Journal of Plant Sciences
2016; 4(3): 52-57
http://www.sciencepublishinggroup.com/j/jps
doi: 10.11648/j.jps.20160403.14
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

Virus Detection and Elimination in Cocoa (*Theobroma cacao* L.) Through Somatic Embryogenesis

Rebicca Edward[1, 2], Andrew Wetten[2]

[1]Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, University Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia

[2]School of Biological Sciences, Harborne Building, University of Reading, Whiteknights, Reading, Berkshire, UK

Email address:

erebicca@unimas.my (R. Edward)

To cite this article:

Rebicca Edward, Andrew Wetten. Virus Detection and Elimination in Cocoa (*Theobroma cacao* L.) Through Somatic Embryogenesis. *Journal of Plant Sciences*. Vol. 4, No. 3, 2016, pp. 52-57. doi: 10.11648/j.jps.20160403.14

Received: May 1, 2016; **Accepted**: May 11, 2016; **Published**: May 26, 2016

Abstract: *Cacao swollen shoot virus* (CSSV) is a major pathogen that has seriously constrained cocoa production in West Africa, particularly Ghana and Nigeria. The objective of this study was to assess the efficacy of cocoa somatic embryogenesis to produce virus-free clonal propagation material both for replanting and to facilitate the safe international exchange of germplasm. Polymerase Chain Reaction (PCR)-based screening, is employed in this study because of its capacity for CSSV detection prior to the appearance of visual symptoms. Degenerate PCR primers were developed in order to improve the CSSV-strain dependence of earlier tests. The degenerate primers were capable of detecting 37 out of a putative 56 CSSV strains, four more than the sequence specific primers. For tissue culture studies, cocoa staminodes cultures were established from flowers of CSSV-infected cocoa genotypes CL 19/10 strain 1A and Amelonado Plant 2 to produce callus, primary and secondary somatic embryos, with genotype AMAZ 15 used as a virus-free control. PCR-based CSSV detection proved that virus could be detected at callus, primary somatic embryos and secondary somatic embryo stages, indicating that the progress of the virus was progressively impeded. These findings support the use of somatic embryogenesis as a mean of improving CSSV-free clonal propagation of cocoa. Somatic embryogenesis is indeed effective for virus elimination in cocoa and it has been demonstrated to function for a range of cocoa genotypes. This also means that a likely mechanism for the interruption of CSSV movement has been identified.

Keywords: *Cacao swollen shoot virus*, Cocoa, Somatic Embryogenesis, Staminodes, Polymerase Chain Reaction

1. Introduction

Cacao swollen shoot virus (CSSV) has a long history in Ghana. The first report of the condition caused by CSSV known as Cocoa Swollen Shoot Disease (CSSVD) in Ghana was made in 1936 [15]. All affected trees and neighbouring contact trees were cut down and burned, even before identifying the pathogen as a virus [15]. Early identification of cacao swollen shoot disease is important since its viral infection at present cannot be cured. Failure to detect the infection of trees is known to be a problem and it has been established that latently infected trees – those not yet showing symptoms - can act as a source for further transmission [5]. CSSVD was later discovered in other West African cocoa producing countries: Nigeria in 1944 [16], Ivory Coast in 1946 [7], Togo in 1949 [12] and Sierra Leone in 1963 [1]. The disease was also reported to be found in Sri Lanka and in Indonesia (Java and North Sumatra) [17]. However, neither the disease nor its pathogen has been reported in the Amazon Basin (South America), from where cocoa was originally exported into West Africa. Quarantine measures to prevent the spread of CSSV by the movement of infected cocoa pods and cutting materials, is now possible with the improved PCR-based detection for the virus [10]. Genomic variability of CSSV isolates collected from different West African locations indicate however that without appropriate precautions PCR-based screening could result in false positives. Therefore, the aim of development of strain-independent CSSV detection by PCR in this study, would not only contribute to the quarantine procedures for the diagnosis of CSSV in cocoa trees, but also to inform the

wider field of safe plant germplasm exchange. This study also aims to examine the efficacy of somatic embryogenesis for the interruption of virus movement during clonal multiplication of cocoa.

