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Full Length Research Paper

Early signs of infection in *Cacao swollen shoot virus* (*CSSV*) inoculated cocoa seeds and the discovery of the cotyledons of the resultant plants as rich sources of CSSV

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Despite the huge efforts and funds expended to try and bring Cacao swollen shoot virus (CSSV) disease under control, the disease has remained largely intractable. Inspired by the need to inject new ideas into the current breeding programmes for CSSV resistance, the interactions between cocoa and the virus in CSSV-infected plants from 3 days post inoculation (dpi) to about 30 dpi were studied using both microscopic and molecular methods. Cellular modifications such as nucleic acid-rich inclusion bodies, apoptosis, and the *in situ* localisation of the virus in novel tissues that is cotyledons and hypocotyl, were discerned by microscopic examinations. These cellular modifications were observed in cotyledons of seedlings as young as 8 dpi. By validating these microscopic results using PCR it was also discovered that the cotyledons of the inoculated plants were virus-rich. CSSV was amplified or detected in total DNAs extracted from all 4 CSSV-isolates studied, and in some cases the detection was from as early as 3dpi plants. Compared to cocoa leaves, the traditional source from which CSSV or its DNA is usually extracted, the cotyledons were much easier to process and analyse. The significance of these findings to the CSSV-resistant breeding programmes, and to CSSV research in general is discussed.

Key words: CSSV, apoptosis, *in situ* localisation, nucleic acid-rich inclusion bodies, microscopic, examination.

INTRODUCTION

Cacao swollen shoot virus (CSSV) causes a devastating disease of cocoa (*Theobroma cacao* L.), the main economic crop of some West African countries, thus causing significant economic loss to those countries. CSSV is transmitted by over 20 species of mealybugs from a single family Pseudococcidae (Dale, 1962; Dzahini-Obiatey, 2008; Leston, 1970; Thresh and Tinsley, 1959), but the predominant species currently found in the field are *Planococcus citri* and *Planococcoides njalensis* (Dzahini-Obiatey, 2008). To control CSSV in the field, either the mealybug or the virus has to be targeted but attempts to target these two agents have so far not yielded the desired results. Breeding for CSSV resistance, a virus targeted strategy, has however been recognised as the most sustainable among the known control measures-at least in Ghana (Dzahini-Obiatey et al., 2006; Posnette, 1981). Yet, lack of rapid and reliable screening methods is hampering progress. Crucial information on the interaction between the virus and cocoa, which can feed into the development of such rapid screening methods are lacking.

It is presumed that when CSSV, like any other pathogen comes in contact with its host (cacao also known as cocoa, an interaction that may either culminate in pathogen ingress and establishment leading to a disease condition (syndrome), or the failure to enter the

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Table 1. Cumulative infectivity rates and the symptoms that were expressed by viruliferous mealybug-inoculated plants as at the end of experiment for the batches of seeds used for PCR in Figure 4.

Isolates	Infectivity rates	Symptoms expressed
1A	90[a]/100[b]	Red vein banding that later transformed into chlorotic patterns like pepper and salt mosaic, fern pattern and vein clearing. There were also shoot and root swellings, and shoot die back
Kpeve	55/100	Red vein banding that transformed into chlorotic mottle symptoms. There were also shoot die back.
Bisa	32/100	Transient red vein banding that disappeared. This was later followed by pronounced stem swellings at the base of the stem.
N1	27/100	Transient red vein banding only, and this disappeared with time.
Healthy Control	0/100	No symptoms

[a]Number of symptomatic plants; [b]total number of seeds planted.

host and cause any damage or disease ensues between them. Failure of the virus to cause disease in the plant may be due to resistance. Although cocoa is known to be resistant to some pathogens, which includes CSSV (Adu-Ampomah et al., 1996; Kenten and Legg, 1971; Posnette, 1951; Thresh et al., 1988), very little is known about the mechanisms or substances (factors) involved in this resistance. A rare example of a reported resistant factor in cocoa was in the form of a phytoalexin, an elemental sulphur, found in *Verticillium dahliae* resistant genotypes of cocoa (Cooper et al., 1996).

It is conceived that efficient processing of infected cocoa tissues for microscopy would enable the exploration of resistant factors, which could in turn lead to the prediction of probable resistance mechanisms in CSSV infected cocoa. Knight and Tinsley (1958), and recently, Jacquot et al. (1999) demonstrated that CSSV-infected cocoa tissues could be efficiently processed for microscopy. Sagemann et al. (1983) used enzyme-linked immunosorbent assay (ELISA) to explore the distribution of CSSV in various tissues from 1 - 7 month-old cocoa plants while Muller et al. (2001) studied the capability of detecting CSSV from *agroinoculated* plantlets over a 3-month period using PCR. These are the developmental studies that have so far been carried out on CSSV-infected cocoa plants.

Inspired by the need to explore sensitive and reliable resistant markers in cocoa, and the urge to address the dearth of microscopic and molecular information on the interactions between CSSV and cocoa, CSSV-infected cocoa seeds were studied from 3 day post inoculation (dpi) to about one month-old plants. Tissue-processing techniques of Knight and Tinsley (1958) and Jacquot et al. (1999) were modified and used.

The microscopic studies enabled the discernment of inclusion bodies, *in situ* localisation of CSSV-like structures in novel tissues, and several other cellular modifications. These findings, particularly on the localisation of the virus in novel cocoa tissues, were further explored using molecular techniques. This paper reports on the results so far obtained by highlighting their significance to CSSV research, and in the breeding for CSSV resistance.

MATERIALS AND METHODS

All cocoa materials used were inoculated as seeds in Ghana using viruliferous mealybugs as described by Dzahini-Obiatey et al. (2005), and carried to Reading, UK, for planting and use in the studies.

Tissue preparation and analysis by microscopy

Cocoa tissues were essentially processed as described earlier (Dzahini-Obiatey and Fox, 2006). Briefly, tissues from parts of a germinating seed such as the embryo, radicle, hypocotyl, cotyledons, tap root and leaves were taken from 3 dpi to about one-month old plants. Four CSSV-isolates namely, CSSV 1A, CSSV Kpeve, CSSV Bisa and CSSV N1, and a healthy or un-inoculated control were studied. The isolates were selected based on the uniqueness of the symptoms they induced in an infected plant (Table 1): CSSV 1A induces red-vein banding that transforms into chlorosis as the leaf matures, and stem and root swellings in an infected plant. This isolate is vigorous and can kill the plant within 3 years - hence termed virulent or severe. CSSV Kpeve, which is also vigorous or severe, causes leaf-symptoms only. CSSV Bisa is mild but mostly causes stem swellings in an infected plant. Rarely does it cause leaf symptoms. CSSV N1 causes the transient red-vein banding symptoms only.

