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Full Length Research Paper

Limitation in detecting *African cassava mosaic geminivirus* in the lignified tissues of cassava stems

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Field-infected cassava stems whose leaves were identified with differential symptoms of *African cassava mosaic geminivirus* (ACMV) disease were assessed for index of severity of symptoms (ISS) to determine their infection status by scoring young resultant plants in the laboratory. Extracts of young stem tissues and leaves of emergent shoots from topped stems were also assayed for occurrence of ACMV by the enzyme-linked immunosorbent assay (ELISA). Geminivirus disease incidence and severity of symptoms were most abundant on leaves of plants from cuttings of field-symptomatic (I) stems of the moderately-resistant TMS 4(2)1425 (63.2%) and the susceptible TMS 60506 (51.1%) genotypes while those from apparently symptom-free (H) stems of the resistant genotype TMS 30001 recorded significantly the least disease (0%). Similarly, the index of severity of symptoms on all plants (ISS_{AP}) and diseased plants only (ISS_{DP}) were significantly highest on leaves of TMS 4(2)1425 (2.42 and 2.83), and were significantly the least (1) on leaves of TMS 30001, respectively. ACMV was not detected in extracts of all sections of lignified cassava stems by ELISA as all absorbance values were below threshold (0.0890). However, the virus was detected at greater concentrations in leaves of emergent axillary shoots regenerating on topped plants of all node types of TMS 60506 and TMS 4(2)1425 as well as the base (node 1) of TMS 30001 stems but not on those from middle and uppermost nodes (10 and 20, respectively) of the latter genotype. The highest absorbance values were recorded on shoots on node 1 of TMS 60506 (0.1720 ± 0.096), TMS 4(2)1425 (0.1640 ± 0.115) and TMS 30001 (0.1580 ± 0.080) in that order, while the least values were on nodes 10 (0.0298 ± 0.020) and 20 (0.0289 ± 0.019) of TMS 30001.

Key words: *African cassava mosaic geminivirus*, infection status, cassava genotypes, detection limitations, enzyme-linked-immunosorbent assay.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz subsp. *esculenta*) is a semi-woody perennial plant and staple crop with great economic importance and the only edible cultivated dicotyledonous species in the genus *Manihot* (family Euphorbiaceae), where its evolutionary and geographical origins have remained both unresolved and controversial (Olsen and Schaal, 1999).

Geminiviruses, which belong to the family Geminiviridae, are small plant viruses with circular single- stranded DNA (ssDNA) genomes encapsidated in twinned (geminate) particles (Harrison, 1985). The begomoviruses constitute the largest genus of the family and the vast majority of its members that infects dicotyledonous plants, are whitefly-transmitted and have bipartite genomes DNAs A and B (Rybicki et al., 2000). DNA A encodes all viral functions required for replication, control of gene expression and encapsidation (Stanley, 1983). The second genome, DNA B, encodes two products involved in movement of the virus between and within plant cells (Noueiry et al., 1994; Lazorowitz et al., 2003. Begomoviruses are mainly distributed in tropical and subtropical regions where the whitefly vector *Bemisia tabaci* Genn (Figure 1a) is prevalent (Figure 1b) and dis-

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Figure 1a. Two whiteflies [*Bemisia tabaci*: the vector of cassava mosaic geminiviruses (CMGs)] feeding under a cassava leaf.