2. Materials and Methods

2.1. CSSV Detection by Polymerase Chain Reaction (PCR)

2.1.1. CSSV Isolates

A total of 56 putatively distinct isolates of CSSV were obtained from the Cocoa Museum, Cocoa Research Institute of Ghana (CRIG). The trees were maintained in a segregated area separated from the nearest neighbouring cocoa trees by insect proof netting. The isolates originate from widely distributed cocoa growing areas across Ghana and were classified by symptom expression according to the presence or absence of swellings and the absence or presence of (and intensity) leaf symptoms [11]. Among the most visually CSSV-infected cocoa leaf samples were those that come from Ghana's Eastern region which is reported to be the region where the most virulent strain of the causal virus occurs [2]. This is followed by Western and Ashanti regions, while only one infected leaf sample derives from the Central region.

All leaf samples from Cocoa Museum, Cocoa Research Institute of Ghana were frozen at -80°C as soon as they were delivered to the University of Reading via courier. Positive controls used in this experiment were two cocoa genotypes from CL 19/10 strain 1A and ICS 68. They were symptomatic leaves from CSSV-infected cocoa trees maintained in an insect-proof netting cage within a glasshouse at the University of Reading. Another two positive controls used were CSSV-infected cocoa seedlings maintained separately at the Plant Science Glass house (strains New Juaben and Kpeve). For the negative control, leaf material was collected from cocoa genotype AMAZ 15 from the International Intermediate Cocoa Quarantine Centre, University of Reading. DNA extraction was conducted using the basic protocol described by manufacturers of the DNeasy® Plant Mini Kit (Qiagen Ltd., UK) with some minor modifications.

2.1.2. PCR Amplification

For the first generation primer, PCR primer pairs were designed based on regions of 100% homology across the six published CSSV DNA sequences of the CSSV genome available from the National Center for Biotechnology Information database (NCBI) (Accession nos.: AJ534983.1, AJ608931.1, AJ609019.1, AJ609020.1, AJ781003.1 and L14546.1) [4, 9]. The primers were generated using software, Geneious version 5.4 (Biomatters Ltd.) and manufactured by Sigma, UK as follows: CSSV forward primer (AACCTTGAGTACCTTGACCT) and the CSSV reverse primer (TCATTGACCAACCCACTGGTCAAG).

The primer product is approximately 375 base pair (bp) depending on viral strain and runs from position 350 to 725 bp on accession AJ608931.1. Whereas for the second and third experiments, CSSV second generation primers with a mixture of nucleotide bases designed using software, Geneious version 5.4 (Biomatters Ltd.) consisted of a mixture of 48 variables with redundant nucleotide bases as follows: CSSVM13uniF: 5' (ACAGCTATGACCATGAGYATHCARGARTGGTAYGA) and CSSVM13uniR: 5' (AAAACGACGGCCAGTCAYTGNCCNACCCAYTGRTC). [R = G or A, Y = T or C, H = A or C or T, N = G or A or T or C]. A final volume of 10 µl for each PCR reaction mixture consisted of a ratio 5:3:1:1 comprising 5 µl of master mix containing Taq polymerase and dNTPs (Multiplex PCR kit, Qiagen, UK), 3 µl of Nanopure water, 1 µl of primer solution containing 2 µM of CSSV forward and reverse primers and 1 µl of DNA sample (concentration range of 4-16 ng/µl). Once all of the PCR tubes contain all 4 of the reactants in their correct ratios the PCR tubes were and then placed into a PCR thermocycler which facilitates the amplification of the DNA. The amplification cycle conditions used were as follows: 94°C for 30 s to denature the DNA, 57°C for 90 s to anneal, 72°C for 60 s for elongation. The cycle was repeated 35 times with a final extension at 10°C indefinitely until removed from the machine.

2.2. CSSV Elimination Through Somatic Embryogenesis

2.2.1. Plant Materials

Unopened immature flowers from three cocoa genotypes infected with CSSV, namely CL 19/10, ICS 68 and Amelonado Plant 2 maintained in the insect-proof netting glasshouse were collected as research samples. Healthy cocoa flowers, as negative control were collected in the University of Reading Cocoa Intermediate Quarantine Unit. Floral buds from these trees were collected for the induction of somatic embryogenesis.