All together, five plants each from each isolate and the un-inoculated control were randomly sampled. The remaining plants were allowed to grow to develop the normal symptoms of the respective isolates (Table 1) so that a future comparison could be made between the microscopic results, infectivity rates, and the types of symptoms expressed by the infected plants. The sampled tissues were initially fixed in Trump's reagent (McDowell and Trump, 1976) overnight at 4°C or at times stored in this buffer at 4°C until ready for further processing. This was followed by secondary fixation in 0.1M Osmium tetroxide (made in 0.1 M sodium cacodylate buffer pH 7.2) The tissues were thereafter washed, stained enbloc with 8% aqueous uranyl acetate, and washed again with 0.1 M sodium cacodylate buffer for 5 min. The

Table 2. Primers used in PCR amplification of CSSV templates.

Name	Sequence*	Reference
Badna 2.1 degenerate – forward primer	$_{5597}$TAYATTGATGAYATWYTKGT	Thomson et al., 1996
Badna 3.1 degenerate – reverse primer	CATCBSTYTCWATKATRATG$_{6054}$	
Agou 1 specific 2.1 – forward primer	$_{5597}$TACATTGATGACATTTTGGT	Muller et al., 2001
Agou 1 specific 3.1 – reverse primer	CATCGCTTTCAATGATAATG$_{6054}$	

*Where K, R, S ,W, Y are IUPAC's uncertainty codes for degeneracy in the oligonucleotide sequence, and figures in subscript representing product region on Agou 1 genome (L14546).

tissues were then dehydrated as follows: 50% acetone for 15 min; 70% acetone for 15 min; twice with 90% acetone for 15 min; 100% acetone for 15 min; twice with 100% acetone for 20 min; 100% acetone for 1 h. Finally, the tissues were embedded with Epon resin (Agar resin-100 7.43 g, dodecenyl succunic anhydride (DDSA) 2.6 g, methyl nadic anhydride (MNA) 5.03 g, benzyldimethylamine (BDMA) 0.15 ml) in a fume cupboard in a graded manner as follows: a mixture of Epon and acetone (Epon/acetone 1:3) overnight at 4°C; Epon/acetone 1:1 for 1 h at room temperature; Epon/acetone 3:1 for I hr at room temperature; pure Epon (100 %) for 2 h at room temperature; pure Epon (100% and freshly prepared) for 3 h at room temperature; followed by the embedment of the fixed tissues in polyprolene moulds using 100% Epon (freshly prepared), and incubating the moulds in an oven at 60°C for 48 h for the resin to set.

Two types of sections, semi thin (10 µm) and thin section (20 nm) were cut from the embedded tissues and used for light microscopy and transmission electron microscopy (TEM) respectively. About 5 sections were made and stained for each tissue type from a sampled plant for each isolate, and at each interval of sampling for TEM and light microscopy respectively. The light microscopy sections were stained with periodic acid Schiff toluidine blue (PASCT) (Yeung, 1990) and azure bromide blue (Cole and Ellinger, 1981), and analysed with Zeiss Axioskop 2 with AxioCam digital camera attached. For TEM, double staining with 8% uranyl acetate followed by Reynolds (1963) lead citrate was used and the analysis made with Phillips CM 20 TEM.

Tissue preparation and analysis by molecular techniques

For the PCR analysis to validate microscopic results, approximately 100 mg mass of tissues from the cotyledon of each CSSV isolate was extracted using Dneasy Plant Mini Kit (Qiagen). However, to avoid cross-contamination between samples, the freezing and grinding of tissues in liquid nitrogen was done in sterile 2 ml Eppendorf tubes using tough and round tip plastic rods. Fresh tubes and rods were used for each sample. Thus, the tissues were put in Eppendorf tubes before dipping each tube into the liquid nitrogen to scoop a small quantity of the nitrogen on top of the tissue. A pre-chilled plastic rod (in liquid nitrogen) was then used to grind the tissues into fine powder. The powder was thereafter processed into total DNAs as stated in the manufacturer's protocol. Total DNAs obtained from this process were quantified using Ultrospec 3000 uv/visible spectrophotometer (Pharmacia Biotech) prior to PCR analysis. The final concentrations of total DNAs used in each PCR were between 0.5 - 1 ng/µl. Two sets of primer pairs with distinct features (that is degenerate primers by Thomson et al. (1996) and strain specific primers by Muller et al. (2001)) were used in the analysis, and these are presented in Table 2. For the degenerate primer pair, the temperature cycles used were an initial denaturation step of 94°C for 2 min, 40 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 1 min, and a final DNA synthesis step of 72°C for 5 min. The strain-specific primer treatment was 94°C for 2 min, 40 cycles of 94°C for 30 s, 49°C for 30 s and 72°C for 1 min, and a final DNA synthesis step of 72°C for 5 min. Either BioMix (Bioline) at a primer final concentration of 4 µM, or Diamond Mix (Bioline) with final primer concentration of 1 µM was used for the degenerate and strain-specific primer pairs reactions. All amplification reactions were carried out in a 2700 GeneAmp Thermocycler (Applied Biosystem).

Where a comparison is made between cotyledon and the leaf (the traditional source of virus and DNA) equal quantities (i.e. 100 mg) of both tissues were used as starting materials.

A small quantity (2 µl) of CSSV Agou 1-clone *p2p14* (Hagen et al., 1994) kindly supplied by Professor Mario Keller of CIRAD, France, was multiplied by transformation in electroporate competent cells (Qiagen) and used as positive control in PCR. All PCR amplified products were analysed on 1% agarose gel stained with ethidium bromide, and in Syngene's gel documentation system.

Monitoring accessibility of CSSV in the cotyledons

The availability, ease to extract and analysis of CSSV from the cotyledons were studied using a single CSSV 1A infected plant, which was sampled from 3dpi to 24 dpi. The seed that was used and which developed into the said plant was tagged when the batch of seeds from which it was taken arrived from Ghana, i.e. 3 dpi. Eighty milligrams of tissue was immediately chipped off the large purple cotyledon of the seed for CSSV DNA indexing, that is by extracting total DNA for analysis by PCR. The remaining part of the seed, which was still viable was planted with the rest of the batch and labelled. The same quantity of tissue (80 mg) was periodically collected for CSSV DNA indexing from the same plant at 7 days after planting (10 dpi), 14 days after planting (17 dpi) and 21 days after planting (24 dpi) a time by which the first symptoms of CSSV (red-vein banding) would have appeared on the leaves. The total DNA extraction was carried out using DNeasy Plant Mini Kit (Qiagen) as described in the previous section.

