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Investigation on *Cacao swollen shoot virus* (CSSV) pollen transmission through cross-pollination

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DNA- and RNA-based polymerase chain reaction (PCR) systems were used with *Cacao swollen shoot virus* (CSSV) primers designed from conserved regions of the six published genomic sequences of CSSV to investigate whether the virus is transmissible from infected trees through cross-pollination to seeds and seedlings. Pollen was harvested from CSSV infected cocoa trees and used to cross-pollinate flowers of healthy cocoa trees (recipient parents) to generate enough cocoa seeds for the PCR screening. Adequate precautions were taken to avoid cross-contamination during duplicated DNA extractions and only PCR results accompanied by effective positive and negative controls were scored. Results from the PCR analyses showed that samples of cocoa pod husk, mesocarp and seed tissues (testa, cotyledon and embryo) from the cross-pollinations were PCR negative for CSSV DNA. Sequential DNA samples from new leaves of seedlings resulting from the cross-pollinated trees were consistently PCR negative for presence of portions of CSSV DNA for over 36 months after germination. A reverse transcription-PCR analysis performed on the seedlings showed negative results, indicating absence of functional CSSV RNA transcripts in the seedlings. None of the seedlings exhibited symptoms characteristic of the CSSV disease, and all infectivity tests on the seedlings were also negative. Following these results, the study concluded that although CSSV DNA was detected in pollen from CSSV infected trees, there was no evidence of pollen transmission of the virus through cross-pollination from infected cocoa parents to healthy cocoa trees.

Keywords: badnavirus, CSSV, PCR, pollen, seed transmission, *Theobroma cacao*

Introduction

Seed transmission of plant viruses is an important phenomenon that occurs in a wide range of viral host species (Desjardins *et al.*, 1979; Greber *et al.*, 1991; Daniels *et al.*, 1995; Wang & Maule, 1996; Wang *et al.*, 2003). In most cases, seed transmission happens when embryos become infected and then germinate to produce infected seedlings. However, in some instances seedlings may become infected after germination due to contamination from virus-infected seed coat tissues, as is the case with *Tomato mosaic virus* (TMV; Broadbent, 1965). Wang & Maule (1994) outlined the processes that could lead to embryo infection and subsequently result in seed transmission into seedlings, using *Pea seed-borne mosaic virus* (PSbMV; genus *Potyvirus*, family Potyviridae) in pea (*Pisum sativum*) as a model crop. That work indicated that embryo infection could occur via two routes, either indirectly from infected gametes at fertilization or by direct invasion of the immature embryo from virus-infected testa tissues. The authors concluded that seed transmission occurred when the virus used the embryonic suspensor as a conduit from the micropylar region of the embryo sac to infect the tip of the embryonic radical. Their work indicated that this route was closed by the programmed degeneration of the embryonic suspensor.

Pathipanowat *et al.* (1995) studied seed and pollen transmission of *Alfalfa mosaic virus* (AMV). They took pollen from virus-infected alfalfa plants to pollinate healthy alfalfa plants. The resultant seeds and seedlings were collected and tested for evidence of AMV presence in the seed and seedling tissues using serological detection methods. They showed that AMV transmission to seedlings through seed produced on infected plants was possible, though differed amongst the isolates tested. The possibility of pollen transmission of AMV through seeds was considered a cause for concern in annual medic pasture breeding, evaluation and control programmes. This was because of the likelihood of the virus being carried-over outside the growing season in medic pastures through seed.

Cacao swollen shoot virus (CSSV; family Caulimoviridae, genus *Badnavirus*) was first identified in Ghana (Stevens, 1936; Posnette, 1940) and is now present in all the other West African cocoa producing countries including Nigeria, Cote d'Ivoire, Togo and Benin

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(Thresh & Owusu, 1986; Thresh *et al.*, 1988; Dzahini-Obiatey *et al.*, 2010). CSSV is mainly transmitted from infected to healthy cocoa trees through the feeding activities of different mealybug species (Roivainen, 1976). Other means of transmission such as graft and mechanical inoculation methods are mainly used for laboratory screening and indexing for CSSV presence and multiplication (Dzahini-Obiatey *et al.*, 2010). Studies on whether the virus is seed transmissible have been inconsistently reported over the years, sometimes with inconclusive results. Whereas work by Posnette (1941) suggested the possibility of CSSV seed transmission using physiological and growth data, further work by Crowdy & Posnette (1947) showed that CSSV was not seed transmissible.