2.2.2. Induction of Somatic Embryos

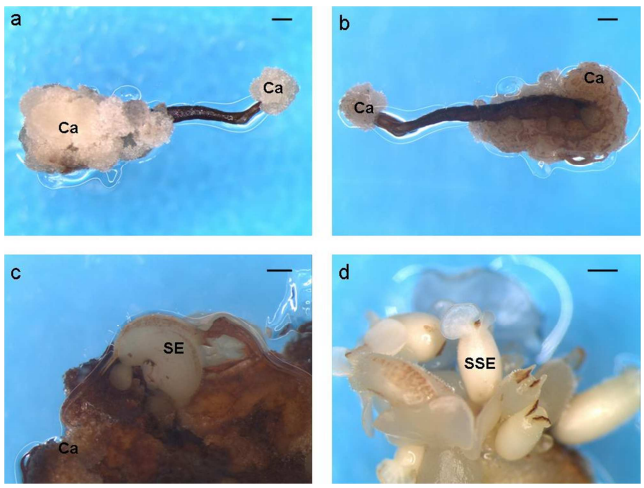

Figure 1. *Cocoa staminode producing callus (a and b); callus started to develop from the cut edge of staminode (b shows the underside of staminode in a). Primary somatic embryo developing on callus tissues (c) and secondary somatic embryo (d) generated from cotyledonary explants of primary somatic embryo. Ca = callus, SE = primary somatic embryo, SSE = secondary somatic embryo. Bars represent 1 mm (a and b), 2 mm (c) and 3 mm (d).*

Induction of cocoa somatic embryos was generated according to the protocol of [6]. After the surface sterilization procedure was conducted, the flower buds were sliced perpendicular to their longitudinal axis about 1/3 of the flower length from the base with a sterile scalpel blade. Staminode (five per floral bud) explants were extracted from the upper part of the flower bud. Twenty staminode explants were placed into a Petri dish containing primary callus growth (PCG) medium and sealed with sealing film (Nescofilm). Cultures were maintained in the dark at 25°C for 14 days. Explants were then transferred into a Petri dish containing secondary callus growth (SCG) medium, sealed and cultured for another 14 days under the same conditions. Callus usually formed at the cut edge of each staminode by the end of this culture period. Explants were then transferred to Petri dishes containing embryo development (ED) medium and cultured in the dark at 25°C for 14 days. Subsequent subcultures were carried out at intervals of 14 days, by transferring explants onto fresh ED medium to induce somatic embryos which arose via the callus. Secondary somatic embryos were induced as described by [8]. Mature primary embryos with developed cotyledons were selected and the cotyledons were separated from the embryo hypocotyls and sliced into 4×4mm pieces. Cotyledon explants were placed in Petri dishes containing SCG medium, sealed with Nescofilm (Azwell, Osaka, Japan) and cultured in the dark at 25°C for 14 days. Explants were subcultured onto fresh ED medium every 14 days and maintained under the same conditions to generate secondary somatic embryos. Secondary embryos were produced within two to three months after culture initiation, with minimal additional callus development. Images of the production of cocoa callus, primary somatic embryos and secondary somatic embryos were presented in Figure 1.

2.2.3. DNA Extraction and PCR Amplification

Total genomic DNA of the experimental materials was extracted using a slightly modified DNeasy Plant mini kit (Qiagen Ltd., UK) protocol. The DNA was extracted from the leaves of CSSV infected cocoa trees (CL 19/10) and a CSSV-free (AMAZ 15); callus tissues induced from the floral buds of the CSSV infected cocoa trees; somatic embryos induced from the callus tissues and secondary somatic embryos induced from the primary somatic embryos. PCR was run as described in section 2.1.2.

3. Results and Discussion

3.1. CSSV Detection by Polymerase Chain Reaction (PCR)

Results of the PCR detection of CSSV presented in Table 1 show leaf samples of CSSV-infected cocoa plants from the Cocoa Museum, Cocoa Research Institute of Ghana (CRIG). To confirm the results, PCRs were repeated for DNA samples that gave negative results and faint positive bands, both for the first generation CSSV primers and also degenerate primers CSSVM13uni. This means that PCRs for both primers were only repeated when two consecutive results were not the same.

