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Development of an efficient virus transmission technique to screen cassava genotypes for resistance to cassava mosaic disease

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Abstract

The most important disease affecting cassava (*Manihot esculenta* Crantz) production in Africa is Cassava mosaic disease (CMD), caused by several whitefly-transmitted begomoviruses. Advancement in cassava breeding for virus resistance is hampered because screening for resistance to CMD is tedious, relying on natural infection conditions and on virus types at a given time and location. Therefore, developing an efficient inoculation technique with defined viruses at an early stage in breeding for resistance would provide a major improvement to the resistance development in cassava. All major begomoviruses in African cassava genotypes were collected, typed by sequence analysis and maintained as reference in cassava cultivars. For inoculation of begomoviruses into cassava, a graft inoculation approach and biolistic inoculation method to deliver DNA from virus infected plants or cloned viral genomes were developed. Graft inoculation technique was only effective in inducing cassava with defined viruses at a later date (3 weeks post inoculation, w.p.i.), in plants with successful graft union. Although, 100 percent of the treated plants survived after biolistic inoculation experiments, the delivery of cloned viral DNA was not sufficiently effective to reach higher number of infected cassava plants compared to the delivery of DNA extracts. Occurrence of infection symptoms was earliest in cassava plants biolistically inoculated with DNA extracts (10 d.p.i.) and much more delayed with inoculation of infectious virus clones. The effectiveness of biolistic inoculation of DNA extracts over other transmission techniques in screening cassava genotypes is discussed.

Keywords: Biolistic inoculation, cassava genotypes, cassava mosaic begomoviruses, cloned virus, DNA extracts, graft inoculation

Introduction

Cassava (*Manihot esculenta* Crantz), a favoured root and tuber crop of the tropics (Nweke, 1994, Henry, 1995) is seriously affected by several distinct Cassava mosaic begomovirus species causing mosaic diseases. This presents a serious constraint to high cassava production.

Resistance breeding is still the most significant and only strategy to counteract the serious effects of these viruses in Cassava (Thresh and Otim-Nape, 1994). The progress in breeding is hampered by the requirement for defined virus materials reflecting those viruses present in a respective geographic zone or cultivation area. This would be a significant step towards the deployment of planting material, which holds up against the adverse effects of the begomoviruses present in the field. It would represent a significant progress in the selection process, if screening for resistance in breeding lines could be improved by a system at a Molecular level, which will ensure a successful delivery of defined virus variants into the breeding lines to be screened for resistance. This will at an early stage in screening provide an indication on the resistance status of the cassava breeding lines against a particular virus type/variant and there will be no dependence on the number of individuals or groups to be screened. Besides, defined inocula will permit accurate virus testing. Also simulation of mixed infections, pseudo-recombinant virus infections and infections of geographic types will be ensured.

Thus, the study was initiated to;

i. develop a reliable and efficient virus inoculation system to screen cassava genotypes for resistance to cassava mosaic disease and

ii. screen promising cassava breeding lines for their reactions to different available cassava mosaic begomoviruses for proper deployment of stable planting materials to pandemic areas.

Materials and Methods

Propagation and maintainance of cassava tissue culture plantlets

Tissue culture plantlets were obtained from six newly developed cassava genotypes (96/1089A, 96/0160, 95/0166, 96/0304, 96/1039, 96/1087) from IITA, Nigeria, three Nigerian local landraces (TME 117, TME 3, TME 4) and one old IITA improved genotype (91/02324) (Table 1). The resistance status to CMD of these landraces and the old IITA improved genotype had been known. They were therefore used in these experiments to have a basis for resistance comparison.

