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Optimisation of regeneration systems for a range of Cassava (Manihot Esculenta Crantz) cultivars suitable for growth in South Africa and transformation with SACMV N-REP gene

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Chapter 3

BIOLISTIC INOCULATION OF CASSAVA (*MANIHOT ESCULENTA* CRANTZ) WITH *SOUTH AFRICAN CASSAVA MOSAIC VIRUS*

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3.1 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the primary food crop in sub-Saharan Africa. Cassava mosaic disease (CMD) is one of the most important diseases of this crop and it is caused by whitefly-borne viruses of the genus *Begomovirus* (family *Geminiviridae*) (Fargette *et al*. 1988; Briddon and Markham 1995; Fondong *et al*. 2000). These include *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and *South African cassava mosaic virus* (SACMV) (Hong *et al*. 1993; Berrie *et al*. 1998; 2001).

The distinguishing feature of most begomoviruses is that they consist of two DNA components, DNA-A and DNA-B. DNA-A is autonomously replicating and encodes five to six genes (one or two on the virion and four on the complementary strand) (Sunter *et al*. 1987; Pita *et al*. 2001a; 2001b) whereas the dependant DNA-B component encodes two genes (one on each strand) necessary for efficient systemic spread of the virus throughout the plant (Brough *et al*. 1988; Ingham *et al*. 1995). The two genomic components share approximately 200 bp of sequence (encompassing the conserved TAATATTAC sequence) homology within the intergenic region (IR) which is then termed the “common region” (CR). Both DNA-A and B components are essential during infection (Eagle *et al*. 1944; Chatterjii *et al*. 1999).

Presently, several clones including ACMV/NG (Briddon *et al*. 1998); ACMV/CM, EACMV/CM and ACMV/CM (Fondong *et al*. 2000); pseudorecombinant EACMV-UG2 DNA-A + EACMV-UG3 DNA-B (Pita *et al*. 2001b) were reported to be infectious to cassava by biolistics. However, some clones including ACMV/KE and ACMV/NG were shown to be infectious to

various tobacco species but the original isolate of ACMV/KE was not infectious to cassava (Stanley 1983). This lack of infectivity was attributed to a probable mutation in the DNA-B component. Since many begomoviruses are not mechanically transmissible, including SACMV, it is essential that a reliable method for virus inoculation is available for large screening of transgenic plants or new cultivars. SACMV dimers A and B have been shown previously to be infectious by agroinoculation but not by biolistics. In this study, we report the results of infection of tobacco and cassava by SACMV dimer A and B using the particle inflow gun.

3.2 MATERIALS AND METHODS

3.2.1 Plasmid Constructs

SACMV dimers A and B were used in both tobacco and cassava infectivity tests. Full-length head-to-tail dimers of DNA-A and DNA-B components of SACMV were constructed by digestion with *Sal*I or *Eco*RI, respectively (Berrie *et al*. 2001). Relevant fragments were then relegated to form dimers in pBluescript KS^+. DNA-A and DNA-B dimers were then subcloned into pBIN19 (pBINSA-A and pBINSA-B, respectively).

3.2.2 Preparation of gold particles

To 30 mg of gold particles, 500 µl of 100 % ethanol was added and the mixture was vortexed for 3 min and the gold particles pelleted by spinning at 12 000 rpm for 30 sec. The supernatant was discarded and 500 µl of fresh 100% ethanol

added followed by vortexing for 90 sec. The gold particles were pelleted by spinning the mixture again at 12 000 rpm for 1 min and ethanol was discarded. A volume of 500 µl sterile distilled water was added to the gold particles pellet. The gold particles were resuspended by vortexing for 1 min and then pelleted with the supernatant discarded. This step was repeated followed by the addition of 500 µl of 100% ethanol per 30 mg of gold particles.

While vortexing at low speed both components of pBINSA-A and pBINSA-B were added to give 100 ng of each component per shot. Then 20 µl spermidine (0.05 M), 50 µl $CaCl_2$ (1M) were added successively as the mixture was vortexed. The mixture was incubated for 10 min at room temperature. The particles were pelleted by centrifuging for 10 sec at 10 000 rpm, then the supernatant was discarded and 50 µl of cold 100% ethanol added. After resuspending the pellet the mixture was added to microcarriers and left to dry at room temperature.