RESULTS

Microscopy

Out of all the tissue types studied, the cotyledons of germinating seeds and young plants were the only tissues from which very few noticeable changes were discerned by light microscopy. Cells of the cotyledons were mostly undifferentiated parenchyma cells. Some of the cells from CSSV-infected cotyledons were shown to contain

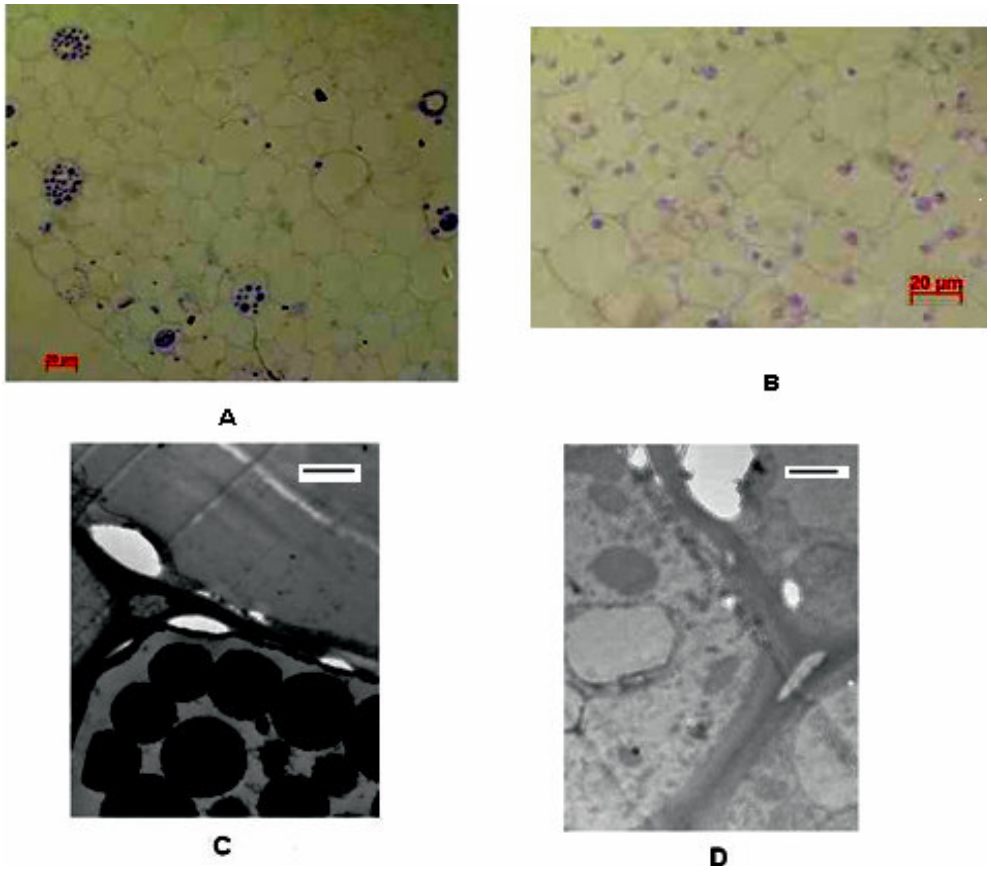

Figure 1. A and B are light microscopic images from 8-dpi cotyledons stained with PASCT. "**A**" is CSSV Bisa-infected plant showing nucleic acid-rich spherical inclusion bodies. Note how the inclusion bodies filled some of the cells. "**B**" is from a healthy (control) plant of a comparable age. **C** and **D** are TEM micrographs from plants of similar ages as in A and B, but which are stained with uranyl acetate. "C" shows the spherical inclusion bodies from CSSV Kpeve-infected plants as seen by TEM. "D" is from healthy (control) plant containing conspicuous starch granules. Scale bar correspond to: 600 nm (C), 700 nm (D).

spherical inclusion bodies, which stained for nucleic acids using the PASCT and azure bromide blue staining methods. The PASCT results are present in Fig 1A. The inclusion bodies varied in size (planar surface diameter ranging from 1.3 - 12.4 µm), number (from a single large inclusion per cell to about 27 per cell), and in some cases filled the entire cell. Affected cells were few (that is, an average of 4% (8 out of 185) cells per microscope viewing field), and randomly dispersed within the entire tissue. All 4 CSSV isolates studied had these inclusion bodies. Some of them were also discernable by TEM (Figure 1C). Another change noticed by light microscopy was apparent aggregation of starch granules in infected tissues as compared to those of the healthy plant (data not shown).

Interestingly, TEM analysis of similar tissues revealed slightly more cellular modifications, which were again mostly confined to the cotyledons. These included *in situ* localisation of CSSV-like particles (Figure 2), which were seen in criss-crossed pattern (Figure 2A and 2B). Particles resembling CSSV were localised in tissues from 3 (that is, CSSV 1A, CSSV Kpeve, CSSV Bisa) out of the 4 isolates studied. One of these was however, in a different tissue-type, the hypocotyls of a CSSV 1A-infected plant (Figure 2C). Others had numerous particles (Figure 2A and 2B) some of which were completely surrounded by deep black stained osmium matrixes (Figure 2B), which may probably consist of phenolic substances. Osmium stained, deep black inclusions, in histological preparations had been determined elsewhere to consist of phenolic substances (Beckman and Mueller, 1969). Other noticeable cellular modifications as seen by TEM were presence of few randomly placed cells in the tissues probably undergoing apoptosis (Figure 3). The probable apoptotic cells were in 2 out of the 4 CSSV-isolates-CSSV 1A, which is a virulent isolate (Figure 3A)

