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The effectiveness of somatic embryogenesis in eliminating the cocoa swollen shoot virus from infected cocoa trees

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Abstract

Investigations were undertaken on the use of somatic embryogenesis to generate cocoa swollen shoot virus (CSSV) disease free clonal propagules from infected trees. Polymerase chain reaction (PCR) capillary electrophoresis revealed the presence of CSSV in all the callus tissues induced from the CSSV-infected Amelonado cocoa trees (T1, T2 and T4). The virus was transmitted to primary somatic embryos induced from the infected callus tissues at the rate of 10 (19%), 18 (14%) and 16 (15%) for T1, T2 and T4, respectively. Virus free primary somatic embryos from the infected callus tissues converted into plantlets tested CSSV negative by PCR/capillary electrophoresis 2 years after weaning. Secondary somatic embryos induced from the CSSV-infected primary somatic embryos revealed the presence of viral fragments at the rate of 4 (4%) and 9 (9%) for T2 and T4, respectively. Real-time PCR revealed 23 of the 24 secondary somatic embryos contained no detectable virus. Based on these findings, it is proposed that progressive elimination of the CSSV in infected cocoa trees occurred from primary embryogenesis to secondary embryogenesis.
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Keywords: Cocoa swollen shoot virus (CSSV); Primary embryogenesis; Secondary embryogenesis; Somatic embryos; PCR/capillary electrophoresis; Real-time polymerase chain reaction

1. Introduction

The tree *Theobroma cacao* also known as cocoa or cacao is a tropical crop with origins in the Amazon basin and dominates the economies of most West African countries. The cocoa swollen shoot virus (CSSV) is restricted mainly to West Africa where it has resulted in the loss of several millions of cocoa trees (Thresh, 1980). CSSV, a badnavirus, is transmitted by at least 14 species of mealybugs of the family *Pseudococcidae* within the *Coccoidae* (Roivainen, 1976). It is believed that the CSSV was present in the forest regions of West Africa before the introduction of cocoa (Posnette, 1950; Thresh, 1961; Thresh et al., 1988).

In Ghana, virulent and mild strains of CSSV are known to occur with severe strains killing cocoa within 2 years (Posnette, 1947). CSSV disease was first discovered in the Eastern Region of Ghana in 1936 and is now found in all cocoa growing areas of the country (Steven, 1936; Ollenu et al., 1989).

Various attempts have been made in the past to control CSSV with at most only partial success. Control measures attempted included biological control of the mealybug vector (Posnette, 1948), chemotherapy and heat therapy of planting materials (Todd, 1951; Lister and Thresh, 1957), removal of wild hosts (Thresh et al., 1988), breeding for CSSV resistance (Adu-Ampomah et al., 1994), destruction of visibly infected cocoa trees termed "zero tolerance" (Sackey, 2000), and the use of mild strain in cross-protection (Ollenu, 2001).

Despite these attempts, there are to date no effective control measures for CSSV. However, tissue culture techniques such as somatic embryogenesis have been applied to a number of perennial crops to eliminate viruses. Somatic embryogenesis from stigma and style cultures of citrus was used to eliminate *Citrus psorosis* from three citrus species (D'Onghia et al., 1997, 2001). Somatic embryogenesis was also effective in eliminating fan leaf viruses and leaf roll-associated viruses from grapevines (Goussard et al., 1991; Goussard and Wiid, 1993). This technique was applied to cocoa in an attempt to eliminate the CSSV.

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The aim of this research was to test the efficacy of somatic embryogenesis as a means of limiting the movement of CSSV from viral infected plants to clonal propagules. The long term objective is to apply somatic embryogenesis to the sanitary improvement of cocoa potentially infected with CSSV. Diagnostic tools like PCR/agarose electrophoresis and PCR/capillary electrophoresis was used for the qualitative assessment of the presence of the CSSV in the clonal propagules of cocoa. In addition, a real-time polymerase chain reaction (PCR) probe was used to quantify CSSV copy number per cocoa cell in the clonal propagules.