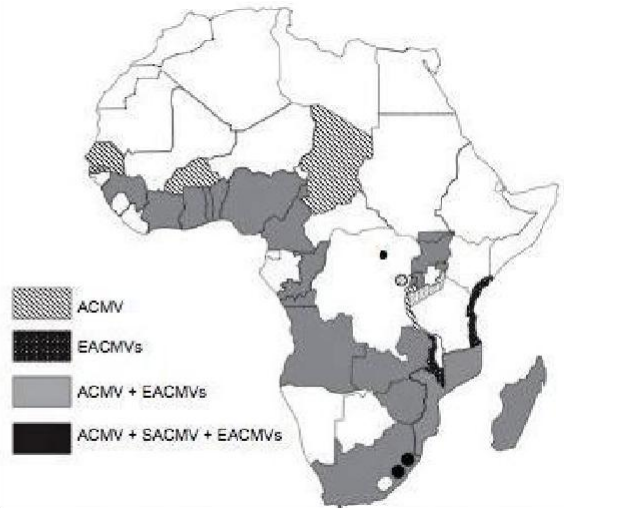

Figure 1b. Known distribution of cassava mosaic geminiviruses (CMGs) and the whitefly vector *Bemisia tabaci* in Africa (Legg and Thresh, 2002).

eases caused by them are important constraints on crop production (Bock, 1982). African cassava mosaic Geminivirus (ACMV) affects seven species of *Manihot* (Fargette et al., 1994) and is transmitted by *B. tabaci*. ACMV is distributed in vegetative propagules and causes the most prevalent and economically-important disease of cassava in Africa (Hahn et al., 1980). Furthermore, the whitefly vector has the most important role of virus dissemination and ACMV became the most important vector-borne disease of any crop in Africa recently (Thresh et al., 1994). Several control options are available (Thresh, 1987) of which the use of resistant and tolerant genotypes has received the greatest attention (Thresh and Otim-Nape, 1994; Thresh et al., 1994), however the nature of the resistance is still not understood (Jennings, 1994). Immunity to ACMV has not been reported (Jennings, 1994). However, ACMV does not become fully systemic in resistant genotypes (Hahn et al., 1989; Rossel et al., 1992), and uninfected cuttings can therefore be obtained from the stems of infected plants (Fauquet et al., 1988) . In resistant genotypes the virus seems to occur mainly towards the base of shoots indicating that uninfected cuttings for use as planting material could be obtained from shoot tips (Cours-Darnes, 1968; Jennings, 1994). Spread within and between plantings of resistant varieties is relatively slow (Thresh et al., 1994), and some cuttings propagated from infected plants may produce healthy progeny (Storey and Nichols, 1938; Jennings, 1960, 1994; Cours-Darnes, 1968; Pacumbaba, 1985; Rossel et al., 1992; Fauquet et al., 1988). Modeling studies suggest that this reversion is of great significance in decreasing disease progress and losses sustained by infected plants (Fargette et al., 1994; Fargette and Vie, 1994, 1995).

Posttranscriptional gene silencing (PTGS) is a sequence-specific defence mechanism that can target both cellular and viral mRNAs for degradation and is widely used as a tool for inactivating gene expression (Baulcombe, 1999; Vance and Vaucheret, 2001). Both transgenes and viruses can induce gene silencing in plants, and it has been proposed as a natural defense mechanism against virus accumulation (Hamilton and Baulcombe, 1999). The process is initiated by double-stranded RNA (dsRNA) or by aberrant RNAs, which become dsRNA by host-encoded RNA-dependent RNA polymerase activity (Waterhouse et al., 2001; Dalmay et al., 2000; Ahlquist, 2002). These dsRNAs are cleaved by dicer-like enzymes into short interfering RNAs (siRNAs) of between 21 - 26 nt (Tang et al., 2003) in length, which then promote RNA degradation by forming a multi-component RNA-induced silencing complex that destroys cognate mRNA (Tuschl et al., 1999; Zamore et al., 2000; Elbashir et al., 2001).

In this study, the infection ("health") status of field-ACMV infected cassava and detection of virus in tissue extracts of lignified stems were determined for cassava genotypes varying in susceptibility to ACMV. This was to understand field tolerance qualities of the genotypes and to assess sensitivity of the ELISA technique and its limitations in detecting ACMV in tissue extracts of lignified cassava stems.

