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Transmission of cocoa swollen shoot virus by seeds

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ABSTRACT

A study was undertaken to determine whether cocoa swollen shoot virus is transmitted by seeds, to improve the robustness of quarantine procedures for international exchange and long term conservation of cocoa germplasm. PCR/capillary electrophoresis, using cocoa swollen shoot virus primers designed from the most conserved regions of the six published cocoa genome sequences, allowed the detection of cocoa swollen shoot virus in all the component parts of cocoa seeds from cocoa swollen shoot virus-infected trees. PCR/capillary electrophoresis revealed the presence of cocoa swollen shoot virus in seedlings raised from seeds obtained from cocoa swollen shoot virus-infected trees. The high frequency with which the virus was transmitted through the seedlings suggested that cocoa swollen shoot virus is transmitted by seeds. This has serious implications for cocoa germplasm conservation and distribution.

1. Introduction

Cocoa (*Theobroma cacao*) is a tropical crop, with origins in the Amazon basin, and dominates the economies of most West African countries. Cocoa swollen shoot virus (CSSV) causes a devastating disease of cocoa, thereby threatening the economies of producing countries. 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).

Currently, cocoa is propagated largely by seeds. Over the years there have been contradictory reports on the transmission of CSSV through seeds. Posnette (1940) reported on the discovery of swollen shoot virus in 2-month old squirrel-sown seedlings under infected cocoa trees, indicating transmission by seeds. Further experiments in insect-proof cages, using only dwarfed pods from trees almost killed by swollen shoot virus, were carried out. Based on visual symptoms, it was suggested that some beans from infected trees carried the virus. It appears that, in anyone dwarfed pod, either all or none of the beans from infected trees carried the virus. No transmission was obtained with beans from normal pods taken from infected trees (Posnette, 1940). Further investigation by Posnette (1947) has suggested that seed transmission does not occur. Frison and Feliu (1989) also reported that CSSV is not transmitted through seeds except to cocoa cotyledons.

Globally, there is considerable evidence suggesting that viruses have been introduced into many geographical regions by infected seeds and through increasing international germplasm exchanges (Bashir and Hampton, 1995). Generally seeds are the passive carriers of pathogens which are transmitted when infected seeds are sown and emerge under suitable environmental conditions (Noble, 1957). Many plant viruses are known to be borne by seeds (Singh, 1989; Mandahar, 1990). Approximately 20% of plant viruses are transmitted from generation to generation in the seed (Hull, 1991; Mink, 1993). The presence of a virus in a seed (Sdoodee and Teakle, 1988), even in the embryo (Nolan and Campbell, 1984; Varma et al., 1992), does not always lead to seedling infection. This property distinguishes a seed-borne virus, that is carried by the seed but does not infect the seedling, from a seed transmitted virus that does infect the seedling produced from the seed (Neegaard, 1979). Virus may invade any seed part (Agarwal and Sinclair, 1996), but seed transmission results most frequently from embryo infection (Meinke, 1994; de Assis Filho and Sherwood, 2000).

According to Maule and Wang (1996), embryo infection can occur by two routes, either directly from infected gametes at fertilization or by direct invasion of the immature embryo from virus-infected testa tissues. Wang and Maule (1994), working on seed transmission of pea seed-borne mosaic virus, proposed that transmission occurred by the virus using the embryonic suspensor, as a conduit from the micropylar region of the embryo sac, to infect the tip of the embryonic radicle, and that this route was closed by degeneration of the suspensor.

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In the light of conflicting reports on the transmission of CSSV through the cocoa seeds, based mainly on symptoms detected visually, a PCR-based study was conducted to determine whether CSSV is seed transmitted. The outcome of this research would improve the robustness of quarantine procedures for the international exchange and conservation of cocoa germplasm.

2. Materials and methods

The experiment was conducted in insect-proof cages within the University of Reading glasshouses. CSSV (strain 1A) infected Amelonado cocoa trees (T2 and T4) and a CSSV-free Amelonado cocoa tree (H), were the experimental trees used.