2. Materials and methods

CSSV (strain 1A) infected Amelonado cocoa trees (T1, T2 and T4) and CSSV-free Amelonado cocoa tree (H), were sources for somatic embryo production. The protocol of Li et al. (1998) for the induction of primary somatic embryos and the protocol of Maximova et al. (2002) for the induction of secondary somatic embryos were the basic procedures employed. Primary and secondary somatic embryos reaching the cotyledonary stage of development were used and converted into plantlets. Conversion was scored when the development of primary roots and the first true leaf were observed.

2.1. DNA extraction

Total genomic DNA was extracted from five sources: the leaves of CSSV-infected Amelonado cocoa trees, the CSSV-free Amelonado cocoa tree, callus tissues induced from the floral buds of the Amelonado cocoa trees, primary somatic embryos induced from the callus tissues and secondary somatic embryos induced from the primary somatic embryos. A slightly modified DNeasy TM 96 Plant kit (Qiagen Ltd., UK) protocol was used. 400 μl of lysate Buffer AP1 (preheated to 65 °C) to dissolve and 2 μl of RNase were added to the eppendorf tubes containing the experimental materials and shaken with a tungsten pellet on a Retsch disrupter for 1.4 min × 2 at 25 cycles s^{-1}. DNA extraction quality from the experimental materials was checked on an ethidium bromide-stained agarose gel.

2.2. CSSV primer design and PCR amplification

Primers for the CSSV were designed in the conserved regions of the six published sequences of the CSSV genome available from the National Center for Biotechnology Information database (NCBI) (AJ608931, AJ609019, AJ609020, AJ781003, CSHCG and CSW534983) (Hagen et al., 1993; Muller and Sackey, 2005). The product is 375 base pairs (bp) and runs from position 350–725 bp on accession AJ608931. Both primers were manufactured by Sigma, UK as follows:

- Forward primer AACCTTGAGTACCTTGACCT
- Reverse primer TCATTGACCAACCCACTGGTCAAG

A master mix containing Taq polymerase and dNTPs was supplied by Qiagen (Multiplex PCR kit), UK. The PCR consisted of 5.0 μl 2× master mix, 1.0 μl primer solution containing 2 μM of the CSSV forward and reverse primers, 3.0 μl of water and 1.0 μl of DNA and was run at 95 °C for 15 min, 94 °C for 30 s, 56 °C for 90 s, 72 °C for 60 s at 35 cycles and with a final extension of 60 °C for 30 min. Unlabelled primers were used for the PCR/agarose electrophoresis while the forward primer was labelled with HEX™ for PCR/capillary electrophoresis. Fragment analysis of the PCR products was run on ABI P RISM 3100 genetic analyzer capillary sequencer.

2.3. Qualitative screening of CSSV

To differentiate between a successful and a failed PCR, an internal control targeting a nuclear cocoa microsatellite was used in a multiplex PCR with the CSSV assay. The single copy microsatellite marker named mTcCIR25 (EMIL accession number Y16997) designed for *Theobroma cacao* by Lanaud et al. (1999) was used. The primer sequence for the marker is as follows: forward primer: CTTCGTAGTGAATGTAGGAG and reverse primer: TTAGGTAGGTAGGGTTATCT. The PCR protocol is the same as described with the addition of mTcCIR25 primers in the fragment analysis.

2.4. Quantitative screening of CSSV

Conventional PCR at best allowed only a qualitative assessment of the presence of the virus. In order to quantify the CSSV in the experimental materials (T2) (different sets of CSSV DNA from the one used for qualitative screening), two PCR assays were designed to quantify a CSSV copy number per cocoa cell. The first assay provided a CSSV number while the second assay provided a cocoa cell number. The CSSV sequence was used to design a TaqMan probe-based quantitative PCR assay with the aid of Primer Express Software Version 2. In order to determine the cocoa cell number a quantitative TaqMan assay was designed from the nuclear marker (mTcCIR25) (Lanaud et al., 1999). The following are the primer and marker sequences used:

- CSSV forward primer CCTTAAGAGGCTAACCAAGC
- CSSV reverse primer GGGCTATCTCTTCTACTAGTC
- TaqMan probe TTCCGAGAAAACAAACCACTGTCT-GAATCTG
- mTcCIR25 forward primer CAGATAAGGAAAGGTG-GAGTTTGG
- mTcCIR25 reverse primer CAAGAATGTCTCCTA-CATTCACTACG
- mTcCIR25 probe TTCCCGTAAGCTTCGTCCCAGATGC

Artificial fragments from the CSSV genome and the microsatellite were used for standard dilution (quantification) using a calibration curve generated ranging from 10^1 to 10^5 fragments per μl. These samples were included in each real-time PCR assay. Each CSSV quantitative PCR reaction contained 12.5 μl of PCR sensimix (QUANTACE), 1.25 μl (400 mM) forward and reverse primers, 1.25 μl (400 mM) TaqMan probe 6 Carboxyl Fluorescein Amidite (FAM), 1.25 μl (6.5 mM) $MgCl_2$,

Table 1
Callus tissues from CSSV-infected Amelonado cocoa trees subjected to PCR/agarose electrophoresis and PCR/capillary electrophoresis for virus screening

Cocoa trees	No. of callus tissues tested	Positive test results by	
		PCR/agarose electrophoresis	PCR/capillary electrophoresis
T1	64	48 (75%)	64 (100%)
T2	50	25 (50%)	50 (100%)
T4	54	54 (100%)	54 (100%)
H	30	0 (0%)	0 (0%)

Table 2
Primary somatic embryos from CSSV-infected Amelonado cocoa trees subjected to PCR/agarose electrophoresis and PCR/capillary electrophoresis for virus screening

Cocoa trees	No. of somatic embryos tested	Positive test results by		No. of embryos converted to plantlets	Weaned plantlet
		PCR/agarose electrophoresis	PCR/capillary electrophoresis		
T1	52	0 (0%)	10 (19%)	0 (0%)	0 (0%)
T2	128	0 (0%)	18 (14%)	28 (22%)	22 (17%)
T4	104	0 (0%)	16 (15%)	28 (27%)	13 (12%)
H	30	0 (0%)	0 (0%)	15 50%)	9 (30%)

3.75 Sigma ultra pure water and 5 μl of DNA to bring the final volume to 25 μl. To ensure minimal pipetting error the reactions were all set up using a liquid handling robot (Corbett CAS 1200). The quantitative assay was then run on a Rotor-Gene 6000 (Corbett, Australia) and the data analysed using Rotor-Gene 1.7.28 software. The quantitative PCR reaction conditions with mTcCIR25 were the same as that of the CSSV assay except for the $MgCl_2$ concentration, which was decreased to 3.0 mM.

3. Results

3.1. Viral screening of primary somatic embryos

PCR/agarose electrophoresis on the callus tissues from which the primary embryos were derived indicated 48 (75%), 25 (50%) and 54 (100%) of T1, T2 and T4, respectively tested PCR positive for CSSV but all callus tissues tested CSSV positive for PCR/capillary electrophoresis (Table 1).

All the primary somatic embryos induced from CSSV-infected Amelonado cocoa trees T1, T2 and T4 tested negative for CSSV when the PCR products were screened by PCR/agarose electrophoresis. However, when the same PCR products were screened by PCR/capillary electrophoresis, 10 (19%), 18 (14%) and 16 (15%) of the somatic embryos of T1, T2 and T4, respectively tested positive for CSSV (Table 2). Primary somatic embryos that tested negative to the CSSV, and were converted into plantlets, tested negative to the virus by PCR/capillary electrophoresis 2 years after weaning.

All the secondary somatic embryos induced from primary somatic embryos infected with CSSV tested negative for PCR/agarose electrophoresis. The same PCR products revealed CSSV infection at the rate of 4 (4%), and 9 (9%) for T2 and T4, respectively (Table 3).