MATERIALS AND METHODS

Virus status of cassava stems of different infection types

The origin and pedigree of cassava genotypes used in this study are given in Table 1. In order to investigate the virus status of infec-

Table 1. Resistance rating of cassava genotypes to *African cassava mosaic geminivirus.*

Cassava genotypes[a]	Pedigree	Origin	Resistance rating[b]
TMS 30001	Not available	IITA[c]	Resistant
TMS 4(2)1425	58308X Oyarugba funfun	IITA	Moderately resistant
TMS 60506	Locally improved clone	Moor plantation	Susceptible

[a]TMS = Tropical *Manihot* Selections.
[b]IITA, 1986: Resistance rating based on symptomatology.
[c]International Institute of Tropical Agriculture.

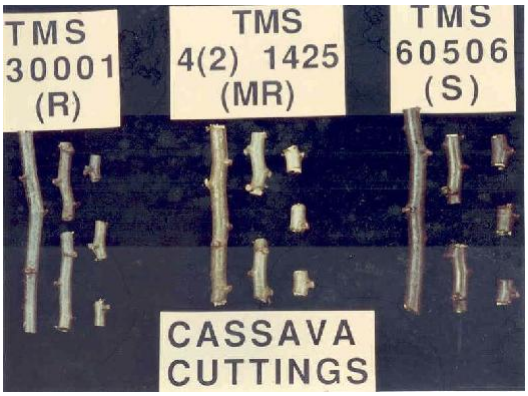

Figure 2. Single-node, three-node and multiple-node-cuttings of the resistant (R), moderately-resistant (MR) and susceptible (S) cassava genotypes ofTMS 30001, TMS 4(2)1425 and TMS 60506.

Figure 3. *African cassava mosaic virus*-infected cassava leaves depicting the disease (ACMD) severity scoring scale 1 - 5: 1 = no symptoms; 2 = a mild chlorotic pattern over the entire leaf while the latter appears green and healthy; 3 = a moderate mosaic pattern throughout the leaf, narrowing and distortion in the lower one-third of the leaflets; 4 = severe mosaic, distortion in two-thirds of the leaflets and general reduction in leaf size; and 5 = severe mosaic and distortion in the entire leaf.

ted and apparently disease-free stems under high disease pressure in the field, different infection types from which cuttings were collected were the following: plants which showed symptoms of infection on leaves of some or all branches were considered to be infected and designated (I); those which did not show symptoms and originated singly from a parent cutting were said to be apparently healthy (H). The third category comprised plants that did not show symptoms of infection but were from a common parent cutting like other infected stems designated (HI). Four stems, of known infection history therefore, of the three genotypes TMS 30001, TMS 4(2)1425 and TMS 60506 were collected from field plots at the IITA for each infection type per genotype. Three-to-four -node cuttings (Figure 2) were made from the stems and planted in rows 5 cm apart inside large trays (45 x 60 x 20 cm) containing sterile loam soil maintained in an insect-proof greenhouse. There were eight rows per tray and a row occasionally overlapped into another tray depending on the number of cuttings resulting from an infection type. There were thus a total of 36 units randomly assigned to rows in a completely randomized design. This procedure was replicated four times at different times with cuttings from different plants. Before planting, cuttings were dipped in 0.5% benomyl (Benlate, 50% WP Du Pont) to prevent fungal attack. They were watered and the trays covered with translucent polythene sheets to prevent desiccation. The sheets were, however, removed after four days when most cuttings had sprouted and the resultant plants which started emerging were subsequently watered about twice and sprayed with the insecticide endosulfan at 5 mL/L weekly to keep away potential contaminating whiteflies. The incidence, index of severity of symptoms (ISS) based on all plants (AP), whether or not they were showing symptoms, and on diseased plants (DP) only, were scored four weeks after planting. The calculations were based on the following formulae deduced from the disease severity scoring scale 1 - 5 (Hahn et al., 1989; Njock, 1994) (Figure 3).

a) Index of severity of symptoms based on all plants (ISS_{AP}):

$$ISS_{AP} = \left(\sum_{s=1}^{5} [SXs]\right) / \left(\sum_{s=1}^{5} [Xs]\right)$$