Table 1. *PCR screening of leaf samples of CSSV-infected cocoa plants from CRIG.*

No.	Genotype	First generation CSSV primer F&R			CSSVM13uni F&R		
	PCR trial	1	2	3	1	2	3
1	Abo Boya (CC) - WR		-	-	-	-	
2	Achechere (CC) - W/R	-	-	-	+	-	
3	Achiasi W/R	-	+ (faint)	-	+	-	-
4	AD 135 ER		+		-	+	+
5	AD 7 E/R		+		-	+	+
6	AD 75/ER		-	-	-	-	-
7	Adiembra/CC WR	-	-	-	+	+ (faint)	
8	Agyepomaa	+	+		+	+	
9	Aiyim (CC) – WR		+		+	+	
10	Amanfie W/R		+		+	+	
11	Anibil (CC) WR		-	-	-	-	
12	Asamankese Isolate	-	+ (faint)	-	+	+	
13	Ayiboso – W/R		+		+	+	
14	Bakukrom /CC/W/R		+		+	+	
15	Bechem B/A	+	+		+	+	
16	Bisa		+		-	+	+
17	Bobiriso/Juaso 1 ASH		+		-	+	+
18	Bosomtwe/I J ASH	-	+		+	+	
19	Bosomuoso 2 W/R		+		-	+	+
20	Datano W/R		-	-	-	-	
21	Dawa/1H/ER	-	-	-	+	+ (faint)	
22	Dochi/IG/ER		-	-	-	+ (faint)	+
23	Enchi E1/A/3 W/R		+ (faint)	-	-	+ (faint)	-
24	Gavepetodzi - U/R	-	+		+	+	
25	Jamesi – W/R		+		+	+	
26	Koben - ASH		+		+	+	
27	Kofi Pare Isolate (1A)	+	+		+	+	
28	Konongo (IK) ASH		+		+	+	

No.	Genotype	First generation CSSV primer F&R			CSSVM13uni F&R		
	PCR trial	1	2	3	1	2	3
29	Kpeve Isolate	-	-	-	-	-	
30	Krofa / Juansa F2T2	-	+		+	+	
31	Kwadzo Kumkrom J2/A	-	-	-	-	-	
32	Kwakoko Juansa North A/R		+		+	+	
33	Kwaku Anyan T1 B/A		-	-	-	+ (faint)	+
34	Madjeda Nkwanta Agogo/F1/T2/ASH		+		+	+	
35	Mampong (1m) - ER	-	-	-	+ (faint)	+ (faint)	
36	Miaso Isolate	-	+		+	+	
37	N1 Isolate	-	-	-	+	+	
38	New Juaben Isolate (1A)	+	+		+	+	
39	Nkrankwanta Isolate		+		+	+	
40	Nsaba Isolate	-	-	-	+	+	
41	Oyimso Agogo 5 ASH		+		-	-	
42	Pa Men (1e) - ER	-	-	-	+	+	
43	Peki - U/R	+	+		+	+	
44	Punekrom – W/R		-	-	-	-	
45	Sankore T3/3		+		+	+	
46	SS 365B Isolate	-	+		+	+	
47	SS167 – E/R (mildstrain)		-	-	-	-	
48	Suhuma W/R	+	+		+	+	
49	Surowno /WR		+		+	+	
50	Tafo Yellow	-	-	-	+	-	-
51	Tease Adeakyi		-	-	-	+ (faint)	-
52	Tease Atomsu-Abuom		-	-	-	+ (faint)	+ (faint)
53	Techimantia outbreak 3T-15		+		+	+	
54	Virus AD 14/ER		+		+	+	
55	Virus AD 196		+		+	+	
56	Worawora	+	+		+	+	