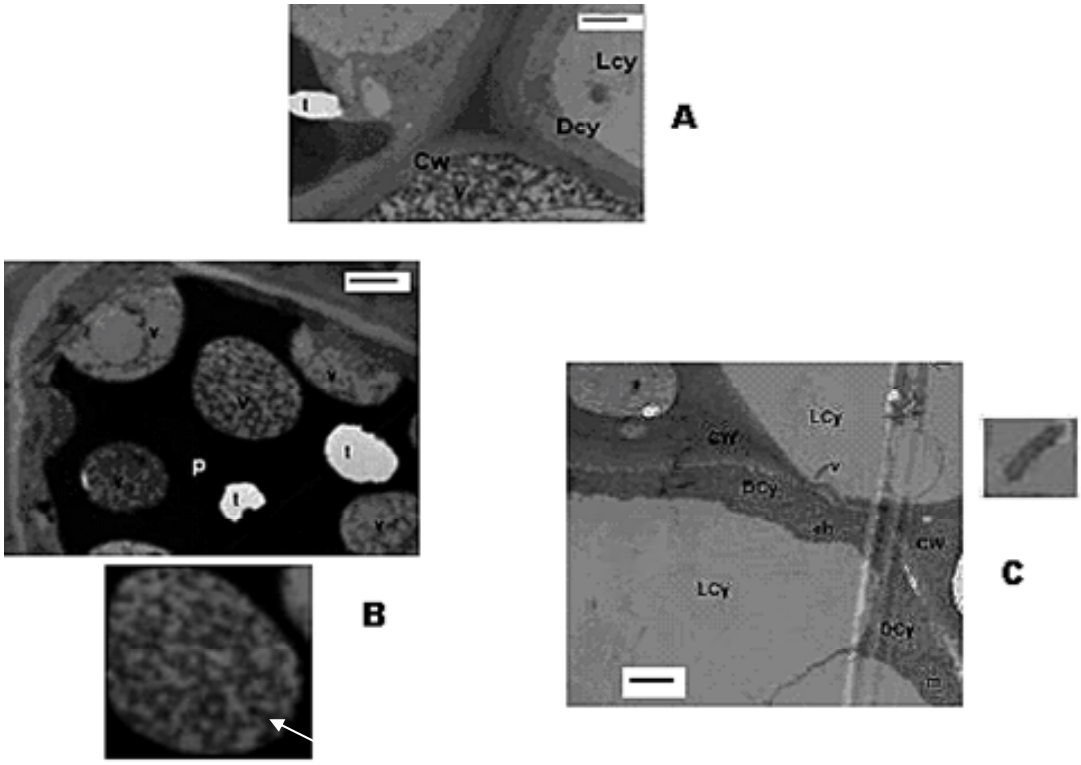

Figure 2. Uranyl acetate stained TEM micrographs from CSSV-infected plants. In contrast to the healthy (control) shown in Figure 1D, note the viral particles "*v*" located in tissues from the infected plants. **A**, cotyledon from CSSV Bisa; **B**, cotyledon from CSSV Kpeve (also showing an enlarged section of the micrograph showing viral particles in more detail as indicated by an arrow); and **C**, hypocotyls from CSSV 1A (also showing the enlarged viral particle, *v*, detailing its bacilliform structure). *Dcy* - densely stained cytoplasmic stream, *Lcy* - lightly stained cytoplasm, *CW* - cell wall, *t* - torn section of tissue caused by partly damaged diamond knife, *p* – dense matrix of phenolic mass surrounding viral particles, *ch* – chloroplast, *m* – mitochondria. Arrow shows score marks of diamond knife probably caused by a bad knife. Scale bar correspond to 270 nm (A), 300 nm (B), 140 nm (C).

and CSSV Bisa, a mild isolate (Figure 3B). The cells looked shrunken and appeared to have viral particles embedded in them. The cell walls of the surrounding cells appeared thickened while those of the affected cells were diminished or disintegrated. Generally, the cellular modifications reported here, were discernable in tissues from 8 dpi plants, and in some cases remained detectable up to the end of the studies.

None of the above cellular modifications was observed either by light microscopy (Figure 1B) or TEM (Figure 1C) in any of the un-inoculated/healthy control tissues examined.

The subsequent infectivity rates and the types of symptoms induced in the plants are presented in Table 2.

PCR amplification of CSSV genome

The degenerate primer pair amplified approximately 450 bp products to a varying degree of efficiency from total DNAs extracted from the cotyledons of seeds inoculated with any of the 4 isolates of CSSV studied; although the product from one, CSSV Kpeve appeared slightly larger than 450 bp (Figure 4A and B). The diffe-rent PCR products will in future be sequenced to enable a clearer comparison to be made between the genomes of all four isolates of CSSV. The strain specific primer was however very efficient by consistently amplifying a 450 bp product from the CSSV 1A isolate. By comparison, the 450 bp corresponded in size to the expected 450 bp product from the primer pair, which spans the badnavirus reverse transcriptase and the RNase H domains of the CSSV Agou 1 genome. Running a PCR of the CSSV Agou 1 clone alongside the total DNAs from different tissues of a CSSV 1A infected plant yielded the 450 bp CSSV amplicon across the samples as presented in Figure 5B.

The efficiency of the strain specific primers in amplifying the expected product also enabled a comparison to be made between the cocoa leaves, a traditional source from which CSSV or its DNA are usually sourced for

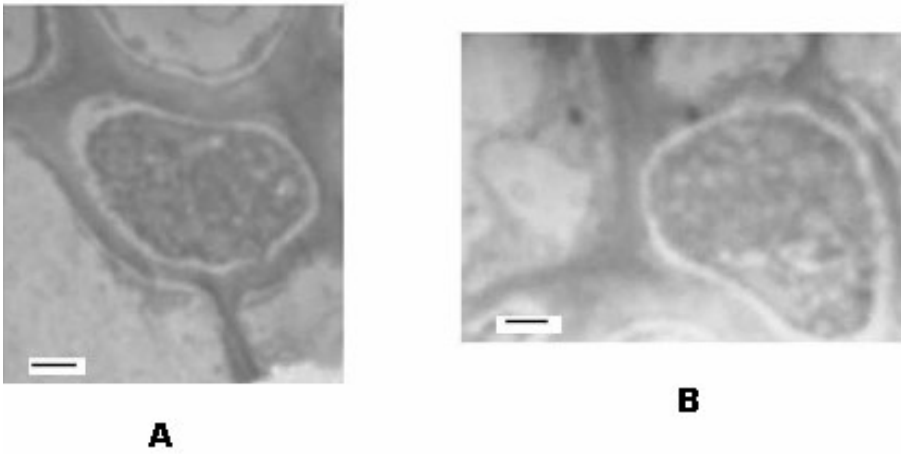

Figure 3. TEM micrographs of uranyl acetate stained tissues from CSSV-infected plants showing cells probably undergoing apoptosis. "**A**" is from CSSV Bisa-infected plant and "**B**" is from CSSV severe 1A-infected plant. No such cells were seen in any of the control plants examined as can seen from Figure 1D.

