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Evaluation of the infection of cassava and *Nicotiana benthamiana* with cassava mosaic geminiviruses and their combinations for potential synergism

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Five test plants each of cassava and *Nicotiana benthamiana* were inoculated in vitro with 50 ng cassava mosaic geminivirus (CMG) deoxyribonucleic acid (DNA) of components A and B and their random combinations for strains African cassava mosaic-Cameroon virus (ACMV-CM), East African cassava mosaic Cameroon virus (EACMV-CM), East African cassava mosaic Uganda virus (EACMV-UG), Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV) were investigated for synergistic interactions by bombardment with gold particle-coated viral DNA using the biolistic particle delivery system giving a total of 80 plants. The plants were maintained in an insect-proof greenhouse and scored for infection one week post inoculation (wpi) for 8 weeks on a scale of 1-5. The experiment was replicated twice. Incidence of infection was higher on *N. benthamiana* than cassava plants. The highest infection was with ACMCV/ICMV (100 %) on *N. benthamiana* and this was not significant over ACMV-CM (98.8 %) and the positive control ACMV-CM/EACMV-CM (96.3 %). Thus the combination ACMV-CM/ICMV probably had a synergistic effect on *N. benthamiana*. On cassava, however, the highest infection was ACMV-CM/EACMV-CM (50 %) which was not significant over ACMV-CM/SLCMV (46.3 %). Similarly, viral effect was more severe on *N. benthamiana* than on cassava plants. The most severe effects were by SLCMV (2.81), SLCMV/EACMV-CM (2.42) and ACMV-CM (2.32). ACMV-CM/EACMV-CM had earlier been demonstrated on cassava to have a synergistic effect. This study demonstrated that the ACMV-CM/ICMV combination had a synergistic effect on *N. benthamiana* and thus a potential threat of significance in plant virus disease epidemiology and therefore subject to further investigation.

Keywords: cassava mosaic disease (CMD), cassava mosaic geminiviruses (CMGs), CMG combinations, synergism, cassava, *N. benthamiana*.

INTRODUCTION

Cassava mosaic disease (CMD) occurs in all cassava (*Manihot esculenta* Crantz)-producing regions of Africa, India and Sri Lanka, resulting in annual yield losses estimated at 1 billion pounds sterling (Fargette et al., 1988). CMD is caused by viruses belonging to the genus *Begomovirus* of the family *Geminiviridae*, which are characterized by small, geminate particles containing circular, single-stranded DNA molecules (Briddon and Markham, 1995). The cassava mosaic geminiviruses are transmitted by the whitefly *Bemisia tabaci* and spread

through infected cuttings, which are the usual mode of cassava propagation.

The bipartite genomes have DNA-A and DNA-B components, which share a common region of approximately 200 bp with high sequence identity (90-100 %) and both components are required for infection. DNA-A encodes all viral functions or proteins necessary for replication, control of gene expression and encapsidation of both DNAs (Sunter et al., 1987) and the second genome, DNA-B, encodes two proteins necessary for efficient systemic spread of the virus between and within plant cells (Noueiry et al., 1994; Ingham et al., 1995; von Arnim et al., 1993; Rybicki et al., 2000; Lazorowitz et al., 2003). The begomoviruses constitute the largest genus of the family and the vast majority of its members infect dicotyledonous plants and are mainly distributed in tropical and subtropical regions where the whitefly vector *Bemisia tabaci* Genn is prevalent and diseases caused by them are important constraints to crop production (Bock, 1982). African cassava mosaic geminivirus (ACMV) which affects seven species of *Manihot* (Fargette et al., 1994) and transmitted by *Bemisia tabaci* and distributed in vegetative propagules, causes the most prevalent and economically-important disease of cassava in Africa (Hahn et al., 1980). Furthermore, the whitefly vector plays the most important role in 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 and immunity to ACMV has not been reported (Jennings, 1994). However, ACMV does not become fully systemic in resistant genotypes (Rossel et al., 1992), and disease-free cuttings and plantlets 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 (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 (Jennings, 1994; Rossel et al., 1992). Modeling studies suggested 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).

CMGs are responsible for cassava mosaic disease (CMD), the most devastating disease of cassava in Africa and the Indian sub-continent. Annual yield losses due to this disease were estimated to be more than $ 1.5 billion (Thresh et al., 1994). Several distinct CMGs have been identified, cloned and sequenced. They are clustered in eight separate virus species named African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), East African cassava mosaic Cameroon virus (EACMCV), East African cassava mosaic Malawi virus (EACMMV), East African cassava mosaic Zanzibar virus (EACMZV), Indian cassava mosaic virus (ICMV), South African cassava mosaic virus (SACMV) and Sri Lankan cassava mosaic virus (SLCMV) (Fauquet and Stanley, 2003).