2.1. Harvesting of pollen grains

Fifty newly opened flowers from each of the experimental trees were harvested at various times using different forceps for each tree. The flowers were tied individually in nylon mesh and placed in separate 1.5 ml Eppendorf tubes. The Eppendorf tubes containing the experimental samples were introduced into a Retsch disrupter for 1.4 min × 2 at 25 cycles per second to separate the pollen grains from the flowers through the nylon mesh. The nylon meshes containing the flowers were removed and the Eppendorf tubes and their contents (pollen grains) were stored at −22 °C for DNA extraction.

2.2. Hand pollination of cocoa trees

Pollination of flowers was carried out between March 2004 and January 2006. Before pollination was carried out, all opened flowers on the cocoa trees were removed. Next day the newly opened flowers were emasculated with fine tip forceps by removing the staminodes to expose the stigma. To avoid problems of self-incompatibility in cocoa and eliminate chances of self-pollination, pollen grains were picked from a flower and deposited on the stigma of a flower on a different tree for fertilization to take place. Successful fertilization resulted in the formation of young cocoa fruits (cherelles), some of which developed into mature fruits.

2.3. Separation of cocoa seeds into component parts

Approximately 6 months after pollination, five mature cocoa pods were harvested from the two CSSV-inoculated trees (T2 and T4) and six pods from the control tree (H). The pods were carefully opened without disturbing the seeds. Using thin sterile metallic plates (each used once) the extracted seeds from each pod were separated longitudinally into two portions. Seeds in the first portion were separated carefully into their component parts (coat, cotyledon, and embryo) using different sterile scalpel blades for each seed. Small portions (about 2 mm × 2 mm in size) of each component part of the seeds were taken, using different sterile scalpel blades for each sample, and placed separately in labelled 1.5 ml Eppendorf tubes. These were stored at −22 °C for DNA extraction.

2.4. Sowing of cocoa seeds

Seeds from the second portion were sown individually inside 4-in. round plastic pots containing a mixture of 3:1 seed sowing compost (Sinclair, UK) and vermiculite and placed in the glasshouse (temperature 25–41 °C). The seeds germinated 2–3 weeks after sowing. The needle leaves (the very first leaves to emerge from each seed) were sampled using discs punched with 0.5 ml Eppendorf tube lids by inserting a leaf across an opened Eppendorf tube and closing the tube to harvest equal size leaf samples (one for each leaf) and stored at −22 °C for DNA extraction.

2.5. DNA extraction

Total genomic DNA was extracted from four sources: pollen grains from CSSV-infected Amelonado cocoa trees (T2 and T4), CSSV-free Amelonado cocoa tree (H), component parts of seeds from cocoa trees (testa, cotyledon and embryo) and needle leaves from cocoa seedlings. A slightly modified DNeasy TM 96 Plant kit (Qiagen Ltd., UK) protocol was used. Buffer AP1 (400 μl) preheated to 65 °C to dissolve and 2 μl of RNase was 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 per second. DNA extraction quality from the experimental materials was checked on an ethidium bromide-stained agarose gel.

2.6. CSSV primer design and qualitative screening of CSSV

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 (position 350–725, accession number 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.

2.7. Qualitative screening of CSSV

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 *T. cacao* by Lanaud et al. (1999) was used. The primer sequence for the marker is as follows: forward primer: CTTCGTAGTGAATGTAGGAG and reverse primer: TTAGGTAGGTAGGGTTATCT.

3. Results

3.1. Screening of pollen grains for CSSV

All the pollen grains isolated from the anthers of cocoa trees T2 and T4 tested 100% positive to the CSSV by PCR/agarose electrophoresis and PCR/capillary electrophoresis. The control (H) tested negative to the CSSV (Table 1).

3.2. Hand pollination of cocoa trees

During the period of hand pollination, flower production was variable on the cocoa trees while the control tree (H) produced more

Table 1
CSSV status of pollen grains from cocoa trees

Experimental materials	Number of flowers tested	Positive test results	
		PCR/agarose electrophoresis	PCR/capillary electrophoresis
T2	50	+	+
T4	50	+	+
H	50	–	–
Total	150		

+ = Positive CSSV pollen grains.
– = Negative CSSV pollen grains.