Later studies by Quainoo *et al.* (2008) on CSSV transmission concluded that cocoa seeds obtained from CSSV infected parents were PCR positive for CSSV DNA in the various seed tissues. However, in their study the temperate conditions under which the experimental material was generated meant that only a limited number of pods were available for analysis. Even though that work reported the presence of CSSV DNA in the seed and seedling tissues, no further analysis was performed to test for expression of the CSSV DNA in the seed tissues or the emergent seedlings. Considering the widespread use of cocoa seedlings for crop regeneration, the inconsistent and conflicting reports on CSSV seed transmission is considered an area in need of further research (Posnette, 1941; Crowdy & Posnette, 1947; Quainoo *et al.*, 2008).

CSSV disease (CSSVD) continues to have a serious effect on cocoa growth and yield and ranks as the most important virus disease of cocoa in West Africa due to its ability to kill the cocoa plant within 3–5 years of infection when the severe strain is involved (Owusu, 1983; Muller, 2008). Due in part to the devastating effects of CSSV disease, cocoa production in Ghana has declined over the past years and the virus is still prevalent and keeps spreading to new areas. The continuous upsurge of CSSVD outbreaks in Ghana and other West African cocoa producing nations calls for further investigation to assess whether the virus could be transmitted through means other than the natural mealybug insect vector transfer.

The aim of this study was therefore to carry out further investigations into the possibility of CSSV transfer from infected trees to seeds and seedlings by cross-pollination with pollen from CSSV infected cocoa trees. This aim was achieved by the use of DNA- and RNA-based PCR systems using CSSV primers designed from published genomic sequences of the virus.

Materials and methods

Establishment of cross-pollination trials

Cocoa hybrid trees of varying susceptibility to CSSV infection which had been previously inoculated with CSSV isolate CSSV 1A (New Juabeng) and mild strains (N1 and SS365B) and carrying the virus for over 12 years under various CSSV trials at the Cocoa Research Institute of Ghana (CRIG) were used for the study (Table 1). The crossing activity involved harvesting of pollen from CSSV infected trees and transferring it onto flowers of counterpart healthy cocoa trees for pod initiation and development. The presence of CSSV DNA in the pollen was confirmed through a PCR test on pollen harvested from CSSV infected trees. As a precaution to prevent unwanted pollination and fertilization from adjacent trees, all opened flowers were removed the night before pollination. Only newly opened flowers on the morning of the day of pollination were considered as a source for pollen harvesting and target for receiving pollen. Pollinated flowers were immediately covered in pollination tubes until cherelles set.

Table 1 CSSV susceptibility classification of the cocoa hybrid trees used for the cross-pollination experiments to generate pods for CSSV seed transmission evaluation

Cocoa cultivars	CSSV susceptibility
[a]T85/799 × Amelonado[a]	Intermediate
T85/799 × T79/501	Tolerant
T79/467 × T87/1312	Tolerant
T63/971 × IMC 60	Tolerant
T85/799 × Pa7/808	Tolerant
T63/967 × T17/524	Tolerant
Amel selfed	Most susceptible

[a]T Code was used by Posnette (1951) to denote seedling material taken from Trinidad to Ghana in 1944. For example, T85/799 denotes seedling 799 of T85 parentage.
Parinari (Pa7) and Iquitos Mixed Calabacillo (IMC 60) from the Upper Amazon, were also introduced into Ghana as clones in the Pound's collections in 1938.

Sampling of cocoa seeds and raising of seedlings for CSSV screening

In order to assess for possible CSSV pollen transmission into the seeds and seedlings from the infected trees as a result of the cross-pollination, 83 samples of pulp tissue were taken from individual pods and 10 seeds were subsequently aseptically sampled from the proximal, medial and distal portions of each pod (totalling 830 seeds). The sampled seeds were segregated into seed tissue components of testa, cotyledon and embryo for DNA extraction and PCR analysis. The segregated seed tissues were individually PCR screened to assess whether CSSV DNA could be detected. To avoid DNA contamination, seed tissue samples were removed from each seed using individual sterile scalpel blades.