The etiology of CMD appears to have recently increased in complexity, particularly in the African continent, reflected by the number and types of CMGs isolated from different locations. Comparison of all known CMGs indicated that ACMV isolates, irrespective of their geographical origin, show little or no variation in their genomic sequence (Pita et al., 2001a). In contrast, CMGs with a backbone of EACMV (i.e. EACMV, EACMCV, EACMMV and EACMZA) are more genetically diverse due to frequent recombination within their two components (Fondong et al., 2000; Pita et al., 2001a; 2001b). The presence of two such EACMV-based recombinants, EACMV-UG2 and EACMV, has been correlated with the severe CMD epidemic in Uganda (Deng et al., 1997; Pita et al., 2001b; Zhou et al., 1997) and to the occurrence of extremely severe CMD symptoms in Cameroon and Ivory Coast (Fondong et al., 2000; Pita et al., 2001a), respectively. In addition to recombination, pseudo-recombination can occur naturally within a species, whereby the geminivirus derives from the combination of heterologous DNA-A and DNA-B components (Pita et al., 2001b). Thus, recombination and pseudo-recombination among geminiviruses has probably played an important role in CMG epidemiology and evolution by creating additional sources of (bio) diversity.

It is common to find cassava plants of the same cultivar, growing in the same field, expressing different levels of CMD symptom severity, with infected leaves showing distinguishable chlorotic and mosaic patterns (Pita et al., 2001a; 2001b). Such variability in symptom phenotype may be explained by the presence of different CMG strains or species.

In this study, we investigated the infection of cassava and *Nicotiana benthamiana* plants to the geminiviruses and their combinations to determine their susceptibility and if synergistic interactions occurred between the strains in vitro in order to better appreciate if devastating field infections of cassava could be the result of synergistic effect.

MATERIALS AND METHODS

Culture of *E. coli* bacilli with plasmids harboring cassava mosaic geminiviruses

The deoxyribonucleic acid (DNA) of components A and B of the cassava mosaic geminiviruses i.e. African cassava mosaic-Cameroon virus (ACMV-CM), East African cassava mosaic-Cameroon virus (EACMV-CM), East African

Table 1. Optical density (OD_{260nm}), concentration and amounts of deoxyribonucleic acid used in inoculating cassava and *Nicotiana benthamiana* plants.

CMG component		OD_{260nm}	µg/µl	ng/µl	10^{-1}ng/µl	10^{-2}ng/µl (µl)!	50-ng aliquot
ACMV-CM	A	0.051	0.258	258	28.8	2.9	17.4*
"	B	0.102	0.515	515	51.8	5.2	10.0*
EACMC-CM	A	0.061	0.308	308	30.8	3.1	16.2*
"	B	0.030	0.152	152	15.2	1.5	33.0*
EACMV-UG	A	0.001	0.005	5	0.5	-	100+
"	B	0.007	0.035	35	3.5	-	14.5+
ICMV	A	0.002	0.010	10	1.0	-	50.0+
"	B	0.003	0.015	15	1.5	-	33.0+
SLCMV	A	0.095	0.479	479	49.9	4.8	10.4*
"	B	0.119	0.601	601	60.1	6.0	8.3*

!=Aliquots were taken from $^{+}10^{-1}$ or $*10^{-2}$ng/µl dilutions of the original DNA preparation.

cassava mosaic-Uganda virus (EACMV-UG), Indian cassava mosaic (ICMV) and Sri-Lankan cassava mosaic (SLCMV) geminiviruses, was maintained in *Escherichia coli* (*E. coli*) plasmids as permanent glycerol stocks at –80 ^{0}C at the International Laboratory for Tropical Agricultural Biotechnology (ILTAB). Aliquots of the components of ACMV-CM, EACMV-CM, EACMV-UG and SLCMV were collected from the glycerol stocks with pipette tips and streaked separately on sterile LB (10 g Bacto Tryptone, 5 g Bacto Yeast Extract and 10 g NaCl) agar media containing 100 mgL^{-1} of the antibiotic ampicillin (amp). Similarly, components of ICMV were streaked on LB media containing 50 mgL^{-1} kanamycin (km) in 100 x 15 mm Petri-dishes and incubated overnight at 37 ^{0}C. The resultant *E. coli* colonies on LB media were collected with pipette tips and transferred (with the tip) into ten corresponding 10-ml tubes containing 2.5 ml LB liquid media (broth) with the appropriate antibiotic. Eight of the tubes received ampicillin while two received kanamycin. All the tubes were incubated overnight at 37 ^{0}C with shaking for bacterial growth. The *E. coli* colonies on LB media in the Petri-dishes were returned and stored in the cold room maintained at 4 ^{0}C.