When the values of the cycle threshold (Ct, Y-axis) obtained by the quantitative PCR are plotted against the corresponding known concentrations of the target gene (expressed as logarithmic form, X-axis), the result is a line representing the linear correlation between the two variables. The equation describing this relationship is used to extrapolate the gene copy number in experimental samples. The assay proved to be highly reproducible, as demonstrated by a high correlation coefficient ($R^2 = 0.98951$ and $R^2 = 0.98960$) of the standard curves (Fig. 1). The implication of this is that the cycle threshold decreases exponentially as the DNA concentration increases.

3.2. CSSV quantification

1.05E + 06 CSSV copies were detected in the DNA from the leaf sample which corresponded to a concentration of 3.69E + 01 virus/cell. A lower level of virus per cell was detected in the staminodes (6.52E-02 virus/cell). The virus was detected also in somatic embryos from both callus 188 and 192 but at very

Table 3
Secondary somatic embryos induced from primary somatic embryos infected with CSSV subjected to PCR/agarose electrophoresis and PCR/capillary electrophoresis for virus screening

Cocoa trees	No. of somatic embryos tested	Positive test results by	
		PCR/agarose electrophoresis	PCR/capillary electrophoresis
T2	100	0 (0%)	4 (4%)
T4	100	0 (0%)	9 (9%)
H	30	0 (0%)	0 (0%)

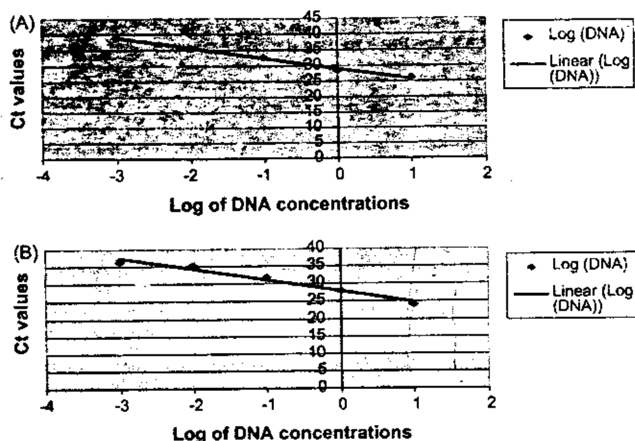

Fig. 1. Real-time PCR standard curves: (A) CSSV TaqMan real-time PCR assay and (B) mTcCIR25 TaqMan real-time PCR assay.

different levels (5.57E-05 virus/cell and 1.75E-03 virus/cell, respectively). CSSV was not detected in 3 out 5 primary somatic embryos derived from callus 188 and 192. Only 1 out of 24 secondary somatic embryos exhibited a very low presence of virus (6.86E-04 virus/cell) (Table 4).

4. Discussion

4.1. Qualitative screening of somatic embryos for CSSV

Somatic embryos were successfully induced from different CSSV-infected cocoa trees (Fig. 2). The callus tissues recorded high viral infections by both PCR/agarose electrophoresis and PCR/capillary electrophoresis (Table 1). Primary somatic embryos originating from the callus tissues subjected to PCR/agarose electrophoresis did not reveal the presence of CSSV. However, PCR/capillary electrophoresis of the same CSSV DNA samples revealed the presence of CSSV in the primary somatic embryos (Table 2). Assuming no accidental viral contamination of primary embryos occur it appears that CSSV is capable of movement from the callus tissues into the somatic embryos but at a reduced frequency.

PCR/capillary electrophoresis on the secondary somatic embryos induced from the infected primary embryos revealed the presence of viral fragments at a low concentration (Table 3). The implications are that from the point of induction of primary somatic embryos to secondary somatic embryos, the virus has gradually been excluded. The point at which the virus was eliminated is yet to be determined.