The remaining seeds from the pods were subsequently germinated in seed trays containing seed sowing compost (Sinclair). After germination they were immediately transferred into disposable plastic pots (7·5 cm^2) containing autoclaved potting mixture (Sinclair), and maintained at 25°C per day and 20°C per night in a glasshouse isolated from any CSSV infected plants. A total of 918

seedlings were obtained. The first true leaves from the emergent seedlings were sampled and PCR screened for the presence of CSSV DNA. For all PCR-based screenings, negative controls taken from quarantine assured CSSV-free trees were included. All PCRs were replicated three times and any reactions accompanied by failed negative controls were rejected.

CSSV primers for PCR analysis

CSSV specific PCR primers were designed from the 100% conserved regions of six published genomic sequences of CSSV currently available in the GenBank database (positions 350–375, AJ608931; Hagen *et al.*, 1993; Muller & Sackey, 2005; Quainoo *et al.*, 2008). The primers could reliably detect a 370 bp DNA fragment of the severe CSSV strain New Juabeng and the mild isolates N1 and SS365B. The primers Joelvirus Forward and Reverse were pre-tested on samples showing CSSV symptoms with consistent positive PCR results. They were considered suitable for the present study as they consistently failed to generate amplicons when tested against CSSV-free healthy (negative control) cocoa plants, an indication of the robustness of the primers to eliminate false positive PCR reactions from the cocoa DNA.

The primer sequences as modified from Quainoo *et al.* (2008), amplifying a 370 bp fragment of ORF 1 on the CSSV Juabeng isolate genome (AJ608931), were as follows: Joelvirus Forward 5′-AACCTTGAGTACCTTGACCT-3′, Joelvirus Reverse 5′-TCATTGACCAACCCACTGG-3′.

Other primer pairs from the various portions of the CSSV genome of the six published sequences were randomly obtained for confirmation of the PCR results from the Joelvirus CSSV Forward and Reverse primers.

DNA extraction and PCR systems for CSSV screening

Total genomic DNA was isolated from the sampled seed tissues and seedlings using QIAGEN DNeasy Kit for mini DNA extraction according to the manufacturer's protocol. The extracted DNA was then used to initiate a PCR test using QIAGEN multiplex PCR master mix buffer (dNTP mix, $MgCl_2$ and Hotstart *Taq* DNA polymerase) with the CSSV specific primers. In order to confirm the results for the presence of CSSV DNA in the seed tissues and seedlings, full-length CSSV primers (5′-CGCTGCAGTATTTCAAAGAA-3′) and (5′-CTGCAGGAGCGTTTTTGA-3′; Muller & Sackey, 2005) designed to amplify the full-length CSSV genome were also included in the PCR system for comparison and as an internal control for the experiment. Ten microlitres of PCR reaction volume were prepared by combining 5 μL QIAGEN multiplex master mix, 3 μL of Nanopure water, 1 μL of 2 μM primer mix and 1 μL of the extracted DNA. The PCR included a preliminary initiation step at 95°C for 15 min (*Taq* activation) followed by 35 cycles comprising a denaturation step at 94°C for 30 s, an annealing step at 57°C for 90 s and an extension step at 72°C for 60 s, followed by a final extension step at 72°C for 10 min, and a storage step at 10°C for 30 s. In the case of the full-length primers (Muller & Sackey, 2005), an elongation step of 8 min was included in the protocol as described in that paper.

The generated PCR products were analysed through TAE agarose gel electrophoresis (1%, 100 V for 1 h). In order to further confirm the results, a parallel PCR reaction was set up to generate products with the same PCR assay using a labelled CSSV forward primer (HEX™, Sigma) for analysis through capillary electrophoresis. DNA was extracted from individual seed tissues and used for PCR analysis to assess for presence of CSSV DNA in the seed and seedling tissues. Following agarose gel electrophoresis analysis of the PCR products, apparent CSSV-free DNA extractions were re-analysed through PCR with HEX™-labelled CSSV primers and capillary electrophoresis. A total of 918 germinated seedlings from all the cultivars were similarly studied for over 2 years for CSSV symptoms, even though all PCR tests on the seedlings were consistently negative in independent DNA extractions at 6, 15 and 24 months after germination.