Bombardment of the cassava and *Nicotiana benthamiana* plants with CMGs and their combinations

The resulting *E. coli* broth cultures were centrifuged at 14000 rpm for 5 minutes and the pellets of the bacterial cells were re-suspended and applied to a QIA-prep Spin Mini-prep kit protocol for plasmid DNA extraction. The optical density at 260 nanometer wavelength (OD_{260nm}) (Pharmacia Biotech; Ultrospec 3000 UV/Visible Spectrophotometer) of the DNA was calculated thus: Viral DNA (µg/µl) = (OD_{260nm} x dilution factor x 50)/1000, where; Dilution factor = dilution coefficient of bacterial growth before taking OD, 50 = a constant.

Based on the DNA concentration of the cassava mosaic geminivirus (CMG) components (Table 1), amounts of aliquots were then computed for a 10-ng viral DNA per component per plant. Components A and B of each CMG were mixed together to produce a single virus and

Table 2. Mean proportion (%) of cassava and N. *benthamiana* plants infected by cassava mosaic geminiviruses and their combinations.

N. benthamiana		cassava	
CMG/combination	% infection	CMG/combination	% infection
EACMV-UG/ICMV	0.0a	EACMV-UG	0.0a
EACMV-UG	5.0a	SLCMV	0.0a
ICMV	5.0a	SLCMV/EACMV-CM	0.0a
EACMV-CM/ICMV	33.8b	SLCMV/ICMV	0.0a
SLCMV/EACMV-UG	52.5c	ICMV	1.3a
ACMV/SLCMV	62.5cd	SLCMV/EACMV-UG	2.5a
SLCMV/ICMV	67.5cd	EACMV-UG/ICMV	3.8ab
EACMV-CM	68.8cde	EACMV-CM	6.3ab
EACMV-CM/EACMV-UG	71.3de	EACMV-CM/ICMV	6.3ab
SLCMV/EACMV-CM	81.3ef	ACMV/ICMV	12.5b
SLCMV	82.5f	EACMV-CM/EACMV-UG	12.5b
ACMV/EACMV-CM (+C)	96.3fg	ACMV	27.5c
ACMV	98.8g	ACMV/SLCMV	46.3d
ACMV/ICMV	100.0g	ACMV/EACMV-CM (+C)	50.0d

subsequently, based on the CMG combinations in question, the appropriate components were mixed accordingly.

Thirty milligrams of gold particles were sterilized in ethyl alcohol and partitioned into ten 50-ul aliquots in 1.5 ml eppendorf tubes with sterile water and the DNA was coated according to a standard protocol adopted by the International Laboratory for Tropical Agricultural Biotechnology (ILTAB, 2003). Using the Biolistic particle delivery system, five healthy *Nicotiana benthamiana* and cassava plants each were inoculated by bombardment with the appropriate CMG or their combinations. There were thus a total of 14 CMGs and their combinations administered each on five plants (i.e. 80 plants altogether). The plants were maintained in an insect-proof greenhouse and watered daily. Symptom severity due to viral infection was scored one week post inoculation (wpi) for 8 weeks based on a scale of 1-5. The experiment was replicated twice.

RESULTS

The optical density at a wavelength of 260 nm (OD_{260nm}) of cassava mosaic geminivirus DNA samples of components A and B of ACMV-CM, EACMV-CM, EACMV-UG, ICMV and SLCMV were determined and the concentration in microgram per microliter (µg/µl) of the DNA computed from the OD_{260} values (Table 1). The corresponding concentration in nanograms per microliter (ng/µl) was computed and this was subsequently diluted tenfold (10^{-1} ng/µl) and hundredfold (10^{-2} ng/µl) and used in the preparation of the inocula on either cassava or *Nicotiana benthamiana* test plants.

The mean proportion (incidence), in percentage, of cassava and *Nicotiana benthamiana* test plants infected with the five cassava mosaic geminiviruses and their random combinations is shown in Table 2. The infection of the test plants was higher on *N. benthamiana* than cassava plants. In the *N. benthamiana* plants, the highest infection (100 %) was recorded with the combination ACMV/ICMV. This was, however, not significantly different from ACMV-CM (98.8 %) and the positive control considered the combination ACMV/EACMC-CM (96.3 %). Furthermore, the positive control was not significantly different from SLCMV (82.5 %) and SLCMV/EACMV-CM (81.5 %). The least infection was recorded on plants inoculated with EACMV-UG/ICMV (0 %), EACMV-UG (5.0 %) and ICMV (5.0 %) and this was significantly different from all other infection types. However, in cassava plants, the highest infection, which was far lower than what was recorded on cassava, was with the positive control ACMV-CM/EACMV-CM (50.0 %) but this was not significantly different from ACMV-CM/SLCMV (46.3 %) as shown in Table 2. Both infection types were significantly different from all other types. The lowest infection (0 %) was with EACMV-UG, SLCMV, SLCMV/EACMV-CM and SLCMV/ICMV.