Table 4
Summary of quantitative real-time PCR showing CSSV DNA copies and the number of viruses per cell as reveal by mTcCIR25 TaqMan assay (T2)

DNA samples	Mean CSSV real number	Mean cocoa cell number (mTcCIR25/2)	CSSV cocoa numbers/cell
Leaf	1.05E+06	2.85E+04	3.69E+01
Staminode	2.15E+00	3.30E+01	6.52E−02
Callus 188	1.11E+02	1.34E+01	8.26E+00
Callus 192	1.76E+01	8.34E+02	2.11E−02
Primary somatic embryo (callus 188)	2.77E+00	4.98E+04	5.57E−05
Primary somatic embryo (callus 188)	[a]	1.92E+04	[a]
Primary somatic embryo (callus 192)	3.86E+01	2.21E+04	1.75E−03
Primary somatic embryo (callus 192)	[a]	2.83E+04	[a]
Primary somatic embryo (callus 192)	3.59E+01	6.83E+04	5.26E−04
Secondary somatic embryo 401	[a]	2.45E+03	[a]
Secondary somatic embryo 402	[a]	7.30E+03	[a]
Secondary somatic embryo 403	[a]	4.18E+03	[a]
Secondary somatic embryo 404	[a]	4.69E+03	[a]
Secondary somatic embryo 405	[a]	1.35E+04	[a]
Secondary somatic embryo 406	[a]	1.63E+04	[a]
Secondary somatic embryo 407	1.62E+00	2.36E+03	6.86E−04
Secondary somatic embryo 408	[a]	7.51E+03	[a]
Secondary somatic embryo 409	[a]	1.25E+04	[a]
Secondary somatic embryo 410	[a]	1.68E+03	[a]
Secondary somatic embryo 411	[a]	5.77E+03	[a]
Secondary somatic embryo 412	[a]	5.09E+03	[a]
Secondary somatic embryo 413	[a]	6.04E+03	[a]
Secondary somatic embryo 414	[a]	3.86E+03	[a]
Secondary somatic embryo 415	[a]	8.39E+03	[a]
Secondary somatic embryo 416	[a]	3.95E+03	[a]
Secondary somatic embryo 417	[a]	7.02E+03	[a]
Secondary somatic embryo 418	[a]	4.30E+03	[a]
Secondary somatic embryo 419	[a]	2.56E+03	[a]
Secondary somatic embryo 420	[a]	6.77E+03	[a]
Secondary somatic embryo 421	[a]	3.65E+03	[a]
Secondary somatic embryo 422	[a]	5.06E+03	[a]
Secondary somatic embryo 423	[a]	1.41E+04	[a]
Secondary somatic embryo 424	[a]	6.56E+03	[a]

[a] Effectively zero.