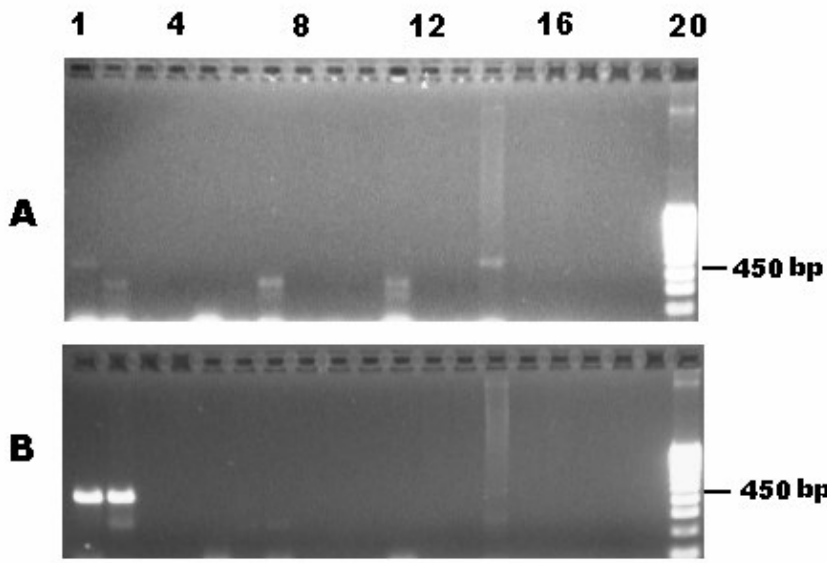

Figure 4. One percent agarose gel electrophoretic analysis of PCR products from total DNAs from the cotyledons of 4 randomly selected 3-dpi seeds from a batch of 100 seeds each from all four CSSV isolates. Bioline's BioMix was used for the reaction, and the final concentrations of the total DNAs and primers in each reaction mixture were 100 ng/µl and 4 µM respectively. A degenerate primer (**A**) or strain-specific primer (**B**) was used in priming the DNAs. Lanes are: CSSV 1A (1-4), CSSV Bisa (5-8), CSSV N1 (9-12), CSSV Kpeve (13-16), un-inoculated (healthy) control (17-19) and Bioline's HyperLadder IV (20).

molecular, biochemical or serological studies (Hagen et al., 1993, 1994; Hoffmann et al., 1997; Jacquot et al., 1996, 1999; Muller et al., 2001) and the cotyledons, which have just been found to contain CSSV in extractable quantities. The results which have been presented in Figure 5 and Table 3 showed that the cotyledons could be a better source of CSSV than the leaves. Although equal quantities (100 mg mass of leaves or 50 ng/µl total DNA) of tissues from different parts of the same plant were used under the same PCR conditions, the strain specific primer pair amplified the expected 450 bp product more efficiently from the total

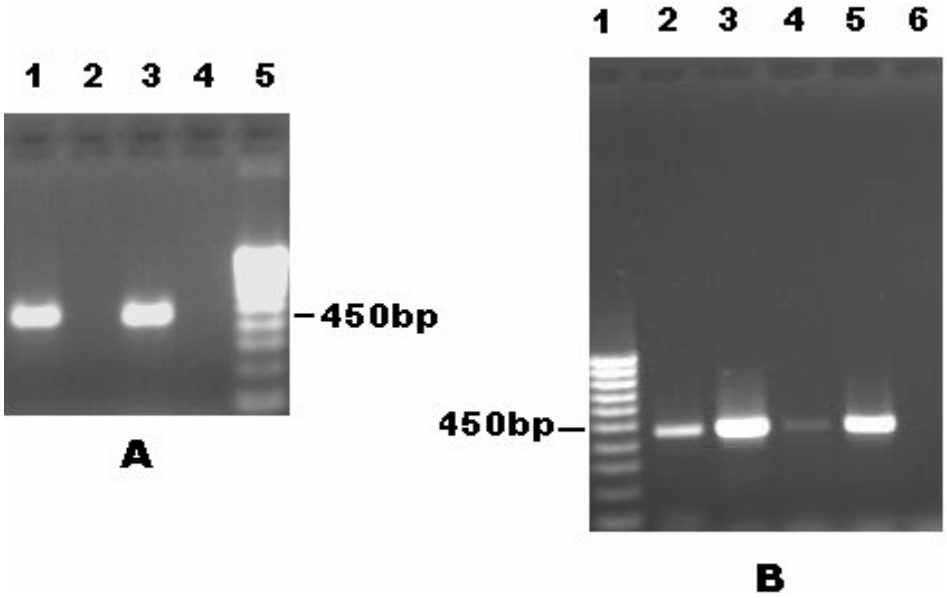

Figure 5. 1% agarose gel analysis of PCR products obtained using strain-specific primer pair on total DNAs from different parts of a CSSV 1A-infected plant. Bioline's BioMix was used, and the final concentrations of total DNAs and primers in the reaction mixture were 50 ng/µl and 4 µM respectively. **A**: Lanes 1-2 are different parts of the same leaf: 1, was from the infection lesion while 2 was from asymptomatic part of the leaf. Lane 3 was from the cotyledon of the same plant – it was still attached to the plant at the time of sampling, and tissues were taken from the stalk and green parts of the cotyledon. Lane 4 was DNA from an un-inoculated (healthy) control plant of comparable age. Lane 5 is Bioline's HyperLadder IV marker. **B**: PCR products from two colonies (a slow and a fast growing respectively) of positive control plasmid, CSSV Agou 1 clone p2p14 (Hagen *et al*., 1994) in lanes 2 and 3, as compared to the products from total DNAs from symptomatic leaf (lane 4) and cotyledons (lane 5). Lane 1 is Bioline's HyperLadder IV marker, and 6 is a negative un-inoculated control.

DNAs from the cotyledons than those from the leaves (Figure 5A and B). Thus, while total DNAs from the cotyledons of the infected plants shown in Fig 5 were strongly amplified on both occasions (lane 3 Figure 5A and lane 5 Figure 5B), the corresponding total DNAs from leaves of the same plants were amplified only from the symptomatic part of the leaf (lane 1 Figure 5A) but not from the asymptomatic part of the same leaf (lane 2 Figure 5A) or were only mildly amplified or detected in the second plant (lane 4 Figure 5B). These results suggest that CSSV may not be evenly distributed in all organs of the infected plant, thus, reaffirming previous reports that CSSV is not fully systemic in an infected plant (Muller et al., 2001; Sagemann et al., 1983). The results showing faint detection of CSSV in Figure 5B also suggests that either total DNA extracted from the cotyledons may be richer in CSSV template than those from the leaves or there may be PCR inhibitors in the leaf extracts. Real time or quantitative PCR using fresh primers, which targeted the same region as the strain specific primers, enabled the viral load from both tissues to be calculated and compared. The results which are yet to be published showed that the total DNAs from the cotyledons contained 4-fold more CSSV template than those from the leaves-confirming the cotyledons to be richer in CSSV. A more extensive studies between cotyledons and leaves of infected plants presented in Table 3 also showed the cotyledons to be a better source of CSSV: Out of 23 PCR analysis carried out on 23 total DNAS extracted using Dneasy Plant Mini Kit (Qiagen) from the two types of tissues (that is cotyledons or leaves) from five batches of infected plants planted at different times, 83% were CSSV positive, that is 83% (19/23) of CSSV DNA was amplified from the cotyledons as compared to the 9% (2/23) that was amplified from the leaf extracts. The results put together highlight the difficulty involved in working with cocoa leaves, thus corroborating previous reports (Adomako et al., 1983; Dzahini-Obiatey and Ollennu, 2000; Hagen et al., 1994; Hoffmann et al., 1997; Jacquot et al., 1999; Muller et al., 2001; Sagemann et al., 1983, 1985). It was generally easier to extract total DNA by Qiagen's DNeasy Plant Mini Kit system from the cotyledons than from the cocoa leaves. The leaves were slimy and difficult to pipette after addition of the buffers to

Table 3. Extraction and amplification of total DNA for PCR from leaves and cotyledons of randomly selected cocoa plants from five batches of planted beans.