Table 1. Newly developed cassava genotypes and local landraces propagated from tissue culture for artificial virus transmission

Accession number	Local name	Source/locality	Parents	Resistance to CMD
TME 117	Isunikankiyan	SW Nigeria	unknown	HS
TME 3	2nd Agric	SW Nigeria	unknown	HR
TME 4	Atu	SW Nigeria	unknown	HR
91/02324		IITA Nigeria	unknown	R
95/0166		IITA Nigeria	92/0429 HS	nd
96/1089A		IITA Nigeria	M94/0461*90/01554	nd
96/1039		IITA Nigeria	M85/00680*90/01554	nd
96/1087		IITA Nigeria	91/02327*M94/0461	nd
96/0160		IITA Nigeria	30572 * Atu Iwo	nd

where, HS = Highly resistant, R = Resistant, nd = not determined, CMD = Cassava mosaic disease

The different Cassava begomoviruses subjected to transmission studies were:

1. African cassava mosaic begomovirus (ACMV)
2. East African cassava mosaic begomovirus (EACMV)
3. EACMV- UG2 (Uganda variant virus)
4. Mixed/double infections of ACMV and EACMV- UG2

Possible synergistic effects of multiple virus infections in *Nicotiana benthamiana*

Single and mixed infections of the aforementioned were first introduced into *Nicotiana benthamiana* to study possible synergistic effects of multiple virus infections.

Leaf samples from cassava plants infected with authenticated viruses and grouped into different infection categories were used to inoculate 10 *N. benthamiana* seedlings mechanically. Mixed infections were introduced by mixing sap from the same samples infected by ACMV and EACMV alone; ACMV and EACMV-UG2 alone to obtain a doubly infected sample.

Sap was obtained from the leaves of the plants at a dilution of 1:10 (w/v) in a 0.1 M phosphate buffer pH 7.0. Inoculated plants were grown in the growth chamber at 25^0C, 16h photoperiod and the symptoms were noted. Symptomatic leaf samples were collected two weeks after inoculation and

screened for the presence of viruses by DNA-A and DNA-B PCR amplification. Attempts were also made to inoculate cassava mechanically with different cassava mosaic virus combinations.

Development of artificial inoculation system for screening cassava breeding lines against different associated begomoviruses

Artificial inoculation systems were therefore attempted namely;

1. Virus transmission through wedge grafting
2. Virus transmission through biolistic inoculation (BI) of total DNA extracted from diseased cassava plants and
3. Virus transmission through biolistic inoculation of cloned virus DNA A + B genomic component.

Infectious virus clones were generated by head-to-tail partial repeats constructed for ACMV [NG]; ACMV [KE]; EACMV-UG2 [KE]; EACMV [NG] and tested for infectivity in *N. benthamiana*. Infectivity tests were carried out by either biolistic delivery of cloned DNA or total DNA extracts. Total plant DNA (2µg/shot) or mixtures of begomovirus cloned DNA A and DNA B multimers (0.5µg A + 0.5µg B)/shot were coated onto 1 micron gold particles. BI was carried out with the Helios gene gun at 200-300 psi, applying 2-3 shots/plant.

Results

Synergistic effects of multiple virus infections in *Nicotiana benthamiana*

In order to determine the occurrence of synergism at the level of viral DNA accumulation, *N. benthamiana* plants were mechanically inoculated with sap from cassava samples singly infected by ACMV [DRC] or EACMV [KE] or EACMV-UG2 [KE], with a mixture of sap from ACMV [DRC] and EACMV [KE] or ACMV [DRC] and EACMV-UG2 [KE], and sap prepared from plants infected with the respective viruses and with sap from a naturally co-infected cassava plant. Disease symptoms shown by doubly infected plants with ACMV [DRC]//EACMV [KE] and ACMV [DRC]// EACMV-UG2 [KE] were more severe showing a characteristic stunting with short internodes compared to disease symptoms of plants with single infection. Plants subjected to these two categories of double infections remained severely affected throughout the period of observation. With these two categories of double infections, all the ten plants mechanically inoculated got severely infected, eight out of ten got infected with EACMV-UG2 [KE] alone and ACMV alone, but nine out of ten got infected with EACMV alone. All cassava plants mechanically inoculated tested negative in both TAS-ELISA and PCR.

