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Regular Article

Age of callus tissues and cotyledonary materials on the selection of cocoa swollen shoot virus-free somatic embryos

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Research was conducted to investigate the effect of age of callus tissue and cotyledonary material on the selection of CSSV-free cocoa somatic embryos. Polymerase chain reaction (PCR) capillary electrophoresis was more sensitive and quick in detecting the CSSV than PCR/agarose electrophoresis. PCR/capillary electrophoresis revealed the presence of CSSV in callus tissues in 1wk at the rate of 53(82 %), 47(94 %) and 46(85 %) for three infected Amelonado cocoa trees, T1, T2 and T4, respectively while PCR/agarose electrophoresis recorded 23(36 %), 19(38 %) and 26(48 %) for T1, T2 and T4), respectively. Primary somatic embryos induced from the callus tissues revealed the presence of CSSV between 12-24 wks by PCR/capillary electrophoresis while PCR/agarose electrophoresis failed to detect the virus. Secondary somatic embryos induced from the cotyledonary materials revealed the presence of CSSV between 20-24 wks by PCR/capillary electrophoresis while PCR/agarose electrophoresis failed to detect the virus. The age of the callus tissues and cotyledonary materials seem to influence the selection of CSSV-free somatic embryos. The appropriate age of the callus tissues to select CSSV-free primary somatic embryos seemed to be 4-8 wks and cotyledonary materials at 4-16 wks for secondary somatic embryos.

***Keywords*:** cocoa swollen shoot virus (CSSV), primary embryogenesis, secondary embryogenesis, somatic embryos, PCR/capillary electrophoresis, PCR/agalose electrophoresis.

The cocoa swollen shoot virus (CSSV) is mainly restricted to West Africa and it is believed that the virus was present in the forest regions of West Africa before the introduction of crop (Posnette, 1950; Thresh, 1961 and Thresh *et al.*, 1988). CSSV, a badnavirus, is transmitted by at least 14 species of mealybugs of the family *Pseudococcidae* within the *Coccoidae* (Roivainen, 1976). The 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 and Ollenu, 1989).

Various attempts made in the past to control CSSV at most resulted in only partial success. Recently tissue culture technique was used to generate virus free somatic embryos from CSSV infected cocoa trees. The virus free somatic embryos were converted into plantlets and tested CSSV negative two years after weaning (Quainoo, 2006). PCR/agarose electrophoresis and PCR/capillary electrophoresis was used for the qualitative assessment of the presence of the CSSV in primary and secondary somatic embryos which revealed differences in sensitivity in detecting the

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virus (Quainoo *et al.,* 2008a). The age of the experimental materials tested seem to influence the detection of the virus in the somatic embryos. Determining the age of the experimental materials at which to harvest virus free somatic embryos will be appropriate especially for quarantine purposes and distribution of cocoa germplasm materials.

The aim of this research is to establish the best time (age) of selecting somatic embryos from the callus tissues and cotyledonary materials in order to avoid the presence of CSSV in the somatic embryos. The long term objective is to optimise the selection time for somatic embryos thereby enhancing the sanitary improvement of cocoa potentially infected with CSSV. The outcome of this research would improve upon the robustness of cocoa quarantine procedures.

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.

DNA extraction

Total genomic DNA was extracted from four sources: leaves of 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. 400µl of lysate Buffer AP1 (preheated to 65^{o}C) to dissolve and 2µl 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 x 2 at 25 cycles s^{-1}. DNA extraction quality from the experimental materials was checked on an ethidium bromide-stained agarose gel.

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) as described by (Quainoo *et al.,* 2008b). The product is 375 base pairs (bp) and runs from position 350 to 725 bp on accession AJ608931. Both primers were manufactured by Sigma, UK as follows:

Forward primer: AAC CTT GAG TAC CTT GAC CT; Reverse primer: TCA TTG ACC AAC CCA CTG GTC AAG

A master mix containing Taq polymerase and dNTPs was supplied by Qiagen (Multiplex PCR kit), UK. The PCR consisted of 5.0 µl 2x 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^{o}C for 15 min, 94^{o}C for 30 sec, 56^{o}C for 90 sec, 72^{o}C for 60 sec at 35 cycles and with a final extension of 60^{o}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.

