clarification by butanol-chloroform (1 part butanol:9 parts chloroform) or 1% triton. By contrast, chloroform treatment did not increase the A_{405} values for infected *N. benthamiana* tissue extracts.

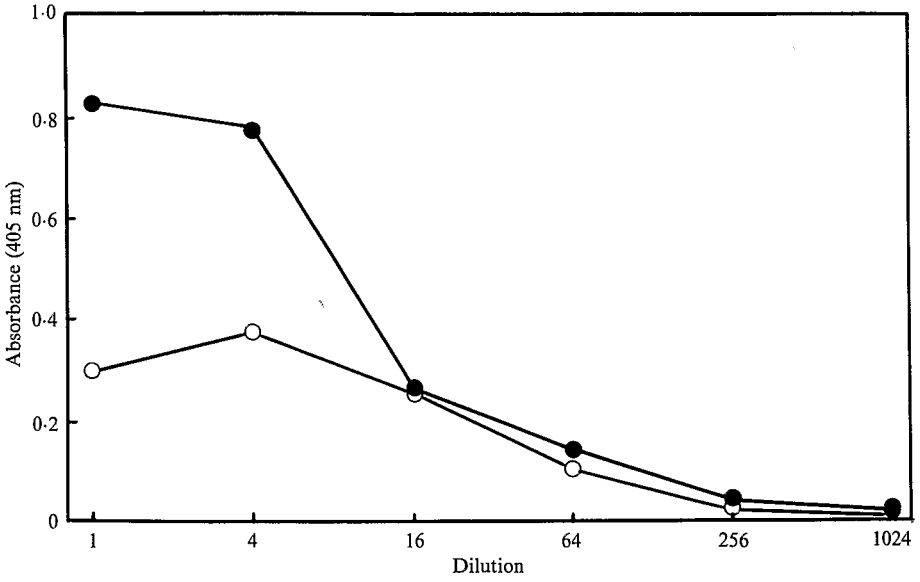

Fig. 3. Absorbances (405 nm) obtained in ELISA with a four fold dilution series of ACMV-infected cassava leaf extracts either clarified with chloroform (●) or unclarified (○).

In seven of the nine tests, the maximum absorbance for clarified extracts was given by undiluted extracts (Fig. 3), although the slope of the dilution curve was still shallower than that for *N. benthamiana*. In two instances, although clarification increased the absorbances, maximum values were still obtained after some dilution.

In another experiment, diseased cassava leaf extract, clarified with chloroform, was mixed with an equal volume of healthy cassava leaf extract before testing by ELISA. Healthy cassava leaf extracts were either clarified with chloroform or unclarified and were used at a series of dilutions in extraction buffer. Unclarified sap had a much greater inhibitory effect than clarified sap (Table 1). The amount of inhibition caused by unclarified sap decreased with dilution until at 1/256 its effect was similar to clarified sap for which the amount of inhibition was little affected by dilution.

Effect of leaf age on inhibition. The inhibitory effects of young growing healthy cassava leaves (leaves 2 and 3) and old matured ones (leaves 20–25) were compared. The A_{405} values were decreased about equally by extracts from young and old leaves, dropping from 1.58 to 0.21

Table 1. *Absorbances (405 nm) obtained in ELISA when testing mixtures of one volume of clarified diseased cassava leaf extract with one volume of healthy cassava leaf extract diluted in extraction buffer*

Type of healthy extract*	Further dilution of healthy extract					
	1/1	1/4	1/16	1/64	1/256	Control†
Unclarified	0·20‡	0·31	0·49	0·70	0·94	1·58
Clarified	0·75	1·09	1·03	1·07	0·98	

* Extracts were prepared by grinding 1 g of tissues in 5 ml extraction buffer and were then either left unclarified, or clarified by shaking with chloroform and centrifuging.
† Control wells received one volume of diseased leaf extract mixed with one volume of extraction buffer.
‡ Each value is the mean of four experiments.

when young leaf extracts were added to diseased extracts and to 0.18 by extracts of old leaves (mean of two experiments).

Symptom expression. In one experiment, the development of symptoms from the young shoot stage until leaf fall was followed, in one leaf of each of 25 diseased cassava plants. Symptoms became obvious when the length of the leaflet was on average 9 cm and were classified on the modified Cours scale. Although these leaves were still growing, the symptoms on them did not intensify and the correlation between the first recorded symptom score and that 20 days later was 0.96. Different leaves on the same shoot often showed symptoms ranging from undetectable to severe.