Evaluation of the resistance status of selected cassava breeding lines to different Cassava mosaic begomoviruses transmitted by wedge grafting

Successful graft contacts were observed about 10 days after grafting in graft transmission studies of ACMV and EACMV-UG2, as buds were seen sprouting on the grafted scions. These buds eventually developed mild to severe symptom depending on the breeding line (Plate 1). For each

breeding line up to five plants were successfully grafted. Cassava mosaic disease (CMD) symptom was observed at about third week after grafting (WAG). In graft transmission study of ACMV, two plants of breeding line 96/0160 out of the five plants with successful graft contacts, were observed to be symptomless uptill 8 WAG, though these two plant stands got infected eventually 10 WAG, but the infection severity levels remained low with mild symptoms all through the period of screening. Similar observation was recorded for grafted plants of 96/1087, with three successfully grafted plants showing no symptoms till 8 WAG. Ten percent of successfully grafted plants of 96/1089A under ACMV transmission remained asymptomatic throughout the period of screening, but disease symptoms remained mild in the infected plants. One grafted plant each of TME 3 and TME 4 were asymptomatic throughout the period of screening and CMD symptoms remained mild in the infected plants. Twenty percent grafted plants of 91/02324 were observed to recover from CMD 10 WAG, as the ISS and % DI values were 1 and 0, respectively, 10 to 12 WAG. A similar trend of reduction in DI and ISS scores was observed in 96/1087, TME 3 and TME 4 (Figure 1). Plants of ISU and 95/0166 were heavily infected with severe symptoms, though the symptom severity levels observed in plants of ISU is greater than that observed in plants of 95/0166.

In the graft transmission study of EACMV-UG2, one plant each of 96/1087, 96/1089A and TME 4 remained symptomless and mild symptoms were observed in the diseased plants. Successfully grafted plants of Isu recorded the highest DI and ISS scores. Similar results were obtained for ACMV transmission by wedge grafting (Figure 1). Positive PCR amplification results were obtained (Plate 2) on diseased leaf samples collected from different breeding lines. Results with TAS – Elisa were also positive for diseased showing leaves. But lower ELISA titre values were obtained for plants of breeding lines 91/02324, 96/1087 and TME 3 compare to values obtained for 96/1039 and Isu (results not shown).

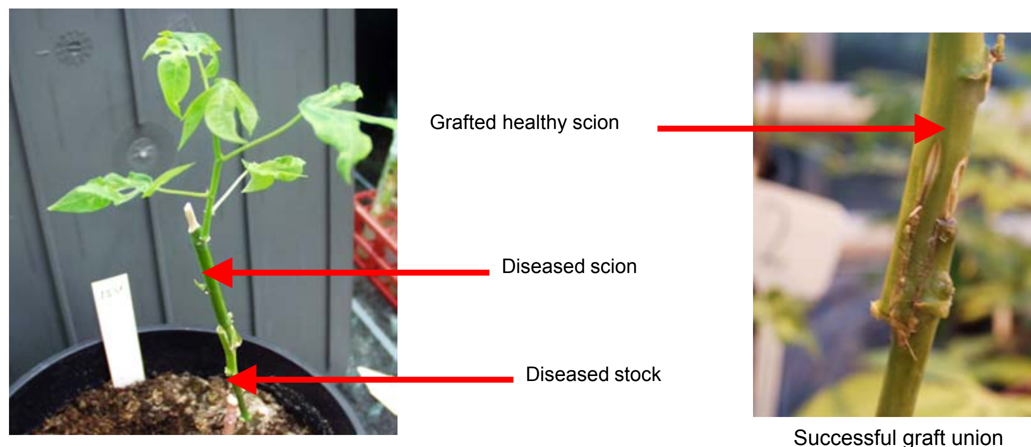

Plate 1. Successful graft contact initiated for virus transmission in cassava plants

Analysis of variance (ANOVA) showed that the disease parameters for the two begomoviruses (ACMV and EACMV-UG2) were significantly different ($P<0.01$). There was also a highly significant difference ($P<0.01$) among breeding lines in terms of disease incidence all through the observation. There was no significant difference among the replicates, but there was a highly significant difference in time ($P<0.01$). The overall means of DI across all the breeding lines (in percentage) for ACMV and EACMV-UG2 across the breeding lines were 16.5 and 26.6, respectively.