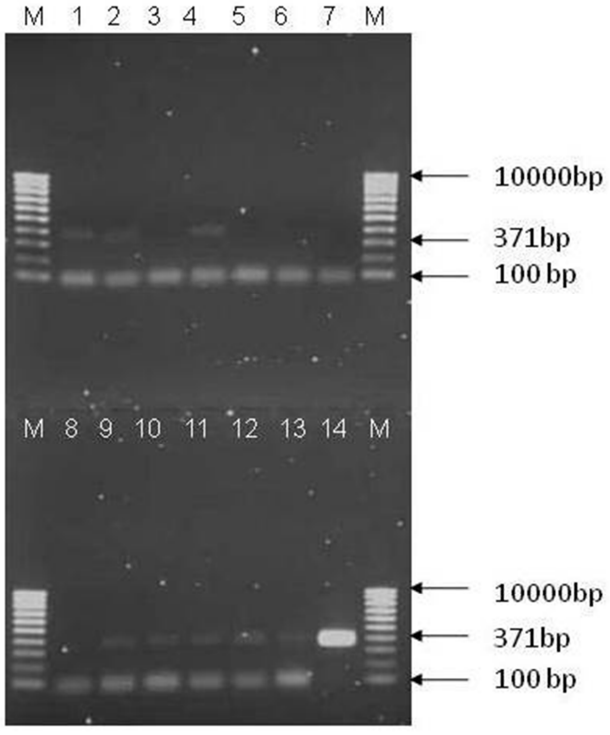

Figure 2. *Third trial for PCR-based screening of DNA leaf samples with degenerate primer (CSSVM13uni). Lane 1 AD 135 ER, Lane 2 AD 7 E/R, Lane 3 Achiasi W/R, Lane 4 Bisa, Lane 5 AD 75/ER, Lane 6 Enchi E1/A/3 W/R, Lane 7 Tafo Yellow, Lane 8 Tease Adeakyi, Lane 9 Bobiriso/Juaso 1 ASH, Lane 10 Bosomuoso 2 W/R, Lane 11 Dochi/IG/ER, Lane 12 Kwaku Anyan T1 B/A, Lane 13 Tease Atomsu-Abuom, Lane 14 positive control CL 19/10 (CSSV-infected cocoa leaf). Marker = HyperLadder™ I. Expected CSSV band = 371bp.*

For the first generation primer, leaf samples were assessed in two batches. In the first PCR trial, a batch of 24 CSSV-infected cocoa leaf samples was received from CRIG. This was the initial experiment and 7 out of 24 cocoa leaf DNA samples were PCR positive with CSSV. The second PCR trial was the result for the batch of 32 CSSV-infected cocoa leaf samples and repeated trial for the former batch of 24 cocoa leaf samples. 33 samples were positive with CSSV and 3 samples gave faint bands. For the third trial, the 3 samples that gave faint bands were repeated and the 20 samples that gave negative amplifications were also repeated. Given the inclusion of a positive CSSV control, the results showed that all samples in the third trial were not positive with CSSV with this primer pair. Whereas for the second generation primer (degenerate primer CSSVM13uni), 36 out of 56 cocoa DNA leaf samples were positive with CSSV in the first PCR trial, while 1 sample gave a faint band using the degenerate primer pair. The second PCR trial was to repeat the first trial. 37 samples gave positive amplification and 7 samples gave faint bands. The third trial (Figure 2) was repeated to confirm the results in the first and second trials and results were similar to those from the second trial.

The first generation primers designed for CSSV detection based on the alignment of the six published nucleotide sequences derived from the virus strain 1A isolates [4, 9] suggested that more than half of the 56 CSSV-infected leaf samples from Cocoa Research Institute of Ghana (CRIG) gave positive CSSV detection. Further screening experiments with CSSV second generation primers, which consisted of a mixture of 48 variable redundant nucleotide bases, suggested its capability to detect 37 strains of the 56 CSSV-infected leaf

samples, four more virus strains than the first generation primers (33 strains). Thus, the design of the second generation CSSV primers showed its potential for improving the quarantine procedures for the international exchange and long term conservation of cocoa germplasm. Further research might be devoted to improvement of the PCR performance by developing a real-time PCR assay for CSSV detection. The quantitative real-time PCR assay might be possible for the reproducible and specific detection of CSSV from the banana and plantain explants, as reported for the detection of episomal Banana streak virus (BSV) in banana and also in plantain [3] An initial study for developing a real-time PCR assay for cocoa was carried out by [13]. His findings showed that real-time PCR assays are appropriate for the detection and estimation of the CSSV concentrations in the cells of cocoa, which conforms to the findings of real-time PCR developed for the rapid detection of episomal BSV in banana [3].

3.2. Viral Screening of Cocoa Embryogenic Tissues Derived from CSSV Infected Cocoa Trees

Statistical significance of differences of the effect of cocoa genotypes on virus elimination was assessed by Chi-square (SPSS software version 19, IBM SPSS Statistics).

Table 2. *Virus screening for 4 weeks old callus tissues derived from staminodes of CSSV-infected cocoa trees.*

Genotype	Number of callus tissues tested	PCR Positive test results
CL 19/10	30	(18/30) 60%
ICS 68	30	(11/30) 36.66%
Amelonado Plant 2	30	(9/30) 30%

PCR-based CSSV detection on callus samples derived from staminodes (Table 2) showed that CL 19/10, ICS 68 and Amelonado Tree 2 (Plant 2) were tested positive with CSSV. The most CSSV detection was found with CL 19/10 calluses (60%), followed by calluses of ICS 68 (36.66%) and Plant 2 (30%). From the chi-square test, the P-value was 0.047. Therefore, there was a significant difference between the cocoa genotypes on virus elimination. PCR image in Figure 3 showed the viral screening of some of the callus derived from staminodes from CSSV infected CL 19/10 on agarose.