Virus content and leaf age. In one experiment, clarified and unclarified extracts of the first 12 leaves of a plant were tested and, in a second, we tested clarified and unclarified extracts of the first four, the sixth and every fourth leaf thereafter of a plant up to the 34th (Table 2). Finally, we tested unclarified extracts of the first 12 leaves of another four 10–month-old and two 3–month-old cassava plants (data not shown). In all plants, ACMV was detected in unclarified extracts of at least the first three leaves, in three out of eight plants down to the fourth, and in one plant down to the sixth leaf. In six plants out of eight, the greatest absorbance was obtained with leaf 1, in one plant with leaf 2 and in one plant with leaf 3.

After clarification, ACMV was detected in more of the older leaves, e.g. in leaves 4, 5, 6 and 7 of plant 1. Leaf size was estimated from the length of the longest leaflet, which is strongly correlated with the surface area of the leaf (Hammer, 1980). Maximum length is usually reached at leaf 4, although it could vary among individuals between leaf 3 to 5. The rate of growth is steeper between leaf 1 and 2 than in older leaves.

Table 2. *Absorbances (405 nm) in ELISA of extracts from different leaves of two cassava plants*

Leaf position*	Leaflet length (cm)†	Plant 1		Plant 2	
		Clarified	Unclarified	Clarified	Unclarified
1	13·1	0·87‡	0·40	0·21	0·09
2	16·8	0·81‡	0·23	0·25	0·14
3	17·6	0·59	0·23	0·31	0·11
4	18·8	0·27	0·03	0·25	0·05
5	19·1	0·27	0·05	—	—
6	19·2	0·25	0·01	0·12	0·02
7	19·2	0·37	0·02	—	—
8	18·4	0·16	0·01	—	—
9	20·0	0·23	0·03	—	—
10	19·5	0·17	0·01	0·12	0·01
11	20·1	0·17	0·01	—	—
12	20·2	0·09	0·01	—	—
14	19·7	—	—	0·14	0·01
18	19·2	—	—	0·14	0·01
22	19·9	—	—	0·10	0·01
26	20·1	—	—	0·11	0·01
30	—	—	—	0·11	0·02
34	—	—	—	0·11	0·03
Healthy		0·14	0·03	0·14	0·03

* Leaf 1 is the youngest unfolded leaf

† Leaflet length was measured on healthy cassava plants from leaves of equivalent leaf position, using the longest leaflet from the leaf. Values are the means of 10 such measurements.

‡ A_{405}; each extract was divided and half clarified by chloroform treatment before testing.

— not tested.

Table 3. *Absorbances (405 nm) in ELISA for extracts from yellow and green parts of infected cassava leaves. Each sample pair (1–13) came from a different leaf. Green areas from virus-free leaves were also tested (H1, H2, H3)*

Sample pair number	Unclarified		Clarified*	
	Yellow	Green	Yellow	Green
1	0·09	0·03	0·37	0·20
2	0·07	0·02	0·41	0·20
3	0·08	0·04	0·28	0·25
4	0·17	0·04	0·31	0·18
5	0·08	0·01	0·29	0·16
6	0·27	0·04	0·47	0·16
7	0·18	0·01	0·27	0·13
8	0·10	0·01	0·49	0·18
9	0·12	0·04	0·37	0·10
10	0·17	0·03	0·54	0·10
11	0·11	0·04	0·24	0·14
12	0·10	0·02	0·36	0·16
13	0·07	0·04	0·41	0·15
Mean	0·12	0·03	0·37	0·16
H1		0·04		0·15
H2		0·05		0·09
H3		0·03		0·15

* Each sample (1 g of leaf material in 5 ml of buffer) was divided and one half clarified by shaking with chloroform and centrifuged before testing.

In other experiments, ACMV was detected in rare instances (2/20) in apices and in very young leaves although some did not show symptoms.

Virus distribution within leaves showing mosaic patterns. Leaves from position 2 with symptoms of class 1 and class 2 (see Materials and Methods) were collected. Yellow areas and dark green areas were separated and extracts from each tested by ELISA to assess the relative virus content (Table 3). Without clarification, ACMV was detected in the yellow areas of 11 of the 13 samples tested. After clarification, ACMV was detected in the yellow areas of all samples. ACMV was not detected in any unclarified extracts of dark green areas but was detected in one of 13 clarified extracts (sample pair 3).