Where S is the severity class (1 - 5), X, the number of plants given the score S, and AP = all plants.

b) Index of severity of symptoms based on diseased plants (ISS_{DP}):

$$ISS_{DP} = \left(\sum_{s=2}^{5} [SXs]\right) / \left(\sum_{s=2}^{5} [Xs]\right)$$

Where DP = diseased plants.

c)

$$\text{Incidence (\%)} = 100 \{\left(\sum_{s=2}^{5} [Xs]\right) / \left(\sum_{s=1}^{5} [Xs]\right)\}$$

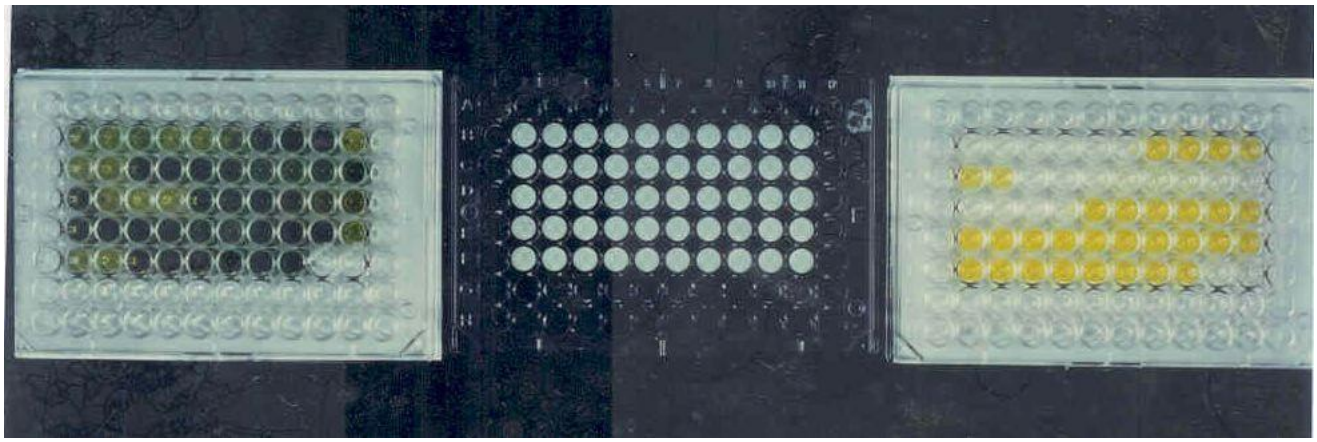

Figure 4. The enzyme-linked immunosorbent assay (ELISA): microtiter plates containing crude test samples (left), low-fat milk blocking (middle) and final antigenic reaction (right).

Figure 5. Potted cassava plants detopped (cut-back) at nodes 1, 10 and 20 from the base of each stem and allowed to regenerate new shoots.

Data collected for disease incidence and index of severity of symptoms based on all plants (ISS_{AP}) and diseased plants only (ISS_{DP}) was subjected to analysis of variance (ANOVA) by the general statistical package (GENSTAT 4) at IITA, Ibadan, Nigeria. Tables of effects and means were generated for each treatment. Treatments were separated using Duncan's multiple range test (DMRT) at 5% level of significance ($P > 0.05$).