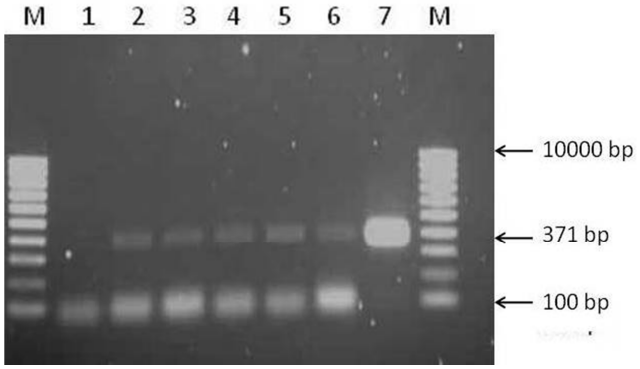

Figure 3. *PCR screening of callus derived from staminodes from CSSV infected CL 19/10 on agarose. Lane 1 DNA of leaf sample of virus-free AMAZ 15 as negative control, Lanes 2 to 6 DNA samples from callus, Lane 7 positive control from DNA leaf sample of CSSV infected CL 19/10. Marker = HyperLadder™ I. Expected CSSV band = 371bp.*

Table 3. *Virus screening of primary somatic embryos of CSSV-infected cocoa trees.*

Genotype	Number of primary somatic embryos tissues tested	Positive test results (%)
CL 19/10	30	(15/30) 50%
ICS 68	22	(9/22) 40.9%
Amelonado Plant 2	27	(11/27) 40.74%

Results of the PCR detection of CSSV presented in Table 3 indicated that half of the primary somatic embryos of CL 19/10 tested positive for CSSV. Primary somatic embryos of ICS 68 and Plant 2 gave positive results at 40.90% and 40.74% respectively. From the chi-square test, the P-value was 0.728. Therefore, no significant difference was found in the effect of cocoa genotype on virus elimination.

Table 4. *Virus screening of secondary somatic embryos induced from primary somatic embryos of CSSV-infected cocoa trees.*

Genotype	Number of secondary somatic embryos tested	Positive test results (%)	Number of embryos converted to plantlets (%)	Weaned plantlets (%)
CL 19/10	26	(5/26) 19.23%	(12/26) 46.15%	(10/26) 38.46%
ICS 68	10	0	(2/10) 20%	(2/10) 20%
Amelonado Plant 2	23	(4/23) 17.4%	0	0

Not all secondary somatic embryos induced from cotyledonary explants of primary somatic embryos infected with CSSV gave positive results. From Table 4, 19.23% of secondary somatic embryos of CL 19/10 were PCR positive for CSSV, followed by secondary somatic embryos of Plant 2 (17.4%). All secondary somatic embryos of ICS 68 tested PCR negative for CSSV. From the chi-square test, the P-value was 0.035. Therefore, significant difference was found in the effect of cocoa genotype on virus elimination. Somatic embryogenesis from cocoa staminodes induced from two CSSV-infected trees, CL 19/10 and ICS 68, successfully resulted in the production of a number of clonal plantlets that could be weaned to the glasshouse. These CSSV PCR-negative plantlets have not shown any symptoms of CSSV infection in over one year since weaning.

A recent report suggested that somatic embryogenesis was capable of the progressive interruption of the movement of CSSV from primary somatic embryos to secondary somatic embryos [14]. From the callus derived from the CSSV-infected staminodes, to the primary somatic embryos and later to the secondary somatic embryos, the presence of CSSV was reduced, indicating that the progress of the virus was progressively impeded. These findings can contribute to the further improvement of the crop by generating disease free materials before they are widely distributed.

During the course of this study, one of the major findings

would be the investigation on effectiveness of the application of somatic embryogenesis technique to interrupt movement of CSSV. Somatic embryogenesis is indeed effective for virus elimination in cocoa and it has been demonstrated to function for a range of cocoa genotypes. This also means that a likely mechanism for the interruption of CSSV movement has been identified.