3.2.3 Bombardment of SACMV dimers into tobacco and cassava plants

The DNA-coated particles were then used to shoot 3-week-old cassava plantlets (cv. TMS60444) in magenta boxes at a pressure of 1500 psi using the Bio-Rad biolistic device. Thirty-day-old *N. benthamiana* seedlings were also inoculated in the same manner. In both cases young tender uppermost leaves were targeted (five plants inoculated and another 5 as control). Disease symptoms were recorded daily on the first emerging leaves. The symptom severity on fully expanded leaves was scored based on a 0-5-point scale described earlier by Fauquet and Fargette (1990).

After bombardment, both cassava plantlets and tobacco seedlings were maintained at 28°C and 16 h photoperiod greenhouse for symptom development.

3.3 RESULTS AND DISCUSSIONS

Cassava plantlets and tobacco seedlings inoculated with SACMV dimers showed infection by visibility of symptoms. On the other hand, control plantlets that were not inoculated were symptomless. Symptoms caused by these dimers are shown in Fig. 3.1(A-B). Symptoms appeared 7 dpi in tobacco whereas mosaic symptoms became visible 14 dpi in cassava (Fig. 3.2A and B). Infected cassava leaves displayed yellow chlorosis and at 14 dpi symptoms were assigned a severity score of 4. Cassava symptom severity scores never went up to 5 and remained constantly at 4 until the experiment was aborted. In the case of *N. benthamina* leaves were assigned symptom severity score of 5 at 7 dpi causing severe stunting on the growth of the plant (Fig. 3.2A). Though severity of cassava symptoms was not comparable to field conditions, symptoms caused by the dimers were phenotypically the same as those in field conditions (Fig. 3.1C). The results of the study could also mean SACMV dimers inoculated by means of a biolistic device proved to be infectious. Previously reported studies by Briddon *et al*. (1998) using cloned ACMV-Nigeria with a hand held biolistic device at a pressure range between 200 to 300 psi proved infectious whereas the current study used pressures at 1500 psi.

Table 3.1 Primers used to amplify AC1 N-Rrep from both cassava and tobacco infected plants.

Name	**Sequence**	**Virus**	**Target**
PN-REPC2611	AATGAATTCCTCACGTATCCG	SACMV	AC1
PN-REPV1990	CGATGAGGATCCTACTCGG	SACMV	AC1