DNA quality analysis

In order not to compromise the quality and reliability of the screening system, PCR reactions were performed using DNA whose quality was assessed and standardized to 10 ng μL^{-1} using a NanoDrop ND-1000 (v. 3.3.0) UV spectrophotometer. In measurements where the NanoDrop value was more than 10 ng μL^{-1}, the DNA was diluted to attain the standardized value. This was done to ensure that all PCR reactions were carried out with samples of DNA of high and uniform quality to eliminate false negative PCR results. Mean values of 10 repeated DNA extractions from the various cocoa plant tissues as assessed with the NanoDrop ND-1000 (v. 3.3.0) UV spectrophotometer were used for the PCR reactions.

PCR product sequencing

In order to confirm the identity of the amplicons from the PCR reactions, DNA sequencing of the PCR products was performed at the Bio-Centre of the School of Biological Sciences, University of Reading, using ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) with the protocol as described below.

The 10 μL BigDye reaction volume contained 4 μL BigDye buffer, 1·6 μL of primer (1 μM) and 4·4 μL of purified PCR DNA. The BigDye sequencing reaction was carried out using a G-storm thermal cycler (Genetic Research Instrumentation) using the following reaction protocol: 95°C for 30 s, 50°C for 15 s, 60°C for 1 min, for 30 cycles. Analysis of the sequencing products was carried out on an ABI PRISM 3130xl sequencer. When more than 20 samples were required for analysis, the BigDye reaction products were commercially sequenced at the Macrogen Sequencing Company (South Korea).

RNA extraction for RT-PCR on seedlings

To establish whether any detected CSSV DNA was being actively translated, a reverse transcription PCR (RT-PCR) analysis was carried out on the germinated seedlings to assess whether any CSSV RNA transcripts were present in the seedlings. Screening for CSSV RNA was deemed important as it is required from the viral DNA for replication and translation necessary for protein synthesis. Any presence of CSSV RNA would have been considered an indication of presence of active CSSV in the seedlings. Total RNA was extracted from leaf tissues using the CTAB (hexadecyltrimethylammonium bromide) method modified from the methods of Murray & Thompson (1980). Reverse transcription (RT) of the extracted RNA to synthesize complementary DNA (cDNA) was performed using Superscript II (Invitrogen) reverse transcriptase. The 10 μL RT reaction volume used to synthesize the cDNA consisted of 1 μL of extracted total RNA, 4 μL of 5 × first strand buffer, 2 μL 0·1 M DTT, 1 μL 10 mM dNTPs, 1 μL 100 μM reverse primer and 10 μL RNase-free water. The reaction mixture was incubated at 65°C for 10 min then at 45°C for a further 70 min for the reverse transcription reaction. The synthesized cDNA was used for further downstream qualitative RT-PCR tests and analysed using TAE agarose gel and capillary electrophoresis.

Infectivity tests on the seedlings using *Nicotiana benthamiana*

To further assess the activeness of CSSV DNA and its capacity to initiate infections in the seedlings, 100 *Nicotiana benthamiana* cuttings were inoculated with sap preparation from 20 different PCR positive cocoa seedlings for the 12 week period and were PCR assayed for presence of CSSV DNA through infectivity tests.

Results

Cross-pollination success and pod set

The cross-pollination programme yielded a total of 83 pods within the entire trial period, with the number of seeds per pod ranging from 22 to 47 (average 36 seeds per pod). It is noteworthy that there was low pod set, there being extensive flower fall and cherelle wilting across all the hybrid cultivars (Table 2).

PCR analysis and fragment sequencing results

Preliminary PCR tests on the DNA extractions from pollen samples from the CSSV infected trees used as pollen donors for the cross-pollinations were consistently positive (Fig. 1). Nonetheless, the PCR analysis through the agarose and capillary electrophoresis systems on all 83 samples of pulp (mesocarp) material taken from the resultant cocoa pods as well as the sampled seed tissues were all negative, indicating absence of CSSV DNA in the pulp tissues from the pods obtained from the cross-pollination programme.

The 100 repeated RT-PCR tests carried out on the seedlings were all negative, indicating the absence of any CSSV viral RNA transcript in the seedlings. Also, none of the seedlings exhibited any of the characteristic symptoms of CSSV. The CSSV DNA detection data presented in Tables 3 and 4 are a combined result from capillary and agarose electrophoresis based screening. All the PCR products from the seedlings were negative for CSSV DNA presence and were therefore not sequenced.