The results also showed that ICMV tended to have enhanced synergism with ACMV-CM but attenuated EACMV-UG and EACMV-CM on *N. benthamiana*. Similarly, SLCMV tended to have enhanced synergistic effect with ACMV-CM on both test plants especially on cassava plants. ACMV-CM was the most virulent strain on *N. benthamiana* followed by SLCMV, EACMV-CM, ICMV

Table 3. Mean severity (1-5) on cassava and *N. benthamiana* plants infected by cassava mosaic geminiviruses and their combinations.

N. benthamiana		cassava	
CMG/combination	severity	CMG/combination	severity
EACMV-UG/ICMV	0.0	EACMV-UG	0.0
EACMV-UG	0.05	EACMV-UG/ICMV	0.0
ICMV	0.15	SLCMV	0.0
EACMV-CM/ICMV	0.94	SLCMV/EACMV-CM	0.0
ACMV/SLCMV	1.53	SLCMV/ICMV	0.0
EACMV-CM/EACMV-UG	1.73	ICMV	0.01
SLCMV/EACMV-UG	1.86	SLCMV/EACMV-UG	0.04
SLCMV/ICMV	2.13	EACMV-CM/ICMV	0.10
ACMV/ICMV	2.16	EACMV	0.16
EACMV-CM	2.19	ACMV/ICMV	0.38
ACMV/EACMV-CM (+C)	2.29	EACMV-CM/EACMV-UG	0.39
ACMV	2.32	ACMV	0.76
SLCMV/EACMV-CM	2.42	ACMV/SLCMV	1.23
SLCMV	2.81	ACMV/EACMV-CM (+C)	1.51

Table 4. Ranking of cassava mosaic geminiviruses and their combinations on infection of *N. benthamiana* plants.

category	CMG/combination	infection/10	% infection
1	ACMV-CM	10	100
	ACMV-CM/ICMV	10	100
	SLCMV/EACMV-CM	10	100
	ACMV-CM/EACMV-CM	10	100
2	EACMV-CM	9	90
3	SLCMV	8	80
	SLCMV/EACMV-UG	8	80
	SLCMV/ICMV	8	80
	EACMV-CM/EACMV-UG	8	80
4	ACMV-CM/SLCMV	7	70
5	EACMV-UG	1	10
	ICMV	1	10
6	EACMV-CM/ICMV	0	0

and EACMV-UG in that other. ACMV-CM was also most virulent on cassava followed by EACMV-CM, ICMV, SLCMV and EACMV-UG in that other. Furthermore, ACMV/ICMV and ACMV were more virulent than the positive control ACMV/EACMV-CM on *N. benthamiana* while on cassava, the positive control was most virulent. Finally, the most virulent strain, ACMV-CM, on cassava was significantly lower than the positive control.

The mean severity on both plant types is shown in Table 3. The viral effect was more serious on *N. benthamiana* than on cassava where the most severe viral effect on the former plant type was by SLCMV (2.81) followed by SLCMV/EACMV-CM (2.42), ACMV-CM (2.32) which were all more severe than the positive control (2.29) in that order. However, the least severe situation was by EACMV-UG/ICMV where no symptoms were recorded (0.0). On cassava, however, the most severe situation was by the positive control (1.51) followed by ACMV-CM/SLCMV (1.23) and ACMV-CM (0.76) in that order. EACMV-UG, EACMMV-UG/ICMV, SLCMMV, SLCMV/EACMV-CM, SLCMV/ICMV all did not show any symptoms of disease (0.0).