4. Conclusions

In conclusion, using CSSV primers to the conserved sequence regions, one can determine the presence of CSSV from the CSSV-infected leaf samples. Similarly, the use of degenerate primers to target the conserved sequence regions of other strains of CSSV, has slightly improved the PCR-based CSSV detection. The outcome of these trials is potentially useful with respect to future breeding work aimed at improving the crop in West Africa as a means to generate clean test materials. It will also be of value for the accelerated testing of cocoa materials for CSSV before they are distributed and conserved globally. Propagating system by somatic embryogenesis technique is useful not only in generating disease-free clonal materials from virus-infected plants, but also useful in improving breeding, where improving can be conducted with clean test materials.

Acknowledgement

This work is supported by a scholarship from the Ministry of Higher Education of Malaysia. The authors are grateful to the staffs at Cocoa Research Institute of Ghana for the leaf samples, staffs at Cocoa Intermediate Quarantine Unit, and Harborne Building and University of Reading, UK for their technical assistance.

References

[1] Attafuah, A., Blencowe, J. W. and Brunt, A. A., 1963. Swollen shoot disease of cocoa in Sierra Leone. *Tropical Agriculture (Trinidad),* 40: 229-232.

[2] Dale, W. T., 1962. Virus diseases. In: *Agriculture and Land Use in Ghana* (Ed, Wills, J. B.) Oxford University Press, London, pp. 286-316.

[3] Delanoy, M., Salmon, M., and Kummert, J., 2003. Development of real-time PCR for the rapid detection of episomal *Banana streak virus* (BSV). *Plant Disease*, 87: 33-38.

[4] Hagen, L. S., Jacquemond, M., Lepingle, A., Lot, H. and Tepfer, M., 1993. Nucleotide sequence and genomic organization of cacao swollen shoot virus. *Virology*, 196: 619-628.

[5] Legg, J. T., 1982. The Cocoa Swollen Shoot Research Project at the Cocoa Research Intitute, Tafo, Ghana, 1969-1978. Overseas Development Administration, London.

[6] Li, Z., Traore, A., Maximova, S. and Guiltinan, M., 1998. Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. *In vitro Cellular and Developmental Biology-Plants*, 34: 293-299.

[7] Mangenot, G., Alibert, G. and Basset, A., 1946. Sur les caractères du swollen shoot en Cote-d'lvoir. *Review international Botany Application Agriculture Tropical*, 283: 13.

[8] Maximova, S. N., Alemanno, L., Young, A., Feffiere, N., Traore, A. and Guiltinan, M. J., 2002. Efficiency, genotopic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. *In vitro Cellular and Developmental Biology-Plant*, 38: 252-259.

[9] Muller, E. and Sackey, S., 2005. Molecular variability analysis of five new complete cacao swollen shoot virus genomic sequences. *Archives of Virology*, 150: 53-66.

[10] Muller, E., Jacquet, E., and Yot, P., 2001. Early detection of cacao swollen shoot virus using polymerase chain reaction. *Journal of Virology Methods*, 93: 15-22.

[11] Ollenu, L. A. A., 2001. *Synthesis: case history of cocoa viruses.* Retrieved from http//www.iita.org/info/viology/pdf_files/33-49.pdf.

[12] Partriot, M., Amefia, Y. K., Djiekpor, E. K. and Bakar, K. A., 1978. Le "swollen shoot" du cacaoyer au Togo: inventaire preliminaire et première estimation des partes causes par la maladie. *Café cacao The,* XXII: 217-228

[13] Quainoo, A. K., 2006. Germplasm conservation of cocoa (*Theobroma cacao* L.) and virus elimination through tissue culture. PhD thesis, University of Reading, UK.

[14] Quainoo, A. K., Wetten, A. C. and Allainguillaume, J., 2008. The effectiveness of somatic embryogenesis in eliminating the cocoa swollen shoot virus from infected cocoa trees. *Journal of Virological Methods,* 149 (1): 91-96.

[15] Steven, W. F., 1936. A new disease of cocoa in the Gold Coast. *Gold Coast Farmer*, 5 (122): 144.

[16] Thresh, J. M., 1959. The control of cacao swollen shoot disease in Nigeria. *Tropical Agriculture (Trinidad)* 36: 35-44.

[17] Thresh, J. M., Owusu, G. K., Boamah, A. and Lockwood, G., 1988. Ghanaian cocoa varieties and swollen shoot virus. *Crop protection*, 7 (4): 219-231.