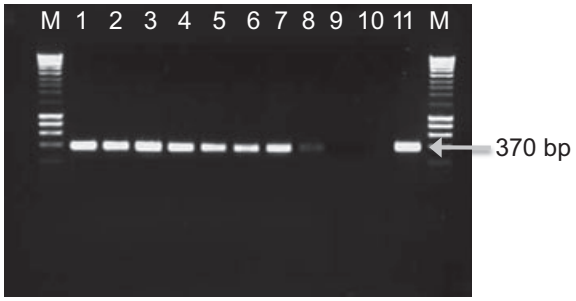

Figure 1 Agarose gel electrophoresis analysis of PCR screening of pollen DNA from CSSV infected cocoa trees as pollen donor. Lanes 1–8, DNA from pollen of flowers of CSSV infected cocoa trees; 9, negative control; 10, blank lane; 11, positive control (leaf); M, Hyperladder IV marker.

Table 2 Total pod set per genotype among self-pollinated cocoa trees showing CSSV symptoms across pollination series 1, 2 and 3

Parent cross (hybrid cultivar)[a]		No. of flowers pollinated	No. of flowers fallen	No. of cherelles set	Total no. of pods harvested
T79/467 × T87/1312 ♂	T85/799 × Amel♀	208	182	17	9
T63/967 × T17/524 ♂	T85/799 × T79/501♀	196	157	26	13
T85/799 × T79/501♂	T63/967 × T17/524♀	292	265	19	8
T63/971 × IMC 60 ♂	T79/467 × T87/1312♀	271	239	21	11
T63/967 × T17/524 ♂	Amel selfed♀	189	156	23	10
Amel selfed ♂	T85/799 × Pa7/808♀	315	288	13	14
T85/799 × Pa7/808♂	T63/967 × T17/524♀	206	174	22	10
Amel selfed ♂	T63/971 × IMC 60♀	194	169	17	8
Total		1871	1630	158	83

[a]♂ – paternal parent (pollen donor); ♀ – maternal parent (pollen recipient).

Table 3 PCR analysis for CSSV DNA presence in embryos from seeds derived from the crossing experiment using CSSV infected pollen onto flowers of healthy cocoa trees

Parent cross			
Infected pollen donor (paternal parent)	Healthy recipient (maternal parent)	Number of PCR positive embryos over total number of embryos tested	Percentage of positive embryos
T79/467 × T87/1312	T85/799 × Amel	0/90	0
T63/967 × T17/524	T85/799 × T79/501	0/130	0
T85/799 × T79/501	T63/967 × T17/524	0/80	0
T63/971 × IMC 60	T79/467 × T87/1312	0/110	0
T63/967 × T17/524	Amel selfed	0/100	0
Amel selfed	T85/799 × Pa7/808	0/140	0
T85/799 × Pa7/808	T63/967 × T17/524	0/100	0
Amel selfed	T63/971 × IMC 60	0/80	0
Total		0/830	0

Table 4 CSSV DNA detection via PCR analysis in seedlings (sampled between 1 and 24 months post-germination) derived from cross-pollination with cocoa hybrid cultivars showing CSSV symptoms onto healthy maternal parent

Parent cross			
Infected pollen donor (paternal parent)	Healthy recipient (maternal parent)	Number of PCR positive seedlings over total number of seedlings tested	Percentage of PCR positive seedlings
T79/467 × T87/1312	T85/799 × Amel	0/131	0
T63/967 × T65/326	T85/799 × T79/501	0/101	0
T85/799 × T79/501	T63/967 × T65/326	0/140	0
T63/967 × IMC 60	T79/467 × T87/1312	0/122	0
T63/967 × T17/524	Amel selfed	0/99	0
Amel selfed	T85/799 × Pa7/808	0/118	0
T85/799 × Pa7/808	T63/967 × T17/524	0/110	0
Amel selfed	T63/967 × IMC 60	0/97	0
Total		0/918	0

None of the *N. benthamiana* inoculated cuttings were PCR-positive for CSSV DNA for the 12 week period in which they were assayed with the primers, nor did they exhibit any symptoms related to CSSV.