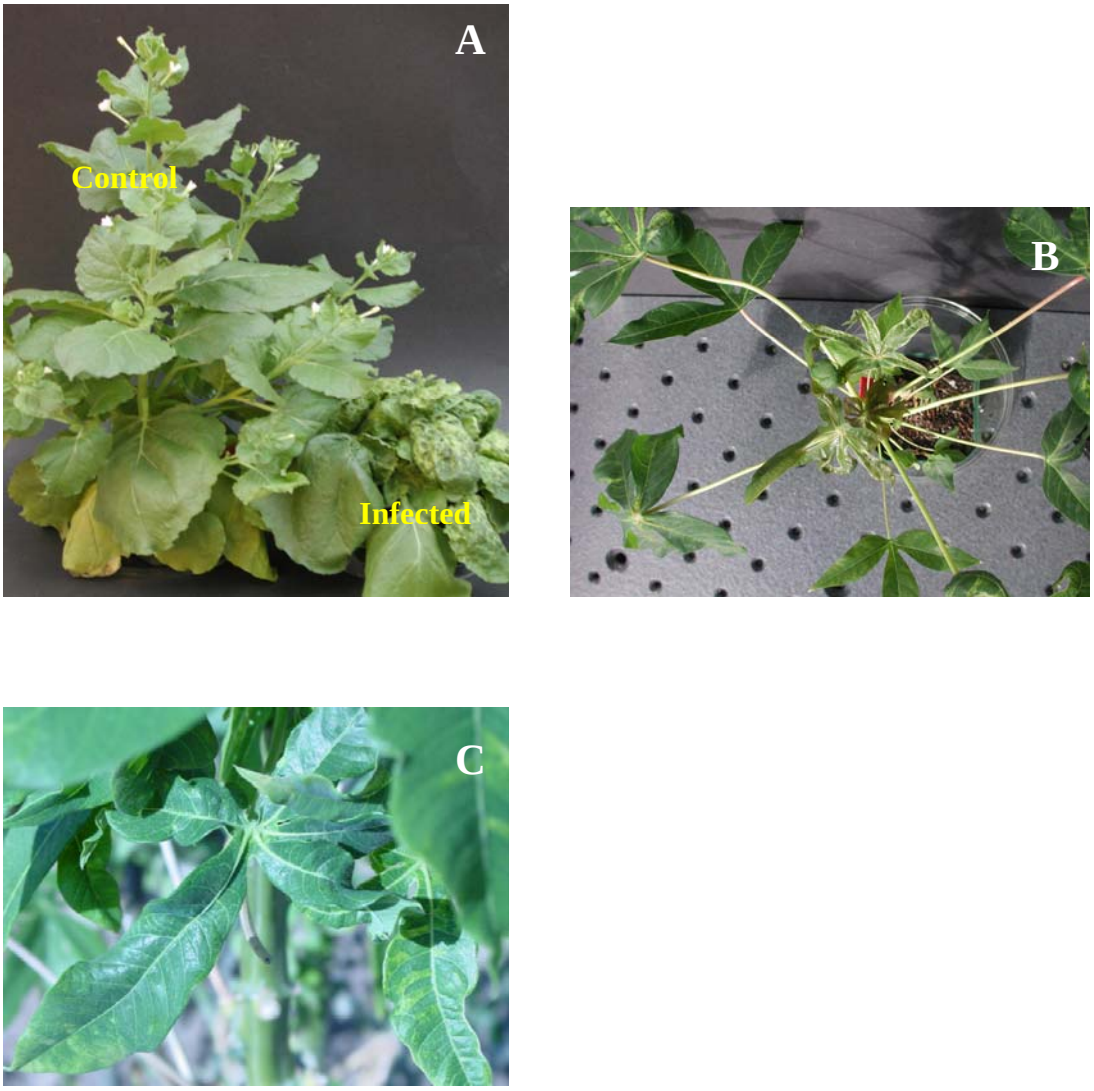
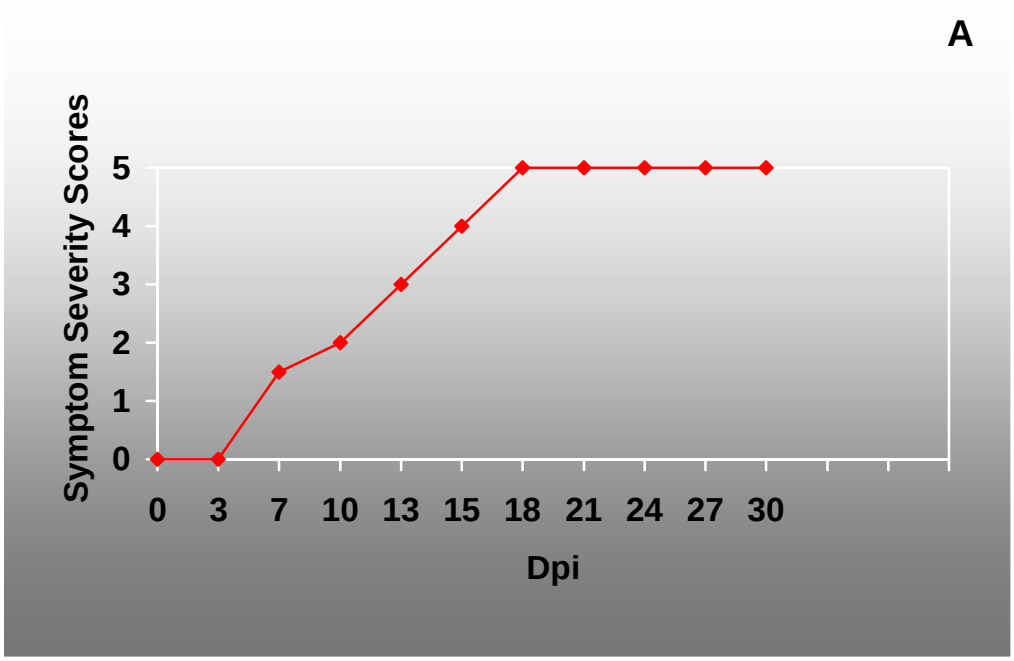