Occurrence of ACMV in extracts of lignified cassava stem tissues

Infected cassava plantlets from the foregone experiment above were transplanted into 26 cm diameter (height, 25 cm) pots filled with dry-heat sterilized soil. Five plants were chosen for each of the genotypes TMS 30001, TMS 4(2)1425 and TMS 60506. This procedure was replicated four times giving a total of 20 plants per genotype. The plants were maintained for six months in an insect-proof greenhouse and sprayed weekly with the insecticide endosulfan. Three stems were collected per genotype from the 6 month old plants. Nodes on each stem were counted and the latter divided up into three equal sections based on the total number of nodes: the top (T), middle (M) and base (B). Node discs approximately 0.5 cm in length, were made from each node per section. Five discs were randomly selected per section per genotype, bulked, weighed, ground in a mortar, diluted 1:5 (w/v) with ACMV extraction buffer (50 mM Tris-Hcl, pH 8.0) and ACMV was assayed using double-antibody sandwich ELISA (Clark and Adams, 1977) (Figure 4). Purified gamma globulin from an ACMV antiserum with a titre of 1:28 in agar gel diffusion test, prepared at the Biotechnology Research Unit, IITA, Ibadan, Nigeria, was diluted 1:1000 (v/v) in coating buffer (0.05 M sodium carbonate containing 0.02% sodium azide, pH 9.6). Cassava plantlets derived from meristem culture and indexed for ACMV were used as virus-free controls. The enzyme reaction was assessed after two hours at room temperature with an ELISA reader (Dynatech MR 5000, USA). The experiment was repeated three times. Positive reactions were considered as those with absorbance (405 nm) values at least twice those of the virus-free controls.

Occurrence of ACMV in shoots regenerating from different nodes of the main stem of infected plants

For each genotype, the rest of the six-month-old plants maintained in the greenhouse, with the same level of disease severity, were topped (i.e. the upper parts of the shoots were cut off) at the base, middle and uppermost nodes corresponding approximately to nodes 1, 10 and 20 from the bottom of the stem (Figure 5). Four plants were topped per node position in each genotype. The cut-off portions were discarded and the original rooted material was maintained in the greenhouse for two weeks, during which time axillary shoots developed from the cut ends. Four two-leaf samples were randomly collected from each node position per genotype, bulked, ground in a mortar, diluted 1:5 (w/v) with ACMV buffer (50 mM Tris-Hcl, pH 8.0) and ACMV was assayed using ELISA as reported above.

Table 2. Occurrence of *African cassava mosaic geminivirus* disease on plants derived from cuttings of different infection types.

Cassava genotype[λ]	Infection type[+]	Disease incidence(%)	ISS_{AP}[!]	ISS_{DP}[†]
TMS 30001 (R)	I	18.3b	1.16a	1.81b
	H	0c	1.0b	nil
	HI	04.6c	1.03ab	1.25c
	Mean	7.63	1.06	1.53
TMS 4(2)1425 (MR)	I	63.2d	2.42c	2.83a
	H	34.3a	1.57c	2.51a
	HI	27a	1.37c	2.64a
	Mean	41.5	1.79	2.66
TMS 60506 (S)	I	54.1d	1.98c	2.57a
	H	14.9b	1.13ab	1.77b
	HI	05.5c	1.03ab	1.33c
	Mean	24.83	1.38	1.89

*Means followed by the same letter in a column among genotypes are not significantly different according to Duncan's multiple range test (P = 0.05).
[λ]R = resistant; MR = moderately resistant; S = susceptible.
[+]Infection types: I = symptomatic stems, H = apparently healthy stems growing singly and HI = apparently healthy stems growing together with other infected ones.
[!]ISS_{AP} = index of severity of symptoms on all plants.
[†]ISS_{DP} = index of severity of symptoms on diseased plants only.

RESULTS

Virus status of cassava stems of different infection types

The disease situation of plants derived from cuttings of cassava stems of different infection types is shown in Table 2. The highest disease incidence (63.2%) was recorded on plants from infected (I) stems of the genotype TMS 4(2)1425. This was not significantly different (P = 0.05) from that on plants of I stems of TMS 60506 (54.1%). The incidence on plants of H stems of TMS 4(2)1425 (34.3%) was quite high but not significantly different from that on plants of HI stems of the same genotype (27%). No disease (0%) was recorded on plants derived from cuttings of H stems of the resistant genotype TMS 30001. Indices of symptom severity calculated for all plants (and diseased plants only) of each genotype (ISS_{AP} and ISS_{DP}, respectively) followed a similar trend as disease incidence. Differences between cuttings from the different infection types were not significant for ISS_{AP} and ISS_{DP} when considering TMS 4(2)1425. Like for disease incidence, symptoms were not recorded either as ISS_{AP} (1.0) or ISS_{DP} (nil) on plants of cuttings of H stems of TMS 30001.