Relation between symptom intensity and A_{405} in ELISA. Leaves from position 2 were collected, their symptoms recorded and the relative virus content assessed by ELISA. For each symptom class, 15 samples were tested. ACMV was detected in most leaves with symptoms, even those in class 1. Indeed ACMV was even detected in one of the 15 symptomless leaves. There was a close relation (correlation = 0.98) between symptom intensity and the mean A_{405} value (Fig. 4). However, individual values differed greatly and there was much overlap between one class and another as indicated by the confidence interval (Fig. 4). For individual samples the correlation between symptom intensity and absorbance was 0.69, which means that 47% (R^2%) of the variation of the absorbance could be explained by the variation of symptom intensity.

The experiment was repeated with 25 extracts treated with chloroform. Fig. 5 shows that absorbances were lower than in former experiments with unclarified extracts because another, less reactive conjugate was used. There was however, a close relation between symptom intensity and the mean absorbance for each class (correlation = 0.98).

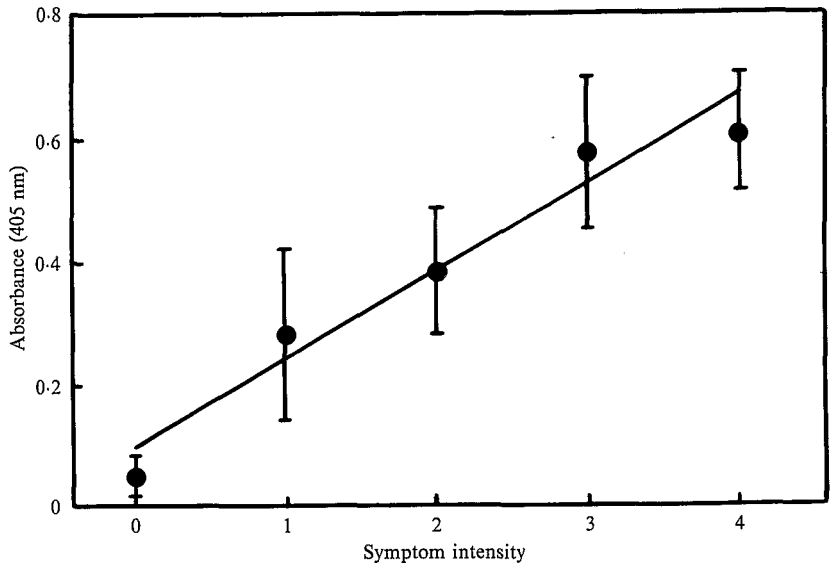

Fig. 4. Mean absorbances (405 nm) obtained in ELISA with unclarified extracts of cassava leaves with symptom intensity ranging from class 0 to 4. Each point is the mean of 15 samples and the confidence intervals are indicated.

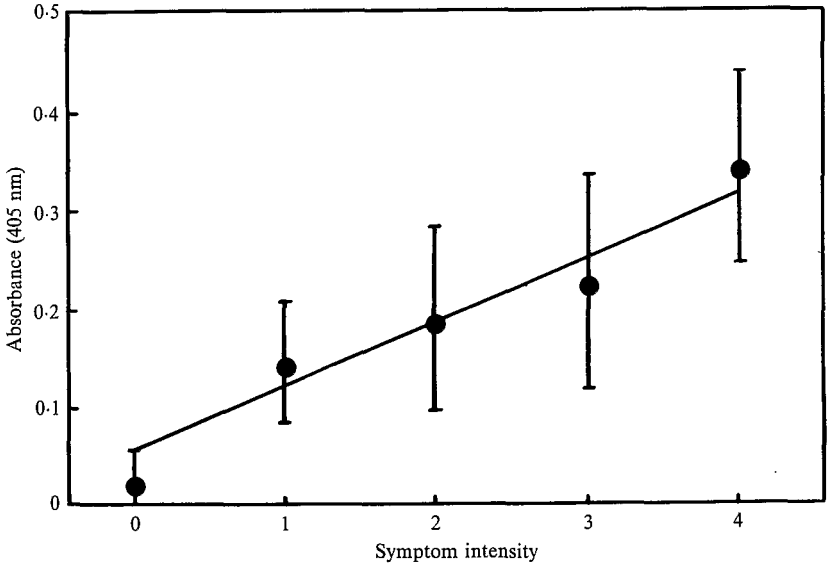

Fig. 5. Mean absorbances (405 nm) obtained in ELISA with clarified extracts of cassava leaves with symptom intensity ranging from class 0 to 4. Each point is the mean of 5 samples and the confidence intervals are indicated.