With Duncan grouping test for significant differences, mean DI values for breeding lines 91/02324 and 95/0166 were grouped together, while mean DI value for Isu was grouped separately and 96/1039 was grouped next to Isu. Mean DI values for 96/1087, 96/0160, 96/1089A, TME 3 and TME 4 were grouped together. Mean DI value for Isu being the highest was observed in the first grouping with Duncan, followed by 96/1039 alone in the next grouping (Table 2).

Symptoms of begomovirus infections in cassava were scored on a scale of 1-5 at bi-weekly intervals (Table 3). For all viruses in general, single virus infection resulted in a lower severity score than mixed virus infections. With all single and mixed virus combinations, the high resistance status of TME 4 and TME 3 proved stable to resist infections. Also, the newly improved breeding lines 96/1089A and 96/0160 were found with similar stable resistance status.

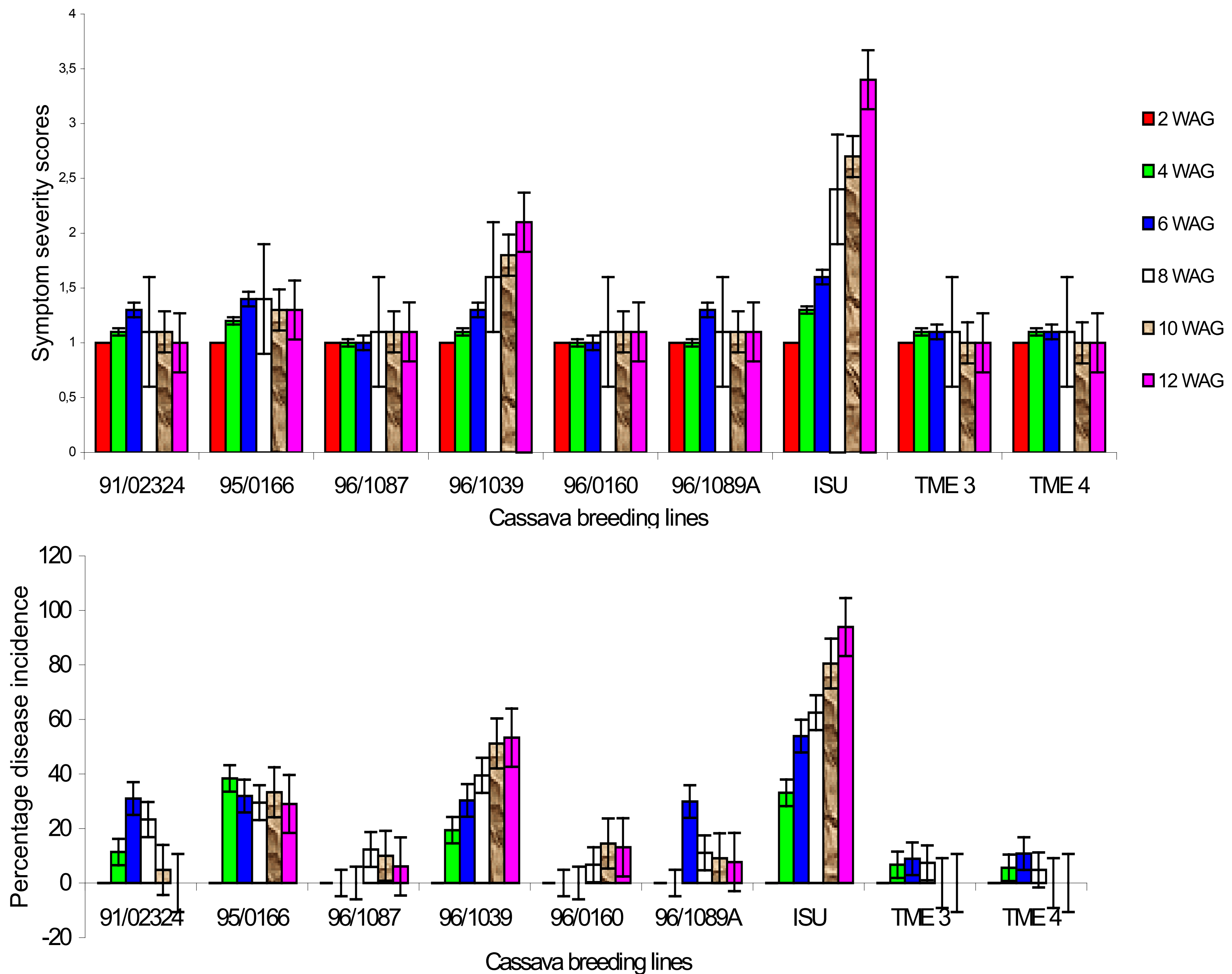

Figure 1. Mean bi-weekly variation in symptom severity scores (above) and percentage disease incidence (below) of nine cassava genotypes artificially infected with ACMV by wedge grafting.

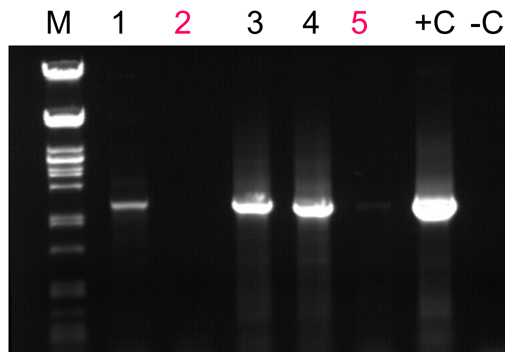

Plate 2. Gel electrophoresis of PCR amplified DNA fragments with primers specific for amplification of ~1380bp DNA B components of EACMV-UG2 and EACMV.