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, 1999) was used. The primer sequence for the marker is as follows: forward primer: CTT CGT AGT GAA TGT AGG AG and reverse primer: TTA GGT AGG TAG GGT TAT CT. The PCR protocol is the same as described with the addition of mTcCIR25 primers in the fragment analysis.

RESULTS

Viral screening of primary somatic embryos

PCR/agarose electrophoresis and PCR/capillary electrophoresis on callus tissues from which the primary embryos were derived varied in CSSV test sensitivity (Table 1). PCR/capillary electrophoresis was more sensitive and earlier in detecting the CSSV than PCR/agarose electrophoresis.

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 somatic embryos tested	Positive test results by				
		PCR/agarose electrophoresis			PCR/capillary electrophoresis	
		1wk	2wks	3wks	1wk	2 wks
T1	64	23(36%)	48(75%)	56(88%)	53(82%)	64(100%)
T2	50	19(38%)	25(50%)	42(84%)	47(94%)	50(100%)
T4	54	26(48%)	54(100%)	54(100%)	46(85%)	54(100%)
H	30	0(0%)	0(0%)	0(0%)	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						
		PCR/capillary electrophoresis						PCR/agarose electrophoresis
		4wks	8wks	12wks	16wks	20wks	24wks	24 wks
T1	52	0(0%)	0(0%)	0(0%)	3(6%)	5(10%)	10(19%)	0(0%)
T2	128	0(0%)	0(0%)	0(0%)	5(4%)	9(7%)	18(14%)	0(0%)
T4	104	0(0%)	0(0%)	1(1%)	8(8%)	13(13%)	16(15%)	0(0%)
H	30	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)

All the primary somatic embryos from CSSV-infected Amelonado cocoa trees T1, T2 and T4 tested negative for the virus 24 weeks after induction, when screened by PCR/agarose electrophoresis. Somatic embryos induced from cocoa tree T4 tested positive for CSSV 12 to 24 weeks after induction when the PCR products were screened by PCR/capillary electrophoresis. Somatic embryos from T1 and T2 also tested positive for CSSV 16 to 24 weeks after induction when the PCR products were screened by PCR/capillary electrophoresis. (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 two years after weaning Fig. 1).

All the secondary somatic embryos induced from primary somatic embryos infected with CSSV tested negative 4 to 16 weeks after induction when subjected to by PCR/capillary electrophoresis. Somatic embryos from T2 revealed CSSV infection at the rate of 1% and 4% at 20 and 24 weeks respectively while T4 revealed CSSV infection at the rate of 1% and 9% at 20 and 24 weeks respectively (Table 3). The same somatic embryos failed to reveal the presence of the virus when subjected to PCR/agarose electrophoresis (Table 3).

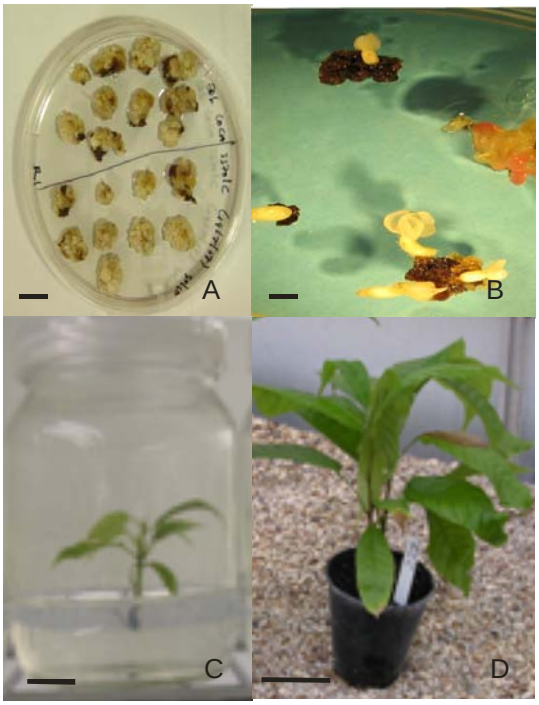

Figure 1. Somatic embryos at different stages of development. A Callus tissues on callus inducing medium. B Somatic embryos at different development stages. C Somatic embryo derived plantlet cultured on primary embryo conversion medium with normal multiple leaves on root induction medium. D Plantlet weaned in the glasshouse. (Scale bars: A and B = 2 mm; C = 1 cm; D = 3 cm).