Tissue	Batches and dates of planting					
	1 (11:03:06)	**2 (15:06:06)**	**3 (10:08:06)**	**4 (12:09:06)**	**5 (28:02:07)**	**Cumulative Total**
Cotyledon	4[a]/5[b]	3/5	5/5	5/5	2/3	19/23 (83)[c]
Leaves	1/5	0/5	0/5	0/5	1/3	2/23 (9)

[a]Selected plants from which tissue extracts were CSSV positive by PCR.
[b]Total of number of symptom bearing plants randomly selected for testing.
[c]Cumulative percentage of CSSV detection.

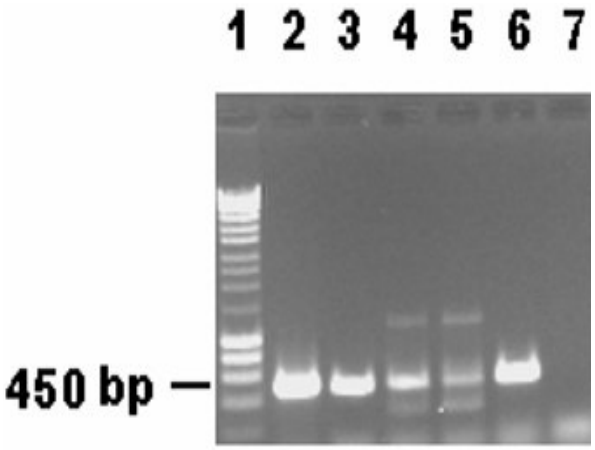

Figure 6. Accessibility of CSSV template or genome in a CSSV 1A-infected plant. Bioline's Diamond Mix was used, and the final concentrations of total DNAs and primers (strain-specific) in the reaction mix were 50 ng/µl and 2 µM respectively. Lane 1: Bioline's DNA hyper ladder IV. Lane 2: positive control (CSSV Agou 1 clone p2p14 (17). Lanes 3-6 are the cotyledons sampled at periodic intervals (3: 3 dpi, 4: 10 dpi, 5: 17 dpi, 6: 24 dpi). Lane 7 – cotyledon of an un-inoculated plant. Note the additional 1300 bp products in the 10 dpi and 17 dpi reactions.

the finely ground tissues, while the cotyledons were much easier to handle since they were brittle and not slimy. These slimy substances may be the source of PCR inhibition.

Thus, the cotyledons can now be seriously considered for use in studying CSSV, and in the making of CSSV diagnostic tools.

Accessibility of CSSV genome in cotyledons of an infected plant

The result of the systematic study on the accessibility of the virus from a 3 dpi to 24 dpi on a CSSV-1A infected plant showed a continuous PCR amplification of the expected 450 bp product at all times that samples were taken for analysis using the strain-specific primer pair (Figure 6). This means that CSSV genome or template could be accessed from the cotyledon at any time in its development. Interestingly, on a few occasions when Bioline's Diamond Mix (that is, polymerase + dNTPs + $MgCl_2$) instead of the BioMix was used in the reaction, an additional product of about 1300 bp was formed along side the 450 bp product (Figure 6). This additional product will be sequenced and analysed to determine if it is also CSSV coded.

DISCUSSION

Cotyledons of germinating seeds or young plants have emerged from the studies carried out here as vehicles that could be used to deliver rapid and reliable screening methods for CSSV infected cocoa. For many years now, attempts to develop rapid diagnostic methods for screening CSSV-resistant cocoa cultivars during breeding programmes have been elusive; hence the painfully slow pace of breeding for CSSV-resistant cocoa in West Africa. The cocoa leaves, the only tissue, which has so far been tried for the development of previous diagnostic assays, is fraught with numerous problems (Adomako et al., 1983; Dzahini-Obiatey and Ollennu, 2000; Hoffmann et al., 1997; Jacquot et al., 1999; Muller et al., 2001; Sagemann et al., 1983, 1985), notable among which are the fact that the leaves contain inhibitors of PCR (Hoffman et al., 1997; Muller et al., 2001; Sackey et al., 1995, 1996, 1999) immunological (Hoffmann et al., 1997; Sagemann et al., 1983, 1985) and other molecular and biochemical assays (Adomako et al., 1983; Dzahini-Obiatey and Ollennu, 2000; Sackey et al., 1999). This is why the discernment of cellular-modifications by microscopy, which is reported in this study, is so important. One of these, the nucleic-acid-rich spherical inclusion bodies, may have immediate diagnostic values, which can be exploited for the development of genetic markers. If induced after inoculation of CSSV into the CSSV-resistant cocoa cultivars currently available, the size, number per cell and distribution of the inclusion bodies within the affected tissues can be measured or counted as have been done in this study (Figure 1A). A correlation between the parameters named above and the known

level of resistance among the cultivars can constitute a selection criterion, and since the diagnostic assay can be carried out on plants as young as 8 dpi, it has the potential of cutting down on the breeding and selection processes of the CSSV-resistant programmes by several months.

The fact that the inclusion bodies have also been determined to be nucleic-acid-rich by histochemical analysis also presents an opportunity for exploring the pathogenesis of CSSV. Although the functions of the inclusion bodies are not clear from the studies conducted, they may be precursors of the virus or may supply nucleic acid for their synthesis. Previous microscopic studies did not discover inclusion bodies in CSSV infected tissues (Jacquot et al., 1999; Knight and Tinsley, 1958), and it was therefore concluded that CSSV do not induce inclusion bodies (Jacquot et al., 1999). Differences in results may be due to differences in the tissue types used for the respective studies. Knight and Tinsley (1958) and Jacquot et al. (1999) studied leaves, stems and roots of infected plants, and discovered proliferation of xylem and phloem cells together with other tissue modifications, but found very little on cellular modification by light microscopy. One interesting parallel about the inclusion bodies reported in this study is that similar spherical inclusions have been reported in another study on another virus from the para-retroviral family (Caulimoviridae) that is *Cauliflower mosaic virus* (Conti et al., 1972). The inclusion bodies were in that case determined to be sites for virus assembly. Could the CSSV inclusion bodies have similar function?

The reason for or the function of the other cellular modification that was observed by light microscopy, that is the apparent aggregation of starch granules in infected tissues, is also unclear.