Discussion

The present study used cross-pollination experiments by taking pollen from CSSV infected cocoa trees onto corresponding healthy cocoa trees to generate cocoa pods for PCR assessment, to determine whether the virus could be transmitted into the resultant seeds and seedlings. The study was considered important because adequate information about the seed transmission status of CSSV could greatly facilitate breeders' decisions on seed usage in cocoa breeding programmes to generate CSSV-resistant cultivars. Furthermore, clarification on CSSV seed transmission could also contribute to the general knowledge on CSSV epidemiology to improve quarantine procedures to limit and manage the spread of the virus. Finally, more information about CSSV seed transmission could also help to determine the risks associated with cocoa seed transfer, both locally and internationally, in spreading the virus from CSSV endemic areas.

There are numerous reports of pollen functioning as a means of vertical virus transfer to seeds and horizontal transfer to previously healthy plants, although effectiveness of pollen and seed transmission varies between and within some plant viruses (Smith & Stubbs, 1976; Desjardins *et al.*, 1979; Hearon & Locke, 1984; Wang & Maule, 1994, 1996; Wang *et al.*, 2003; Barba *et al.*, 2007). Wang & Maule (1996) investigated pollen and seed transmission of an isolate of *Pea seed-borne mosaic virus* (PSbMV) in two pea cultivars, one with high incidence of seed transmission and one which is not seed-transmissible. They noted that although PSbMV could infect the floral tissues (sepals, petals, anther and carpel) of both cultivars, the virus was not detected in ovules prior to fertilization. Analysis of virus incidence and concentration in the pea seeds at different developmental stages showed that the cultivar with high incidence of seed transmission had the virus directly invading the immature embryos and multiplying in the embryonic tissues which persisted during seed maturation. In contrast, the cultivar without seed transmission did not show invasion of the virus into the immature embryos and there was no evidence for virus multiplication or persistence during embryo development and seed maturation. That study therefore concluded that for virus transfer into

seeds to result in seedling infection, the virus needs to find a means of entering the embryonic tissues.

Pollen transmission of *Avocado sunblotch viroid* (ASVd) in avocado seedlings was also studied by Desjardins *et al.* (1979). This was prompted by the observation that infected avocado seedlings had germinated and grown under conditions that precluded the possibility of transmission by natural root graft. The most logical explanation for their infection was that the virus was transmitted by pollen. The authors had demonstrated in a preliminary study that the virus was present in pollen grains of infected avocado plants. This was accomplished by implantation of pollen grains, completely free of other floral parts, from flowers of infected trees under the bark of the test seedlings. Although ASVd was successfully transmitted to resultant seedlings through this technique, the study was careful to conclude that horizontal and vertical transmission of ASVd occurs in nature through infected pollen.

Taking into account the numerous examples and occurrences of pollen and seed transmission in other plant viruses, it was therefore considered important to evaluate this phenomenon in seed and seedling tissues from the cross-pollinated cocoa pods. The presence of CSSV DNA in the test tissues would give a clearer understanding of the infective status of the pollen from CSSV infected parents and its ability to initiate and cause CSSV spread.

Results from the present study showed that pollen from CSSV infected trees were PCR positive for portions of CSSV DNA, and this concurred with earlier observations by Quainoo *et al.* (2008). However, seeds and seedlings from the crossing experiments were PCR negative for the presence of the virus. Attempts to inoculate the indicator plants with sap preparations from the seedlings were unsuccessful, as the subsequent PCR tests were negative. Even though CSSV DNA was initially detected in the pollen from the flowers of CSSV infected trees, the results have provided limited evidence to support its ability to cause CSSV infection in cocoa seeds obtained from healthy cocoa trees pollinated with pollen from infected trees. None of the seedlings showed symptoms of CSSV for over 2 years during the study period and all the infectivity tests to assess whether sap preparations from the resultant seedlings could transmit CSSV DNA to healthy indicator cocoa and *N. benthamiana* tobacco species were PCR-negative.

Combining the PCR results from the cross-pollinations and the infectivity analysis, this study concludes that although CSSV DNA was detected in the pollen from CSSV infected trees, there was no clear-cut evidence to indicate that the virus is transmissible to developing seeds and seedlings from healthy cocoa trees pollinated with pollen from infected cocoa trees.. The implication of these results is that the role of cocoa pollinating midges (Diptera: Ceratopogonidae) as a possible vector for carrying infected pollen is at present not important, and that infected pollen is not a potential source of CSSV spread.

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