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/capillary electrophoresis						PCR/agarose electrophoresis
		4wks	8wks	12wks	16wks	20wks	24wks	24 wks
T1	100	0(0%)	0(0%)	0(0%)	3(6%)	1(1%)	4(4%)	0(0%)
T4	100	0(0%)	0(0%)	0(0%)	5(4%)	1(1%)	9(9%)	0(0%)
H	30	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)

DISCUSSION

Qualitative screening of somatic embryos for CSSV

The callus tissues induced from the staminodes of the CSSV-infected cocoa trees successfully generated somatic embryos. The callus tissues recorded high viral infections by both PCR/agarose electrophoresis and PCR/capillary electrophoresis (Table 1). PCR/capillary electrophoresis was sensitive and quick taking 2 wks to detect the CSSV in all the callus tissues. PCR/agarose electrophoresis on the other hand seemed to be less sensitive and slower in detecting the virus at a lower concentration (Table 1). The implications are that the PCR/capillary electrophoresis may be a faster and more reliable method of detecting the CSSV than PCR/agarose electrophoresis. Furthermore, it seemed that the age of the callus tissues determines the threshold of CSSV as the percentage infection increases with the age of the callus tissues (Table 1).

The primary somatic embryos induced from different aged callus tissues did not reveal the presence of CSSV by PCR/agarose electrophoresis. However, PCR/capillary electrophoresis of the same CSSV DNA samples revealed the presence of CSSV in somatic embryos induced from callus tissues aged between 12-24 wks (Table 2). It appears the PCR/capillary electrophoresis is more robust in detecting the CSSV.

The age of the callus tissues at which somatic embryos are induced seemed to determine whether somatic embryos are infected with the virus or not. The implications are that somatic embryos induced from callus tissues aged between 4-8 wks recorded no viral infection (Table 2).

PCR/capillary electrophoresis on secondary somatic embryos induced from infected primary embryos revealed the presence of viral infection at low concentration. The CSSV was not detected in the primary cotyledonary materials aged between 4-16 wks which could be a range of time for selecting CSSV-free somatic embryos (Table 3).

The effectiveness of somatic embryogenesis as a technique in eliminating and selecting CSSV-free somatic embryos seem to be influenced by the age of the callus tissues. These results conform to the finding of (D'Onghia, 2001 and Goussard, 1991) who used somatic embryogenesis to eliminate the *Citrus psorosis* virus, and fan leaf virus and leaf roll-associated viruses from grave vine.

Results indicated that the older the callus tissues the higher the frequency of inducing CSSV infected somatic embryos. Therefore, the appropriate age range to select CSSV-free primary somatic embryos is when the callus tissues are between 4-8 wks old and 4-16 wks old for primary and secondary somatic embryos, respectively with PCR/capillary electrophoresis as the technique of detection. This phenomenon may be accounted for by the fact that the viruses may remain in their locations (cells) multiplying until they reach a threshold where the cells can no longer contain them. At this stage the viruses may move into adjacent cells or infect the somatic embryos possibly through the plasmodesmata.

CONCLUSION

Somatic embryogenesis was effective in generating and selecting CSSV-free cocoa clonal materials over a period of time. The effectiveness of somatic embryogenesis generating CSSV-free clonal materials seemed to be influenced by the

age of the callus tissues and cotyledonally materials from which the somatic embryos are induced. Indexing of CSSV was more reliable using PCR/capillary electrophoresis.

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