Distribution of Bemisia *on cassava plants.* The numbers of *Bemisia* on cassava leaves from the tips to the old mature leaves on 25 cassava plants were counted. The experiment was repeated with 4–, 5– and 6–month-old cassava plants. Most whiteflies were found on young growing leaves and progressively fewer were found on older leaves (Table 4). No *Bemisia* were found on leaves 9 to 25.

Table 4. *Number of whiteflies* (Bemisia tabaci) *on cassava leaves of different ages*

	Leaf number*										
Age of plant	−1	0	1	2	3	4	5	6	7	8	9
4 months	4†	16	15	6	1	1	0	0	0	0	0
5 months	14	9	18	14	9	2	3	0	1	1	0
6 months	6	14	13	7	6	4	4	2	0	0	0

* Leaf 1 is the youngest unfolded leaf (mean leaflet length 12 cm); leaves 0 and −1 are younger and folded.
† Total number on 25 leaves; although all leaves up to leaf 25 were examined, no whiteflies were found on leaves older than leaf 8.

DISCUSSION

Many plants contain compounds which inhibit serological or enzymatic reactions (Clark, 1981), and such effects have been reported with cassava leaves (Sequeira & Harrison, 1982; Thouvenel *et al.*, 1984). These inhibitory effects occurred with both growing and matured cassava leaves but not with *N. benthamiana*. Sequeira & Harrison (1982) reported a greater inhibitory effect of extracts from older than younger leaves. Although inhibitory effects varied among samples, we did not observe this difference in our experiments. The inhibitors are normal constituents of cassava leaves and are not induced by virus infection. They greatly reduce ELISA absorbance values and prevent their translation into virus concentration. Although inhibition decreased with dilution, it persisted in extracts diluted to 1/256.

Chloroform treatment of extracts decreased the inhibitory effects but did not completely remove them. Although the background reaction was increased, clarification allowed ACMV to be detected in some samples which gave a negative reaction with unclarified extract. Chloroform treatment was the most effective of the treatments tested.

ACMV concentration decreased with increasing leaf age and the virus was not detected in mature leaves (more than about 10 days after unfolding). By contrast, cassava brown streak virus may reach a greater concentration in mature cassava leaves than it does in young ones (Lister, 1959).

Symptom severity in very young leaves (leaflet length < 9 cm) was difficult to assess, but ACMV was detected in some young leaves that seemed to be symptomless. However, symptoms were easily classified 2 days before the stage of leaf 1, and they remained unchanged, except for general enlargement, as the leaf grew older. Dark green areas in the mosaic pattern of diseased leaves contained very little ACMV compared to the yellow or yellow green areas. These results are consistent with those of Robinson, Sequeira & Harrison (1983) obtained with the Angolan ACMV isolate. Early establishment of the symptom pattern and restriction of virus to the yellow areas of the mosaic pattern is a feature of diseases of the mosaic type (Matthews, 1973).

Increase in severity of symptoms is sometimes but not always associated with increased virus production (Matthews, 1981). In cassava, ACMV concentration is positively correlated with symptom intensity. Leaves produced during the dry season show no symptoms or only very slight ones (Dubern, 1976; Fauquet, Fargette, Thouvenel & Monsarrat, 1983). Sequeira & Harrison (1982) found that symptoms in *N. benthamiana* were less severe and ACMV concentration much less at 30°C than at 20–24°C.

Potency of sources of infection and vector transmission efficiency are critical for virus spread (Thresh, 1980). Epidemics of ACMV are characterised by rapid virus spread in healthy cassava fields caused by incoming viruliferous whiteflies (Fargette, Fauquet & Thouvenel, 1985). Young cassava leaves not only contain more virus but also are more susceptible to

infection that mature ones (Storey & Nichols, 1938; Dubern, 1976), so the prevalence of *Bemisia* on the young growing leaves of cassava will help both acquisition and inoculation and thus the field spread of ACMV.