Fig 3.1 Infection with SACMV (A) Stunting and leaf distortion of tobacco leaves in infected plants (right) and normal looking healthy control (left) Plants were photographed at 28 dpi (B) CMD symptoms on young systemically infected leaves of cassava (right) and young healthy leaves (left) Plants were photographed at 30 dpi (C) Leaf showing CMD mosaic symptoms in a cassava field in Mpumalanga Province, South Africa.



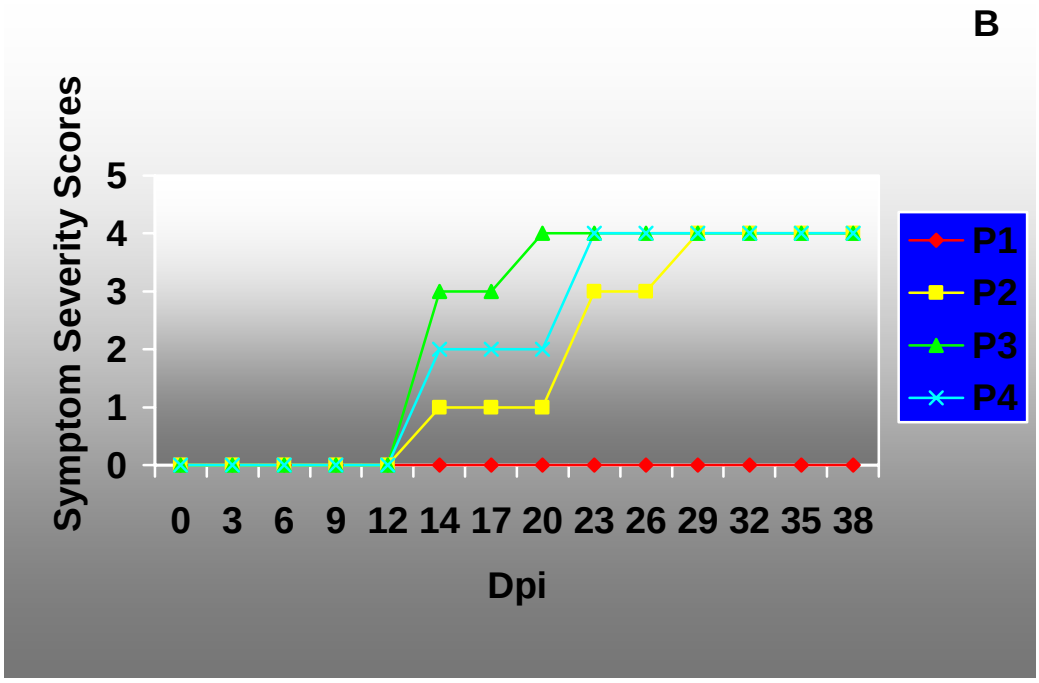


Fig 3.2 Symptom development after particle bombardment with SACMV

(A) tobacco and (B) cassava, P (Plant)

Symptoms spread systemically from first inoculated leaves to young tender developing ones confirming the fact that both components are required for infection and movement from one cell to another. Plants displaying symptoms were screened for the presence of SACMV-DNA-A by PCR amplification using primers designed to amplify the N-Rep region of the SACMV AC1 gene (Table 3.1). N-Rep fragments (621bp) were amplified successfully from plants that displayed symptoms but not obtained from plants that had no symptoms.

We report here successful biolistic infection of SACMV dimer A and B in cassava using the Bio-Rad PDS-1000/He biolistic device for the first time. SACMV symptoms have visual similarity to EACMV but no early recovery after SACMV infection in contrast to other geminiviruses. The results of this study are useful, as our laboratory is currently investigating various strategies that aim to engineer local South African cassava cultivars for resistance to SACMV. We believe that biolistic inoculation of SACMV dimers infectious to cassava will save time by quickly being able to screen for SACMV resistance without having to rely on whitefly inoculation or agroinoculation.

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