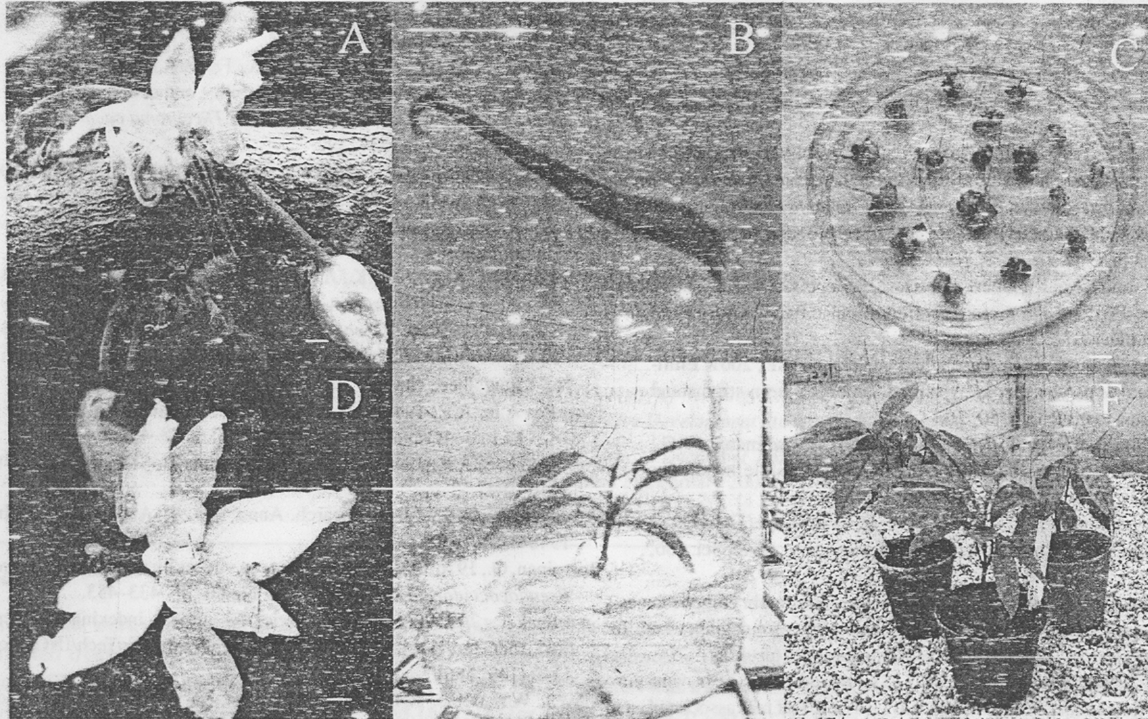

Fig. 2. Primary somatic embryo production from cultured staminodes of CSSV-infected Amelonado trees. (A) Opened flower showing staminodes. (B) Freshly isolated staminode cultured on primary callus growth medium. (C) Cocoa calluses with roots 8 weeks on embryo development (ED) medium. (D) Cocoa somatic embryos at different developmental stages 12 weeks on ED medium. (E) Plantlet cultured on primary embryo convention medium with multiple true leaves and roots ready for acclimatization. (F) Plantlets from somatic embryos 24 months after weaning (scale bars: A, B and D = 1 mm; C and E = 1 cm; F = 3 cm).

The effectiveness of somatic embryogenesis as a technique in eliminating the CSSV was further demonstrated by plantlets testing CSSV negative by PCR/capillary electrophoresis 2 years after weaning. This result conforms to the findings of (D'Onghia et al., 2001; Goussard et al., 1991) who used somatic embryogenesis to eliminate the *Citrus psorosis* virus, and fan leaf virus and leaf roll-associated viruses from grave vine.

Although one round of somatic embryogenesis (primary embryogenesis) was an efficient procedure for producing and selecting CSSV-free cocoa materials, its multicellular origin and unsynchronised embryo production does not make it ideal for research purposes and improvement of the crop. The unicellular origin and synchronised embryo production of secondary somatic embryos was more efficient for clonal propagation of cocoa for breeding programmes and the development of genetic transformation (Pence, 1989; Maximova et al., 2002).

4.2. Development of real-time PCR assay for CSSV

While capillary screening provided a good qualitative assessment, quantitative PCR assays were used to determine absolute CSSV levels during the somatic embryogenesis process. 23 out of 24 secondary somatic embryos exhibited no virus copies per cell. The presence of CSSV in the leaves, staminodes and callus tissues gave further evidence that somatic embryogenesis is capable of generating disease free planting materials from CSSV-infected trees (Table 4).

The performance of the real-time PCR assay enabled the detection and estimation of CSSV concentrations in the cells of cocoa and conforms to the findings of real-time PCR developed for the rapid detection of *Banana streak virus* (Delanoy et al., 2003). It can also be used to monitor the evaluation of viral tolerance levels in new breeding programmes (Dahal et al., 2000).

With the virtual absence of the CSSV in the secondary somatic embryos, which is the basis of enhancement of cocoa germplasm conservation through cryopreservation (Fang et al., 2004), these results have significant implications for the rapid and efficient generation of disease free clonal propagules, germplasm movement and cocoa improvement programmes.

5. Conclusion

Somatic embryogenesis was effective in eliminating the CSSV from infected cocoa trees producing disease free clonal stock materials. The CSSV was drastically reduced from primary to secondary embryogenesis. Indexing of CSSV was more reliable using PCR/capillary electrophoresis, as PCR/agarose electrophoresis was prone to false negatives. Real-time PCR TaqMan assay allowed sensitive quantification and estimation of CSSV concentrations in the cocoa cells.

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