Lane M on the gel contained λ Pst I digested phage DNA (Fermentas NBI, Germany). Also, lanes 1 and 2 represent PCR amplification results of DNA extracted from treated leaves of genotypes 96/1089A, lane 3 for sample from infected TME 3, lanes 4 and 5 for TME 4. Lane 6 contained DNA from EACMV-UG2, isolate 55 (for gel B) to serve as positive control (+C). Lane 7 contained DNA of healthy leaf from propagated cassava tissue culture, to serve as negative control (-C). Lanes in red colour contained DNA samples from non symptomatic cassava leaves that had recovered from infection.

Table 2. Resistance status of some newly developed cassava genotypes based on their reactions to single infection with ACMV and EACMV-UG2, artificially transmitted by wedge grafting

Reaction to ACMV (A)		Reaction to EACMV-UG2 (B)	
Cassava genotypes	Mean DI (%)[a]	Cassava genotypes	Mean DI (%)[b]
ISU	53.9a	ISU	68.6a
96/1039	32.2b	95/0166	56.2b
91/02324	24.4b	96/1039	43.5b
95/0166	11.8c	91/02324	32.1c
96/1089A	7.2c	96/1087	13.8d
96/0160	6.4c	96/0160	10.3d
96/1087	4.7c	TME 3	7.5d
TME 3	3.8c	96/1089A	5.1d
TME 4	3.5c	TME 4	2.5d

Based on the number of leaves per plant that developed cassava mosaic symptoms after artificial inoculation. Means with the same letter are not significantly different based on Duncan Multiple range test at P=0.05, where, HS=highly susceptible, S=susceptible, MR=moderately resistant, HR=highly resistant, DI(%)=disease incidence in percentage, ACMV= *African cassava mosaic begomovirus*, EACMV-UG2=*Ugandan variant.*

Table 3. Severity of infection symptoms induced by mixed infections of ACMV with EACMV-UG2 and EACMV-UG2 alone, artificially transmitted by biolistic inoculation of virus-laden DNA extracts on cassava genotypes, recorded visually at biweekly intervals.