Table 2
Outcome of artificial pollination of cocoa trees

Experimental materials	Number of flowers pollinated	Number of cherelles formed	Mature cocoa pods
T2	86	25	5
T4	102	19	4
H	112	43	10
Total	300	87	19

A total of 300 cocoa flowers were hand pollinated involving three cocoa trees resulting in the formation of 87 cherelles. Most of the cherelles formed wilted, resulting in 19 cocoa pods.

flowers. The peak for flower production was between April and September with limited number of flowers, or no flower production, around October and February. All the successful pollinations that resulted in pod formation occurred between May and September. Pollination outside this period at best resulted in cherelle formation which wilted later. Table 2 represents the outcome of hand pollination of cocoa trees.

3.3. Screening for CSSV in component parts of cocoa seeds

A total of 287 cocoa seeds from T2, T4 and H were screened destructively (separated into component parts: testa, cotyledon and embryo) for CSSV by PCR/agarose electrophoresis and PCR/capillary electrophoresis. The component parts of the seeds from T2 and T4 revealed varying levels of CSSV infection by PCR/agarose electrophoresis while PCR/capillary electrophoresis revealed 100% CSSV infection from the same DNA products (Table 3).

3.4. Screening for CSSV in cocoa seedlings

PCR/capillary electrophoresis detected CSSV viral products among T2 and T4 seedlings. All seedlings were maintained in insect-proof glasshouses. Except seedlings that tested CSSV positive, no additional seedlings tested positive for the viral particles at least 1 year following germination.

Only those multiplex PCRs which proved positive for the cocoa microsatellite internal control were scored for presence/absence of viral DNA. Absence of the microsatellite PCR product was taken to indicate a failed PCR though this occurred only infrequently (Fig. 1).

4. Discussion

4.1. Cocoa pod formation

Artificial pollination was carried out because the insect, midge and the attendant ants *Crestomagaster*, responsible for the pollination of cocoa, were absent in the University of Reading glasshouses and there was no guarantee that natural pollination would take place. Artificial pollination was facilitated by the apparent self-compatibility of Amelonado cocoa trees (Wood and Lass, 1987; Mossu, 1992). In cocoa flowering is profuse with a tree producing a minimum of 50,000 flowers in its lifetime with less than 5% setting pods (cherelle) (Lass, 1999). The low frequency of cherelle

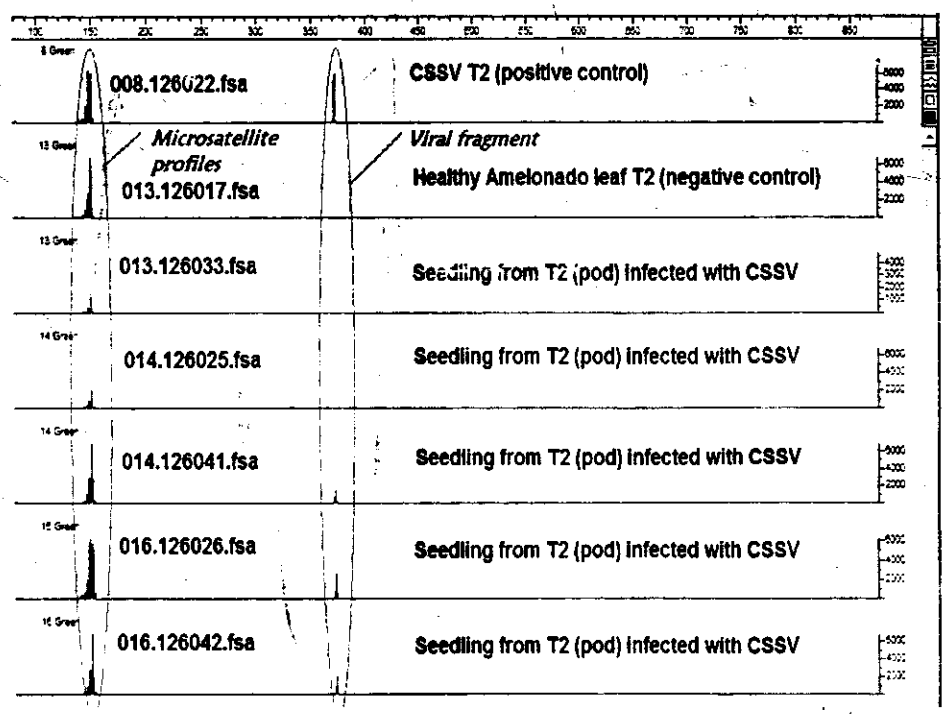

Fig. 1. PCR profile of cocoa seedlings from CSSV-infected tree subjected to capillary electrophoresis.