Table 5. Ranking of cassava mosaic geminiviruses and their combinations on their infection of cassava plants.

category	CMG/combination	infection/15	% infection
1	ACMV-CM/SLCMV	10	67
2	ACMV-CM/EACMV-CM	8	53
3	ACMV-CM	7	47
4	EACMV-CM	5	33
	EACMV-CM/EACMV-UG	5	33
	EACMV-CM/ICMV	5	33
5	ACMV-CM/ICMV	4	27
6	SLCMV/EACMV-CM	2	13
7	SLCMV/EACMV-CM	1	7
	SLCMV/ICMV	1	7
	EAMCV-UG/ICMV	1	7
8	EACMV-UG	0	0
	ICMV	0	0
	SLCMV	0	0

A ranking of the infection of *N. benthamiana* test plants by the CMGs and their combinations revealed categories among the CMGs where ACMV-CM, ACMV-CM/ICMV, SLCMV/EACMV-CM and ACMV-CM/EACMV-CM proved to be the most infectious category infecting all test plants (100 %) inoculated (Table 4). On the contrary, EACMV-CM/ICMV proved to be most non-infectious infecting no test plants (0 %). Similarly, the ranked infection of cassava test plants by the CMGs and their combinations revealed that ACMV-CM/SLCMV proved to be most infectious infecting 67 % of the test plants while EACMV-UG, ICMV and SLCMV proved most non-infectious infecting no test plants (0 %) (Table 5).

DISCUSSION

Relative to the cassava mosaic geminiviruses, synergism has earlier been reported between ACMV-CM and EACMV-CM (Fondong et al., 2000), thus the latter combination was used in this study as the standard. Our data showed that the mean percentage incidence of infection of cassava and *N. benthamiana* test plants in vitro by cassava mosaic geminiviruses and their combinations showed that infections were more severe on *N. benthamiana* than on cassava test plants. This was probably due to the higher susceptibility of *N. benthamiana* as a routine in vitro assay host than cassava. In *N. benthamiana* the highest infection (100 %) was recorded with the ACMV/ICMV virus combination compared to, but not significantly different from, the infection by ACMV-CM (98.8 %) and the confirmed standard combination (positive control) ACMV-CM/EACMV-CM (96.3 %). Similar findings using infectious clones of ACMV-CM and EACMV-CM in *N. benthamiana* were reported (Fondong et al., 2000). They maintained that it was possible that, even though EACMV-CM was capable of infecting *N. benthamiana*, the latter was not an adaptable host compared to cassava. That in the synergism between ACMV-CM and EACMV-CM there were increases in DNA accumulation of both viruses in mixed infections. Furthermore, their preliminary results suggested that there was trans-complementation between ACMV-CM and EACMV-CM and confirmation of the results could partly explain the observed synergism. Our results indicated that the ICMV virus species had a positive enhancing effect in the infectivity of ACMV-CM than EACMV-CM.

In cassava, the highest infection, which was far lower than that recorded on *N. benthamiana*, was the standard combination or positive control ACMV/EACMV-CM (50 %) and was not significantly different from the ACMV-CM/SLCMV combination (46.3 %). The fact that the SLCMV species alone recorded no infection (0 %) on cassava, but significant infection occurred when it was combined with ACMV-CM just as when ACMV-CM was combined with EACMV-CM, showed that SLCMV and EACMV-CM viral species probably possessed genomic similarities. Also ICMV viral species appeared to synergize with ACMV-CM but probably attenuated EACMV-UG and EACMV-CM on *N. benthamiana*. Similarly, the SLCMV species also appeared to synergize with ACMV-CM on both test plants especially on cassava plants. This enhancement of ACMV-CM by both ICMV and SLCMV suggests the likely genomic similarity of these viruses from a similar sub-continental origin. This similarity was confirmed by Saunders et al. (2002) that SLCMV was most closely related to ICMV and considered DNA-A sequence diversity which was 84.2 % identical with ICMV DNA-A.

Mean disease severity on both test hosts showed that viral effect was more severe on *N. benthamiana* than on cassava. The most severe viral effect on *N. benthamiana* was that by the SLCMV (2.81) species followed by SLCMV/EACMV-CM (2.42) and ACMV-CM (2.32) which were more severe than the standard positive control (2.29). However, on cassava, the most severe effect was by the standard positive control ACMV/EACMV-CM (1.51) followed by ACMV/SLCMV (1.23) and ACMV (0.76). Again, SLCMV viral species tended to show enhanced synergism with ACMV on cassava indicating that SLCMV may have a similarity with EACMV-CM.

Confirmation of synergism and trans-complementation among the cassava mosaic geminiviruses would have serious epidemiological implications of double infections of ACMV-CM with ICMV, SLCMV, EACMV-CM or EACMV-UG. Understandably, the viruses belong in the same genus *Begomovirus* and are transmitted by the same whitely vector *Bemisia tabaci* thus there are higher chances for co-infecting same host as earlier indicated (Fondong et al., 2000). Consequently, doubly infected plants have considerable potential as sources of inoculum for both viruses and, whiteflies feeding on such plants would, therefore more easily acquire and transmit both viruses to virus-free plants.

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