Other cellular modifications discerned by TEM were both confirmatory and interesting. The *in situ* localisation of CSSV-like particles confirms previous report about localising the virus in the vascular tissues of cocoa leaves by Jacquot et al. (1999). The images were also similar to those found in a related genus, Kalanchoe top-spotting virus (KTSV) (Hearon and Locke, 1984). Added to that, the current studies discerned the viral particles in novel tissues, that is in the cotyledons and hypocotyls of infected plants. The probable encasement of viral particles by a matrix believed to consist of phenolic substances as seen in a CSSV Kpeve infected plant Figure 2B), and by the apparently apoptotic cell from CSSV Bisa and CSSV 1A (Figure 3) are interesting and seem to suggest plant reactions to the pathogen, which may probably be defensive. Phenolic compounds in the form of phytoalexins have been reported to be used by many plants to fight against an invading pathogen or pathogen ingress (Beckman and Mueller, 1969; Benhamou and Belanger, 1998; Benhamou and Lafontaine, 1995; Benhamou et al., 1994; Hammershmidt, 1999; Snyder and Nicholson, 1990), and this included cocoa (Cooper et al., 1996). So it will be useful to explore the defence role of these events or substances. It is probable that the dense matrix of phenolic substances observed completely encasing some viral particles in cells from CSSV-infected cocoa may be a defence response from cocoa to try and prevent CSSV movement and spread into other cells, tissues and organs. Apoptosis is another known defense mechanism that is invoked by plants when they come in contact with incompatible pathogens (Bailey and Mansfield, 1982; Darvill and Albertsheim, 1984; Hammershmidt, 1999). It could be that the few dying cells observed in this study are apoptotic cells, but these were very few and scattered as compared to those observed by Mittler et al. (1995). Could it be an event to cull the numbers of the viral particles so as to prevent virus spread from cell-to cell, and to reduce the virus load to a manageable level in the plant since CSSV and cocoa occasionally do coexist, at least for some time? This point may further support the fact that CSSV Bisa is mild and often co-exists with the virus for long periods before causing any effect on them. This isolate also rarely affect yield or cause death to the infected plants in the field (Crowdy and Posnette, 1947).

The molecular confirmation of the presence of CSSV in extractable quantities from the cotyledons of inoculated seeds is encouraging. These findings more or less confirm the microscopic observations. By showing that CSSV could be amplified or detected in inoculated seeds as young as 3 dpi, coupled with the proof that PCR amplification of CSSV from the cotyledons are more efficient and reliable than from cocoa leaves, presents yet another opportunity for developing rapid diagnostic tools for CSSV detection or screening, which can then be used in CSSV-resistant breeding programmes for cocoa. It is also noteworthy that, there was no need to increase the number of PCR cycles for the virus to be amplified from the different isolates of CSSV studied in this project as against what was reported by Muller et al. (2001), nor was it necessary to further process the CSSV DNA prior to amplification as was carried out by Hoffmann et al. (1997). An added advantage of working with cotyledons was that, total DNAs were easier to extract from them than was the case for the leaves. There was little or no mucilage to grapple with, and pipetting was easier. Judging from the ease with which CSSV DNA was regularly extracted and amplified from the cotyledons in this study, as compared to the problems others faced working on cocoa leaves (Hagen et al., 1994; Hoffmann et al., 1997; Jacquot et al., 1999; Muller et al., 2001; Sagemann et al., 1983, 1985). It could then be said that mucilage may be the culprit in cocoa leaves, which could have interfered with biochemical and molecular analysis previously reported (Hagen et al., 1994; Hoffmann et al., 1997; Jacquot et al., 1999; Muller et al., 2001; Sagemann et al., 1983, 1985). This is because it was the main sub-

stance that was less encountered during the DNA or viral extraction and purification from the cotyledons. Although not specifically studied in this project, long term storage of cotyledons at -80°C did not have any adverse effect on the probability of extracting and amplifying CSSV DNA from the stored tissues. Thus, infected cotyledons could be stored at such temperatures until a convenient time that they can be analysed. The capability to extract amplifiable DNA from the cotyledons at any time in the development of the plant has also been demonstrated from the studies. This gives the cotyledons another advantage over the leaves as the most suitable tissues to use for CSSV research.

It is worth noting that the comparative works cited in this paper, were on seedlings inoculated using mealybugs (Hoffmann et al., 1997), *Agrobacterium tumefaciens* (Jacquot et al., 1999; Muller et al., 2001) or particle bombardment methods (Hagen et al., 1994). The mealybug-inoculated seeds have been used because planting materials in Ghana are seeds made from crosses between varieties (hybrids), and the methods for inoculating the seeds are the same as reported in these studies. Thus, if any markers or rapid screening methods are ultimately developed, they will be readily applicable in the field.

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REFERENCES

Adomako D, Lessmann DE, Paul HL, Owusu GK (1983). Improved methods for the purification and detection of Cocoa swollen shoot virus. Ann. Appl. Biol. 103: 109-116.

Adu-Ampomah Y, Owusu GK, Sackey S, Padi B, Abdul-Karimu A (1996). Use of gamma rays to induce mutants resistant to cocoa swollen shoot disease in *Theobroma cacao* L. Plant Breed. 115: 74-76.

Bailey JA, Mansfield JW (1982). Phytoalexins. Blackie Glasgow.

Beckman CH, Mueller WC (1969). Distribution of phenols in specialized cells of banana roots. Phytopathology, 60: 79-82.

Benhamou N, Belanger RR (1998). Induction of systemic resistance to *Pythium* damping-off in cucumber plants by benzothiadiazole: ultrastructure and cytochemistry of the host response. Plant J. 14: 13-21.

Benhamou N, Lafontaine PJ (1995). Ultrastructural and cytochemical characterization of elicitor-induced structural responses in tomato root tissues infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Planta 197: 89-102.

Benhamou N, Lafontaine PJ, Nicole M (1994). Induction of systemic resistance to *Fusarium* crown and root rot in tomato plants by seed treatment with chiton. Phytopathology, 84: 1432-1444.

Cole MB, Ellinger J (1981). Glycol methacrylate in light microscopy: Nucleic acid and cytochemistry. J. Microsci. 123: 75.

Conti GG, Vegetti G, Bassi M, Favali MA (1972). Some ultrastructural and cytochemical observations on Chinese cabbage leaves infected with cauliflower mosaic virus. Virology, 47: 694-700

Cooper RM, Resende MLV, Flood J, Rowan MG, Beale MH, Potter U (1996). Detection and cellular localization of elemental sulphur in disease-resistant genotypes of *Theobroma cacao.* Nature, 379: 159-162.