Table 3
Viral screening for CSSV from component parts of cocoa seeds

Experimental materials	Number of seeds tested	Positive test results by					
		PCR/agarose electrophoresis			PCR/capillary electrophoresis		
		Testa	Cotyledon	Embryo	Testa	Cotyledon	Embryo
T2	88	62 (71%)	50 (57%)	31 (35%)	88 (100%)	88 (100%)	88 (100%)
T4	69	46 (67%)	31 (45%)	16 (23%)	69 (100%)	69 (100%)	69 (100%)
H	130	0 (%)	0 (%)	0 (%)	0 (%)	0 (%)	0 (%)
Total	287	–	–	–	–	–	–

Table 4
Viral screening for CSSV from needle leaves of cocoa seedlings

Experimental materials	PCR/capillary electrophoresis	
	Number of seedlings tested	Number of CSSV positive seedlings
T2	102	40 (39%)
T4	98	53 (54%)
H	135	0 (0%)
Total	335	–

A total of 335 cocoa seedlings from T2, T4 and H were screened for CSSV using PCR/capillary electrophoresis. Seedlings from T2 and T4 tested positive to the CSSV at 40% (39%) and 53% (54%), respectively while all seedlings from H tested negative to the CSSV (Table 4).

formation may be the result of the flowers receiving inadequate pollen grains, which varies with time and the health of the trees (Mossu, 1992), to fertilize the ovules. The low frequency of mature pod formation (Table 2) may be accounted for by the fact that the cocoa trees were not growing in their natural environment where cocoa is planted among trees which provide shade. The presence of CSSV represents an additional stress on the cocoa trees.

4.2. Detection of CSSV in pollen grains and component parts of cocoa seeds

The presence of CSSV in the pollen grains from CSSV-infected Amelonado cocoa trees suggested that the virus may be transmitted through the gametes during fertilization (Table 1). The detection of the virus in all the component parts of the seeds by PCR/capillary electrophoresis further suggested the testa as a possible source of the viral infection of the embryo (Table 3). This is supported by the assertion of Maule and Wang (1996) that seed transmission can occur via two routes, zygotic infection from infected gametes and direct early embryo invasion from infected testa. It is unclear which of the two routes is used by the CSSV to infect the embryo. However, it is possible that the two routes can be used concurrently by the virus to invade the embryo.

4.3. Seed transmission of CSSV

Capillary electrophoresis has high protein separation efficiency and resolution capabilities than agarose electrophoresis(Richard et al., 1998). The sensitivity of PCR/capillary electrophoresis over PCR/agarose electrophoresis (Table 3) made it a better choice for the detection of CSSV in the needle leaves of the seedlings (Fig. 1). Out of the 52 seedlings from T2 and 43 seedlings from T4 screened for the CSSV, 40 (39%) and 53 (54%), respectively tested positive (Table 4). These high infection rates suggested that the CSSV is transmitted at a high frequency in cocoa seeds.

Although embryo invasion by the virus is necessary for seed transmission, this does not always lead to seedling infection. This may explain why the virus was not detected in all the needle leaves of the seedlings, assuming all the embryos of the seeds were infected by the virus. Furthermore, only the initial seedlings from T2 and T4 that tested CSSV positive remained CSSV positive at least 1 year after germination. This property establishes the CSSV both a seed-borne and seed transmitted virus.

5. Conclusion

It is concluded that CSSV can be detected in every component part of the cocoa pod and the CSSV can be transmitted through cocoa seedlings. Indexing of CSSV was more reliable using PCR/capillary electrophoresis, as PCR/agarose electrophoresis was prone to false negative results. However, there remain many unanswered questions on the mode of transmission of the CSSV (pollen-borne or through the gametes). Future research work should focus on the use of genetic markers to identify the movement of the CSSV virus in cocoa seeds.

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