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REFERENCES

ADEJARE, G. O. & COUTTS, R. H. A. (1982). The isolation and characterisation of a virus from Nigerian cassava plants affected by the cassava mosaic disease and attempted transmission of the disease. *Phytopathologische Zeitschrift* **103,** 198–210.

BOCK, K. R. & WOODS, R. D. (1983). Etiology of African cassava mosaic disease. *Plant Disease* **67**, 994–995.

BOCK, K. R., GUTHRIE, E. J. & MEREDITH, G. (1978). Distribution, host range, properties and purification of cassava latent virus, a geminivirus. *Annals of Applied Biology* **90**, 361–367.

CHANT, S. R. (1958). Studies on the transmission of cassava mosaic virus by *Bemisia* spp. (Aleyrodidae). *Annals of Applied Biology* **46**, 210–215.

CLARK, M. F. (1981). Immunosorbent assays in plant pathology. *Annual Review of Phytopathology* **19**, 210–215.

CLARK, M. F. & ADAMS, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475–483.

COURS, G. (1951). Le manioc à Madagascar. *Mémoires de l'Institut Scientifique de Madagascar* **3**, 203–416.

DUBERN, J. (1976). La mosaïque du manioc: bilan des connaissances actuelles. *Rapport, Office de la Recherche Scientifique et Technique Outre-Mer*, 29 pp.

FARGETTE, D., FAUQUET, C. & THOUVENEL, J-C. (1985). Field studies on the spread of African cassava mosaic virus. *Annals of Applied Biology* **106**, 285–294.

FAUQUET, C., FARGETTE, D., THOUVENEL, J-C. & MONSARRAT, A. (1983). Etude de la mosaïque africaine du manioc. *Rapport, Office de la Recherche Scientifique et Technique Outre-Mer*, 28 pp.

HAMMER, G. L. (1980). Estimation of cassava leaf area by a simple, non-destructive field technique. *Journal of Australian Institute of Agricultural Science* **46**, 61–62.

HARRISON, B. D., BARKER, H., GUTHRIE, E. J., MEREDITH, G. & ATKINSON, M. (1977). Plant viruses with circular single-stranded DNA. *Nature, London* **270**, 760–762.

LISTER, R. M. (1959). Mechanical transmission of cassava brown streak virus. *Nature, London* **183**, 1588–1589.

MATTHEWS, R. E. F. (1973). Induction of diseases by viruses with special reference to turnip yellow mosaic virus. *Annual Review of Phytopathology* **11**, 147–170.

MATTHEWS, R. E. F. (1980). Host plant responses to virus infection. In *Comprehensive Virology: virus-host interactions*, pp. 297–359. Eds H. Fraenkel-Conrat and R. R. Wagner. New York: Plenum Press.

MATTHEWS, R. E. F. (1981). *Plant Virology, Second edition.* Academic Press, London and New York. 897 pp.

ROBINSON, D. J., SEQUEIRA, J. C. & HARRISON, B. D. (1983). Identification of viruses in relation to diseases of other crop plants: cassava latent virus. *Report of the Scottish Crop Research Institute for 1982*, 193–194.

SEQUEIRA, J. C. & HARRISON, B. D. (1982). Serological studies on cassava latent virus. *Annals of Applied Biology* **101**, 33–42.

STOREY, H. H. & NICHOLS, R. F. W. (1938). Studies on the mosaic diseases of cassava. *Annals of Applied Biology* **25**, 790–806.

THOUVENEL, J-C., FARGETTE, D., FAUQUET, C. & MONSARRAT, A. (1984). Serological diagnosis of African cassava mosaic by immunoenzymatic method. *Proceedings of the sixth International Tropical Root Crops Symposium.* 21–23 Feb. 1983. Lima. Peru. pp. 353–356.

THRESH, J. M. (1980). An ecological approach to the epidemiology of plant virus diseases. In *Comparative Epidemiology*, pp. 57–70. Eds J. Palti and J. Kranz. Wageningen: Pudoc.

WALTER, B. (1980). Isolation and purification of a virus transmitted from mosaic diseased cassava in the Ivory Coast. *Plant Disease* **64**, 1040–1042.

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