Occurrence of *African cassava mosaic geminivirus* in extracts of lignified cassava stem tissues

The concentration of virus from samples of lignified stem sections of the genotypes TMS 30001. TMS 4(2)1425 and TMS 60506 are shown in Table 3. Among genotypes, samples tested from the base stem section of TMS 4(2)1425 recorded the highest absorbance value (0.0320 ± 0.017) while the least value (0.0253 ± 0.010) was on samples from the middle stem section of TMS 30001. However, all samples from all stem sections of the genotypes and the virus-free controls showed negative enzymatic reactions relative to the threshold (0.089) considered as twice the mean of the virus-free control plus its standard deviation. The infected (positive) control (0.413), however, showed a positive enzymatic reaction about ten times greater than the threshold (Table 3).

Occurrence of ACMV in shoots regenerating from different nodes of the main stem of infected plants

ACMV was detected serologically in leaves from shoots developing at all three node locations along the stems of TMS 4(2)1425 and TMS 60506 although absorbance (405 nm) values tended to be highest in shoots from the most basal nodes (Table 4). Only shoots from the lowest nodes on stems of TMS 30001 contained detectable ACMV titers. There was no evidence of the presence of virus at nodes 10 and 20 of this genotype.

DISCUSSION

This paper describes greenhouse experiments in which *African cassava mosaic geminivirus* (ACMV) disease incidence and severity in different cassava genotypes were

Table 3. ELISA-detection of *African cassava mosaic geminivirus* in extracts of the lignified main stem of infected cassava genotypes differing in resistance to the virus.

Cassava genotypes[a]	Stem section	Absorbance (405 nm)[b] (Mean ± SD)	ELISA reaction (+ or -)[c]
TMS 30001 (R)	Top	0.026 ± 0.014	-
	Middle	0.025 ± 0.010	-
	Base	0.029 ± 0.014	-
TMS 4(2)1425 (MR)	Top	0.029 ± 0.011	-
	Middle	0.027 ± 0.011	-
	Base	0.032 ± 0.017	-
TMS 60506 (S)	Top	0.027 ± 0.014	-
	Middle	0.028 ± 0.013	-
	Base	0.032 ± 0.011	-
ACMV-free cassava (negative controls)		0.042 ± 0.031	-
ACMV-infected cassava (positive controls)		0.413 ± 0.211	+
Threshold		0.0890	none

[a]R = resistant; MR = moderately resistant; S = susceptible.
[b]Values are means of three tests; absorbance values more than twice that of the negative controls were considered positive for the virus.
[c]+ = ACMV positive, - = ACMV negative.

Table 4. ELISA-detection of *African cassava mosaic geminivirus* in shoots regenerating from different nodes along the main stem of infected cassava genotypes differing in resistance to the virus.

Cassava genotypes[a] detopped	Stem position[b]	Absorbance (405 nm)[c] (Mean ± SD)	ELISA reaction (+ or -)[d]
TMS 30001 (R)	Uppermost	0.029 ± 0.019	-
	Middle	0.030 ± 0.020	-
	Base	0.158 ± 0.080	+
TMS 4(2)1425 (MR)	Uppermost	0.144 ± 0.053	+
	Middle	0.146 ± 0.990	+
	Base	0.164 ± 0.115	+
TMS 60506 (S)	Uppermost	0.119 ± 0.075	+
	Middle	0.118 ± 0.042	+
	Base	0.172 ± 0.096	+
ACMV-free cassava (negative controls)		0.042 ± 0.031	-
ACMV-infected cassava (positive controls)		0.413 ± 0.211	+
Threshold		0.0890	none

[a]R = resistant; MR = moderately resistant; S = susceptible.
[b]Stems of the same age divided into equal sections.
[a]Values are means of three tests; absorbance values more than twice that of the negative controls were considered positive for the virus.
[d]+ = ACMV positive, - = ACMV negative.

assessed in shoots regenerated from nodes of cuttings from stems of different infection types as well as laboratory limitations in detecting the virus in lignified cassava stem tissue extracts by enzyme-linked immunosorbent assay (ELISA).