Cassava	EACMV-UG2[#] (Weeks post biolistic inoculation)							
genotypes	2	4	6	8	10	12	Mean	S.E (±)
TME 4	1.0	2.0	1.0	1.0	1.0	1.0	1.2	0.15
96/1089A	1.0	1.1	1.1	1.0	1.0	1.0	1.0	0.02
96/1039	1.2	2.0	2.1	2.1	1.5	1.5	1.7	0.14
TME 117	2.0	2.7	3.2	3.4	3.5	3.5	3.1	0.22
96/0160	1.0	1.2	1.1	1.0	1.0	1.0	1.1	0.03
	ACMV + EACMV-UG2* (Weeks post biolistic inoculation)							
TME 4	1.2	1.2	1.1	1.1	1.0	1.0	1.1	0.03
96/1089A	1.2	1.1	1.1	1.0	1.0	1.0	1.1	0.03
96/0304	1.4	2.2	2.3	2.5	2.4	1.7	2.1	0.16
TME 117	2.0	2.8	3.6	3.9	3.9	4.2	3.4	0.31
96/0160	1.2	1.2	1.0	1.0	1.0	1.0	1.1	0.04

where, TME 117 is locally called Isunikankiyan and abbreviated as "Isu" in the text. [#]mean severity of infection symptoms caused by EACMV-UG2 alone, *mean severity of infection symptoms caused by ACMV + EACMV-UG2. Symptom severity was based on a scale of 1 (no symptoms) to 5 (very severe symptoms) (Terry, 1975; IITA, 1990)

Discussion

The four categories of infection of cassava mosaic begomoviruses introduced by sap inoculation, although, elicited appreciable mosaic symptoms in *N. benthamiana,* but could not induce infection symptoms in cassava plants. These findings agree with those of Berrie *et al.* (1997) whose attempts to transmit SACMV back from *N. benthamiana* to cassava by sap inoculation were unsuccessful. Successful graft union observed about 10 days after grafting is an indication of contact between the xylem and phloem vessels of the stock and scion. The development of mild to severe symptoms depending on the breeding line suggests that the different cassava genotypes used under this experiment have different levels of resistance and susceptibility to CMD.

The delay in infection symptoms of EACMV-UG2 in some grafted plants of cassava genotypes, 96/0160 (40% of all plants treated remained symptomless till 8 WAG) and 96/1089A (50%) could be due to low inoculum potential in disease stock at early stage of the experiment. But the eventual symptoms occurrence 10 WAG in the same set of plants suggest that the virus multiplied in the stock, thus increasing the inoculum amount sufficient enough to induce infection symptoms.

Plants of cassava genotypes TME 4, TME 3, 96/1089A and 96/0160 proved highly resistant (HR) under different transmission techniques. This could be due to the fact that some of these genotypes (96/0160, 96/1089A and 96/1087) were progenies of parents earlier evaluated highly resistant (HR) to CMD (Ariyo *et al*., 2002). Also, preliminary analysis of field data obtained at five different locations in Nigeria revealed those genotypes to be HR, implying the resistant abilities of these genotypes to a wide range of begomovirus infections. Biolistic delivery of cloned virus did not result in sufficient number of infected cassava plants and occurrence of infection symptoms was delayed till 6w.p.i. Graft inoculation technique reached 85% survival rate across all cassava genotypes, with infection symptoms showing up 3w.p.i. The biolistic delivery of DNA extracts with defined begomovirus infections is much faster, eliciting infection symptoms 10 d.p.i. with 100% survival of the treated cassava plants. Therefore, biolistic inoculation of virus-laden DNA extracts is the most efficient virus transmission technique in this study. Thus, recommended for screening cassava germplasm.

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