Crowdy SH, Posnette AF (1947). Virus diseases of cacao in West Africa II. Cross-immunity experiments with viruses 1A, 1B and 1C. Ann. Appl. Biol. 34: 403-411.

Dale WT (1962). Diseases and Pests of Cocoa. A. Virus Diseases *In:* Wills JB (Ed.). Agriculture and land use in Ghana, Oxford University Press, London, pp. 286-314.

Darvill AG, Albertsheim PA (1984). Phytoalexins and their elicitor, A defense against microbial infections in plants. Annu. Rev. Plant Physiol. 35: 243-275.

Dzahini-Obiatey H (2008). Cytopathological and Molecular studies of Cacao swollen shoot Badnavirus (CSSV) infected cocoa plants. PhD thesis, University of Reading, pp. 5-15

Dzahini-Obiatey H, Ollennu LA (2000). Extraction and purification of CSSV isolates. Annual Report Cocoa Research Institute of Ghana 1996/1997, Akim Tafo Ghana pp. 132-134.

Dzahini-Obiatey H, Fox RTV (2006). Early developments in host-pathogen interaction between Cacao swollen shoot virus (CSSV) and the cocoa plant. Proceedings of the 15th International Cocoa Research Conference, Herradura Plazza Hotel, San Jose, Costa Rica, October 9-14, 2006, pp. 885-897

Dzahini-Obiatey H, Aculey PC, Ollennu LA (2005). Inhibition of cocoa swollen shoot badnavirus (CSSV) by an antiviral substance. Proceedings of the 14th International Cocoa Research Conference, Accra, 13th-18th October 2003 pp 1365-1371. Cocoa Producers Alliance, Malaysia.

Dzahini-Obiatey H, Ameyaw Akumfi G, Ollennu LA (2006). Control of cocoa swollen shoot disease by eradicating infected trees in Ghana: a survey of treated and replanted areas. Crop Prot. 25: 647-652

Hagen LS, Jacquemond M, Lepingle A, Lot H, Tepfer M (1993). Nucleotide sequence and genomic organisation of cacao swollen shoot virus. Virology, 169: 619-628.

Hagen LS, Lot H, Gordon C, Tepfer M, Jacquemond M (1994). Infection of *Theobroma cacao* using cloned DNA of cacao swollen shoot virus and particle bombardment. Phytopathology, 84: 1239-1243.

Hammershmidt R (1999). Phytoalexins: What have we learned after 60 years? Annu. Rev. Phytopathol. 37: 285-306.

Hearon SS, Locke JC (1984). Graft, pollen and seed transmission of an agent with top spotting in *Kalanchoë blossfeldiana*. Plant Dis. 68: 347-350.

Hoffmann K, Sackey ST, Maiss E, Adomako D, Vetten HJ (1997). Immunocapture polymerase chain reaction for the detection and characterization of cacao swollen shoot virus 1A isolates. J. Phytopathol. 145: 205-212.

Jacquot E, Hagen LS, Jacquemond M, Yot P (1996). The open reading frame 2 product of cacao swollen shoot badnavirus is a nucleic acid-binding protein. Virology, 225: 191-195.

Jacquot E, Hagen LS, Michler P, Rohfritsch O, Stussi-Garaud C, Keller M, Jacquemond M, Yot P (1999). In situ localisation of cacao swollen shoot virus in *Theobroma cacao*. Arch. Virol. 144: 259-271.

Kenten RH, Legg JT (1971). Varietal resistance of cocoa to swollen shoot disease in West Africa. FAO Plant Prot. Bull. 19: 2-12.

Knight R, Tinsley TW (1958). Some histological observations on virus infected *Theobroma cacao* L. Ann. Appl. Biol. 46: 7-10

Leston D (1970). Entomology of the cocoa farm. Annu. Rev. Entomol. 15: 273-294.

McDowell EM, Trump BF (1976). Histologic fixatives for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. 100: p. 405.

Mittler R, Vladimir S, Eric L (1995). Co-ordinated activation of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. Plant Cell 7: 29-42.

Muller E, Jacquot E, Yot P (2001). Early detection of cacao swollen shoot virus using the polymerase chain reaction. J. Virol. Methods,

93: 15-22.
Posnette AF (1951). Progeny trials with cacao in the Gold Coast. Empire J. Exp. Agric. 19: 242-252
Posnette AF (1981). Viruses and resistance to virus disease in cocoa. In: Proceedings of the 6th International Cocoa Research Conference, Caracas, Venezuela, 1977, pp. 262-266. Nigeria: Cocoa Producers Alliance, Lagos.
Reynolds JA (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17: 208.
Sackey ST, Lowor ST, Dzahini-Obiatey HK, Owusu GK, Adomako D, Hoffmann K, Vetten HJ, Mais E (1995). Polymerase chain reaction and differentiation of cocoa swollen shoot virus isolates. Proceedings of the First International Cocoa Pests and Diseases Seminar. Accra, Ghana, 6-10 November 1995 p. 191
Sackey ST, Dzahini-Obiatey HK, Owusu GK, Adomako D (1996). Variation in cocoa swollen shoot virus isolates. Proceedings of the 12TH International Cocoa Research conference. Salvador, Brazil.18-22 November, 1996, pp. 107-112.
Sackey ST, Dzahini-Obiatey H, Lowor ST (1999). The use of cloned DNA probes for the detection of CSSV 1A and CSSV Nsaba. J. Ghana Sci. Assoc. (Special Edition) 2 (3): 102-109.
Sagemann W, Paul HL, Adomako D, Owusu GK (1983). The use of enzyme linked immunosorbent assay (ELISA) for detection of cacao swollen shoot virus (CSSV). Phytopathologische Zeitschrift 106: 281-284.
Sagemann W, Lesemann DE, Paul HL, Adomako D, Owusu GK (1985). Detection and comparison of some Ghanaian isolates of cacao swollen shoot virus (CSSV) by enzyme-linked immunosorbent assay(ELISA) and immunoelectron microscopy (IEM) using an antiserum to CSSV strain 1A. Phytopathologische Zeitschrift 114: 79-89.
Snyder BA, Nicholson RL (1990). Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. Science 248: 1637-1639.
Thomson KG, Dietgzen RG, Thomas JE, Teakle DS (1996). Detection of pineapple baciliform virus using the polymerase chain reaction. Ann. Appl. Biol. 129: 57-69.
Thresh JM, Tinsley TM (1959). The viruses of cocoa. Technical Bulletin No. 7 West African Cocoa Research Instititute, pp. 6-28.
Thresh JM, Owusu GK, Ollennu LA (1988). Cocoa swollen shoot: An archetypal crowd disease. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 95: 428-446.
Yeung EC (1990). A simple procedure to visualize osmicated storage lipids in semithin epoxy sections of plant tissues. Stain Technol. 65: 45.