Under field conditions of high disease pressure for prolonged periods, some plants were observed to be asymptomatic but they later produced plants when cuttings were made from them and planted in the greenhouse. It is likely that such plants, while in the field, could either have been highly tolerant of viral infection or they may simply have completely escaped it. Also, the symptoms on plants of cuttings derived from stems of the moderately-resistant genotype TMS 4(2)1425 were more severe than those of the resistant and susceptible counterparts TMS 30001 and TMS 60506, respectively. This may suggest

that TMS 4(2)1425 was more of a tolerant, rather than a resistant, genotype to infection under field conditions. The findings of this study therefore portrayed that the genotype TMS 4(2)1425 was readily vulnerable to inoculation and subsequent systemic invasion by virus, on one hand, but it tolerated its rapid replication within its tissues without significant corresponding disease symptom development, on the other hand. However, according to Seif (1981) and Njock (1994), the susceptible genotype TMS 60506 was more vulnerable to inoculation with ACMV by whitefly vectors than the moderately-virus-resistant TMS 4(2)1425 and virus-resistant TMS 30001 genotypes. The low disease associated with plants of the different infection types of the virus-resistant genotype TMS 30001 (Table 2) relative to the moderately-virus-resistant and susceptible genotypes agreed with earlier findings by Barker and Harrison (1985) who demonstrated that low potato leaf-roll virus (PLRV) concentration was consistently associated with high resistance in potato plants inoculated by grafting or aphids, although it is, however, recognized that the latter case represents a different virus-vector -host system. Furthermore, the low disease recorded in the genotype TMS 30001 is consistent with Hahn et al. (1989) who reported that ACMV spreads slowly and is of restricted distribution in resistant cultivars.

In this study, attempts to detect virus in crude extracts of lignified stem cuttings of the cassava genotypes by ELISA failed. This is consistent with our previous findings (Njock, 1994). This difficulty may not probably imply total absence of virus but it is likely that the virus may have been present in the form of naked nucleic and therefore undetectable by the specific polyclonal antibodies prepared against purified virions from samples from actively growing (meristematic) portions of the assay plant (*Nicotiana benthamiana*). This is probably so because lignified (woody) cassava stem tissues are considered dormant and virus may be found in them more in the form of its nucleic acid rather than as complete actively replicating virions unlike the case in the meristematic tissues. Furthermore, this could have been overcome if antisera were initially prepared using purified virions in their native conformational state from corresponding lignified stem samples. This view is consistent with Esau (1958) who reported in yet a different virus-host system that tobacco mosaic virions (TMV) were not found in pores within the walls of infected cells either in the narrow canals of plasmodesmata or the wider openings in the plates probably because TMV passed through the walls in some other form than complete virus particles.

Virus detection was, however, achieved in leaves of young shoots regenerating from all topped stem sections of the moderately-virus-resistant and susceptible genotypes TMS 4(2)1425 and TMS 60506, respectively, and those of the most basal section of the resistant genotype TMS 30001 by ELISA (Table 4) but not those of the middle and uppermost nodes on the latter genotypes.

This phenomenon, observed in the resistant genotype, strongly suggests that either mechanical barriers restricting invasion of uppermost tissues or the possible initiation of antiviral factor(s) which progressively become more efficient in the attenuation and/or deterioration of virions with age of the plant, could be the principal component of the resistance in cassava against ACMV.

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